BBA - Proteins and Proteomics 1868 (2020) 140291

Contents lists available at ScienceDirect

BBA - Proteins and Proteomics

journal homepage: www.elsevier.com/locate/bbapap

Characterization of alkaline phosphatase PhoK from *Sphingomonas* sp. BSAR-1 for phosphate monoester synthesis and hydrolysis

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ARTICLE INFO

Keywords:
Biotransformation
Enzyme
Phosphatase
Phosphate ester
Transphosphorylation

ABSTRACT

The biocatalytic activity of a so far underexploited alkaline phosphatase, PhoK from *Sphingomonas* sp. BSAR-1, was extensively studied in transphosphorylation and hydrolysis reactions. The use of high-energy phosphate donors and oligophosphates as suitable phosphate donors was evaluated, as well as the hydrolytic activity on a variety of phosphate monoesters. While substrates bearing free hydroxy group displayed only moderate reactivity as acceptors for transphosphorylation by PhoK, strong hydrolytic activity on a broad variety of phosphate monoesters under alkaline conditions was observed. Site-directed mutagenesis of selected amino acid residues in the active site provided valuable insights on their involvement in enzyme catalysis. The key residue Thr89 so far postulated to engage in enzyme phosphorylation was confirmed to be crucial for catalysis and could be replaced by serine, albeit with much lower catalytic efficiency.

1. Introduction

Alkaline phosphatases along with nucleotide phosphodiesterases, co-factor independent phosphoglycerate mutases, phosphonate monoester hydrolases and aryl sulfatases are metalloenzymes, members of the large alkaline phosphatase (AP) superfamily that share similar metal-binding site architecture and protein fold, and are responsible for a range of catalytic activities [1–3]. Over the past decades, a number of promiscuous activities across the family members have been observed [4]. Catalytic promiscuity of enzymes along with particular structural features reflects evolutionary relationship and provides understanding of factors that influenced their evolution [5]. This valuable information might help to guide enzyme engineering and de novo enzyme design [6]. A particularly interesting case is the evolutionary interconnection between alkaline phosphatases from *E. coli* (EcAP) [7], *Chryseobacterium meningosepticum* (PafA) [8], and *Sphingomonas* sp. BSAR-1 (PhoK) [9], and the nucleotide pyrophosphatase/phosphodiesterase (NPP) from *Xanthomonas citri* [4i], all characterized by a bimetallic Zn^{2+}-center. EcAP, PafA and PhoK catalyze the hydrolysis of phosphate monoesters, while NPP is specific for phosphate diesters. These enzymes were subject of recent studies shedding light on the evolution and catalytic diversification of enzymes in the AP superfamily, and PhoK was recently suggested to be the first member of a novel class of APs, based on analysis of crystal structure and comparison with EcAP-like enzymes [10].

PafA and PhoK (~26% protein sequence identity) appear evolutionary distant from EcAP (~11% identity to PafA/PhoK) and NPP (~18% identity to PafA/PhoK). While EcAP-like enzymes employ a binuclear Zn^{2+}-center accompanied by a Mg^{2+}-ion as well as a serine residue responsible for the formation of a covalent enzyme-phosphate intermediate, in PafA and PhoK the role of the missing Mg^{2+} is thought to be replaced by that of a protonated lysine, while a threonine appears to replace the catalytic serine residue.[10b,d] In the case of PhoK, an additional Ca^{2+}-ion is suggested to aid in the dimerization of the protein, as inferred from analysis of the crystal structure.[10d] Furthermore, PhoK contains six cysteine residues, all of which are involved in disulfide bond formation within the monomer and are responsible for a rigid and compact structure. Remarkably, PhoK seems to recruit simultaneously several amino acid residues that are differently conserved among other members of the AP superfamily, in particular Thr89, Asn110, Lys171 and Arg173.

Beside biochemical significance, alkaline phosphatases have found useful biotechnological applications, for instance in the synthesis of farnesyl alcohol derivatives [11], in the analysis of sphingosine analogues [12], in enzymatic chemiluminescent assays [13], and in a potential bioremediation process involving precipitation of uranium in form of phosphate salt [9]. Recently, alkaline phosphatases were used in biocatalytic cascade reactions for the assembly of supramolecular

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https://doi.org/10.1016/j.bbapap.2019.140291
Received 26 July 2019; Received in revised form 4 October 2019; Accepted 10 October 2019
Available online 31 October 2019
1570-9639/

materials [14], for equilibrium displacement in disaccharide synthesis [15], and in downstream processing of nucleotide-diphosphosugars [16] and oligosaccharides [17]. Although alkaline phosphatases can theoretically be used in the synthesis mode, similarly to the well-exploited acid phosphatases [18], their phosphate-*transfer* ability has barely been harnessed. One of the rare examples reported so far is the use of calf intestine alkaline phosphatase employing inorganic oligophosphates as phosphate donors (P-donors) and a range of short to medium-chain alcohols as acceptors at high concentration (60 vol%) [19]. The system was optimized for the large-scale production of glycerol-1-phosphate in a continuous flow reactor using immobilized enzyme [7b]. More recently, immobilized bovine alkaline phosphatase was used for the preparation of glycerol-1-phosphate, precursor of dihydroxy acetone phosphate, used in the synthesis of carbohydrates in a cascade setup [20].

We recently became interested in exploiting acid phosphatases in the synthesis mode for the biocatalytic selective phosphorylation of alcohols using cheap inorganic oligophosphates as high-energy phosphate donors at pH 4–5 [21]. This required targeted efforts to reduce unwanted product hydrolysis, which results in low yields of the phosphorylated compounds and is also connected with great variability in optimum conditions to reach high product titer. Following similar motivation, we aimed at studying the unexplored biocatalytic potential of PhoK for synthetic applications by investigating both its phosphotransferase and phosphate ester hydrolase activity (Scheme 1). A number of structurally diverse molecules, precursors of valuable sugar-phosphates, nucleotides and metabolites, such as mevalonate-phosphate, were therefore evaluated as suitable acceptors in transphosphorylation reactions.

2. Results and discussion

2.1. Over-expression

The *phoK* gene was obtained through commercial synthesis (Uniprot accession number: A1YYW7) and was initially cloned into pET30a vector for expression with C-terminal His-tag. The vector was then transformed into *E. coli* BL21(DE3) harboring chaperone 1 of the Takara chaperone plasmid set, and the protein was over-expressed and purified (see supporting information). The enzyme solution was stored at 4 °C for several weeks after purification and dialysis to allow for proper refolding of the protein. This step was necessary due to the use of denaturing conditions in the cell lysis step (0.5 M urea [10g], see supporting information). The specific activity of PhoK-pET30a in the *p*-nitrophenylphosphate (*p*-NPP) assay reached a final value of 39 U mg^{-1} at pH 9.0 in 200 mM Tris-HCl buffer, which is in the range of that of non-specific acid phosphatases (NSAPs) expressed in *E. coli* [21]. During the course of the study, the expression of PhoK was further optimized. PhoK was cloned into pASK-IBA2 vector allowing for periplasmic expression, and purification via C-terminal Strep-Tag (see supporting information). The purified protein PhoK-IBA2 showed a significantly improved specific activity of 1690 U mg^{-1} (43-fold increase compared to the activity of the previous preparation). Initial characterization was performed with PhoK-pET30a before switching to the more active PhoK-IBA2 (see below).

2.2. Characterization

The hydrolytic activity of the enzyme was first tested in different buffer systems. Metal ion supplementation (Zn^{2+} and Ca^{2+}) was also tested as a way to increase activity and overcome possible chelation effects of Tris [22] and glycine [23] (Fig. 1). Surprisingly, the enzyme activity was strongly affected by rising metal ion concentration in carbonate, Tris and CHES (*N*-cyclohexyl-2-aminoethanesulfonic acid) buffers but not in glycine buffer, in which the activity could be significantly improved with up to 5 mM salt. Upon metal supplementation, the enzyme was most likely replenished with metal and displayed improved activity. Similar trends have been observed with carboxypeptidase A, a zinc containing enzyme, in presence of phytic acid [24]. No activity was observed in metal-containing buffer in absence of enzyme (data not shown). As reported in the literature [10], the pair of Zn^{2+} is essential for catalysis in the AP superfamily, not only activating the nucleophilic residue but also stabilizing negatively charged oxygen of the leaving phosphate during hydrolysis. Upon analysis of the crystal structure, Ca^{2+} in PhoK has been proposed to play a structural role, influencing dimerization, which likely explains the importance of sufficient metal availability for activity. No other divalent metals were tested. With more closely related PafA, the addition of Ca^{2+} was shown to have no effect on activity [10b]. Interestingly, in the case of purple acid phosphatase-type enzymes, which do not generate a phosphoenzyme intermediate during catalysis, Ca^{2+} was also found essential for an active enzyme; with diesterase Rv0805 from *Mycobacterium tuberculosis*, Ca^{2+} binding was proposed to regulate activity [25].

The effect of previously reported inhibition of alkaline phosphatases by phosphate [7b,9] was evaluated in the hydrolysis of *p*-NPP by PhoK in the presence of increasing concentration of P_i. PhoK hydrolytic activity decreased by 50% and 90%, respectively, in the presence of 100 mM and 300 mM phosphate, respectively (Fig. 2).

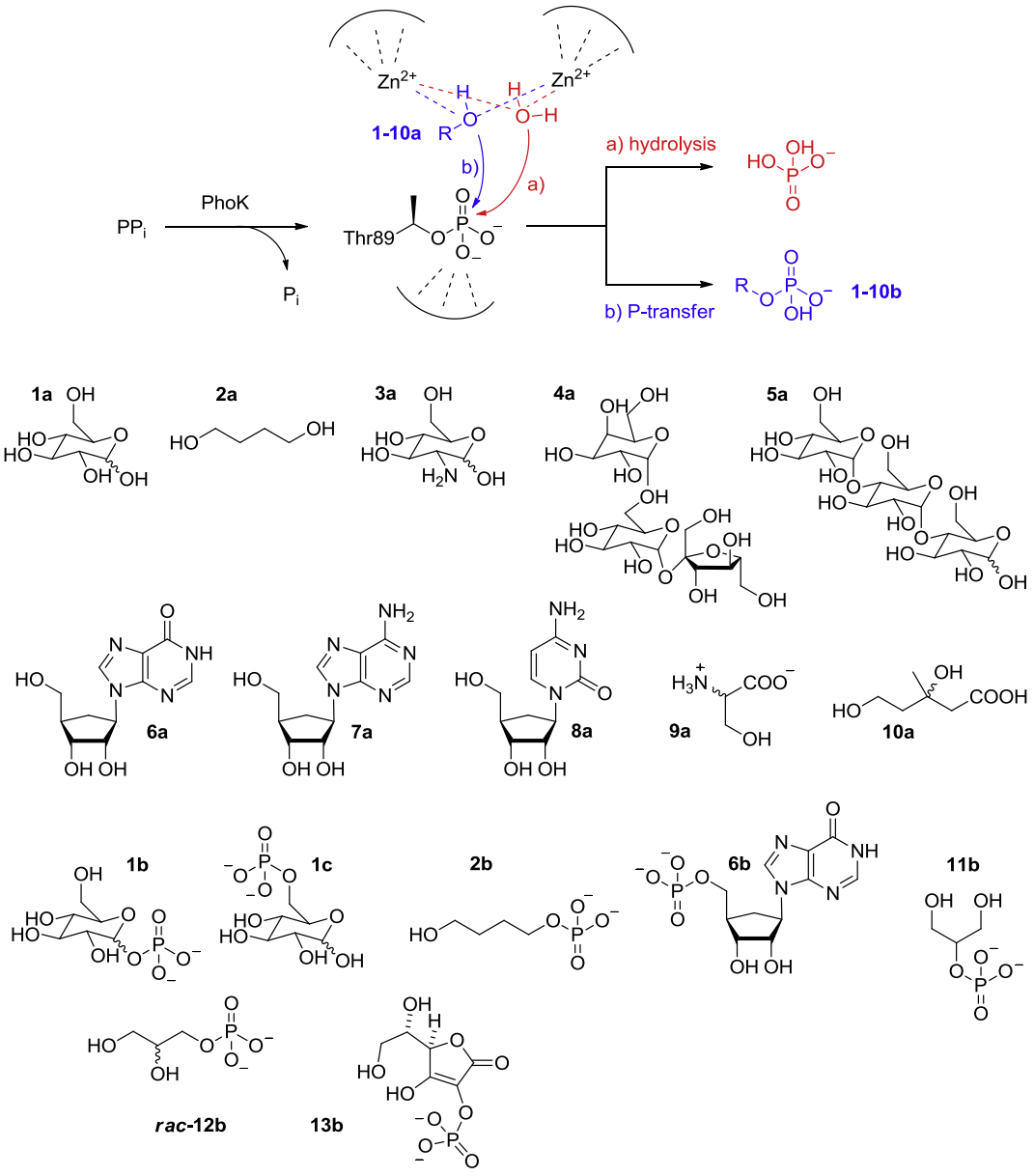
Inorganic oligophosphates are P-donors of choice in transphosphorylation reactions catalyzed by acid phosphatases (for structures of P-donors, see Table S1) [21]. However, oligophosphates are notorious chelators and may cause inactivation of metal-dependent enzymes. The effect of various oligophosphates and increasing concentration of zinc and calcium ions was therefore studied in the *p*-NPP assay (Fig. 3). PhoK displayed no activity in PP_i and trimetaphosphate buffers in absence of added metal ions, however, zinc and calcium ion supplementation resulted in enhanced specific activity, indicating partial restoration of the hydrolytic activity (up to 21 U mg^{-1}). Phytic acid seemed to be less detrimental as PhoK was reasonably active even without added metal ions. The activity could be enhanced at low metal ion concentrations (< 5 mM) but was reduced at elevated concentrations (15 mM). In contrast, metal ions had no effect on the activity in hexametaphosphate buffer, which remained comparatively low (max. 3.1 U mg^{-1}).

PP_i was selected for further investigations, based on previous reports [21], and the more active protein obtained from expression using IBA2 vector was chosen (PhoK-IBA2). The hydrolysis of 250 mM PP_i was first followed at various metal concentrations and pH values (Figs. 4, 5). No conversion was observed without added metals, confirming the data obtained with *p*-NPP (Fig. 3). Addition of 5–10 mM zinc and calcium could however restore the hydrolytic activity. Higher concentrations (from 20 mM) strongly affected the activity of PhoK enzyme. As expected for alkaline phosphatases, a pH study identified pH 9 as optimum value for the hydrolysis of PP_i (up to 90% conversion after 20 h). Higher pH values resulted in lowest conversion levels (< 10%). PP_i could be replaced by PPP_i, another cheap, high energy P-donor, however, reduced reaction rate in the first 5 h was observed at pH 9, in line with the observation made with acid phosphatase PhoN-Sf [21]. After 20 h however, similar conversion level was achieved.

2.3. Transphosphorylation

The transphosphorylation activity of PhoK was first probed with 500 mM D-glucose (**1a**) as substrate and 250 mM PP_i in the presence of 5 mM $ZnCl_2$ and 5 mM $CaCl_2$ at various pHs (Fig. 6).

Similar pH optimum as in the hydrolysis reaction was observed for the phosphotransferase activity (pH 9). Product concentration reached max. 16 mM at 91% consumption of PP_i after 4 h, corresponding to about 7% phosphate transfer efficiency; the major part of PP_i was thus preferentially hydrolyzed without phosphate transfer (Scheme 1, path a) dominating). Beyond this time, significant product hydrolysis took place. Lowering the pH resulted in reduced reaction rates and the



Scheme 1. Competition between hydrolysis (red) and transphosphorylation (blue) catalyzed by alkaline phosphotase PhoK. Both pathways are proposed to proceed through a phosphorylated threonine [10d]. R-OH: phosphate acceptors **1a**-**10a**. Substrates investigated in this study are depicted **1a**-**10a** and **1b**/**1c**-**13b**; PP_i: pyrophosphate, P_i: monophosphate.

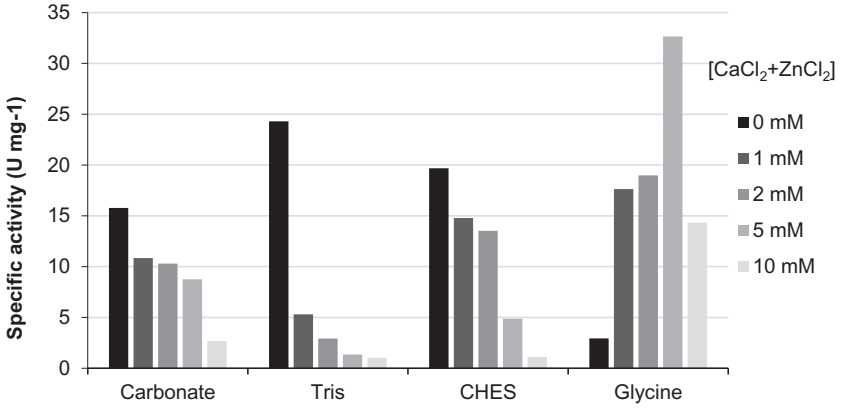


Fig. 1. PhoK-pET30a specific activity based on *p*-NPP assay in various buffers (100 mM) at pH 9 at varying equimolar [$ZnCl_2$ + $CaCl_2$] concentration.

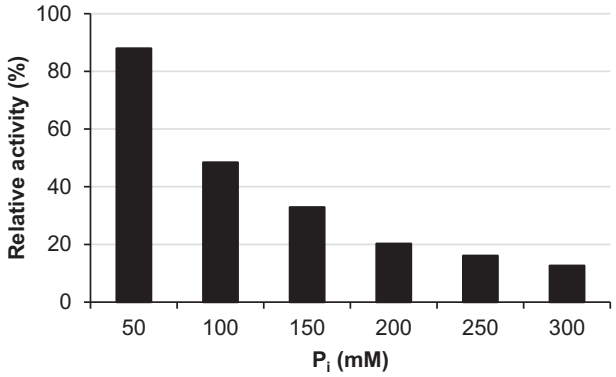


Fig. 2. Influence of P_i concentration on hydrolytic activity of PhoK-pET30a at pH 9. 100% activity refers to enzyme activity in Tris-buffer (200 mM, pH 9) and 0 mM phosphate in the *p*-NPP assay.

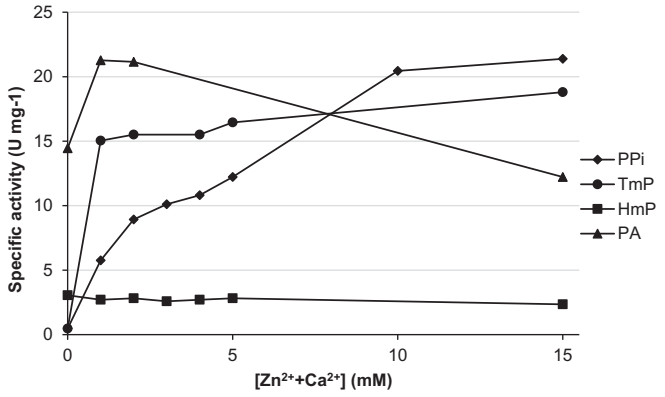


Fig. 3. PhoK-pET30a hydrolytic activity based on *p*-NPP assay in buffers of P-donor salts (100 mM) at pH 9 at varying equimolar [$ZnCl_2$ + $CaCl_2$] concentration (TmP: trimetaphosphate, HmP: hexametaphosphate, PA: phytic acid).

consumption of PP_i similarly slowed down. After 5 days, no remaining product was detected at pH 8 and 9, while at pH 7, ~7 mM residual product was measured. PP_i was almost fully consumed in all cases. At higher pH values (> 10), only traces of product could be observed (< 5 mM) and a very slow rate of PP_i consumption (data not shown). Overall, the transphosphorylation activity follows the same trend upon variation of the pH value as observed in the hydrolysis of PP_i. Enhanced concentration of **1a** (1.0 M) at pH 9 furnished double amount of product (~29 mM) as well as reduced product hydrolysis rate, while 1.5 M **1a** resulted in substantially lower product concentration (Fig. S3). ^{31}P NMR analysis revealed that phosphorylation selectively took place at the primary C6-OH moiety resulting in formation of D-glucose-6-phosphate (**1c**) [21c].

PhoN-Sf from *Shigella flexneri*, PiACP from *Prevotella intermedia* and PhoN-Se from *Salmonella typhimurium* LT2 were used to compare the transphosphorylation activity of PhoK with that of representative acid phosphatases. These enzymes have been shown to exhibit a broad substrate scope, high phosphotransfer activity and robustness in the synthesis of phosphate esters at pH 4–5 [21a,b,26]. All three enzymes were active on D-glucose (**1a**), PhoN-Sf and PiACP yielding 120–140 mM product (**1c**) in 23 h (~9-fold higher product titer compared to that with PhoK), while PhoN-Se furnished moderate product level (~45 mM) (Fig. S4).

Next, the substrate scope of PhoK was explored by testing additional acceptors including 1,4-butanediol (**2a**), mono- and oligosaccharides (**3a-5a**), nucleosides (**6a-8a**), *rac*-serine (**9a**) and *rac*-mevalonic acid (**10a**) at pH 9 (Scheme 1 and Table 1). Unfortunately, only very low phosphate transfer efficiencies (max. 5%) were observed with all tested substrates at 70–80% PP_i consumption after 2 h. In the case of inosine (**6a**) and cytidine (**9a**), the formation of multiple products could be monitored (^{31}P NMR), indicating non-selective phosphorylation. Despite broad substrate acceptance, the moderate level of transphosphorylation activity observed under the tested conditions does not appear suited for preparative-scale synthetic applications using PhoK.

2.4. Hydrolysis

We then turned our attention to the hydrolytic activity of PhoK on a set of phosphorylated compounds. Regioisomers of glycerol-phosphate (2-phosphate: **11b** and 1-phosphate: *rac*-**12b**) and D-glucose-phosphate (1-phosphate: **1b** and 6-phosphate: **1c**) as well as phytic acid, ascorbic acid-2-phosphate (**13b**), 4-hydroxybutyl phosphate (**2b**) and inosine-5′-monophosphate (**6b**) were subjected to hydrolysis at pH 9 at 60–100 mM concentration (Table 2 and Fig. S5). Overall, the rate of hydrolysis of phosphorylated secondary hydroxy group (**1b**, **11b**, **13b**) was found higher than that of primary counterparts. PhoK displayed however poor regiopreference in the hydrolysis of regioisomers of glycerol 11b and 12b (Aspec = 629 vs. 520 U mg^{-1})

regioisomers of glycerol **11b** and **12b** (A_{spec} = 629 vs. 520 U mg^{-1}). With glucose derivatives, **1b** was converted at higher rate than **1c** (A_{spec} = 1417 vs. 477 U mg^{-1}), indicating an almost 3-fold preference for hydrolysis of 1-phosphate compared to 6-phosphate, which is somewhat unexpected given the exquisite regioselective transphosphorylation of glucose **1a** toward **1c**. Nearly full conversion was observed with most substrates after 5 h incubation time except in the case

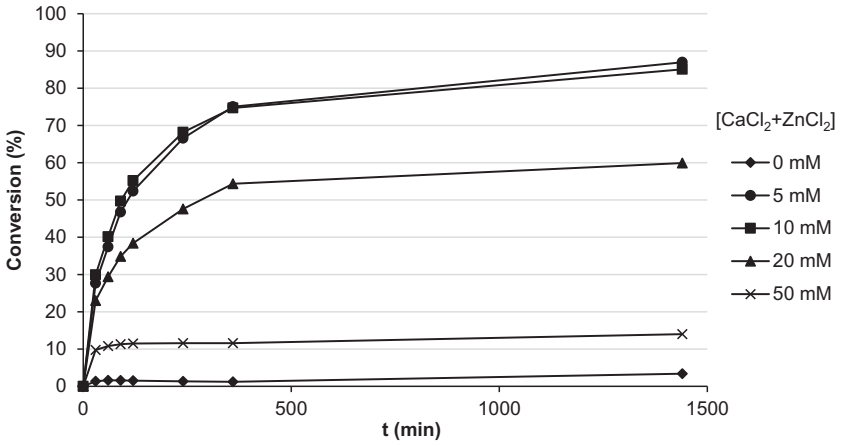


Fig. 4. Hydrolysis of 250 mM PP_i at pH 9 by PhoK-IBA2 (8.2 µg mL^{-1}, ~0.14 µM) in the presence of various amounts of [$ZnCl_2$ + $CaCl_2$] (equimolar).

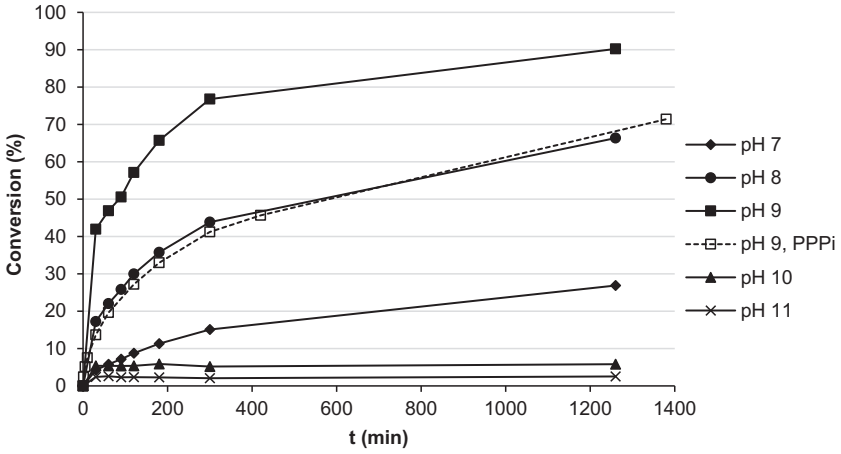


Fig. 5. Hydrolysis of 250 mM PP_i or PPP_i by PhoK-IBA2 (8.2 μg mL^{-1}, ~0.14 μM) at various pHs in the presence of 5 mM [$ZnCl_2$ + $CaCl_2$] (equimolar).

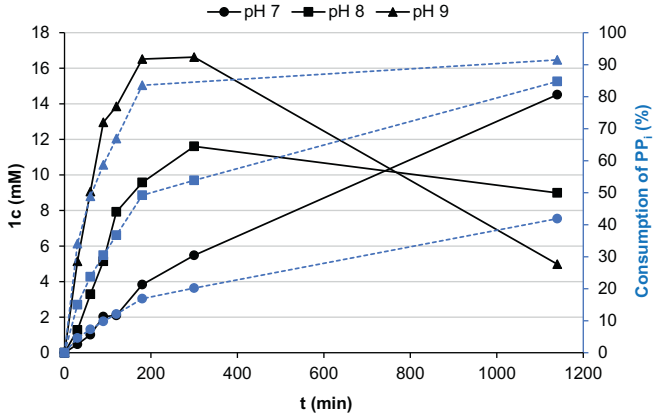


Fig. 6. Transphosphorylation activity of PhoK-IBA2 (8.2 μg mL^{-1}, ~0.14 μM) with 500 mM D-glucose (**1a**) and 250 mM PP_i at pH 9 in the presence of 5 mM $ZnCl_2$ and 5 mM $CaCl_2$ toward formation of D-glucose-6-phosphate (**1c**).

Table 1
Substrate-scope of PhoK-IBA2 in transphosphorylation mode using PPi as donor[a].

Substrate	[2–10a] (mM)[b]	[2–10b] (mM)[c]	$Consumption_{PPi}$ (%)[d]	Efficiency (%)[e]
1,4-butanediol (**2a**)	500	11	82	5
D-glucosamine (**3a**)	500	3	85	1
raffinose (**4a**)	500	n.d.	80	n.a.
maltotriose (**5a**)	500	n.d.	85	n.a.
inosine (**6a**)	80	3[f]	73	2
adenosine (**7a**)	40	n.d.	72	n.a.
cytidine (**8a**)	300	9[g]	67	5
rac-serine (**9a**)	300	9	74	5
rac-mevalonic acid (10a)	280	1[h]	51	< 1

[a] 250 mM PP_i at pH 9 in the presence of 5 mM $ZnCl_2$ and 5 mM $CaCl_2$, samples taken after 2 h.
[b] Starting concentration of substrate.
[c] Product concentration based on ^{31}P NMR analysis.
[d] Consumption of PP_i.
[e] Percentage of consumed PP_i used for product formation (phosphate transfer efficiency).
[f] Total product amount, ratio of products 1:1.1.
[g] Total product amount, ratio of products 4:1:5.5.
[h] Sample taken after 1 h. n.d. dot detected; n.a. not applicable.

Table 2
Phosphohydrolase activity of PhoK-IBA2 on various phosphate monoesters[a].

Substrate	A_{spec} (U mg^{-1})
1b	1417
1c	477
2b	443
11b	629
12b	520
13b	1114

[a] Reaction performed at pH 9 in the presence of 5 mM $ZnCl_2$ and 5 mM $CaCl_2$. See supporting information for details.

of 4-hydroxybutyl phosphate (**2b**, full conversion reached after overnight incubation). Phytic acid was not accepted. The data clearly highlights the potential of PhoK as an efficient phosphohydrolase, enabling rapid hydrolysis of primary and secondary phosphorylated hydroxy groups of diversely substituted substrates in the alkaline range, with TTN up to 7.1 × 10^6 (0.14 μM biocatalyst used on 60–100 mM substrate).

2.5. Mutational study

The analysis of PhoK crystal structure indicates major differences with the structures of other alkaline phosphatases, such as emblematic EcAP from *E. coli* [10d]. Four residues located in the active site have been identified as signature of a suggested new class of alkaline phosphatases – Thr89, Asn110, Lys171 and Arg173 – and the catalytic function of each residue was predicted based on crystal structure comparison. However, no mutational analysis was performed to clearly identify the role of each residue with PhoK, which prompted us to design single alanine mutations of PhoK at the indicated positions. In addition, the alleged phosphorylating threonine residue was replaced by other nucleophilic residues (Ser, Asp, Lys, His, Tyr) known to be responsible for phosphate-transfer in other classes of phosphatases [27].

The variants were cloned, overexpressed and purified according to the procedure applied to the wild-type protein (see supporting information), and characterized (Table 3). In the *p*-NPP assay, a drastic loss of activity was observed upon exchanging any of the four targeted amino acids. Thr89Ser, Lys171Ala and Arg173Ala only showed a residual specific activity (max. 1 U mg^{-1}) three orders of magnitude lower than that of the wild-type. Further Thr89 mutations (including Ala) resulted in complete loss of activity (data not shown). Asn110Ala variant could retain approx. 2% of the wild-type activity (38 U mg^{-1}).

Table 3
Comparison of hydrolytic activity of PhoK wild-type and variants.

Enzyme	A_{spec}*p*-NPP (U mg^{-1})	A_{spec} PP_i (U mg^{-1})	PP_i hydrolysis (%)[a]
PhoK-WT	1359	818	89 (9 h)
PhoK-T89S	1.0	3.1	78 (24 h)
PhoK-N110A	38	14	79 (23*h*)
PhoK-K171A	< 1	4	23 (19 h)
PhoK-R173A	1.1	< 0.1	n.d.

[a] Conversion of PP_i over varying reaction time (indicated in h); n.d. not detected.

The difference in catalytic activity between variants and wild-type was also reflected in the hydrolysis of 100 mM PP_i, with highest activity observed with Asn110Ala (A_{spec} = 14 U mg^{-1}). Despite lower specific activity upon mutation, high conversion level could still be reached after 23 h (79% compared to 87% after 5 h with the wild-type, see Fig. S6). Thr89Ser and Lys171Ala, which displayed even lower specific activity (max. 4 U mg^{-1}), also led to high conversion levels after 24 h (78% with Thr89Ser). Arg173Ala was found inactive in the hydrolysis of PP_i. In comparison, mutation of conserved threonine to serine with NPP led to increased activity on monoester and decreased activity on diester. With PafA, mutation of Thr79 to serine resulted in decreased catalytic activity, regardless of the ester type. Mutation of the three other residues with PafA resulted in 50% of wild-type catalytic activity with Asn100Ala on monoester, and a reduction in catalytic activity by 1 and 4 orders of magnitude with Arg164Ala and Lys162Ala, respectively [10d]

Residues Asn110 and Arg173, which are not conserved in EcAP-like enzymes, can be found in nucleotide phosphodiesterases and phosphonate monoester hydrolases, and were proposed to play a role in binding of the substrate phosphoryl group in PhoK [10d]. Lys171 is thought to replace the third metal ion (Mg^{2+}) of EcAP-like alkaline phosphatases via the protonated ε-amino group. The replacement of Asn110, Lys171 and Arg173 with Ala most likely affected the sophisticated hydrogen-bonding network responsible for the stabilization of the phosphate group during catalysis, as suggested with PafA [10d]. Finally, the drastic loss of activity upon mutation of Thr89 clearly supports the suggested role as phosphorylating residue, previously demonstrated with NPP [28]. Only mutation to homologous serine, present in EcAP, resulted in a functional, although less active, enzyme.

3. Conclusion

In this study, the catalytic potential of the first member of a recently proposed new class of alkaline phosphatases relying on a rare catalytic threonine, PhoK from *Sphingomonas* sp. BSAR-1, was evaluated for synthetic applications. Parameters important for activity under process conditions were identified: i) The deactivating effect on PhoK of oligophosphates via chelation could be alleviated by supplementation of essential divalent metal ions (Zn^{2+}/Ca^{2+} in equimolar amounts); ii) pH value of 9 was determined to be optimum for both phosphotransferase and hydrolase activity; iii) High concentrations of pyrophosphate and triphosphate (up to 250 mM) were well accepted in hydrolysis reactions. Importantly, phosphotransferase activity was identified on a variety of substrates (e.g., alcohols, nucleosides, sugars) using pyrophosphate as donor, however, maximum product levels overall remained below those typically obtained with acid phosphatases and calf intestine alkaline phosphatase. A range of *prim*- and sec-phosphate monoesters was finally successfully hydrolyzed by PhoK, thereby offering a suitable alternative for use for instance in biocatalytic cascade reactions in the alkaline range. Finally, results of the mutation study of selected residues in the active site unambiguously assigned the key catalytic role to threonine 89.

4. Experimental section

4.1. Assay for phosphohydrolase activity (p-NPP assay)

Enzyme activities were assayed spectrophotometrically by measuring the dephosphorylation of 4-nitrophenyl phosphate (*p*-NPP) via release of *p*-nitrophenol (pNP). A protein solution (final concentration 1–200 μg mL^{-1}, ~12.5 nM-2.5 μM) was added to an appropriate buffer (carbonate, Tris-HCl, CHES - *N*-cyclohexyl-2-aminoethanesulfonic acid, glycine, P_i, PP_i, TmP, HmP or PA; 100 mM final concentration or as indicated) supplemented with the appropriate amount of $ZnCl_2$ and $CaCl_2$ at a given pH to a final volume of 480 μL. The solutions were pre-incubated at 30 °C for 5 min followed by addition of 20 μL 250 mM *p*-NPP in H_2O (10 mM final concentration in buffer) and mixed at 30 °C and 450 rpm. After 1 min incubation time, the reaction was quenched with 500 μL of 1 M NaOH and the absorbance of *p*-NP was recorded at 405 nm (ε = 18,500 M^{-1} cm^{-1}). The activity tests were always performed in triplicate and values are mean values. Deviation from mean values was below 5%. One unit of phosphatase activity (U) corresponds to the amount of *p*-NP (micromoles) released per minute under assay conditions. Specific activity (A_{spec}) represents the phosphatase activity (U) of 1 mg protein.

4.2. Phosphotransferase and phosphohydrolase activity using inorganic oligophosphates

A standard reaction mixture contained substrate and/or P-donor (PP_i or PPP_i) in H_2O supplemented with the appropriate amount of $ZnCl_2$ and $CaCl_2$ at a concentration and pH indicated in the footnotes of tables and captions of all figures in 1 mL final volume. The reaction was initiated by adding a given amount of enzyme, obtained via periplasmic expression of PhoK-IBA2: 8.2 μg mL^{-1} (~0.14 μM) wt, 100 μg mL^{-1} Thr89Ser, Arg173Ala and Asn110Ala, 40 μg mL^{-1} Lys171Ala. The mixture was shaken in 1.5 mL screw-cap glass vial at 30 °C and 800 rpm in an Eppendorf thermoshaker. Samples of 25 μL volume from the reactions with PP_i, PPP_i, substrates **1a**, **1b-13b** and **1c** were taken at intervals, diluted with 480 μL of 140 mM aq. H_2SO_4 and analyzed on HPLC-RI. Reactions were performed in duplicate. Data points are mean values of at least duplicate reactions. Deviation from mean values was below 5%, and curves displaying time profile behavior (from duplicate samples) were perfectly continuous (relative error of ≤ 5%). Conversion values in the hydrolysis of PPP_i were calculated by relating P_i concentration to theoretical maximum P_i concentration (i.e. 750 mM) due to the insufficient separation of PP_i and PPP_i on the HPLC column.

In the substrate scope study in transphosphorylation mode with substrates **2a-10a** (Table 1), ^{31}P NMR was used to analyze the reactions. To a standard reaction was added 20 μL conc. HCl after 1 or 2 h, then 600 μL sample was added to 100 μL D_2O and ^{31}P NMR spectrum was recorded using inverse gated decoupling (ns 32, d1 = 30 s, pw = 11 μs).

Details on cloning and characterization of enzymes and analytical methods can be found in the supporting information.

Declaration of Competing Interest

There is no conflict of interest to declare.

Acknowledgements

Funding by the Austrian BMWFW, BMVIT, SFG, Standortagentur Tirol, Government of Lower Austria and ZIT through the Austrian FFG-COMET-Funding Program is gratefully acknowledged. Klaus Zangger (University of Graz) is thanked for NMR measurements.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbapap.2019.140291.

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