Vanadate Inhibition of Fungal PhyA and Bacterial AppA2 Histidine Acid Phosphatases

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ABSTRACT: The fungal PhyA protein, which was first identified as an acid optimum phosphomonoesterase (EC 3.1.3.8), could also serve as a vanadate haloperoxidase (EC 1.11.1.10) provided the acid phosphatase activity is shut down by vanadate. To understand how vanadate inhibits both phytate and pNPP degrading activities of fungal PhyA phytase and bacterial AppA2 phytase, kinetic experiments were performed in the presence and absence of orthovanadate and metavanadate under various acidic pHs. Orthovanadate was found to be a potent inhibitor at pH 2.5 to 3.0. A 50% activity of fungal phytase was inhibited at 0.56 μM by orthovanadate. However, metavanadate preferentially inhibited the bacterial AppA2 phytase (50% inhibition at 8 μM) over the fungal phytase (50% inhibition at 40 μM). While in bacterial phytase the K_m was not affected by ortho- or metavanadate, the V_{max} was reduced. In fungal phytase, both the K_m and V_{max} was lowered. The vanadate exists as an anion at pH 3.0 and possibly binds to the active center of phytases that has a cluster of positively charged Arg, Lys, and His residues below the enzymes' isoelectric point (pI). The active site fold of haloperoxidase was shown to be very similar to fungal phytase. The vanadate anions binding to cationic residues in the active site at acidic pH thus serve as a molecular switch to turn off phytase activity while turning on the haloperoxidase activity. The fungal PhyA phytase's active site housing two distinct reactive centers, one for phosphomonoesterase and the other for haloperoxidase, is a unique example of how one protein could catalyze two dissimilar reactions controlled by vanadate.

KEYWORDS: histidine acid phosphatase, fungal, bacterial, PhyA phytase, AppA2 phytase, vanadate

INTRODUCTION

Aspergillus ficuum phytase, EC 3.1.3.8, belongs to "histidine acid phosphatase" (HAP), which is a well-characterized enzyme.[1-3] The biocatalyst was cloned and overexpressed leading up to commercialization in the mid-1990s by Gist-Brocade to liberate inorganic orthophosphate from phytate that is copiously present in plant derived meals for better utilization of phosphates that are in poultry and swine feed.[4] The enzyme's three-dimensional structure was elucidated in 1997.[5] An unexpected structural relationship between integral membrane phosphatase that belonged to the phosphatidic acid phosphatase (PAP) class of phosphomonoesterase (EC 3.1.3.4) and soluble haloperoxidases (EC 1.11.1.10) was discovered.[6] Moreover, another group described shared signature motifs in a superfamily of proteins that contains PAP and vanadium-dependent chloroperoxidase.[7] Concurrently, a third group also established that the vanadate-chloroperoxidases and acid phosphatases have a similar three-dimensional structure of the active site and the apoenzyme that lacks vanadate in the active site could catalyze phosphomonoesterase activity at acidic pH.[8]

Figure 1 shows the schematic representation of the active site of vanadium haloperoxidase from *Curvularia inequalis*[9] and phytase from *Aspergillus ficuum*.[5] In both the cases, the anionic vanadate is held in place by basic amino acid residues such as Arg, His, Lys, etc. that are present at the active center.[10] For fungal phytase, this creates a unique situation because it could perform both the phosphomonoesterase and haloperoxidase activity depending upon whether vanadate ion is present or absent in the active site pocket of the enzyme. As per the model, when vanadate is absent, the *A. ficuum* PhyA serves as phytase; however, when orthovanadate is present at micromolar level,

the phytase activity is severely inhibited but the vanadate-dependent haloperoxidase activity sets in.[11] While orthovanadate-dependent haloperoxidase activity was reported for the fungal phytase, thus far, no enzymatic characterization of orthovanadate inhibition of fungal phytase was carried out. Furthermore, it is imperative to know whether the concentration at which vanadate inhibits phytase activity has any physiological significance. If the inhibitory concentration of vanadate is at high picomolar or low micromolar level, then it will be meaningful from a physiological standpoint vis-à-vis turning the phosphomonoesterase activity off while turning on the haloperoxidase activity.

In this communication, we present the results of inhibition studies of both ortho- and metavanadate in both fungal and bacterial histidine acid phosphatase (HAP) type of phytases whose kinetic parameters were compared in a recent study.[12] Our findings indicate that, at acidic pH, both ortho- and metavanadate are potent inhibitors of both the fungal and bacterial PhyA phytase activities. Phytase's active site has a preponderance of acidic residues, and perhaps these offer a binding site for both anionic ortho- and metavanadate to serve as a molecular switch. As per this model when vanadate is absent, the phytase activity of HAP sets in, however, in the presence of a low micromolar level of vanadate, the phytase activity is shut down to turn on the vanadate-dependent haloperoxidase activity. The same enzyme performing either as a monoesterase (EC 3.1.3.8)

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or haloperoxidase (EC 1.11.1.10) at acidic pH while vanadate is performing as the molecular switch is a unique example of how simple inorganic molecules could regulate the chemical behavior of biocatalysts.

■ MATERIALS AND METHODS

Source of Phytases. The phytase gene (*phyA*) from *Aspergillus niger* was cloned and overproduced in *Pichia pastoris*.[12] The recombinant phytase was purified using sequential ion-exchange column chromatographies first using MacroPrep S column, a cation exchanger, at pH 3.0 and then a MacroPrep DEAE column, an anion exchanger, at pH 7.0. The specific activity of the purified phytase was about 3000 nKat/mg when assayed at 58 °C.

The *Escherichia coli* phytase coded by *AppA2* gene was a gift from Phytex LLC, Portland, ME, which was overexpressed in *Pichia pastoris*.[12] The crude culture filtrate was dialyzed against 25 mM glycine, pH 2.8 buffer and loaded onto a MacroPrep S column, a cation exchanger, and eluted as a single activity component in a linear salt (0–0.5 M sodium chloride) gradient. The expression level of phytase was so high in *P. pastoris* that it took only one ion exchange column to purify the protein to near homogeneity. The final specific activity of the phytase was about 15,000 nKat/mg of protein at 55 °C.

Phytase Assay. Phytase assays were carried out in triplicate in 1.0 mL of buffer at 55 °C similar to *A. ficuum* phytase assay, and the average value was reported for each data point.[1] The buffers used were 25 mM glycine (pH 1.0 to 3.5), 50 mM acetate (pH 4.0 to 5.5), and 50 mM imidazole (pH 6.0 to 8.0). Liberated inorganic *ortho*-phosphates were quantitated spectrophotometrically using a freshly prepared AMA

(acetone–molybdate–acid) reagent consisting of acetone, 10 mM ammonium molybdate, and 5.0 N sulfuric acid, (2:1:1, v/v). Adding 2.0 mL of AMA reagent per assay tube terminated phytase assay. After 30 s, 0.1 mL of 1.0 M citric acid was added to each tube to fix the color generated by AMA reagent. Absorbance was read at 355 nm after blanking the spectrophotometer with appropriate control. Enzyme activity was expressed as nKat/mL. Kat stands for katal that converts 1 mol s^{-1} of substrate to product.

Acid Phosphatase Assay. The phytase enzyme samples were placed in buffers mentioned above and incubated with 1.25 mmol of *p*-nitrophenylphosphate (pNPP) in a final volume of 1.0 mL at 55 °C for 1 min. The reaction was terminated using 0.1 mL of 1.0 N NaOH, and the liberated *p*-nitrophenol was measured spectrophotometrically at 400 nm using an extinction coefficient of 18.3 mM^{-1} cm^{-1}.[1]

Vanadate Inhibition of Phytase Activity at Various pHs. Phytases (8 μL of PhyA and 5 μL of AppA2) were incubated with 1000 μM sodium orthovanadate in 1 mL total volume (from pH 1.0 to 8.0) at room temperature for 10 min. Then aliquots from the treated enzyme samples were transferred to a 55 °C water bath for 2 min before the commencement of phytase assay.

Kinetics of Vanadate Inhibition of Fungal and Bacterial Phytase. Various concentrations of phytate (0–1000 μM) and pNPP (0–1250 μM) were incubated with PhyA and AppA2 phytases in the presence and absence of sodium orthovanadate or metavanadate to determine K_m and V_{max} of the phytases for substrates and K_i for the inhibitors. The concentration of vanadates used varied, which was based on the concentration at which 50% activity was lost.

Figure 1. Schematic diagram of the active sites of vanadium chloroperoxidase (**a**) from *Curvularia inaequalis*[9] and phytase (**b**) from *Aspergillus ficuum*.[5] The comparison of the active sites was first made by a Dutch group.[10].

■ RESULTS

The Effect of pH on Inhibition of Phytase Activity by Orthovanadate. To test whether pH has any effect on ortho-vanadate-induced inhibition of phytase activity at 1 mM, both the fungal phytase (PhyA) and bacterial phytase (AppA2) were tested and the results are shown in Figure 2. The inhibitor was found to be more effective in the acidic pH range. Fortuitously, the phytases from both fungal and bacterial origin perform optimally at pH 5.0 and 2.5; therefore, the inhibitory effect of vanadate could be studied at the pH where the biocatalysts are active.[1]

Inhibition of Fungal PhyA and Bacterial AppA2 Phytase Activity by Vanadates. Both orthovanadate and metavanadate species were found to be potent inhibitors of phytase activity in fungal PhyA and bacterial AppA2 phosphomonoesterases. Table 1 summarizes the results showing concentration of vanadate at which 50% phytase activity was inhibited. At pH 2.5 it took only 0.56 μM orthovanadate to inhibit 50% activity of the fungal
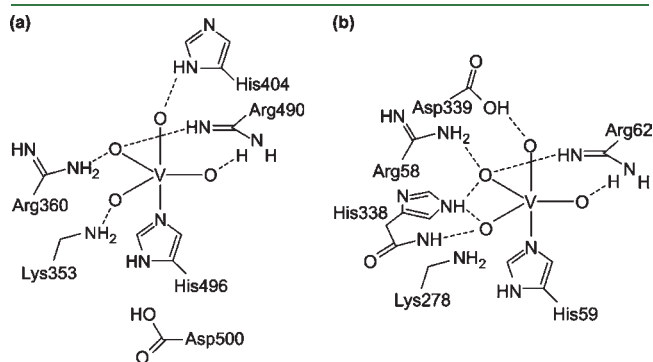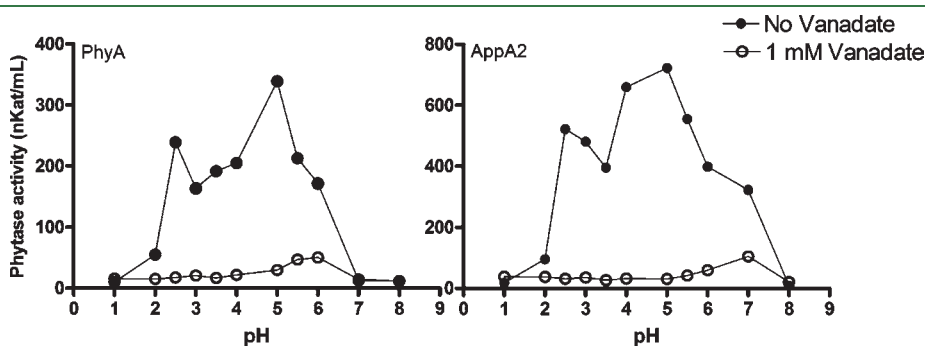

Figure 2. Inhibition of fungal PhyA phytase (left panel) and bacterial AppA2 phytase (right panel) activity by 1.0 mM sodium orthovanadate as a function of various pHs.

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phytase. On the other hand, at pH 4, 2.0 μM orthovanadate could inhibit 50% of AppA2 phytase activity (Table 1). At pH 5, however, a considerably higher orthovanadate concentration (125 μM for PhyA and 12 μM for AppA2) was needed to inhibit 50% activity of the two phytases. Metavanadate also inhibited phytase activity in both the phytases; however, as an inhibitor it is not as potent as orthovanadate at pH 2.5 for PhyA phytase and at pH 4.0 for AppA2 phytase. For example, it took about 40 μM metavanadate to inhibit the fungal phytase at pH 2.5 and 8.6 μM to inhibit the bacterial phytase (AppA2) activity at pH 4. At pH 5.0, metavanadate inhibited PhyA phytase at the same level done by orthovanadate. For AppA2 phytase, metavanadate at pH 4.0 had exerted a 50% inhibition at a concentration four times higher than that of orthovanadate (Table 1). The results shown in Table 1 point out that orthovanadate is more potent as an inhibitor than metavanadate.

The above experiments were repeated, but the synthetic substrate p-nitrophenylphosphate or pNPP designed to measure acid phosphate activity had replaced phytate that specifically measures phytase activity.[1] The results of ortho- and metavanadate inhibition of acid phosphatase activity in both PhyA and AppA2 phytases are shown in Table 2. Based on 50% activity inhibition data, orthovanadate was found to be a potent inhibitor at pH 2.5 and pH 3.0. At both pHs, the orthovanadate was found to inhibit acid phosphatase activity of the two phytases. At pH 5.0, orthovanadate was able to inhibit both the fungal and bacterial acid phosphatase activities, however, it took nearly 30- to 100-fold more metavanadate to exert 50% inhibition as compared the inhibition achieved by the same inhibitor at pH 2.5 and 3.0 (Table 2).

Effect of Vanadates on Kinetic Parameters of Both Fungal and Bacterial Phytases. The orthovanadate at acidic pH affected the K_m, V_{max}, turnover number, and kinetic efficiency of fungal PhyA phytase. The fungal enzyme has two pH optima when phytate is used as substrate and orthovanadate had affected kinetic parameters at both pH 2.5 and 5.0 (Table 3). For general purpose acid phosphatase assay p-nitrophenylphosphate was used at pH 5.0. In this case too the V_{max} was drastically reduced (Table 3). Correspondingly, the turnover number and kinetic efficiency were reduced as well. The metavanadate was found to be less of an inhibitor for fungal PhyA phytase and acid phosphatase at pH 2.5 than it was at pH 5.0 (Table 4). It may seem that vanadate had noncompetitively inhibited PhyA enzyme activity. The turnover number and kinetic efficiency of the fungal enzyme was also reduced by both ortho- and metavanadate (Tables 3 and 4).

The K_m, V_{max}, turnover number, and kinetic efficiency of the bacterial phytase, AppA2, in the presence and absence of orthovanadate and metavanadate are shown in Tables 5 and 6, respectively. While the K_m was unaffected in all cases, the inhibitors affected the V_{max}, turnover number, and kinetic efficiency. The phytate-driven enzyme activity of the AppA2 enzyme was affected more so by both orthovanadate and metavanadate than the p-nitrophenylphosphate-driven acid phosphatase activity of the same phosphohydrolase.

Table 1. Inhibition of Fungal PhyA and Bacterial AppA2 Phytase Activity by Vanadate

phytases	pH	vanadate concn required for 50% inhibition	
		sodium orthovanadate (μM)	sodium metavanadate (μM)
PhyA	2.5	0.56	40
	5.0	125.0	130.0
AppA2	4.0	2.0	8.6
	5.0	12.0	35.0

Table 2. Inhibition of Fungal PhyA and Bacterial AppA2 pNPP Activity by Vanadate

phytases	pH	vanadate concn required for 50% inhibition	
		sodium orthovanadate (μM)	sodium metavanadate (μM)
PhyA	2.5	0.4	1.1
	5.0	40.0	5.3
AppA2	3.0	0.072	0.75
	5.0	2.2	0.90

DISCUSSION

In this communication we report the results of detailed inhibition studies with two members of acid phosphomonoesterses with two species of vanadate, namely, ortho- and metavanadate. To make a semisynthetic peroxidase out of *Aspergillus niger myo*-inositol-hexakisphosphate 3-phosphohydrolase, a group of researchers had incorporated vanadium into the active site of

Table 3. Kinetic Parameters for PhyA WT in the Presence and Absence of Orthovanadate

orthovanadate concn (μM)	pH	substrate	K_m (μM)	K_i (μM)	V_{max} (nKat/mL)	turnover no. (s^{-1})	kinetic efficiency ($\times 10^6$ M^{-1} s^{-1})
0	2.5	phytate	324		455	1260	3.9
10	2.5	phytate	178	5.7	165	457	2.6
0	5.0	phytate	140		610	1690	12.0
100	5.0	phytate	320	178	390	1080	3.4
0	5.0	pNPP	980		219	60	0.060
50	5.0	pNPP	1000	40	96	27	0.027

Table 4. Kinetic Parameters for PhyA WT in the Presence and Absence of Metavanadate

metavanadate concn (μM)	pH	substrate	K_m (μM)	K_i (μM)	V_{max} (nKat/mL)	turnover no. (s^{-1})	kinetic efficiency ($\times 10^6$ M^{-1} s^{-1})
0	2.5	phytate	87		455	1260	3.9
15	2.5	phytate	320	5.6	395	1090	3.4
0	5.0	phytate	140		610	1690	12.0
100	5.0	phytate	76	40	176	487	6.4

Table 5. Kinetic Parameters of AppA2 in Presence and Absence of Orthovanadate

orthovanadate concn (μM)	pH	substrate	K_m (μM)	K_i (μM)	V_{max} (nKat/mL)	turnover no. (s^{-1})	kinetic efficiency ($\times 10^6$ M^{-1} s^{-1})
0	4.0	phytate	425		1056	4470	10.00
0.5	4.0	phytate	406	0.2	268	1136	2.80
0	3.0	pNPP	300		136	289	0.96
0.07	3.0	pNPP	316	0.2	100	215	0.68

Table 6. Kinetic Parameters of AppA2 in Presence and Absence of Metavanadate

metavanadate concn (μM)	pH	substrate	K_m (μM)	K_i (μM)	V_{max} (nKat/mL)	turnover no. (s^{-1})	kinetic efficiency ($\times 10^6$ M^{-1} s^{-1})
0	4.0	phytate	250		1385	2348	9.30
10	4.0	phytate	320	4.0	400	672	2.10
0	3.0	pNPP	275		79	67	0.24
0.07	3.0	pNPP	283	0.2	56	48	0.17

the biocatalyst. The semisynthetic vanadium peroxidase was able to catalyze enantioselective sulfoxidation with hydrogen peroxide as oxidant.[11] The same group of researchers also discovered that the metal-free fungal phytase was able to catalyze enantioselective oxygen transfer reaction. Later, a method was developed to incorporate the fungal phytase as an enantioselective biocatalyst for the synthesis of chiral sulfoxides with hydrogen peroxide as oxidant.[14]

A detailed study of vanadate induced inhibition of microbial phytases belonging to the histidine acid phosphatase subfamily of phosphomonoesterase could point out whether the inhibition caused by the inorganic molecule has any physiological relevance. The observed low micromolar concentration of vanadate (0.56 to 120 μM) that could inhibit 50% of phytase reaction in both fungal and bacterial phytase is indicative of a very high sensitivity of the biocatalysts for vanadates. The notion that incorporation of a metal oxoanion in the active center of phytase should inhibit phosphomonoesterase activity was also predicted, and the K_i was reported to be 3.9 μM for orthovanadate.[13] No inhibition studies were however made against microbial phytases using metavanadate. Our results indicated that at pH 2.5 metavanadate shows about one-sixtieth less inhibition as compared to the inhibition caused by orthovanadate. At pH 5.0 this difference ceases to exist. For Appa2 phytase, the enzyme showed more sensitivity to orthovanadate than metavanadate and the inhibitors were more active at pH 4.0 than at pH 5.0.

The observation that both the fungal and bacterial phytases were inhibited at acidic pH indicates that both orthovanadate and metavanadate are predominantly present as oxoanion $H_2VO_4^-$.[15] Under acidic conditions vanadate could bind to cationic residues such as Arg, Lys, and His. Coincidentally, the active site of *A. niger* phytase, which is located in a positively charged cleft between an α and an α/β domain, has a preponderance of Arg and His residues. In acidic pH, these residues would be cationic and thereby would readily bind oxoanion $H_2VO_4^-$ (Figure 1). In *Klebsiella* sp. ASR1 phytase, which is also a member of the histidine acid phosphatase family, three negatively charged sulfate ions bind to the catalytic pocket of an inactive mutant phytase.[16] There is a possibility that the vanadates bind to the same Arg58, Arg62 or His59 residues in the active site of the phytase that plays a role in binding phytate, which contains six negatively charged phosphate groups.[5]

The fact that vanadate inhibits phytase reaction at pH 2.5 has bearing on feed formulation supplemented with fungal or bacterial phytase. Thus far, results of the phytase supplemented feed trials reported in published papers have not shown any enzyme inhibition due to both cations and anions present in the feed.[17,18] This could be due to slightly higher stomach pH of the monogastric animals (pH 4.5 to 6.0) or that other components present in the feed are preventing vanadate or other anions from binding to phytase's active site.

The oxidation of thioanisole by vanadate free *A. niger* phytase was shown to be very sluggish; however, when orthovanadate was incorporated into the enzyme mix, the reaction rate increased about 42-fold.[14] Therefore, binding of the orthovanadate into the positively charged residues of Arg or His in the active site of fungal phytase is a prerequisite to the oxidation of thioanisole. Under this model, when the Arg or His residues are free, the phosphomonoesterase reaction is favored as is the case with phosphohydrolytic cleavage of phytate molecule; however, when vanadate ion binds to the same positively charged residues in the phytase molecule, no further binding of phytate molecule is allowed and the biocatalyst favors oxidation of thioanisole. In this communication, we have shown that at acidic pH both orthovanadate and metavanadate could inhibit phytase reaction at low micromolar concentration. The observation that vanadate could bind the active site of a well-characterized phosphohydrolase (EC 3.1.3.8) and convert the biocatalyst into a haloperoxidase (EC 1.11.1.10) is a unique example of a switching mechanism to realize two distinct enzymatic reactions emanating from a single polypeptide chain. A cross-linked enzyme aggregate of 3-phytase (EC 3.1.3.8) from *A. niger* was synthesized and incubated with vanadate and then tested as a biocatalyst in the sulfoxidation of thioanisole using hydrogen peroxide as the oxidant. The results indicate that the immobilized phytase exhibited a similar efficiency (approximately 95% conversion) as the free phytase.[19] As more enzymes are being studied at X-ray crystallographic resolution, the structural homology studies will reveal multiple active sites in a single protein molecule. This is what exactly had happened in the case of fungal phytase.[6−8] Without this discovery, the role of vanadate to switch an acid phosphatase into a haloperoxidase could have not been possible.

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