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Phosphorylation and dephosphorylation of polyhydroxy compounds by class A bacterial acid phosphatases

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Received 14th April 2003, Accepted 16th June 2003
First published as an Advance Article on the web 9th July 2003

Nonspecific acid phosphatases share a conserved active site with mammalian glucose-6-phosphatases (G6Pase). In this work we examined the kinetics of the phosphorylation of glucose and dephosphorylation of glucose-6-phosphate (G6P) catalysed by the acid phosphatases from *Shigella flexneri* (PhoN-Sf) and *Salmonella enterica* (PhoN-Se). PhoN-Sf is able to phosphorylate glucose regiospecifically to G6P, glucose-1-phosphate is not formed. The K_m for glucose using pyrophosphate (PPi) as a phosphate donor is 5.3 mM at pH 6.0. This value is not significantly affected by pH in the pH region 4–6. The K_m value for G6P by contrast is much lower (0.02 mM). Our experiments show these bacterial acid phosphatases form a good model for G6Pase. We also studied the phosphorylation of inosine to inosine monophosphate (IMP) using PPi as the phosphate donor. PhoN-Sf regiospecifically phosphorylates inosine to inosine-5′-monophosphate whereas PhoN-Se produces both 5′IMP and 3′IMP. The data show that during catalysis an activated phospho-enzyme intermediate is formed that is able to transfer its phosphate group to water, glucose or inosine. A general mechanism is presented of the phosphorylation and dephosphorylation reaction catalysed by the acid phosphatases. Considering the nature of the substrates that are phosphorylated it is likely that this class of enzyme is able to phosphorylate a wide range of hydroxy compounds.

Introduction

Nonspecific acid phosphatases (NSAPs) are bacterial enzymes which are able to catalyse the hydrolysis of phosphate monoesters. On the basis of amino acid sequences, NSAPs are categorized into three classes, designated as class A, B and C.[1,2] Three domains are present in the amino acid sequences of class A acid phosphatases which contain their active site residues. These are also conserved in mammalian glucose-6-phosphatases (G6Pase), lipid phosphatases, vanadium-containing chloroperoxidases (CPO) and vanadium-containing bromoperoxidases (BPO).[2–9] The amino acid residues in sequence motifs of domain I: KX_6RP, domain II: PSGH and domain III: SRX_5HX_3D play very important roles in catalysis. The active site residues participate in the binding of vanadate or phosphate, act as nucleophiles, stabilize the penta-coordinated transition state and play a role in leaving group protonation.[10]

G6Pase, a key enzyme in glucose homeostasis, catalyses the hydrolysis of G6P to glucose and phosphate, the terminal steps in gluconeogenesis and glycogenolysis. Mutations have been identified[11] in G6Pase that cause glycogen storage disease type Ia which is an autosomal recessive metabolic disorder. It was shown that these mutations alter the active site residues and abolish G6Pase activity demonstrating the importance of these residues in phosphatase action. The importance of these residues was also demonstrated in mutagenesis studies in the conserved domain of two lipid phosphatases.[8,12] Similarly, Renirie *et al.* investigated the effect of 6 mutations of putative catalytic residues on the phosphatase activity of apo CPO.[13] Based on these mutagenesis studies, a model was derived that may serve as a template for G6Pase and other related phosphatases. The kinetic data on the phosphatase reaction catalysed by apo CPO point to a mechanism in which during turnover a phosphorylated enzyme intermediate is present.

Although a crystal structure of the G6Pase is not available yet, the crystal structures of the nonspecific acid phosphatase from *Escherichia blattae*[14] and the vanadium-containing chloroperoxidase from the fungus *Curvularia inaequalis*[3] are available, showing the striking similarity of the active sites of those enzymes.[9,14] A similar architecture of the active site of the acid phosphatases, the lipid phosphatases, the vanadium haloperoxidases and the mammalian G6Pases[15] suggests that in principle this super family of enzymes is able to catalyse essentially the same basic reactions with variations in specificity and catalytic efficiency. This has already been demonstrated by us showing that vanadate substituted acid phosphatases have bromoperoxidase activity[9] and apo CPO has phosphatase activity.[5]

Recently, Asano *et al.*[16–20] reported a new enzymatic method of phosphorylation of inosine to produce inosine-5′-monophosphate (5′IMP) using pyrophosphate (PPi) as a phosphate donor by the recombinant PhoC from *Morganella morganii*.[17,18,20] They investigated the phosphotransferase activity of a number of enterobacteria,[16,19] and using PPi as the phosphate donor they showed that especially class A1 acid phosphatases, which are not inhibited by fluoride, exhibit high regioselective transphosphorylation activity.[19] *M. morganii* PhoC belongs to class A1 acid phosphatase in which the non-specific acid phosphatase from *Shigella flexneri* (PhoN-Sf) is also classified.

G6Pase catalyses not only the hydrolysis of G6P but it has phosphotransferase activity, being able to synthesize G6P from glucose and various phosphate donors including PPi.[21] These studies prompted us to study the phosphorylation and dephosphorylation of glucose and G6P, respectively, by acid phosphatases, and compare the catalytic mechanism and parameters with those of G6Pase. We also compare the phosphorylation of glucose by the acid phosphatase with that of inosine. Although this reaction has been studied by Asano *et al.*,[16–20] some kinetic details for this enzymatic phosphorylation are still lacking. Our data point to the evidence of an activated phosphorylated enzyme intermediate that is able to regiospecifically transfer its phosphate group to an appropriate substrate acceptor. It is concluded that the enzymatic formation of phosphate esters catalysed by the acid phosphatases using pyrophosphate as a cheap phosphate donor may present a viable alternative for the classical chemical synthetic methods using $POCl_3$.

DOI: 10.1039/b304012g

Results and discussion

Expression and purification of recombinant PhoN-Sf

Sh. flexneri phoN was cloned in pET3a and expressed under control of T7 promoter in pET3a.[9] Due to instability of the expression system using pET3a, it was decided to amplify and subclone the mature sequence of *phoN-sf* and fuse it to the gene III signal sequence in pBAD/gIIIB. PhoN-Sf was inducibly expressed in the periplasmic space of the *E. coli* TOP10 host cell. On average, the yield was approximately 30 mg per litre of culture. The final specific activity of acid phosphatase in the strain was about 40 U mg^{-1} with over 95% purity. This expression system was a significant improvement over the previous system.[9]

Phosphorylation of inosine to inosine monophosphate by nonspecific acid phosphatases

The phosphorylation of inosine to inosine monophosphate using pyrophosphate (PPi) by recombinant nonspecific acid phosphatases from *Shigella flexneri* (PhoN-Sf) and *Salmonella enterica* (PhoN-Se) was tested by using different enzyme concentrations. The amount of inosine phosphorylated is linearly dependent upon the enzyme concentration (results not shown). This indicates that this phosphotransferase activity is catalysed by both PhoN-Sf and PhoN-Se. PhoN-Sf catalyses the phosphorylation of inosine to inosine-5′-monophosphate (5′IMP), whereas PhoN-Se synthesizes both 5′IMP and inosine-3′-monophosphate (3′IMP). Fig. 1 shows the time course of 5′IMP synthesis by PhoN-Sf. The phosphorylating activity of PhoN-Sf is highly dependent on pH, while the highest yield of 5′IMP was obtained at pH 5.0. However, the activity of PhoN-Se was hardly affected by pH in the range of 4.5 to 6.0, only at pH 3.5 the activity was much lower. The maximal amounts formed were 6.8 mM 5′IMP for PhoN-Sf (Fig. 1) and approximately 4.3 mM 5′IMP and 1.7 mM 3′IMP for PhoN-Se (results not shown), respectively. The turnover of PhoN-Sf is 76 min^{-1} (2.8 U mg^{-1}) and that of PhoN-Se 5′IMP synthesis is 18 min^{-1} (0.67 U mg^{-1}). These values are comparable to those reported by Mihara *et al.* for various other acid phosphatases. The phenomenon, that class A1 acid phosphatase PhoN-Sf has a much higher regioselectivity for the formation of 5′IMP, has also been observed by Mihara *et al.*[18,19] They imply that class A1 nonspecific acid phosphatases exhibit regioselective PPi-nucleoside phosphotransferase activity.[19] On the other hand PhoN-Se class A2 acid phosphatase catalysed the synthesis of 5′IMP and 3′IMP with a 4-fold lower turnover. The overall sequence similarity of PhoN-Se and PhoN-Sf is about 40%, although the active site domains of those enzymes are identical. Mutagenesis studies of PhoC from *Morganella morganii*[17–19] and non-specific acid phosphatase from *Escherichia blattae*[20] have shown considerable enhancement of phosphorylation activity of these enzymes by changing a few amino acid residues near a potential inosine-binding site. Thus the high regioselectivity in 5′IMP synthesis is determined by amino acid residues that are not involved in binding the phosphate moiety of the substrate.

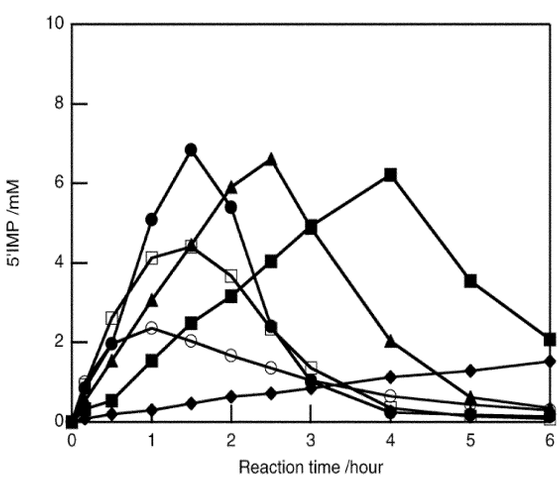


Fig. 1 Time course of 5′IMP synthesis from inosine and PPi by PhoN-Sf at pH 3.5–6.0. The reaction mixture contains 1 μM of phosphatase, 40 mM inosine, 100 mM disodium pyrophosphate and 100 mM sodium acetate buffer pH 3.5 (◆), 4.0 (■), 4.5 (▲), 5.0 (●), 5.5 (○) and 6.0 (□).

The amounts of 5′IMP (Fig. 2; panel A) and 3′IMP (Fig. 2; panel B) produced by PhoN-Se were also dependent on PPi concentrations. It is clear that the total yields of IMPs differ at different concentrations of PPi and that they are very low at low PPi concentrations. Not only was the yield of 5′IMP during phosphorylation higher than 3′IMP, but also the dephosphorylation rate of 3′IMP was faster than that of 5′IMP. A major problem in the study of the phosphorylation by nonspecific acid phosphatases is the hydrolysis of synthesized 5′IMP and 3′IMP that occurs at the same time, which makes analysis of the events difficult.

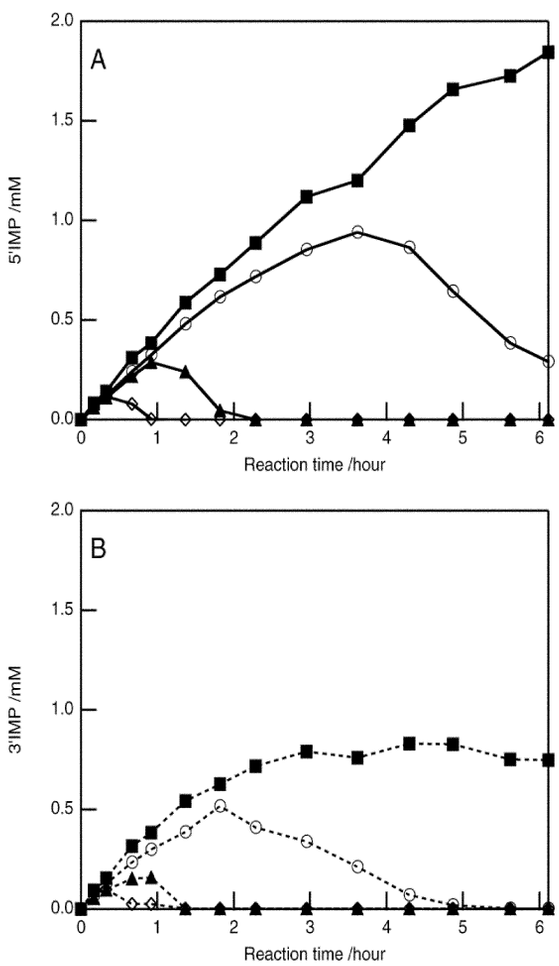
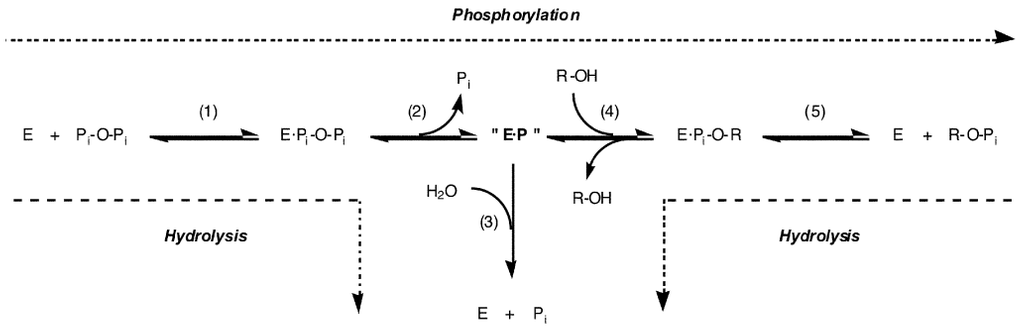


Fig. 2 Time course of 5′IMP (panel A) and 3′IMP (panel B) synthesis from inosine and by PhoN-Se using (◇) 5 mM, (▲) 10 mM, (○) 40 mM and (■) 100 mM PPi at pH 6.0. The reaction mixture contains 500 nM of PhoN-Se, 40 mM inosine, and 100 mM sodium acetate buffer (pH 6.0).

Basically five reactions are thought to occur at the same time (Scheme 1). First the enzyme reacts with PPi to produce a binary pyrophosphate–enzyme complex (step 1). This complex dissociates (step 2) to yield an activated phosphorylated enzyme intermediate ("E·P"). In the next step, a reaction (step 3) with water may occur resulting in dissociation of the activated phosphorylated enzyme as well as the hydrolysis of pyrophosphate.



Scheme 1 Overall mechanism of phosphorylation and dephosphorylation catalysed by acid phosphatases. Steps (1) and (2), formation of phosphorylated enzyme intermediate. Reaction (3), hydrolysis of the intermediate "E·P". Equilibrium (4), forward reaction; reaction of "E·P" to yield a binary enzyme-phosphorylated substrate (E·R–O–Pi), backward; dissociation of the dephosphorylated substrate. Equilibrium (5), forward; dissociation into the phosphorylated substrate and free enzyme, backward; hydrolysis of the phosphorylated substrate.

The intermediate ("E·P") may also transfer (step 4) the phosphate to a bound phosphate acceptor (R–OH, in this case inosine), which dissociates to form E + ROPi, resulting in the production of IMP. Thus competition with water occurs, and a high inosine concentration is required for an effective transphosphorylation reaction. At low PPi concentration, the maximal yields of 5′IMP and 3′IMP are very low because the intermediate concentration of the phosphorylated enzyme intermediate is very low and also rapid hydrolysis by water occurs. Further as the reaction proceeds in time PPi becomes exhausted and dephosphorylation of IMP occurs *via* formation of the activated phosphorylated enzyme intermediate and again hydrolysis by water occurs. At higher PPi concentration, the difference in yields between 5′IMP and 3′IMP became clearer, and 3′IMP was hydrolysed earlier than 5′IMP.

Fig. 3 shows the substrate dependency of 5′IMP and 3′IMP during the dephosphorylation at pH 6.0. There is a striking difference in the dephosphorylating activity of PhoN-Se and PhoN-Sf. The latter enzyme has hardly any activity towards 3′IMP and is selectively hydrolysing 5′IMP. The V_{max} is 24.4 U mg^{-1} with a K_m of 300 μM. The class A2 acid phosphatase PhoN-Se hydrolyses both 5′ and 3′IMP with a similar V_{max} (6.2 U mg^{-1} and 5.8 Umg^{-1}, respectively), but the K_m values differ considerably (225 μM and 34 μM, respectively). This difference in K_m values is probably the reason why the amount of 3′IMP produced by PPi decreases more rapidly during turnover (Fig. 2). The dependence of the phosphorylation rate on the inosine concentration was also studied but it was difficult to obtain an accurate K_m value due to the solubility of inosine which is less than 80 mM under our condition. For the non-specific acid phosphatase *E. blattae* a K_m value of 202 mM was reported.[20]

The effect of vanadate on the phosphorylation of inosine has been studied. Vanadate, which is structurally homologous to phosphate, is known as an inhibitor of a number of acid phosphatases.[22,23] The K_i for vanadate of PhoN-Sf in the dephosphorylation of *p*NPP is about 70 nM.[9] Furthermore, we have shown that vanadate is incorporated into the active sites of PhoN-Sf and PhoN-Se with a high affinity and these vanadate-substituted enzymes show bromoperoxidase activity.[9] When 100 mM PPi was used as phosphate donor in the phosphorylation of inosine, the addition of 100 μM vanadate to the reaction mixture at pH 6.0 unexpectedly did not show inhibition. However, as shown by Tracy *et al.*[24] PPi is known to form a complex with vanadate and it is likely that vanadate is scavenged by PPi, therefore inhibition may be quenched. During the experiment the yields of 5′IMP and 3′IMP (results not shown) were increased somewhat. It is likely that vanadate inhibits hydrolysis of synthesized 5′IMP and 3′IMP. Therefore the effect of 100 μM vanadate was studied on the hydrolysis of 5′IMP. Indeed in the presence of vanadate, the hydrolysis was completely inhibited (results not shown).

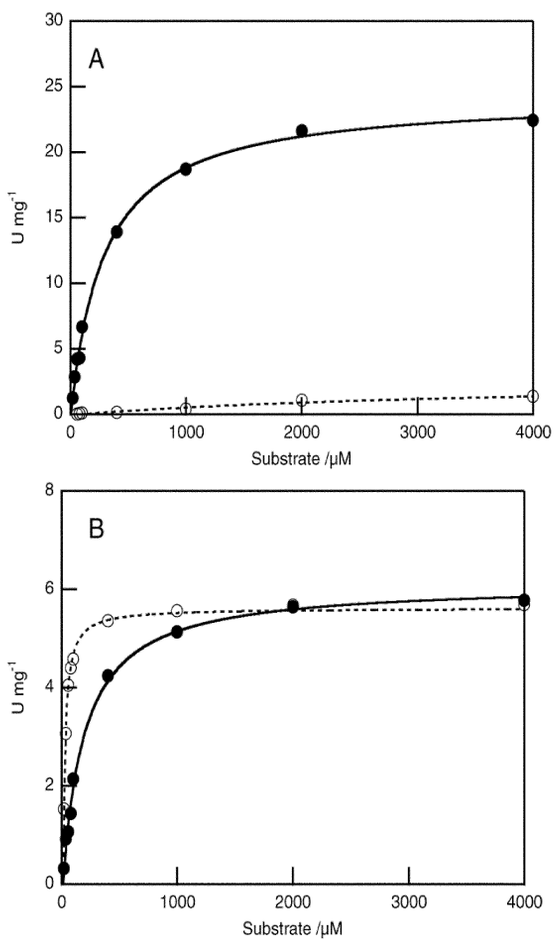


Fig. 3 Dephosphorylation of 5′IMP (●, solid line) and 3′IMP (○, broken line) by (A) PhoN-Sf and (B) PhoN-Se at pH 6.0. The reaction mixture contains 100 nM of phosphatase, 100 mM sodium acetate buffer (pH 6.0) and various concentrations of IMPs. K_m and V_{max} were calculated by a non-linear-regression plot using EnzymeKinetics (Trinity Software). The data points are means of triplicate measurements.

Phosphorylation of glucose to glucose-6-phosphate by nonspecific acid phosphatases

PhoN-Sf and PhoN-Se also mediate the phosphorylation of glucose to glucose-6-phosphate (G6P) using PPi as the phosphate donor. The enzymatic assay system used (see Experimental) allows us to monitor in a convenient way the formation of G6P. The amount of glucose which was consumed by the reaction was also determined by HPLC (results not shown) and is in agreement with that of the enzymatic assay. Glucose-1-phosphate (G1P) was not formed upon phosphorylation according to the enzymatic assay using phosphoglucomutase.

Table 1 Kinetic constants[a] of phoshorylation and dephosphorylation by PhoN-Sf

Reaction	Substrate	pH	K_m/mM	V_{max}/U mg^{-1}
Phosphorylation	Glucose	4.0	8.2 ± 1.9	26.3 ± 1.3
	Glucose	4.5	3.2 ± 0.4	12.9 ± 0.3
	Glucose	5.0	3.2 ± 0.5	8.2 ± 0.2
	Glucose	5.5	3.6 ± 0.2	4.2 ± 0.1
	Glucose	6.0	5.3 ± 1.0	2.5 ± 0.1
	Inosine	5.0	192 ± 22	18.5 ± 1.4
Dephosphorylation	G6P	6.0	0.021 ± 0.004	19.2 ± 0.4
	5′IMP	6.0	0.298 ± 0.004	23.7 ± 1.0
	PPi	6.0	0.087 ± 0.009	20.3 ± 1.0

[a] To obtain initial rate constants of glucose phosphorylation, the reaction mixture containing 1 μM of PhoN-Sf, 100 mM disodium pyrophosphate, 100 mM sodium acetate buffer (pH 4.0–6.0) and various concentrations of glucose was incubated for 5 min at 30 °C, then the amount of G6P was assayed using G6P dehydrogenase. For inosine phosphorylation, the reaction mixture containing 1 μM of PhoN-Sf, 100 mM disodium pyrophosphate, 100 mM sodium acetate buffer (pH 5.0) and various concentrations of inosine were incubated for 10 min at 30 °C, and the amount of the formed 5′IMP was determined using HPLC. Dephosphorylation of G6P, 5′IMP and PPi was carried out using the reaction mixture containing 100 nM of PhoN-Sf, various concentrations of each substrate in 100 mM sodium acetate (pH 6.0). After incubating for 1 min, the reaction mixture was quenched by addition of Biomol Green™ reagent. The kinetic constants were determined by using EnzymeKinetics (Trinity Software). The data points are means of triplicate measurements.

Thus this phosphorylation reaction of glucose is also regiospecific. As shown in Fig. 4, glucose phosphorylation by PhoN-Sf is dependent upon pH, although the behaviour differs from the observation of inosine phosphorylation. Unlike inosine phosphorylation, the hydrolysis of G6P hardly occurred within 8 hours (Fig. 4). However, at low pH after initial formation of G6P, the phosphorylated sugar was re-hydrolysed as the reaction continued (results not shown). Since the PhoN-Se produced only 5 mM G6P at pH 4.0, which is considerably less than the 40 mM of G6P produced by PhoN-Sf under the same conditions, we restricted our study to PhoN-Sf. As Fig. 4 shows the amount of G6P produced reaches a maximum value of approximately 40 mM at pH 4 after 3 hours and then remains constant. Inspection of Fig. 1 and Fig. 2 show that under these conditions nearly all PPi is consumed as a consequence of both phosphorylation of glucose and by the hydrolysis of the activated phosphorylated enzyme intermediate. It is likely therefore that further conversion of glucose can be obtained by further addition of PPi. Indeed a second addition of PPi increased the conversion to about 60% (results not shown). Fig. 5 illustrates the phosphor nuclear magnetic resonance (^{31}P NMR) spectra taken at several time points during the incubation. In line with the experiments in Fig. 4, at pH 4.0 approximately 30 mM G6P was produced after 3 hours incubation. Fig. 5 only shows chemical shifts corresponding to G6P, free phosphate and PPi. Other phosphorylated compounds were not detected, confirming that phosphorylation of glucose by PhoN-Sf is very regiospecific.

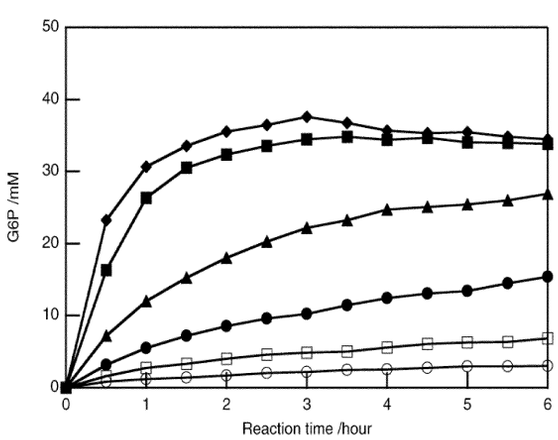


Fig. 4 Time course of G6P synthesis from glucose and PPi by PhoN-Sf. The reaction mixture contains 1 μM of PhoN-Sf, 100 mM glucose, 100 mM disodium pyrophosphate and 100 mM sodium acetate buffer pH 3.5 (◆), 4.0 (■), 4.5 (▲), 5.0 (●), 5.5 (□) and 6.0 (○).

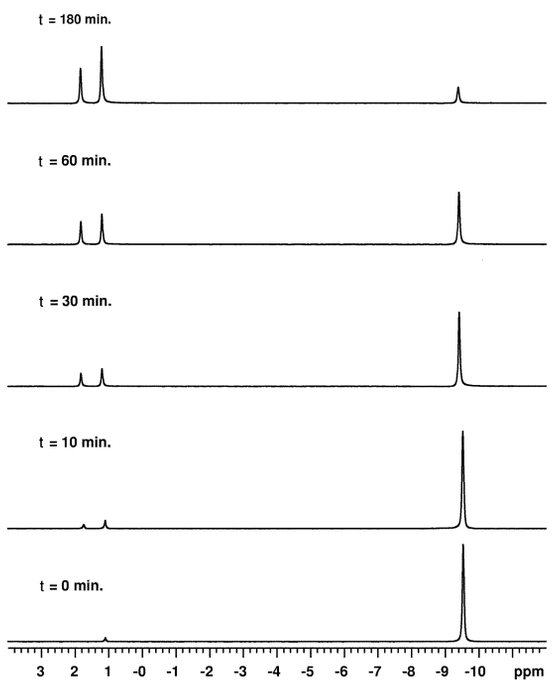


Fig. 5 Time course of G6P synthesis from glucose and PPi by PhoN-Sf followed by ^{31}P NMR. The reaction mixture contains 1 μM of PhoN-Sf, 100 mM glucose, 100 mM disodium pyrophosphate and 100 mM sodium acetate buffer pH 4.0. At zero time a spectrum was taken from the reaction mixture without PhoN-Sf. The reaction was initiated by addition of 1 μM PhoN-Sf in the NMR tube. Concentrations of products and reactants were determined using dimethylmethylphosphonate as an external standard. Peaks at δ −9.39, 1.21 and 1.84 correspond to pyrophosphate, phosphate and glucose-6-phosphate, respectively.

Table 1 shows the kinetic constants for the phosphorylation of glucose by PhoN-Sf that were obtained from initial rate measurements. The K_m value for glucose is only slightly dependent upon pH whereas the V_{max} increases more rapidly at low pH values. The hydrolysis of G6P was also studied, and Table 1 shows that at pH 6.0 the K_m value of G6P is considerably less (21 μM) than for glucose in the phosphorylation reaction (5.3 mM). Similarly the K_m values for inosine in the phosphorylation reaction is higher (192 mM) than the K_m values for 5′IMP (0.3 mM) in the dephosphorylation reaction. The data indicate that non-phosphorylated substrates have a lower affinity for the enzyme than the phosphorylated substrates.

The difference in K_m values for glucose and inosine (5.3 mM *versus* 192 mM) may explain that G6P is hardly dephosphoryl-

ated (Fig. 4) and 5′IMP is relatively easily dephosphorylated (Fig. 1) during phosphorylation. Scheme 1 shows that there is a competition between the phosphate acceptor R–OH and water for the phosphate–enzyme intermediate "E·P". Low values of K_m for the phosphate acceptor R–OH or its high concentration will drive the equilibrium in the direction of the phosphorylated intermediate and inhibit the hydrolytic reaction.

This also explains why glucose inhibits the dephosphorylation of G6P. Fig. 6 shows inhibition of PhoN-Sf on G6P phosphorylation by glucose (pH 6.0). The mechanism of the inhibition is complex. Panel A indicates competitive inhibition by glucose, which gives K_{ic} of 50 μM, and panel B indicates uncompetitive inhibition with K_{iu} of 150 μM. The inhibition of G6P dephosphorylation by glucose gives an interesting clue to the understanding of the scheme of dephosphorylation and phosphorylation. In this case the glucose reacts with the phosphorylated enzyme intermediate resulting in re-formation of G6P. It is interesting to note that inhibition of mammalian G6Pase activity by glucose has been observed and this inhibition gave a clue to the mechanism of G6Pase.[25] The values found here for the K_m of the acid phosphatase for glucose and the G6P of 5.3 mM and 0.021 mM at pH 6.0, respectively (Table 1), are much lower than those for liver microsomal G6Pase. For this enzyme, values reported are a K_m for glucose of 90–120 mM (pH 5.5–7.0) in the phosphotransferase reaction and 0.65 mM for the hydrolysis of G6P (pH 6.5), respectively.[21]

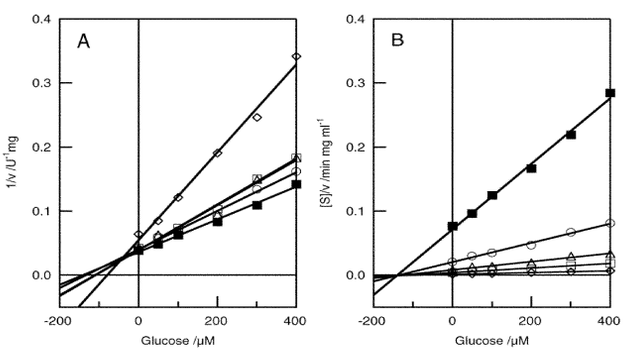


Fig. 6 Inhibition of PhoN-Sf on G6P dephosphorylation by glucose. (A) K_{ic} is given by plots of 1/v against glucose (inhibitor) concentrations at various substrate concentrations. (B) K_{iu} is given by plots of [S]/v against glucose (inhibitor) concentrations at various substrate concentrations. The concentrations of G6P (substrate) were 0.02 mM (◇), 0.10 mM (□), 0.20 mM (△), 0.50 mM (○) and 2.0 mM (■). The combination of intersecting lines in (A) and (B) indicate a mixed type of inhibition. Competitive inhibition was seen as in (A) with K_{ic} of 50 μM and uncompetitive inhibition was seen as in (B) with K_{iu} of 150 μM. The reaction mixture was buffered with 100 mM sodium acetate (pH 6.0), and the reaction was quenched by addition of Biomol Green™ reagent.

It is noteworthy that the V_{max} values for the dephosphorylation catalysed by the PhoN-Sf of G6P, 5′IMP and PPi are about the same (Table 1), despite the difference in size and charge of these substrates. This points to a step in the mechanism where hydrolysis of the phosphorylated enzyme intermediate by water and liberation of phosphate is rate-limiting. Such a rate-limiting step has been suggested for the phosphatase reaction catalysed by apo CPO,[5] and it is likely that it corresponds to step 3 in Scheme 1.

The classical chemical introduction of a phosphate group into a polyhydroxy compound is tedious since it requires a number of protection and deprotection steps. Further $POCl_3$ that is being used should be handled with caution and it may give rise to a number of side products. Employing enzymes may eliminate many of the disadvantages associated with these chemical syntheses. In fact kinases have been used to phosphorylate a number of compounds.[26] A problem associated with the use of these enzymes is that they require expensive ATP as a cofactor. Although recycling of ATP is possible the system has as a major drawback that for each class of substrate converted another enzyme has to be used. The kinetic studies reported here show clearly that these acid phosphatases can be used to regiospecifically phosphorylate inosine and glucose under very mild conditions using pyrophosphate as a phosphate donor. These substrates are structurally very different and both polyhydroxy compounds are apparently able to enter the active site of the acid phosphatases and become phosphorylated as well. If so, the enzymatic method may become a useful alternative to the existing chemical methods.

Dephosphorylation and phosphorylation by apo haloperoxidases

It has been shown now that the binding pocket for vanadate in the haloperoxidases is similar to the phosphate binding site in the acid phosphatases and G6Pase.[5] It has also been demonstrated that apo CPO had phosphatase activity when *p*-nitrophenyl phosphate (*p*NPP) was used as a substrate.[5] Taking this analogy into consideration, we studied the dephosphorylation of substrates for acid phosphatases by apo haloperoxidases. Adenosine- 5′-monophosphate (5′AMP), 5′IMP, guanosine 5′-monophosphate (5′GMP), adenosine diphosphate (ADP), inosine triphosphate (ITP), guanosine triphosphate (GTP), xanthine triphosphate (XTP), *p*NPP, PPi, G6P, phenyl phosphate, phenolphthalein monophosphate, phenolphthalein diphosphate, and carbamyl phosphate were tested, but only *p*NPP and PPi were hydrolysed by apo CPO and apo BPO. Since the active site of the vanadium CPO is similar to that of the vanadium BPO, we also studied the phosphatase activity of apo BPO from *Ascophyllum nodosum*. Indeed as Fig. 7 (panel A) shows, *p*NPP was hydrolysed but the maximal turnover of *p*NPP hydrolysis by apo BPO is 0.08 min^{-1} at pH 8.7. This turnover is about 15 times lower than that

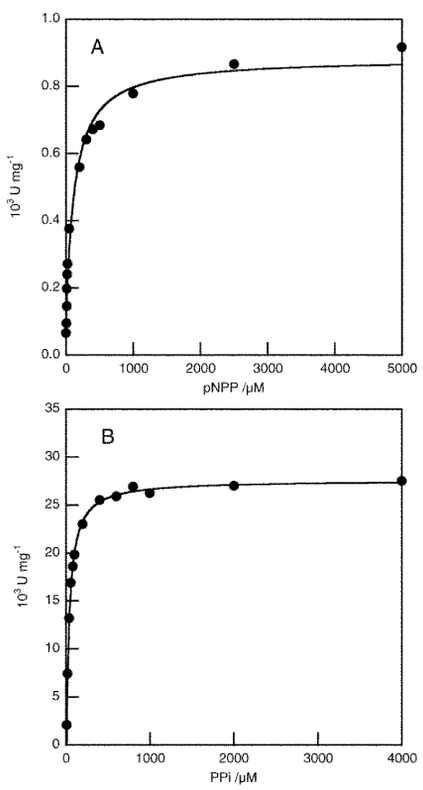


Fig. 7 Phosphatase activity of apo BPO and apo CPO. (A) Dephosphorylation of various concentrations of *p*NPP by 500 nM apo BPO in 100 mM Tris–acetate at pH 8.7. The reaction mixture was incubated for 21 hours. (B) Dephosphorylation of various concentrations of PPi by 500 nM apo CPO in 100 mM sodium acetate buffer at pH 4.5. The reaction mixture was incubated for 30 min and quenched by Biomol Green™ reagent.

for apo CPO.[5] Apo CPO was also able to hydrolyse PPi with a turnover of about 1.9 min^{-1} at pH 4.5 (Fig. 7 panel B). This should be compared to the hydrolysis rate of PPi by PhoN-Sf (V_{max} of 550 min^{-1} at pH 6.0, Table 1), which is about 300 times faster than that of the apo haloperoxidases. The K_m for PPi on hydrolysis by apo CPO was approximately 50 μM, which is nearly in the same order as that of PhoN-Sf (87 μM at pH 6.0, Table 1).

We also investigated whether apo BPO or CPO were able to phosphorylate inosine or glucose using 100 mM PPi as a phosphate donor. Conversion of inosine into 5′IMP or 3′IMP or that of glucose into G6P was not observed even after incubation with these enzymes for 24 hours. Our studies on the acid phosphatases show that an activated phosphorylated enzyme intermediate ("E·P") must be present catalysing the transfer of a phosphate group to an acceptor (R–OH). Considering the very low hydrolysis rate of PPi by apo haloperoxidase, only a very low intermediate concentration of an activated phosphoenzyme ("E·P") is present. This makes phosphate transfer by these enzymes to a phosphate acceptor very unlikely.

The obvious question is why the PPi reacts so sluggishly with the apo haloperoxidases compared to the acid phosphatases despite the fact that the binding pocket for phosphate is identical in the two classes of enzymes. However, the nature of the amino acid residues in the channel giving access to the phosphate binding pocket is probably very different. A comparison of the active site channel of the apo CPO[3] and that of the acid phosphatase from *E. blattae*[14] shows clearly that the active site channel in the former enzyme is much more restricted. This may also be the reason why the NSAPs have such a wide range in substrate specificity.

Experimental

Materials

All standard recombinant DNA procedures were performed as described by Sambrook *et al*.[27] Expression and purification of *Salmonella enterica* PhoN (PhoN-Se) was as described elsewhere.[9] Plasmid pET3a harbouring *phoN-sf* was a kind gift from Prof. A. J. Lange.[9]

The host strains *Escherichia coli* and TOP10 (Invitrogen) were used in subcloning and expression experiments. Bacteria were routinely grown at 37 °C in Luria-Bertani (LB) medium (10 g L^{-1} tryptone, 10 g L^{-1} NaCl and 5 g L^{-1} yeast extract at pH 7.5)[27] containing 100 μg ml^{-1} ampicillin. Expression vectors pBAD/gIIIA and pBAD/gIIIB (Invitrogen) were used to clone the *phoN* gene from *S. enterica* and *Sh. flexneri* respectively. pBAD/gIIIA and pBAD/gIIIB carry the gene III signal sequence for secretion of the recombinant protein into the *E. coli* periplasmic space.

Vanadium-containing bromoperoxidase was purified from the seaweed *Ascophyllum nodosum* as described previously.[28,29] The *Curvularia inaequalis* vanadium chloroperoxidase was purified using the *Saccaromyces cerevisiae* recombinant expression system.[30]

D-glucose, glucose-6-phosphate, inosine, 5′IMP, 3′IMP, tetrasodium pyrophosphate ($Na_4P_2O_7$) and disodium pyrophosphate ($Na_2H_2P_2O_7$) were purchased from Sigma (USA), and glucose-6-phosphate dehydrogenase, phosphoglucomutase and NADP were purchased from Roche (Germany).

Expression and purification of recombinant PhoN-Sf

The gene for PhoN-Sf was cloned in the expression vector pBAD/gIIIB as follows. The mature sequence of *phoN-sf* (*i.e. phoN* gene without the 5′-end coding for the native secretion signal) was PCR amplified from the pET3a *phoN-sf* gene using the forward primer 5′-CATGCCATGGCTCAATTC-CTCCGGGAAATG-3′ and the reverse primer 5′-CCCAA-GCTTATTTTTTCTGATTGTTAGCGAATTC-3′ (the *Nco*I and *Hind*III sites, respectively, are underlined). PCR was performed using the Expand™ High Fidelity PCR System (Roche) with the following conditions: 10 ng pET3a *phoN-sf* gene, 1 μg each primer, 200 μM each dNTP, 1.5 mM $MgCl_2$, 2.6 U high-fidelity polymerase mix in a final volume of 50 μl. A "hot start" of 3 min at 95 °C was followed by 30 cycles of denaturation (1 min at 95 °C), annealing (1 min at 55 °C) and extention (1 min at 72 °C), and 7 min at 72 °C using a programmable heating block (Eppendorf Mastercycler 5330). pBAD/gIIIB (Invitrogen) was chosen for the *phoN-Sf* gene, as it holds the gene III signal sequence for secretion of the recombinant protein into the periplasmic space.[9] The PCR product was restricted with *Nco*I and *Hind*III cloned into the corresponding sites of pBAD/gIIIB, and the resulting clone was confirmed by double-stranded DNA sequencing.

E. coli TOP10 harbouring the recombinant plasmid pBAD/gIIIB *phoN-sf* was grown at 37 °C in LB medium until the absorbance of the culture suspension reached an A_{600} of 0.4–0.6. The expression of recombinant PhoN-Sf was induced by adding 0.02% L-arabinose and the growth was continued at 37 °C for 4 h. The bacterial cells were harvested by centrifugation, and secreted PhoN-Sf was released from the *E. coli* periplasmic space by osmotic shock. The cell pellet was resuspended in osmotic shock solution 1 (20 mM Tris–HCl, pH 8.0, 2.5 mM EDTA and 20% sucrose) to A_{600} = 5, and was incubated on ice for 10 min. After centrifugation for 15 min at 4 °C, the cell pellet was resuspended in osmotic shock solution 2 (20 mM Tris–HCl, pH 8.0 and 2.5 mM EDTA) to A_{600} = 5, and was incubated on ice for 10 min. The secreted PhoN-Sf was obtained in the supernatant (osmotic shock fluid) after centrifuging for 15 min at 4 °C. The osmotic shock fluid was applied onto a DEAE sepharose (Pharmacia Biotech) ion-exchange column which was washed with 20 mM Tris–HCl (pH 8.8), and the enzyme was eluted with 1 M NaCl in 20 mM Tris–HCl (pH 8.8). The active fractions were pooled and exhaustively dialysed against 20 mM sodium acetate (pH 5.0). The dialysate was centrifuged at 20 000 g for 30 min and the supernatant was passed through a 0.45 μm filter (Millipore) and then applied onto a SP Sepharose Fast Flow (Pharmacia Biotech) ion-exchange column. The enzyme was eluted with a linear gradient of NaCl (0–0.3 M) in 20 mM sodium acetate (pH 5.0). The active fractions were pooled and dialysed against 50 mM Tris–acetate (pH 7.5) and concentrated by membrane filter Amicon PM 10 (Millipore). Sephadex G75 column (Pharmacia Biotech) was used to perform a gel filtration step. The recombinant PhoN-Sf was eluted with 50 mM Tris–acetate (pH 7.5) at a flow rate of 0.1 ml min^{-1}, and was concentrated on a membrane filter Amicon PM 10.

The purity of the preparations was checked on SDS-PAGE gels stained with Coomassie Brilliant Blue R-250, and the protein concentration was determined by using a protein assay kit (Bio-Rad) with BSA as the standard.

Enzymatic assay of phosphotransferase activity

The enzymatic phosphorylation of inosine was assayed as described by Asano *et al*.[16–20] A standard reaction mixture contains 40 mM inosine, 100 mM disodium pyrophosphate (PPi) and 0.1–1 μM of enzyme solution in a total volume of 1 ml. It was not convenient to use tetrasodium pyrophosphate under acidic conditions because the pH of the solution was alkaline (about pH 11), therefore disodium pyrophosphate (pH 4.2) was used for the detailed studies unless otherwise mentioned. The pH of all reaction mixtures was adjusted after addition of sodium acetate buffer and PPi. For the time course study, 100 μl of sample was taken from the reaction mixture and diluted 2–10 times before injection into the HPLC. The amount of inosine and phosphorylated products 5′IMP and 3′IMP were determined by HPLC using a Nucleosil 100–5 C_{18} column (0.45 × 12 cm; Macherey-Nagel) equipped with a Pharmacia LKB-

HPLC pump 2248 and an LKB Bromma 2140 rapid spectral detector. The column was eluted with 10 mM CH_3COOH–NH_4OH (pH 5.0) containing 200 μM sodium azide at a flow rate of 0.5 ml min^{-1}. The retention time for 5′IMP, 3′IMP and inosine were 4, 6 and 15 min, respectively. The HPLC effluent was monitored at 254 nm. The Borwin software program (JMBS developments) was used for HPLC data acquisition and evaluation.

During glucose phosphorylation, the formation of G6P was assayed enzymatically by glucose-6-phosphate dehydrogenase. This assay is based on the method of Noltman *et al.*[31] A phosphorylation reaction mixture contains 1 μM PhoN, 100 mM glucose and 100 mM disodium pyrophosphate in 100 mM sodium acetate (pH 3.5–6.0). To determine the amount of G6P, 10 μl of phosphorylation reaction mixture was added to 1 ml of a G6P assay mixture. This assay mixture contains 0.01 mg ml^{-1} glucose-6-phosphate dehydrogenase, 1 mM of $NADP^+$ and 10 mM $MgCl_2$ in 100 mM Tris–acetate (pH 7.5). The formed NADPH can be monitored at 340 nm (molar absorption coefficient 6.22 mM^{-1} cm^{-1}). The formation of glucose-1-phosphate (G1P) was measured by conversion of G1P to G6P using 0.06 mg ml^{-1} phosphoglucomutase in the same assay system as mentioned above. After measuring the absorption at 340 nm and a stable reading was obtained, phosphoglucomutase was added to the same mixture, and the additional increase of absorption at 340 nm was monitored.

The quantity of glucose and free phosphate were also determined by HPLC using an Alltech OA 1000 organic acid column (0.65 × 30 cm) equipped with a Dionex 580 LPG pump and Dionex UVD-340D/Shodex RI-101 detector. The column was eluted with 25 mM H_2SO_4 at a flow rate of 0.4 ml min^{-1}. The Chromeleon software program (Dionex) was used for HPLC data acquisition and evaluation.

G6P, PPi and free phosphate were quantified by phosphor nuclear magnetic resonance (^{31}P NMR). Spectra were determined in D_2O on a Varian Unity Inova at 202 MHz. Chemical shifts (δ) are expressed in ppm relative to 85% phosphoric acid.

At zero time a spectrum was taken from the reaction mixture containing 100 mM PPi, 100 mM D-glucose, 100 mM sodium acetate buffer (pH 4.0) in a 10 mm NMR tube. The reaction was initiated by addition of 1 μM PhoN-Sf, and was carried out in the NMR tube. Concentrations of product and reactants were determined using dimethylmethylphosphonate as an external standard. The peak at δ = 1.84 corresponds to that seen in authentic G6P (80 mM).

Enzymatic assay of phosphatase activity

The phosphatase activity was measured by hydrolysis of 10 mM *p*-nitrophenyl phosphate as a substrate in 100 mM sodium acetate (pH 6.0). The reaction mixtures were quenched with 0.5 M NaOH to change the pH to 12, and the production of *p*-nitrophenol was measured at 410 nm (molar absorption coefficient 16.6 mM^{-1} cm^{-1}).

When PPi, 5′IMP, 3′IMP or G6P were used as substrates, Biomol Green™ reagent (Biomol), which is a modification of a malachite green method,[32,33] was used for the detection of free phosphate. A reaction mixture contained 100 mM sodium acetate (pH 6.0), various concentrations of substrate and 20–100 nM of PhoN in a total volume of 100 μl, and was incubated for 1 min at room temperature. The reaction was quenched by addition of 1 ml of Biomol Green™ reagent. Following 20 min incubation at room temperature, the absorbance values at 620 nm were measured.

Acknowledgements

This work was supported by the Council of Chemical Science of the Netherlands Organisation for Scientific Research. We gratefully acknowledge Dr. W. Hemrika and Dr.V. Dumay for providing us the plasmid harbouring, the *S. enterica* ser. *typhimurium* PhoN gene, and Prof. A. J. Lange, University of Minnesota (USA) for providing us the plasmid pET3a *phoN-sf*.

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