Supporting Information for

"**Diesterase Activity and Substrate Binding in Purple Acid Phosphatases"**

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Experimental Section

General. All chemicals used were of analytical grade unless otherwise stated. Bis (4-nitrophenyl) hydrogen phosphate (BpNPP) was obtained commercially. The bis (cyclohexyl-ammonium) salt of *p*-nitrophenyl phosphate (pNPP) was synthesized as described.[1] Ethyl *p*-nitrophenyl phosphate (EpNPP) was synthesized according to the method of Hendry and Sargeson[2] and methyl *p*-nitrophenyl phosphate (MpNPP) was synthesized similarly, except methanol was used instead of ethanol. Methyl phosphate (MP) and Ethyl phosphate (EP) were synthesized as described by Grzyska.[3] The identities of all substrates were verified by NMR and had no other detectable phosphate species. ^{1}H and ^{31}P NMR spectra were performed with a Bruker ARX 400 MHz spectrometer. pH determinations were done with a calibrated ORION Model 410A Meter in conjunction with a combination glass pH electrode. All substrates were premixed into a stock solution of buffer and the pH was verified and adjusted to the desired pH as necessary. Pig (pPAP) and red kidney bean PAP (kbPAP) were provided by Dr. Schenk's group at the University of Queensland, and were isolated as described previously.[4] All kinetic assays were analyzed by UV-VIS absorption using 96-well plates and a VERSAmax Tunable Microplate Reader from Molecular Devices. The steady state kinetic parameters were determined assuming Michaelis-Menten kinetics and were fit to the Michealis-Menten equation 1 using Kaleidagraph v3.6 where v is the initial rate, [S] is the substrate concentration, and V_{max} is k_{cat} multiplied by the amount of enzyme present.

$$v = \frac{V_{max} \times [S]}{K_m + [S]} \qquad (1)$$

Enzyme Concentration. Enzyme concentration was determined by UV-VIS spectrophotometry at 280 nm ($\varepsilon = 349\ M^{-1}\ cm^{-1}$) and was corrected for the amount of active enzyme by kinetic assay with pNPP in 0.1 M acetate buffer, pH 4.90, at substrate concentrations of 0.625, 2.5, 5 and 10 mM. Once the k_{cat} for pNPP was determined, the ratio of active enzyme was obtained by dividing the measured k_{cat} values by previously reported k_{cat} values for pNPP ($470\ s^{-1}$ for pig PAP and $850 s^{-1}$ for kidney bean PAP).[5] The enzyme concentration obtained from UV-VIS absorption was then multiplied by the ratio of active enzyme to get the final concentration of active enzyme.

Kinetic Assays. Kinetic constants with MpNPP, EpNPP, and Bis pNPP as substrates were performed in 1.5 M acetate buffer, pH 4.90 (0.1 M acetate buffer for Bis pNPP) for pPAP and 1.0 M MES buffer, pH 6.25 (0.1 M MES buffer for Bis pNPP) for kbPAP. Substrate concentrations for MpNPP and EpNPP ranged from 10 mM to 350 mM and from 10 mM to 450 mM, respectively. Substrate concentrations for Bis pNPP ranged from 1 mM to 30 mM and could not be increased due to solubility issues with the Bis pNPP. Rates obtained with varying concentrations of substrate were assayed in 250 μL reaction mixtures. Before addition of enzyme, a blank sample was removed (50 μL aliquot) and quenched with 250 μL 1.0 N NaOH. The reaction was started by addition of enzyme (10 to 50 nM) and 50 μL aliquots were removed periodically and quenched with base as mentioned previously. The amount of *p*-nitrophenolate ion present was measured at 400 nm and an extinction coefficient of $18.3\ mM^{-1}\ cm^{-1}$ was used. In the case of Bis pNPP, the resulting rates were halved and a Lineweaver-Burk equation (2) was used to determine k_{cat}/K_m due to the inability to obtain high substrate concentrations and saturate the enzyme with substrate.

$$\frac{1}{v} = \frac{K_m}{V_{\max}[S]} + \frac{1}{V_{\max}} \qquad (2)$$

Kinetic constants for pPAP and kbPAP with MP and EP as substrates were also performed in 1.5 M acetate buffer, pH 4.90 and 0.75 M MES buffer, pH 6.25, respectively. Substrate concentrations ranged from 10 to 400 mM. The amount of inorganic phosphate (P_i) released during the reaction was detected by a modified method of Jones and Black [6-10] in which a solution of 2 parts 12% w/v ascorbic acid in 1 N HCl to 1 part 2% w/v ammonium molybdate tetrahydrate in water was prepared (solution A) and another solution of 2% w/v sodium citrate tribasic dihydrate and 2% v/v acetic acid in water was also prepared (solution B). Rates obtained with varying concentrations of substrate were assayed in 250 μL reaction mixtures and a 50 μL aliquot was removed as a blank before addition of the enzyme and quenched accordingly. The reaction was started with addition of the enzyme (10 to 50 nM) and 50 μL aliquots were removed periodically and mixed with 125 μL solution A, thereby quenching the reaction (pH < 1), and the solution was allowed to react for 5 minutes before 125 μL of solution B was added. The solution was allowed to equilibrate for 15 minutes before the amount of inorganic phosphate produced was then measured at 655 nm. A phosphate standard curve was established by adding 10 μL aliquots of KH_2PO_4 solutions in 0.1 M acetate buffer pH 4.90 (concentrations ranging from 0.5 to 10 mM) to solution A (150 μL) and then adding solution B (150 μL) as described above.

For studies of the inhibition by MP and EP of the reaction with pNPP, 1.5 M acetate buffer (pH 4.90) was used with the pPAP and 0.75 M MES buffer (pH 6.25) was used with the kbPAP. pNPP concentrations ranged from 0.625 to 10 mM and inhibitor concentrations ranged from 0 to 250 mM. Reactions were started with addition of enzyme (10 to 25 nM) and 50 μL

aliquots were removed at intervals and quenched with 250 μL 1 N NaOH and the release of *p*-nitrophenolate was monitored at 400 nm. MP and EP inhibition of the enzymatic reactions with the substrates MpNPP and EpNPP, respectively, were performed similarly with MpNPP concentrations ranging from 1 mM to 300 mM, EpNPP concentrations ranging from 1 to 200 mM, and MP and EP concentrations ranging from 0 to 200mM. Inorganic phosphate inhibition against the enzymatic reactions of MpNPP and EpNPP were performed in 1.5 M acetate buffer (pH 4.90) with the pPAP and 0.75 M MES buffer (pH 6.25) with the kbPAP, with substrate concentrations ranging from 0.5 to 300 mM and inhibitor concentrations ranging from 0 to 20 mM. Enzyme concentrations were also 10 to 25 nM. To determine the K_i, inverse rates were plotted against the inverse substrate concentrations at each inhibitor concentration, according to the Lineweaver-Burk Equation (2). The slopes (K_m/V_{max}) of the resulting lines were plotted against the inhibitor concentrations and that resulting intercept (K_m/V_{max}) was divided by its slope ($K_m/(V_{max}K_i)$) to obtain K_i.

The kinetic data for the diesters MpNPP, EpNPP, and BpNPP, and for the monoesters MP and EP are reported in Table S1, along with literature values for the substrates pNPP. The ethyl diester EpNPP exhibited K_m values higher than MpNPP, presumably from its larger steric requirement, and much lower k_{cat}, which may result from an inability to bind in a productive manner suitable for in-line attack on phosphorus. The high K_m of EpNPP prevented saturation, resulting in the significant uncertainties in its kinetic constants. BpNPP exhibited only minimal activity at its maximum concentration of 20 mM, allowing only an estimate of k_{cat}/K_m obtained from data obtained over long time courses.

Table S1. Kinetic results for pig and kidney bean PAPs with methyl p-nitrophenyl phosphate (MpNPP), ethyl p-nitrophenyl phosphate (EpNPP), bis(p-nitrophenyl) phosphate (BpNPP), methyl phosphate (MP), ethyl phosphate (EP), and p-nitrophenyl phosphate (pNPP). Pig PAP kinetic deta were measured at pH 4.90 and data for kidney bean PAP were measured at pH 6.25.

Pig PAP	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($mM^{-1}s^{-1}$)
MpNPP	110 (18)	542 (91)	4.9
EpNPP	315(57)	13 (1)	0.04
BpNPP	ND	ND	0.009
MP	138 (10)	17 (1)	0.12
EP	154 (14)	3.0 (0.2)	0.02
pNPP[5]	1.25 (0.25)	470 (40)	380

kbPAP	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($mM^{-1}s^{-1}$)
MpNPP	356 (70)	542 (24)	1.5
EpNPP	529 (57)	8 (1)	0.014
BpNPP	ND	ND	0.008
MP	281 (47)	20 (3)	0.07
EP	191 (35)	2.5 (0.2)	0.013
pNPP[11]	3.7 (0.2)	944 (68)	244

ND = Not determined.

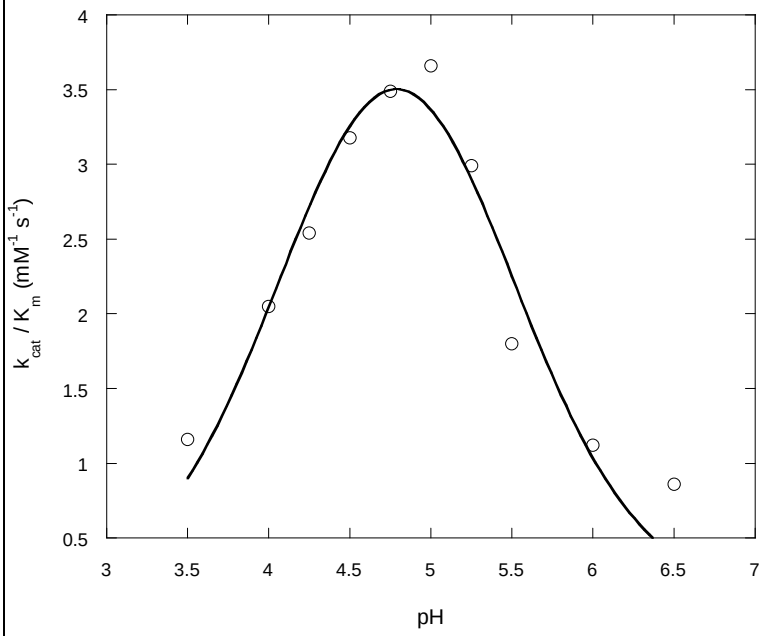
Table S2. Inhibition data.

pPAP	K_i (mM) vs. pNPP	K_i (mM) vs. Methyl pNPP
Inorganic phosphate	2[12]	4.1 (0.9)
MP	106 (11)	108 (22)

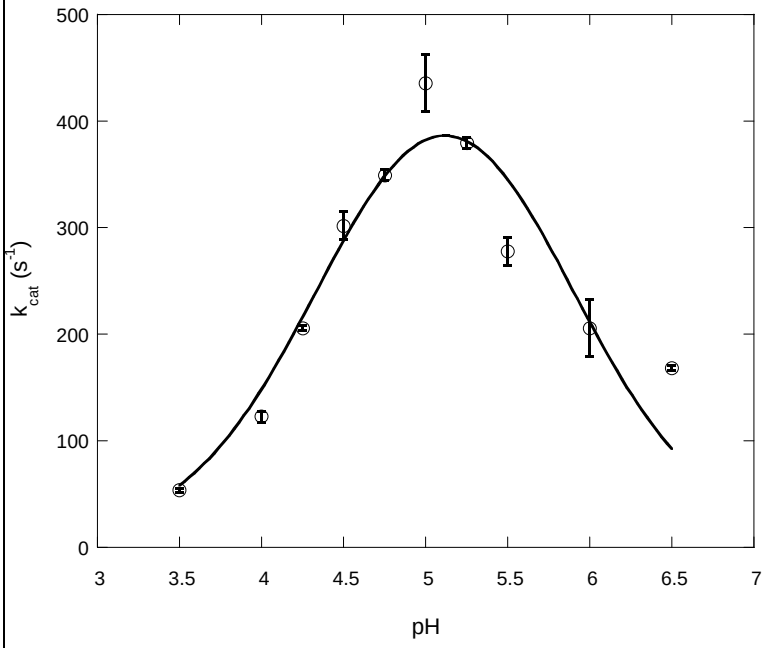
kbPAP	K_i (mM) vs. pNPP	K_i (mM) vs. Methyl pNPP
Inorganic phosphate	1.8[13]	0.8 (0.3)
MP	104 (15)	103 (11)

pH Dependence Studies. A tri-component buffer (1.0 M acetate, 0.5 M Tris, and 0.5 M Bis-Tris) which maintained constant ionic strength over the entire pH range (pH 3.0 to 8.0) was used for the pH profile kinetic assays. All of the assays were performed at ambient temperature (22˚C). For the pig and kidney bean PAP-catalyzed hydrolysis of MpNPP, assay mixtures 100 μL in total volume were set up. Reactions were started by addition of enzyme (20-26 nM for pPAP and 10 nM for kbPAP) to solutions containing various concentrations of MpNPP (50 to 300 mM). The mixture was allowed to react until no more than 3% of the substrate was hydrolyzed. The reactions were sampled over time by taking a 20 μL aliquot and quenching it with 280 μL 1 N NaOH. The absorbance was measured at 400 nm. Assays were repeated every 0.25 to 0.5 pH units in the range of pH 3.5 to 6.5 for pPAP and pH 3 to 8 for kbPAP. The k_{cat}/K_m and k_{cat} versus pH were fit to the following equation using Kaleidagraph, where K_1 and K_2 are apparent acid dissociation constants of the enzyme and/or substrate and H is the proton concentration (see Figure S1):

$$k_{cat}/K_m = \frac{(k_{cat}/K_m)^{max}}{(1 + H/K_1 + K_2/H)} \qquad (3)$$

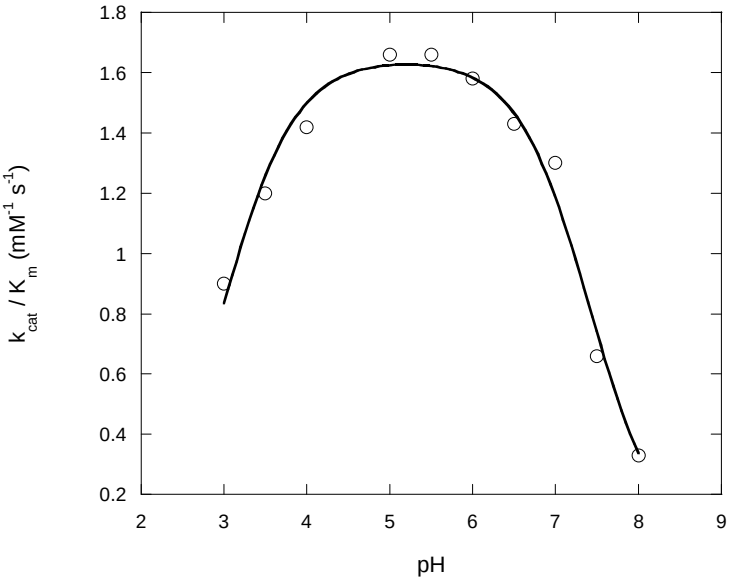


$k_{cat}/K_m = (k_{cat}/K_m)^{max}/(1+H/K_1+K_2/H)$		
	Value	Error
$(k_{cat}/K_m)^{max}$	5.2422	0.80948
K_1	6.5565e-05	2.3698e-05
K_2	4.0504e-06	1.4731e-06
Chisq	0.64103	NA
R	0.96621	NA

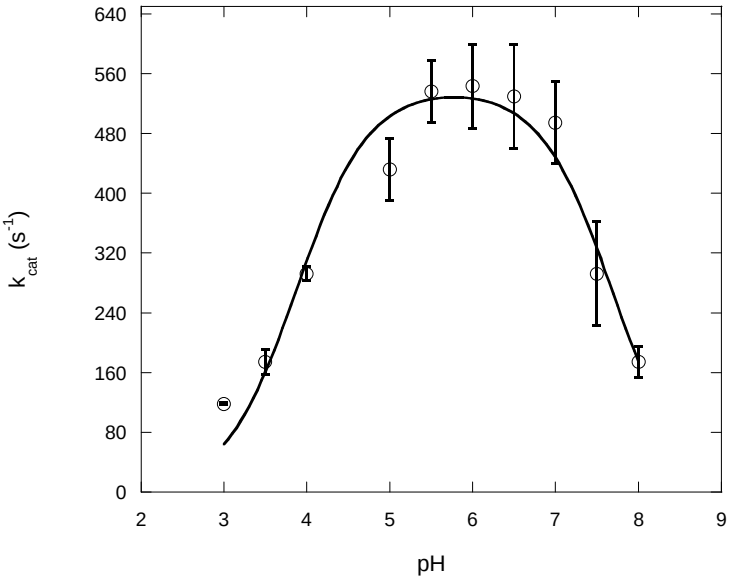


$k_{cat} = k_{cat}^{max}/(1+H/K_1+K_2/H)$		
	Value	Error
k_{cat}^{max}	541.84	91.272
K_1	3.7946e-05	1.5552e-05
K_2	1.5365e-06	7.1876e-07
Chisq	14137	NA
R	0.94398	NA

A



$k_{cat}/K_m = (k_{cat}/K_m)^{max}/(1+H/K_1+K_2/H)$		
	Value	Error
$(k_{cat}/K_m)^{max}$	1.6469	0.038742
K_1	0.0010253	0.00016557
K_2	3.8985e-08	5.4275e-09
Chisq	0.037526	NA
R	0.98961	NA



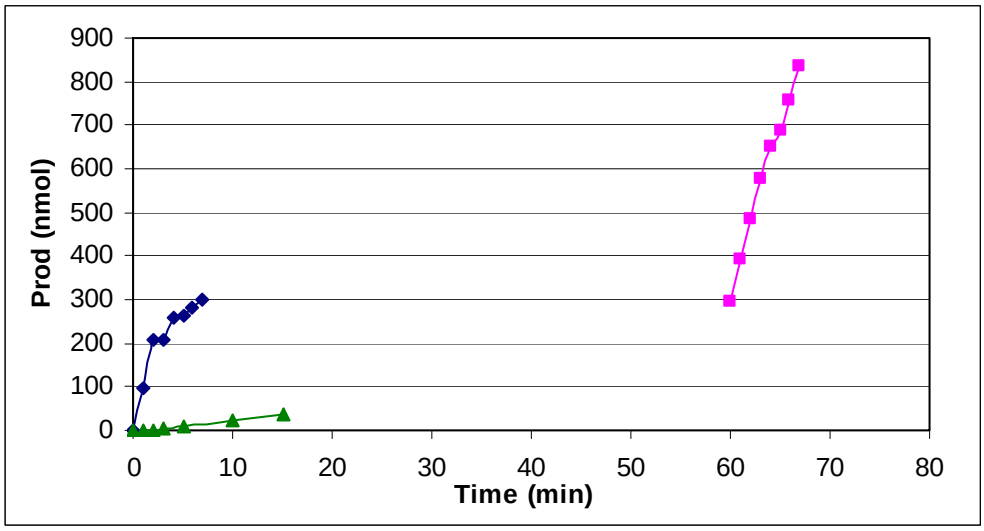
$k_{cat} = k_{cat}^{max}/(1+H/K_1+K_2/H)$		
	Value	Error
k_{cat}^{max}	541.78	24.176
K_1	0.00013377	3.4086e-05
K_2	2.0909e-08	5.4553e-09
Chisq	12718	NA
R	0.97457	NA

B

Figure S1. pH-rate profiles for k_{cat}/K_m and k_{cat} for pig purple acid phosphatase reaction (A) and red kidney bean purple acid phosphatase reaction (B) using the diester methyl p-nitrophenyl phosphate (MpNPP).

MpNPP Processivity. The reaction was initiated in 0.1 M acetate buffer, pH 4.90 (pig PAP) or 0.1 M MES buffer, pH 6.25 (red kidney bean PAP) with a MpNPP concentration of 15 mM. Total reaction volume was 500 μL. A 50 μL aliquot was removed as a blank before addition of enzyme. The reaction was started by addition of enzyme (25 nM) and 50 μL aliquots were removed at intervals and quenched with 250 μL 0.01 N NaOH. From each of these solutions, two 50 μL aliquots were removed. One was added to 250 μL 0.01 N NaOH and measured at 400 nm to detect *p*-nitrophenolate and the other aliquot was added to 125 μL of solution A and allowed to react 5 minutes and then 125 μL of solution B was added to that and the solution equilibrated for 15 minutes to detect inorganic phosphate at 655 nm (as described above). After 60 minutes, pNPP was added to the original reaction mixture and 50 μL aliquots were quenched with 250 μL of 0.01 N NaOH and the amount of *p*-nitrophenolate released was measured at 400 nm. Another reaction mixture was set up with 400 nmol P_i in 500 μL of buffer. Enzyme was added (25 nM) and the reaction was started with addition of MpNPP (15 mM). At intervals, 50 μL aliquots were taken and quenched with 250 μL of 0.01 N NaOH and the amount of *p*-nitrophenolate released was measured at 400 nm. For all reactions that monitored *p*-nitrophenolate concentration, the pH was verified to be >9 before measuring the absorbance. The results are plotted in Figure S2.

A



B

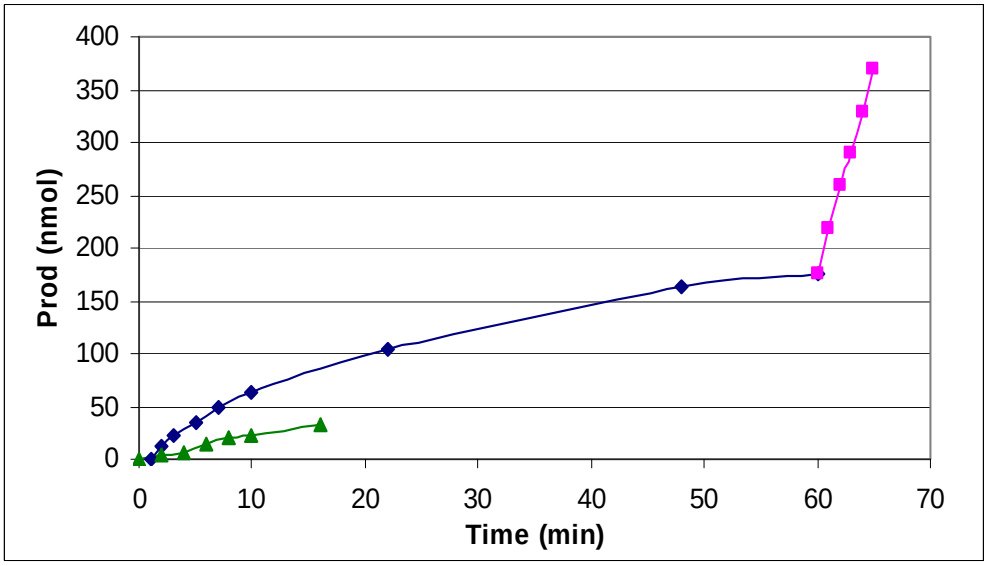


Figure S2. Processivity reactions with pig PAP (A) and red kidney bean PAP (B). In A and B, (♦) is formation of inorganic phosphate and p-nitrophenol from a reaction initiated at [15mM] of the diester substrate MpNPP with 25 nM enzyme, (▲) is formation of p-nitrophenol in a similar experiment but with preincubation with 400 nmol of inorganic phosphate, and (■) is the formation of p-nitrophenol after addition of the monoester substrate pNPP.

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