Crystal structure of *Klebsiella* sp. ASR1 phytase suggests substrate binding to a preformed active site that meets the requirements of a plant rhizosphere enzyme

Kerstin Bo¨hm1,*, Thomas Herter2,*, Jürgen J. Müller1, Rainer Borriss2 and Udo Heinemann1,3

1 Kristallographie, Max-Delbru¨ck-Centrum fu¨r Molekulare Medizin, Berlin, Germany
2 Institut fu¨r Biologie, Humboldt-Universita¨t zu Berlin, Germany
3 Institut fu¨r Chemie und Biochemie, Freie Universita¨t Berlin, Germany

Keywords
dephosphorylation; phytase; plant rhizosphere enzyme; preformed substrate binding site; proton structure

Correspondence
U. Heinemann, Kristallographie, Max-Delbru¨ck-Centrum fu¨r Molekulare Medizin, Robert-Rössle-Str. 10, 13125 Berlin, Germany
Fax: +49 30 9406 2548
Tel: +49 30 9406 3420
E-mail: heinemann@mdc-berlin.de

Database
Structural data have been submitted to the Protein Data Bank under the accession numbers 2WNI (native PhyK) and 2WUO (PhyK H25A)

Note
*These authors contributed equally to this work

(Received 3 November 2009, revised 16 December 2009, accepted 22 December 2009)
doi:10.1111/j.1742-4658.2010.07559.x

Introduction

The extracellular phytase of the plant-associated *Klebsiella* sp. ASR1 is a member of the histidine-acid-phosphatase family and acts primarily as a scavenger of phosphate groups locked in the phytic acid molecule. The *Klebsiella* enzyme is distinguished from the *Escherichia coli* phytase AppA by its sequence and phytate degradation pathway. The crystal structure of the phytase from *Klebsiella* sp. ASR1 has been determined to 1.7 Å resolution using single-wavelength anomalous-diffraction phasing. Despite low sequence similarity, the overall structure of *Klebsiella* phytase bears similarity to other histidine-acid phosphatases, such as *E. coli* phytase, glucose-1-phosphatase and human prostatic-acid phosphatase. The polypeptide chain is organized into an α and an α/β domain, and the active site is located in a positively charged cleft between the domains. Three sulfate ions bound to the catalytic pocket of an inactive mutant suggest a unique binding mode for its substrate phytate. Even in the absence of substrate, the *Klebsiella* phytase is closer in structure to the *E. coli* phytase AppA in its substrate-bound form than to phytate-free AppA. This is taken to suggest a preformed substrate-binding site in *Klebsiella* phytase. Differences in habitat and substrate availability thus gave rise to enzymes with different substrate-binding modes, specificities and kinetics.

Abbreviations
G1P, glucose-1-phosphatase; HAP, histidine-acid phosphatase; Mse, selenomethionine; NMM, new minimal medium; PAP, prostatic-acid phosphatase.

myo-inositol 1,2,3,4,5,6-hexakisphosphate [1]. Phytic acid accumulates during seed development in cereals, legumes, nuts and oil seeds and accounts for 60–90% of the total phosphorus content in mature seeds [2,3].
According to their sequence, most bacterial, fungal and plant phytases belong to the group of histidine-acid phosphatases (HAPs). Within this structural classification, there are two phytase subgroups: some members show broad substrate specificity but low specific activity for phytate, whereas others have narrow substrate specificity and high specific activity for phytic acid. All members of the HAP class share two conserved active-site motifs, RHGXRXXP and HD, and hydrolyze metal-free phytate with pH optima in the acidic range. They consist of two domains, a large α/β domain and a small α domain with the catalytic site at the interface of the two domains [4,5]. HAPs can initiate hydrolysis of phytate at either the C³ (EC 3.1.3.8) or C⁶ (EC 3.1.3.26) position of the inositol ring and produce myo-inositol monophosphate, in particular myo-inositol 2-phosphates because of its axial position, as the final product [6–8].

HAPs share a common two-step mechanism for catalysis [9,10]. The reaction starts with a nucleophilic attack on the phosphoester bond by a conserved histidine in the long active-site motif. The histidine side chain from the conserved HD motif protonates the leaving group [11]. The second step comprises hydrolysis of the resulting covalent phospho-histidine intermediate. The final product of histidine-acid phytases is myo-inositol monophosphate, whereas alkaline phosphatases are only able to hydrolyze three phosphate groups resulting in myo-inositol triphosphates as product. In addition to their ability to make inorganic phosphorus available for metabolism, the elimination of phytate, which is known to chelate nutritionally important minerals, is another beneficial effect of phytases [1]. The phytase enzyme with the highest specific activity currently known is the pH 2.5 acid phosphatase AppA from \textit{E. coli} [12]. Initially, the flexible AppA binding pocket is not fully occupied by phytate. Upon substrate binding, the active-site pocket closes, allowing successive dephosphorylation of phytate [5]. Although the amino acid sequence of \textit{E. coli} glucose-1-phosphatase (G1P) is related to AppA, the crystal structure suggests that phytate can bind to the active site of G1P only in an orientation with the 3-phosphate as a scissile group. Leu24 and Glu196 in G1P are proposed to act as ‘gating residues’ that narrow access to the comparatively stiff and small substrate-binding cleft [13].

The phytase from \textit{Klebsiella} sp. ASR1 (PhyK) is a 3-phytase with myo-inositol 2-phosphate as the final product [14]. A virtually identical phytase from \textit{Klebsiella terrigena} has also been described [6]. PhyK contains both the conserved long active-site motif, RHGXRXXP (residues 24–30), and the catalytically active dipeptide, HD (residues 290–291). \textit{Klebsiella} sp. ASR1 has previously been isolated from an Indonesian rice field during a survey for phytase-producing bacteria associated with plant rhizospheres. It is assumed that the presence of such bacteria within the vicinity of plant roots serves to improve plant growth by supplying additional inorganic phosphate [15]. According to its sequence, PhyK belongs to a group of phytases synthesized by plant-associated bacteria such as \textit{Xanthomonas campestris}, \textit{Pseudomonas syringae} and \textit{Erwinia carotovora}. Despite some sequence similarity, this group is distinct from that of the AppA-related enzymes, mainly produced by human pathogenic \textit{E. coli}, \textit{Salmonella} and \textit{Yersinia} spp. and also from that of the glucose-1-phosphatases found in several enterobacteria [16].

Structural information about phytases with different environmental functions is important for understanding their specific role within the microenvironment of which they are a part (human gut or plant rhizosphere, for example). Here, we present the crystal structures of the ligand-free PhyK at 1.7 Å resolution and of a catalytically incompetent PhyK mutant that suggest a model for substrate binding. Comparison with the structures of the related enzymes AppA and G1P of \textit{E. coli} suggests the existence of a common ancestor (‘prototype’) of HAPs, endowed with the potential to develop specific enzymatic features in response to selective pressures arising from individual environmental conditions. According to the crystal structures reported here, PhyK seems to have a preformed substrate-binding site and to be less optimized for efficient substrate hydrolysis than AppA.

**Results and Discussion**

**Overall structure**

The crystal structure of PhyK was determined by single-wavelength anomalous diffraction to 1.7 Å resolution. Recombinant PhyK crystallized with two molecules in the asymmetric unit of its tetragonal unit cell. The conformation of the two molecules is similar with a rmsd of 0.5 Å for the superposition of 394 Cα atoms [17]. The globular fold is composed of two domains: an α/β domain and an α domain (Fig. 1A). The known active-site motif is found in a cavity between the two domains. The α/β domain consists of a central six-stranded β sheet of mixed topology surrounded by α helices on each side. These major structural features are well conserved throughout the HAPs of bacteria, fungi and mammals. Moreover, two short β strands with antiparallel topology are found in this domain.
The smaller α domain is formed by several α helices, where the two central helices are part of the catalytic pocket of the enzyme. A β-hairpin motif, which to date has been described only in AppA [5] and G1P [13], is also found in PhyK. These two proteins display the closest structural similarity.

There are three disulfide bonds involving all six cysteine residues (85/116, 176/182 and 370/379) (Fig. 1A). Formation of disulfide bridges was assured by periplasmic localization of the heterologously produced proteins. The C-terminal loop linker, cysteines 370/379, is conserved in all HAP structures [13]. However, the other disulfide bridges are not fully conserved. Despite the very similar structure, the human prostatic-acid phosphatase (PAP) has, in addition to the C-terminal loop linker, only one disulfide bridge, which is not found in PhyK. G1P shows the same disulfide bond pattern as PhyK, whereas AppA has an additional disulfide bond between Cys133 and Cys408. In all proteins, the disulfide bridges are not directly involved in catalysis. However, they were shown to be essential for the folding and stability of the molecular structure for fungal phytases [18].

The catalytic center is located between the two domains of PhyK. The catalytic motif, 24-RHGXRXP-30, and the substrate binding motif, 290-HD-291, are conserved and in close proximity. In order to orient His25 for the nucleophilic attack on the substrate, the N\(^\ominus\) atom donates a hydrogen bond to the backbone oxygen atom of Gly26. The other important histidine side chain in the catalytic pocket is also fixed with a hydrogen bond. The distance between the N\(^\ominus\) of His290 and the O\(^\ominus\) of Ser96 is 2.81 and 2.89 Å for the two molecules in the asymmetric unit, respectively.

**Comparison with *E. coli* phytase AppA**

Overall, PhyK bears significant structural similarity to other HAPs. A structure-based search with dali [17] revealed several similar structures. With rmsd values of 2.3 Å for both enzymes, *E. coli* AppA (PDB entry 1DKL) and *E. coli* G1P (PDB entry 1NT4) are the closest structural matches (Fig. 1B). The dali Z-score was 47.0 for 402 superimposed C\(^\alpha\) atoms of AppA and 41.9 for 391 superimposed C\(^\alpha\) atoms of G1P. Despite the structural similarity, the sequence identity with PhyK is only 22.6 and 23.2%, respectively.

The overall fold of PhyK is evolutionarily highly conserved. The human enzyme PAP can be superimposed with an rmsd value of 3.2 Å (342 C\(^\alpha\) atoms, Z-score 28.7) although only 19% of the sequences are identical [19]. Nevertheless, human PAP does not contain the β-hairpin motif present in PhyK as well as in AppA and G1P.

The α/β domain of HAPs is evolutionarily more conserved than the α domain. For example, the phytase of *Aspergillus fumigatus* shows closer similarity to PhyK in the α/β domain than in the α domain [20]. This is also reflected in the rigidity of the protein. The atomic displacement factors of the PhyK structure are smaller for the α/β domain than for the α domain. This has also been observed for other HAPs. The helices directly involved in substrate recognition are well conserved among species.
Binding model

A crystal structure of the inactive mutant H25A of PhyK was determined for four molecules in the asymmetric unit. The exchange of a single amino acid residue was sufficient to inactive the enzyme without affecting the structure (mean rmsd of 0.48 Å for 394 Cα atoms in all possible superpositions of the four mutant protein chains with the two wild-type PhyK molecules). Thus, the differences between wild-type and mutant structure are in the same range as the differences between the two molecules of the asymmetric unit of the wild-type structure. Neither the mutation nor the different crystallization conditions evoked structural differences. The crystal was grown in the presence of phytate, as well as 80 mM ammonium sulfate. Although phytate is the natural substrate of PhyK, we do not observe phytate binding at the active site. Instead, there is electron density for three sulfate ions at the active sites of the four protein molecules in the asymmetric unit which presumably occupy binding sites for phosphate groups of a substrate phytate molecule. The preferred binding of sulfate over phytate is attributed to a 53-fold molar excess of sulfate ions over phytate, which was necessary to obtain crystals.

Based on the electron density for three sulfate ions bound at the active site, a model of phytate bound to PhyK was calculated, so that the sulfate positions mark the sites of phytate phosphate groups. In this model, the 3-phosphate was arranged to point towards the exchanged catalytic residue 25, because this phosphate was biochemically identified as the first site of hydrolysis [14]. This leaves only one choice for the orientation of a phytate (standard 5eq/1ax ring pucker) in the binding pocket that places two more phosphates into sulfate density. An independent calculation for each of the four proteins in the asymmetric unit gave rise to very similar binding modes (Fig. 2A). In the model, the phosphate groups 1, 3 and 4 fill the observed electron density. In the following, only the model for chain B is discussed, because in this region the electron density was best defined, and the sulfates have the lowest atomic displacement factors. The electrostatic surface potential representation shows a positively charged pocket between the two domains where the conserved active-site motifs are found (Fig. 2B). Coulomb charges as well as the helix dipoles of helices A and L would serve to enhance the catalytic activity by lowering the pKₐ value of His25. Hence, the catalytically important histidine side chain would be rendered a more potent nucleophile, and binding of the negatively charged substrate would be facilitated. This explains the acidic pH optimum and the substrate specificity towards metal-free phytate of PhyK. In comparison with AppA and G1P the binding pocket of PhyK shows an even more positively charged surface. Notably, the catalytic pocket is surrounded by a patch of positive charges which may direct the substrate towards the active site. Surface charge patterns are not that prominent in other HAPs such as the phytases from *E. coli*, *A. niger* or *A. ficuum*, or human PAP.

Because the sulfate ions mimic a phytate molecule, the sites with the highest affinity for sulfate ions are likely to be important for substrate recognition. Indeed, the scissile 3-phosphate is involved in seven

---

**Fig. 2.** Model for the binding of phytate to PhyK. (A) For each of the four protein molecules in the asymmetric unit a phytate-binding model was calculated based on the positions of three sulfate ions. Superposition of the proteins reveals a very similar binding mode for all models. For clarity only one protein chain is shown. Colors are the same as in Fig. 1A indicating the two domains. (B) Electrostatic surface potential of the active site of PhyK as calculated with APBS [38] is displayed in a range from −10 kT (red) to +10 kT (blue). The binding model of phytate is represented as a stick model. The positively charged catalytic pocket favors binding of the negatively charged substrate. Phytate does not fully occupy the pocket, explaining the potential to bind other substrates. The scissile 3-phosphate is located deep inside the catalytic pocket.
hydrogen bonds, whereas the other phosphate groups are bridged by single hydrogen bonds only. The recognition of phosphate groups 1 and 4 involves the conserved Arg100 and Tyr249, respectively.

Nevertheless, all six phosphate groups of phytate are involved in a hydrogen bond network connecting the substrate with PhyK (Fig. 3), explaining why the first dephosphorylation step is faster than the subsequent steps. Phytate does not occupy the whole cavity (Fig. 2B), leaving enough freedom for the bulky phytate to rotate for further dephosphorylation steps or for alternative substrates to bind to the active site. Indeed, PhyK is able to dephosphorylate a number of substrates including nitrophenyl phosphate, naphtyl phosphate, fructose phosphates, glucose phosphates and NADP [14]. By contrast to AppA, PhyK is even able to dephosphorylate nucleoside phosphates.

Of the conserved 24-RHGXRXP-30 motif, Arg24 and Arg28 directly contact the substrate (Fig. 3). The side chain of Arg24 forms two hydrogen bonds with the scissile 3-phosphate. The 3-phosphate is in close contact with Arg28. Therefore, these two conserved arginine residues are important for arranging the substrate in the correct orientation for catalysis, whereas His25 is responsible for the nucleophilic attack on the scissile phosphoester bond. Next to the conserved motif, Thr31 also forms a hydrogen bond with the 6-phosphate. This threonine is conserved in AppA and A. fumigatus phytase as well as in human PAP, showing its importance for substrate binding. By contrast, a leucine is found here in G1P, which functions as a gatekeeper, explaining the narrow substrate spectrum of G1P compared with PhyK [13].

The catalytically active dipeptide 290-HD-291, together with the adjacent Thr292, is also directly involved in substrate recognition (Fig. 3). In the modeled structure of phytate-bound PhyK, the side chain of His290 is locked by Ser96 in the same orientation as in the ligand-free structure. Whereas His290 and Asp291 form hydrogen bonds with the 3-phosphate, Thr292 fixes the 2-phosphate. The conserved HD dipeptide forms the N-terminus of α helix L. The orientation of this helix allows substrate binding by hydrogen bond formation, and its dipole facilitates substrate binding as well. The hydrogen bond between the backbone nitrogen atom of Asp291 and the 3-phosphate of the substrate is the only interaction with the protein backbone; all other contacts are formed using the side chains. The conserved Arg100 forms hydrogen bonds with two of its side chain nitrogen atoms. Its Nε atom and an Nη atom bind the scissile 3-phosphate of phytate, and the other Nη atom fixes
the 1-phosphate. In addition, two more residues are involved in phytate binding. Tyr249 forms a hydrogen bond with phosphate group 4. Another hydrogen bond is found between the side chain nitrogen atom of Asn209 and phosphate group 5.

The model of a PhyK-phytate enzyme-substrate complex explains the broad substrate specificity of the enzyme. Although all six phosphate groups are involved in the hydrogen-bond network, the scissile 3-phosphate of phytate is clearly bound tightest to the enzyme. This is also reflected in the quality of the electron-density map originating from the bound sulfate ion. There are seven hydrogen bonds formed to recognize this group, whereas the other phosphate groups are bound by a single hydrogen bond each. The shape of the area responsible for the binding of the scissile phosphate group is ideal for a phosphate (or sulfate) group and does not allow other esters to bind.

**Comparison with phytate binding by AppA**

Although the PhyK homolog AppA is biochemically characterized as a 6-phytase, a co-crystal structure shows the phytate 3-phosphate as scissile group [5] in a similar position to in the active site of PhyK. Nevertheless, there are some differences in the substrate binding of PhyK. The α helix A is longer in PhyK, presumably making the catalytic pocket more rigid. The N-terminus of this elongated helix points towards the substrate-binding site. Thus, the dipole moment of the helix supports the binding of negatively charged ligands. AppA lacks this long helix. Instead, the side chain from a lysine forms two hydrogen bonds to phytate. These substrate interactions are absent in PhyK, and their loss may explain the broader substrate spectrum for PhyK.

In the structure of phytate bound to AppA, several residues forming water-mediated contacts to phytate were identified. These are not part of the predicted binding mode of phytate to PhyK. Out of the water molecules included in the structure none is bound in the catalytic cleft. However, it cannot be ruled out that there are water-mediated contacts in addition to the direct contacts described here. Nevertheless, all phosphate groups of phytate are recognized through direct interactions by PhyK explaining its high potency to dephosphorylate the substrate. Possible additional water-mediated contacts would thus be of secondary importance.

The two arginine residues of the conserved motif including the nucleophilic histidine are involved in substrate recognition in PhyK as well as in AppA. Although they are responsible for three hydrogen bonds to the 3-phosphate of phytate in PhyK, they also orient the 4-phosphate in AppA. This group is fixed by a hydrogen bond with Tyr249 in PhyK. Formation of this hydrogen bond is not possible in AppA, because there is a phenylalanine at the corresponding position. The adjacent tyrosine in AppA points into the opposite direction from the helix. In G1P of *E. coli* a glutamine residue is at the appropriate position, which might form a hydrogen bond with the substrate.

Thr31 adjacent to the conserved motif is important for substrate binding in both PhyK and AppA. Whereas a hydrogen bond is formed with the 6-phosphate in PhyK, the *E. coli* enzyme recognizes the 5-phosphate with the threonine side chain. This phosphate group is linked with Asn209 by a hydrogen bond which is not found in AppA, where a methionine is present at this position. In the structure of G1P, a serine residue is at the equivalent position and is able to contact a polar substrate.

The hydrogen bonds involving Arg100 are found in both the *Klebsiella* and the *E. coli* phytase. The motif 290-HDT-292 of PhyK is also found in AppA. The histidine side chain is fixed in its position by Ser96 or Asp88, respectively, whereas the histidine is bridged with the scissile phosphate group. There is a single hydrogen bond between the substrate, phytate and the backbone of PhyK involving Asp291. In the structure of phytate bound to AppA, this hydrogen bond is also observed and, moreover, is the only direct contact between the protein backbone and the substrate. The side chain of Thr292 is found to recognize the 2-phosphate in both the binding model of PhyK and the crystal structure of AppA. This is the only phosphate group in an axial position, although five phosphate groups occupy energetically preferred equatorial positions. This phosphate group might therefore be important to distinguish between 3- and 6-phytases. However, the crystal structure of *E. coli* AppA shows phytate bound with the 3-phosphate as a scissile group, although its biochemical characterization classifies it as a 6-phytase. There is another hydrogen bond of this particular phosphate group with Arg267 in AppA. The corresponding Arg262 in PhyK has a different side chain conformation. It seems likely that this residue might change its conformation upon substrate binding.

All HAPs share a positively charged catalytic pocket ideally suited for binding of a negatively charged substrate. In addition, PhyK has a positively charged rim surrounding the catalytic site. This rim is less prominent in other HAPs. The positive charges in close proximity to the catalytic cleft are in agreement with
the observation that the highly negatively charged phytic acid is degraded faster than substrates bearing fewer charges.

**Induced conformational changes upon substrate binding**

For a detailed structural superposition, **lsqman** [21] was used. After an initial least-squares alignment, the superposition of the structures was improved by considering only pairs of C atoms < 2.5 Å apart. Because of the different length of α helix A, the C atoms N-terminal to or inside helix A are separated by large distances. The corresponding region in AppA shows severe conformational changes upon substrate binding. Averaged distances for pairs of C atoms matching in a sequence-based alignment (Fig. 4) were determined for residues of helix A and those being involved in substrate recognition. For the superposition of the substrate-free PhyK with substrate-free AppA the C atoms are 2.41 Å apart, whereas for the substrate-free PhyK and the substrate-loaded AppA the averaged distance is only 1.87 Å.

Distinct conformational changes were observed upon substrate binding to AppA. Residues 20–25, which include a part of the active site, move significantly upon phytate binding. The change in the position of Arg20 was proposed to trigger a shift of Thr23 and Lys24 into the binding pocket [5]. Strikingly, the corresponding Arg28 in PhyK shows the same conformation with and without sulfate bound to the active site (Fig. 5A). This conformation is more similar to the ligand-bound state in AppA (Fig. 5B) than its substrate-free conformation. Arg28 is anchored in the long α helix A in PhyK, whereas it is in a loop region in AppA. Because the helix is more rigid than a loop, a conformational change of this region of PhyK is not very likely. Because the elongated α helix A was observed in all six protein molecules of this study (two in the structure of PhyK and four in PhyK H25A), these structural differences are not caused by crystal lattice contacts.

Another conformational change in AppA involves Glu219. The side chain of this residue is pushed out of the catalytic pocket upon phytate binding. Here, PhyK mimics the phytate-bound structure of AppA, even in the absence of sulfate ions. The side chain of the corresponding Glu212 bends out of the catalytic pocket of PhyK avoiding steric or charge interactions with a substrate molecule. Both PhyK structures resemble that of AppA in the substrate-bound state. It therefore seems that PhyK is always kept in a conformation suitable for phytate binding, whereas AppA undergoes a distinct conformational change upon substrate binding.

**Classification of HAPs**

Phylogenetic trees of bacterial HAPs based on their sequences suggest three branches [14,16]. Besides a G1P branch, two groups of ‘true’ phytases are considered. The group including PhyK consists of phytases mainly produced by plant-associated bacteria, whereas the AppA-like group comprises phytases from pathogenic bacteria. The *Klebsiella* phytase is a member of the PhyK group and, to our knowledge, is the first example of the PhyK group for which structural information is available. The *Klebsiella* PhyK shares some structural and biochemical features with the G1P branch, although other characteristics are closer to the AppA group.

One striking difference between PhyK and *E. coli* AppA is the relative stiffness of the catalytic pocket. For both PhyK and *E. coli* G1P, conformational changes upon substrate binding are not as distinct as for *E. coli* AppA, suggesting a preformed active site that does not adjust its conformation upon phytate binding. The loop region of AppA which moves towards the substrate is part of the elongated helix A in PhyK and G1P. This helix is part of the α domain which is responsible for substrate recognition and specificity, whereas the residues responsible for catalysis are part of the more conserved α/β domain [4]. However, PhyK and AppA show the highest specific activity for phytate and are able to hydrolyze five of the six ester bonds, whereas *E. coli* G1P shows exclusive 3-phytase activity [22]. The responsible gating residues, Leu24 and Glu196, are found in members of the G1P group only. Members of the three groups show different kinetics for the hydrolysis of phytate (Table 1). The $K_m$ value for PhyK is considerably smaller than for AppA, showing that binding is favored by the preformed site. By contrast, catalysis by AppA is faster, as reflected in the values for $k_{cat}/K_m$, indicating that a conformationally flexible phytate active site can support more rapid turnover. The $k_{cat}/K_m$ values increase from G1P over PhyK to AppA by a factor of ~2200. The conformational changes of AppA upon substrate binding facilitate a faster turnover of phytate and are in line with a higher specificity. The relatively stiff catalytic pocket of PhyK does not allow such a fast turnover. However, other substrates not converted by AppA can be hydrolyzed, suggesting considerable freedom of substrate binding and release outside the catalytic site of PhyK.
The three distinct groups of HAPs are adapted to different habitats. To support plant growth, bacteria do not need to release phosphate as fast as the digestive tract of an animal host, where possible substrates might be available for a limited time only. A long-term constant supply of phosphate is more important to...
support plant growth. As a consequence of this evolutionary pressure, phytases of the PhyK group have acquired a broader substrate spectrum [14]. There might be a common ancestor for these three types of bacterial HAPs from which enzymes with different features evolved. Depending on the different microenvironments of the bacteria, molecular evolution of phytases apparently either favored highly specialized enzymes required for fast and specific catalysis or enzymes which liberate phosphate at a constant, moderate rate from different substrates. This hypothesis is supported by HAPs sharing some characteristics with one group and other features with another.

Materials and methods

Cloning of the *Klebsiella* phytase gene (phyK)

The *Klebsiella* phytase gene *phyK* was amplified using primers KlebTH-fw (5′-TCGGATCCGCCGCCGCGAC TGGCAGCTG) and KlebTH-rv (5′-CCGGCGGTAGCATGGTCCTGCCGAAGCTT) and chromosomal DNA of *Klebsiella* strain ASR1 as a template. The PCR product was cloned into the *Bgl*II and *Hind*III sites of plasmid pET22b(+) (Novagen, Nottingham, UK), containing a C-terminal His6 tag and an N-terminal signal sequence for periplasmic localization [14]. The inactive mutant PhyK H25A was generated by using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA). Plasmid pET-1TK was used as template and Kleb(HtoA)fw (5′-GCTTAGCCGCCGCCGCAATTCG) and Kleb(HtoA)rv (5′-CGAATGCCGCCGCCAAGCTT) as primers for
mutagenesis of *phyK*. The H25A mutation was confirmed by sequencing analysis.

**Gene expression and purification of PhyK**

The genes encoding PhyK and the mutant PhyK H25A were expressed in *E. coli* C41 (DE3) as described previously [14]. The genes were expressed in TBY medium by lactose induction (1%) for 18 h at 37 °C. His-tagged native PhyK was purified by affinity chromatography using Ni-NTA (Qiagen, Hilden, Germany) in 25 mM Tris pH 7.5 and 300 mM NaCl. For purification of PhyK H25A, the HiTrap chelating HP-FPLC column and a linear imidazol gradient (5–300 mM) were used according to the manufacturer’s protocol (GE Healthcare, Uppsala, Sweden).

Selenomethionine (Mse) labeled PhyK was produced in *E. coli* B834 (DE3) according to a modified protocol of Budisa et al. [23]. The preculture was incubated for 6 h at 37 °C, and the cells were washed with new minimal medium (NMM) and finally resuspended in 5 mL NMM. The second culture was inoculated with 1 mL of washed cells in 100 mL NMM containing 0.1 mM methionine and 0.4 mM selenomethionine (Sigma, St. Louis, MO, USA). After 12 h the culture was washed again and used to inoculate the main culture containing 0.5 mM selenomethionine. At $D_{600}$ of 0.4–0.8 *phyK* expression was induced by adding 1 mM isopropyl thio-β-D-galactoside. Cells were harvested 8.5 h after induction. After lysis of the cells the affinity purification with Ni-NTA was performed.

**Crystallization**

Prior to crystallization, the buffer was changed to 20 mM sodium acetate (pH 5.0), 50 mM NaCl. Crystals were grown using hanging-drop vapor diffusion at 18 °C within 5–6 weeks. Drops consisted of 1 μL protein solution (6 mg/mL) and an equal volume of 4.0 mM sodium formate. The Mse-labeled protein was crystallized under the same conditions in 4 months. The inactive PhyK H25A with its substrate phytase, the refined structure of an inactive PhyK mutant with its substrate phytase, the refined model was used as search model for molecular replacement with the native data with a resolution of 1.68 Å. Iterative cycles of model building and refinement with Phaser [29], REFMAC [30] and manually tracing and fitting in COOT [31] resulted in a final model containing 796 of 836 amino acids, 804 water molecules, 2 glycerol molecules, 1 magnesium and 4 sodium ions. Ions were verified with the structure analysis server STAN [32]. This model has an $R_{	ext{work}}$ of 0.180 and $R_{	ext{free}}$ of 0.206. As suggested by the electron density, the $S^2$ values of all six cysteine side chains were modeled with a reduced occupancy of 0.6–0.8 to account for the likely effect of radiation damage [33].

In order to determine the structure of the complex of an inactive PhyK mutant with its substrate phytase, the refined structure of the wild-type enzyme was used for molecular replacement with the diffraction data obtained from the cryo-crystallization trials. Further refinement was performed using Phaser [29], REFMAC [30] and manually fitting in COOT [34]. At the expected phytase binding site, three density maxima large enough to accommodate phosphate or sulfate ions were observed. This electron density was observed next to all four protein molecules of the asymmetric unit. Even at very low contour levels no connecting density indicating bound inositol phosphates was revealed. These sites were thus assigned as sulfate ions, because
ammonium sulfate was present in the crystallization drop at 80 mM concentration, i.e. in > 50-fold molar excess over phytate. An excess of sulfate ions over phytate was necessary to crystallize the protein, although it eventually prevented phytate binding. The model was refined without introducing binding partners in the active site cavity to avoid model bias until late in the refinement when the sulfate ions were introduced. Refinement converged at $R_{\text{work}}/R_{\text{free}}$ values of 0.199/0.243. Data collection and refinement statistics are summarized in Table 2.

To derive a model for substrate binding to PhyK, phytate was fitted into the difference electron density of the PhyK H25A structure with the 3-phosphate as scissile group. After manually fitting the substrate, REFMAC was used to refine the stereochemical parameters. We found a unique orientation in which three of six phosphate groups of phytate perfectly fitted into the electron density occupied by sulfate ions in the crystal structure, suggesting the geometry of an enzyme–substrate complex.

**Acknowledgements**

We are grateful to the staff at BESSY (Berlin) and beamline X13 at EMBL/DESY (Hamburg) for their...
help with X-ray diffraction experiments. Data collection in Hamburg was supported by the European Community (RII-CT-2004-506008). Work at MDC (Berlin) was supported by the Fonds der Chemischen Industrie.

References


