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Determination of Inhibitor Specificity for Low Molecular Weight Protein Tyrosine Phosphatase Isoforms A and B

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ABSTRACT

The two active isoforms of Low Molecular Weight Protein Tyrosine Phosphatase (LMW-PTP) have inverse cellular effects on cancer. Isoform A has been found to be anti-oncogenic, while overexpression of Isoform B is oncogenic. Inhibitors of Isoform B could have anti-cancer effects, however, it is of utmost importance that these inhibitors exhibit specificity for Isoform B and do not affect the function of Isoform A. Isoform A was recombinantly expressed, purified and characterized in order to compare its structure and function to Isoform B. It is hoped that this characterization, along with the synthesis and screening of potential inhibitors for both isoforms, will streamline the discovery and optimization of new anti-cancer candidates that specifically target LMW-PTP Isoform B.

INTRODUCTION

In humans, there are over 100 known Protein Tyrosine Phosphatases (PTPs), which play many different regulatory roles in cellular growth, proliferation, motility, and gene expression¹. There are four types of PTPs, and though they all have a similar catalytic phosphate binding loop, they are otherwise structurally different². The smallest of this group, the Low Molecular Weight Protein Tyrosine Phosphatases (LMW-PTPs), are 18 kD soluble proteins that are involved in the regulation and control of several signal transducers that modulate cell growth and proliferation such as platelet derived growth factor receptor (PDGFR), epidermal growth factor (EGF), and Ephrin-A2 receptor (EPHA2). Because of this participation in tumorigenic pathways, overexpression of LMW-PTP is indicative of the onset and proliferation of aggressive tumors in breast, colon, bladder, and kidney cancers in humans¹. These cancers exhibit invasive properties and migrate from their origin to other tissues, causing widespread

damage. In addition, studies have shown that over-expression of LMW-PTP aids in the transition of cells from healthy to cancerous³. There are two active isoforms of LMW-PTP (isoforms A and B) expressed through alternative splicing of the ACP1 gene, which, as shown in figure 1, differ structurally only by a loop flanking the active site¹. Recently it has been shown that the two human isoforms of LMW-PTP have different effects on tumor growth. Isoform A has been shown to have anti-oncogenic properties, whereas Isoform B has been shown to be oncogenic⁵. New specific inhibitors of Isoform B that do not affect the activity of Isoform A could potentially be used to treat and prevent the spread of cancer. In addition, many small molecules have been tested for inhibition of Isoform B, but their effect on Isoform A is unknown. Therefore, further characterization of the nuanced differences between the two isoforms could streamline the discovery and optimization of potential anti-cancer agents that specifically target LMW-PTP Isoform B.

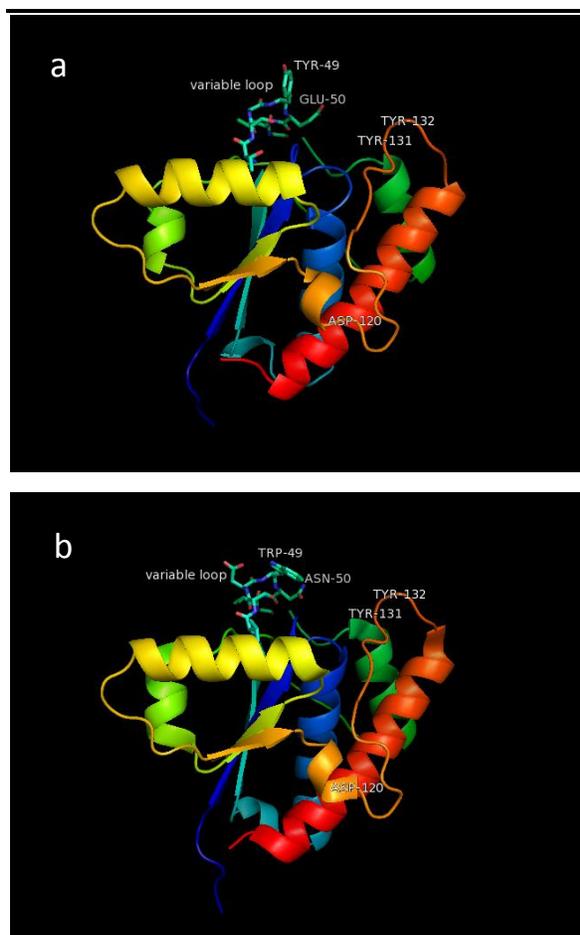


Figure 1. Crystal structures of LMW-PTP: (a) Isoform A (PDB entry 5PNT); (b) Isoform B (PDB entry 1XWW).

Pyridoxal-5'-Phosphate (PLP), the active form of Vitamin B₆, effectively inhibits LMW-PTP Isoform B, but it has numerous cellular roles, such as acting as a cofactor of many enzymes. This lack of specificity makes it an impractical candidate for targeting LMW-PTP. In addition to PLP's lack of selectivity, it is a poor substrate of LMW-PTP because the phosphorus-oxygen bond is easily hydrolyzed by many enzymes present in the cellular environment, leading to limited bioavailability. Using molecular docking software Maestro (Schroedinger, LLC), Pyridoxal 5'-Phosphonate and its analogs, which exhibit similar properties to PLP as

shown in Figure 2, were identified as effective LMW-PTP inhibitors⁴. Pyridoxal- 5'-Phosphonate and its analogs are good candidates because of the stronger, non-hydrolyzable carbon-phosphorus bond⁴. If these phosphonate inhibitors strongly inhibit Isoform B while leaving Isoform A unaffected, they could be potential anti-cancer drug candidates.

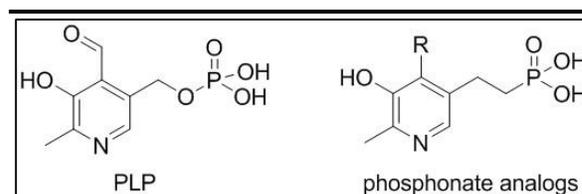


Figure 2. Comparison of PLP and potential inhibitor, Pyridoxal 5'-Phosphonate.

The specificity of inhibitors needs to be evaluated by comparative assays of the two isoforms. Isoform B was previously expressed and many small molecules have been assessed for inhibitory properties. However, no assays have been done with Isoform A, so the specificity of the tested inhibitors is unknown. In order to correct this, Isoform A was expressed, purified, and refolded to yield active enzyme. This makes the Isoform A comparison assays possible, which will lead to a better understanding of the small molecule properties that provide the best specificity for Isoform B.

RESULTS AND DISCUSSION

Expression and Characterization of Isoform A. A pEx expression vector with an amino-terminal polyhistidine tag and synthetic ACP1 mRNA variant 3 gene was obtained from Blue Heron Biotech, LLC, and expressed in BL21 competent *E. coli*. Induction of expression was successful and the protein was purified using a Ni-NTA affinity column. The resulting protein was

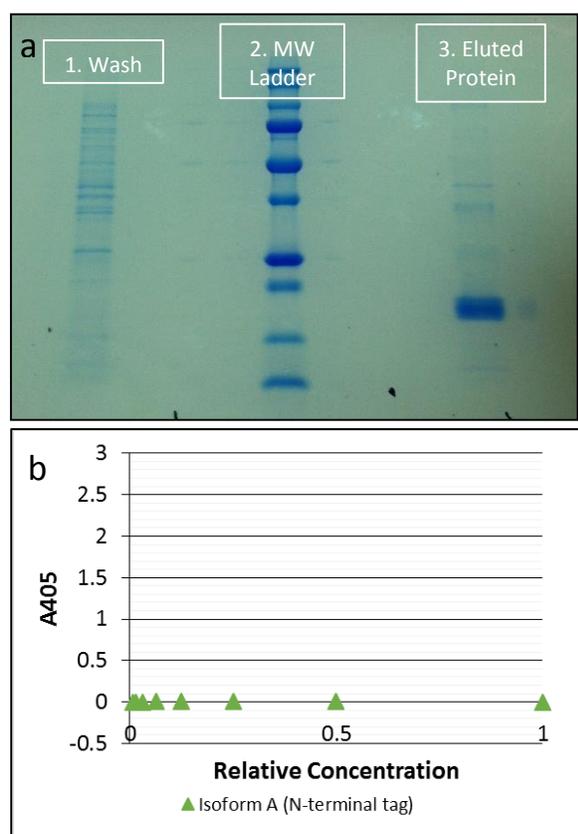


Figure 3. (a) SDS-PAGE analysis of LMW-PTP Isoform A expressed with N-terminal polyhistidine tag. Lane 1 contains protein that did not bind to the column and came through with wash buffer (20 mM Tris, 0.3 M NaCl, 25 mM imidazole), lane 2 contains Precision Plus Protein Dual Color Standard (Bio-Rad), and lane 3 contains protein eluted from the column with elution buffer (20 mM Tris, 0.3 M NaCl, 0.25 M imidazole); (b) Enzyme activity assay results for N-terminally tagged Isoform A (Concentration of 1 = 0.11 mg/mL).

analyzed by SDS-PAGE, and the molecular weight was determined to be approximately 19 kD (Figure 3a). Enzymatic activity was then assessed by an *in vitro* assay that was developed to measure inhibitory properties of small molecules on Isoform B. The

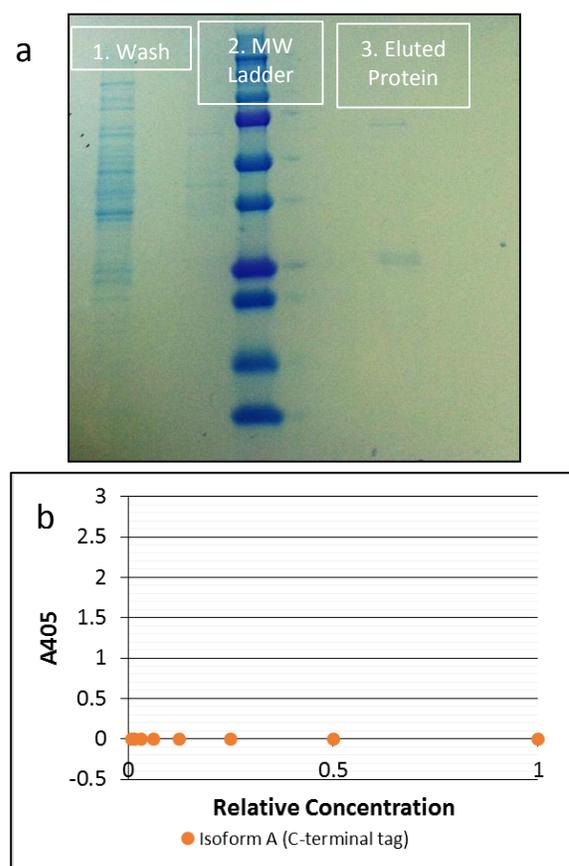


Figure 4. (a) SDS-PAGE analysis of LMW-PTP Isoform A expressed with C-terminal polyhistidine tag. Lane 1 contains protein that did not bind to the column and came through with wash buffer (20 mM Tris, 0.3 M NaCl, 25 mM imidazole), Precision Plus Protein Dual Color Standard (Bio-Rad), and lane 3 contains protein eluted from the column with elution buffer (20 mM Tris, 0.3 M NaCl, 0.25 M imidazole); (b) Enzyme activity assay results for C-terminally tagged Isoform A (Concentration of 1 is approximately 0.01 mg/mL).

substrate used in this assay is p-nitrophenyl phosphate, which is enzymatically hydrolyzed to form the product p-nitrophenol, which, when placed in a basic environment, can be detected by absorbance spectroscopy at 405

nm. This substrate is reportedly compatible with Isoform A⁵. However, when the recombinant Isoform A was assayed, no activity was observed (Figure 3b).

Since the affinity tag was potentially interfering with protein folding during translation, a new pEx vector with a carboxy-terminal polyhistidine tag was obtained and expressed in the same conditions as the amino-terminal vector. However, as shown in Figure 4a, the protein was not as robustly expressed. It was determined through SDS-PAGE analysis that the purified protein had a molecular weight of approximately 30, which is far from the expected molecular weight of 18 kD. The C-terminally tagged protein was then assayed, and once again showed no activity, as shown in Figure 4b.

As moving the affinity tag to the C-terminal end of the protein did not yield active protein, refolding of the protein was attempted. The N-terminally tagged Isoform A was used

since its expression was more reliable and the molecular weight was much nearer to the expected value. The protein was purified in denaturing conditions (6M guanidine) and subjected to nine different conditions with differing levels of the denaturants guanidine and L-arginine as well as differing redox environments. The results of this experiment are shown in Table 1. Refolding of Isoform A measured by recovery of activity in the assay described above was most effectively achieved by a reduced to oxidized glutathione ratio of 10:1 and 0.48 M guanidine. Control experiments were run on Isoform B, which was previously expressed as a glutathione S-transferase (GST) fusion protein, to verify whether refolding conditions interfered with the activity assay. The buffer conditions did not appear to interfere, as Isoform B showed trends very similar to Isoform A in refolding behavior (Table 1).

Table 1. The effect of denaturant and redox conditions on protein refolding measured by recovery of activity (significant recovery denoted by +, modest recovery by 0, and no recovery by -). All buffer conditions also contained 1 mM EDTA, 50 mM Tris, 20 mM NaCl, and 0.8 mM KCl.

Buffer Condition	Guanidine (M)	L-Arginine (M)	Reduced Glutathione (mM)	Oxidized Glutathione (mM)	Isoform A Refolding	Isoform B Refolding
1	0.48	0	20	2	+	+
2	0.48	0.4	20	4	+	+
3	0.48	0.8	10	10	0	0
4	0.98	0	20	4	0	0
5	0.98	0.4	10	10	0	0
6	0.98	0.8	20	2	0	0
7	1.48	0	10	10	-	-
8	1.48	0.4	20	2	-	-
9	1.48	0.8	20	4	-	-

Synthesis of inhibitors. Previous *in silico* docking experiments using Maestro (Schroedinger, LLC) identified pyridoxal 5'

phosphonate and its analogs as good inhibitor candidates. They are being synthesized through the synthetic scheme shown in figure

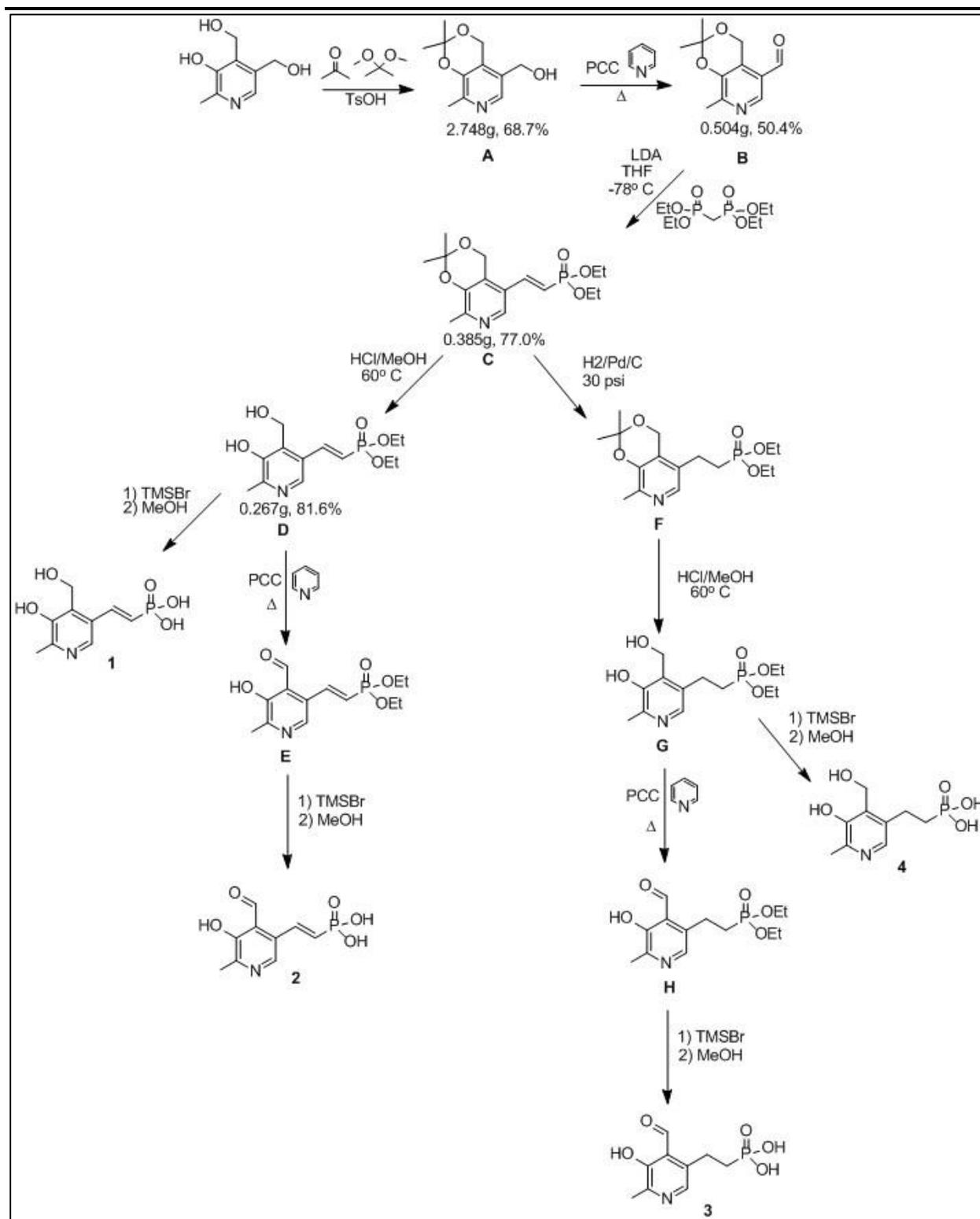


Figure 5. Synthetic scheme for target phosphonate analogs.

5. The addition of the protection group to yield intermediate **A** was unsuccessful unless

everything was completely anhydrous. After distillation of the acetone and 2, 2- dimethoxy

propane, and vacuum drying of the pyridoxine and p-toluenesulfonic acid, intermediate **A** was formed with good yield and verified through ^1H NMR. The oxidation of **A** to **B** catalyzed by pyridinium chlorochromate was fairly successful as indicated by the appearance of an aldehyde peak (10.03 ppm) on a scale less than or equal to 1 gram, but was difficult to scale up. Increased purity of intermediate **A** led to increased yield of intermediate **B**. Tetraethylmethylene diphosphonate was then used in a Horner-Wittig reaction to form **C**, which was effectively purified using a silica gel column with a water/methanol/ethyl acetate v/v=1/10/89 mobile phase. Both **E** and **Z** isomers were formed, but since it is desirable for both the alkane and alkene analogs to be synthesized and tested for inhibitory activity in both isoforms of LMW-PTP, the fractions that were not isomerically pure were saved for hydrogenation. The removal of the isopropylidene protecting group was achieved through reflux with hydrochloric acid in methanol, though in later trials removal of intermediate **D** was required several times to shift the equilibrium and get an acceptable yield. Deprotection of the phosphonate to form compound **1** was difficult due to very limited solubility of intermediate **D** in dichloromethane. Several other solvents were tested, and solubility was very low in all of the solvents compatible with the reactions. Since it was suspected that the pyridyl nitrogen had formed the hydrochloride salt, conversion to the basic form was attempted with sodium hydride, but was not successful. The oxidation of the second alcohol to form intermediate **E** was attempted using the same procedure as the oxidation to form intermediate **B**. Due to the increased polarity of the compound, it was not possible to extract it from the aqueous

layer. The water was therefore removed from the sample on a speedvac in an attempt to isolate the product. However, formation of the aldehyde was not verified either by ^1H NMR or testing with 2, 4-dinitrophenylhydrazine. Once the conditions for the synthesis and purification of compounds **1** and **2** are optimized, the hydrogenation of **C** to **F** will be performed, and the same conditions will be used to obtain compounds **3** and **4**.

CONCLUSION

The expression of LMW-PTP Isoform A and the subsequent refolding procedure allows for production of the active form of the enzyme. This procedure needs to be scaled up before it is a viable method of procuring active Isoform A. Once a larger scale method is optimized, further characterization of this protein, such as the acquisition of circular dichroism (CD) data, will need to be done. This characterization, as well as a detailed comparison to Isoform B, will further streamline the process of finding good, selective inhibitors for Isoform B. Once active Isoform A is produced on a larger scale, all the small molecules previously tested on Isoform B can be tested for inhibition of Isoform A. In addition, once the synthesis of the phosphonate analogs of PLP is complete, they can be tested for inhibition of both isoforms. The results of these assays will help to identify structural motifs that impart inhibitor specificity for one isoform over the other, which will simplify the process of developing effective anti-cancer agents that target LMW-PTP Isoform B.

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EXPERIMENTAL

Introduction of plasmid into cells. The pEx expression vector (0.1 μ L, 50.1 ng/ μ L) obtained from Blue Heron Biotechnology, LLC, was added to BL21 competent *E. coli* cells (New England BioLabs) and incubated on ice for 30 minutes. The cells were then heat shocked at 42°C for 10 seconds, followed by incubation on ice for 5 minutes. Subsequently, 900 μ L of cold SOC medium was added and incubated at 37°C with 225 rpm shaking for 1 hour. Cells (100 μ L, 1X and 0.1X) were plated on LB/ampicillin agar plates and incubated at 37°C for 18 hours.

Protein expression. One colony from the plates from the above step was inoculated into each of 4 sterile aliquots of LB media (5 mL) to which ampicillin (10 μ L, 50 mg/mL) had been freshly added. The cells were then grown in liquid culture at 37°C with 225 rpm shaking for 18 hours. Cells were then archived in 40% glycerol solution and stored at -80°C. The glycerol stock was then grown in liquid culture as previously described. The liquid culture (2 mL) was added to each of 5 aliquots of sterile LB (200 mL) to which ampicillin (400 μ L, 50 mg/mL) was freshly added. The aliquots were then incubated at 37°C with 225 rpm shaking for 4 hours. Absorbance at 600 nm was used to monitor cell growth, and isopropylthio-D-galactoside (IPTG; 200 μ L, 24 mg/mL) was added to each aliquot when A_{600} reached 0.4 to induce protein expression. The aliquots were

incubated at 37°C with 225 rpm shaking for 3 hours, followed by centrifugation at 5000X g for 10 minutes. The pellet was then isolated and resuspended in B-PER Bacterial protein extraction reagent (Thermo Scientific) at 4 mL per gram of cell pellet. The suspension was incubated at room temperature for 15 minutes before being centrifuged at 15000X g for 5 minutes. The soluble protein was then isolated and stored at -80°C.

Purification of protein. The soluble protein extract described above was purified using Ni-NTA resin (Thermo Scientific). Sample was loaded in 1:1 equilibration buffer (20 mM TRIS, 0.3 M NaCl, 10 mM Imidazole, pH 10.0). The column was washed with wash buffer (20 mM TRIS, 0.3 M NaCl, 25 mM Imidazole, pH 10.0) until A_{280} reached baseline. Protein was eluted with elution buffer (20 mM TRIS, 0.3 M NaCl, 0.25 M Imidazole, pH 10.25) and analyzed for purity and molecular weight through SDS-PAGE.

Purification of protein in denaturing conditions. Protein was purified as described above except with 6 M GuHCl present in each buffer.

Enzyme activity assay. E-pure water (180 μ L), 1.0 M sodium acetate/acetic acid buffer solution (25 μ L), and 50 mM p-nitrophenyl phosphate (25 μ L) were added to 0.5 mL microcentrifuge tubes and mixed well. The protein being assayed (20 μ L) was then added at timed intervals and incubated for 30 minutes. The reaction was quenched by transferring 20 μ L of the reaction mixture to a well of a 96 well plate containing 100 μ L of 0.5 M KOH. Absorbance was then measured at 405 nm.

Refolding assay. Protein purified in denaturing conditions was added in 20 μ L increments to each of 9 tubes containing 10 mM EDTA and differing amounts of

guanidine, L-arginine, reduced glutathione, and oxidized glutathione followed by incubation at 4°C for 24 hours. Buffers were obtained from Pierce Biotechnology, Inc. Activity was assessed in the same manner described above except the reaction was run for 18 hours instead of 30 minutes due lower protein concentration.

Synthesis of 3, 4'-o-isopropylidene pyridoxine (A). Distilled acetone (60 mL), distilled 2, 2-dimethoxy propane (40 mL), and vacuum dried pyridoxine HCl (4 g, 19.52 mmol) was added to a 250 mL round bottom flask containing p-toluenesulfonic acid (13.24 g, 78.08 mmol) that had been dried at 115°C on a kugelrohr vacuum distillation apparatus for 6 hours. The yellow-orange suspension that formed was stirred for 24 hours at room temperature in a dry environment maintained by a calcium carbonate filled drying tube. The reaction mixture, now dark brown, was neutralized by solid sodium bicarbonate, filtered, and concentrated. The remaining dark brown solid was dissolved in brine (8 mL) and extracted with dichloromethane. The organic fractions were washed with water and dried over sodium sulfate, and concentrated. The cream colored solid product (2.748 g, 68.7%) was recrystallized by dissolution in ethanol followed by precipitation by the addition of cold diethyl ether. ¹H NMR (CDCl₃, 400 MHz): δ (ppm) 7.84 (s, 1H), 4.93 (s, 2H), 4.55 (s, 2H), 2.37 (s, 3H), 1.54 (s, 6H).

Synthesis of 2,2,8-trimethyl-4H-[1,3]dioxino[4,5-c]pyridine-5-carbaldehyde (B). Compound A (1.0 g, 4.8 mmol), pyridinium chlorochromate (1.03 g, 4.8 mmol), and pyridine (14 mL) were added to a 25 mL round bottom flask and refluxed at 115°C for 90 minutes. After the reaction mixture had cooled to room temperature, diethyl ether (35 mL) was added. The black

precipitate that formed was then filtered off, and the filtrate was then washed with water. The organic fractions were dried with sodium sulfate and concentrated to give a brown oil (0.504 g, 50.4%). ¹H NMR (CDCl₃, 400 MHz): δ (ppm) 10.03 (s, 1H), 8.46 (s, 1H), 5.17 (s, 2H), 2.50 (s, 3H), 1.56 (s, 6H).

Synthesis of diethyl (E)-(2-(2,2,8-trimethyl-4H-[1,3]dioxino[4,5-c]pyridin-5-yl)vinyl)phosphonate (C). Lithium diisopropylamide (1 M in THF, 1.5 mL) in a 25 mL round bottom was cooled to -78°C in a dry ice/acetone bath in a nitrogen environment. Tetraethyl-methylenediphosphonate (0.66 mL, 2.65 mmol) was mixed with tetrahydrofuran (3 mL) and added to the flask and the mixture was stirred for 1 hour. Compound B (0.5 g, 2.414 mmol) was dissolved in 6 mL tetrahydrofuran and added to the flask, followed by stirring for 45 minutes. The reaction mixture was then allowed to slowly warm to room temperature, and it was then refluxed for 2 hours at 100°C. After cooling to room temperature, the reaction was quenched with saturated aqueous ammonium chloride followed by extraction with diethyl ether. The organic fractions were then dried with sodium sulfate, filtered and concentrated to give yellow oil. Purification of crude product was done by silica gel column chromatography (1 x 20 cm, 63-200 mesh silica gel, eluent: water/methanol/ethyl acetate, v/v=1/10/89) to give pure product C (0.385 g, 77%). ¹H NMR (CDCl₃, 400 MHz): δ (ppm) 8.23 (s, 1H), 7.30 (dd, 1H), 6.21 (t, 1H), 4.90 (s, 2H), 4.14 (quint, 4H), 2.43 (s, 3H), 1.55 (s, 6H), 1.36 (t, 6H).

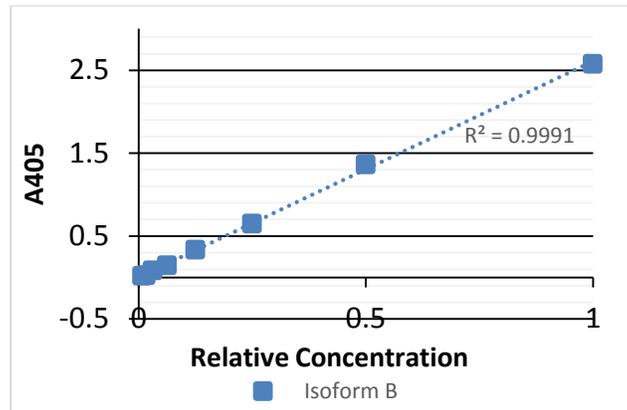
Synthesis of diethyl (E)-(2-(5-hydroxy-4-(hydroxymethyl)-6-methylpyridin-3-yl)vinyl)phosphonate (D). Compound C (.327 g, 9.59 mmol) was dissolved in methanol (0.5 mL) and added to a 25 mL

round bottom flask. Hydrochloric acid (4 mL, 1.25 M in methanol) was added to the flask followed by reflux for 2 hours at 60°C. The reaction mixture was neutralized with solid sodium bicarbonate, filtered, and concentrated. The resulting sticky brown substance was washed with diethyl ether to yield product **D** (.267 g, 81.6%). ¹H NMR (D₂O, 400 MHz): δ (ppm) 7.79 (s, 1H), 7.62 (dd, 1H), 6.32 (t, 1H) 4.74 (s, 2H), 4.06 (quint, 4H), 2.34 (s, 3H), 1.22 (t, 6H).

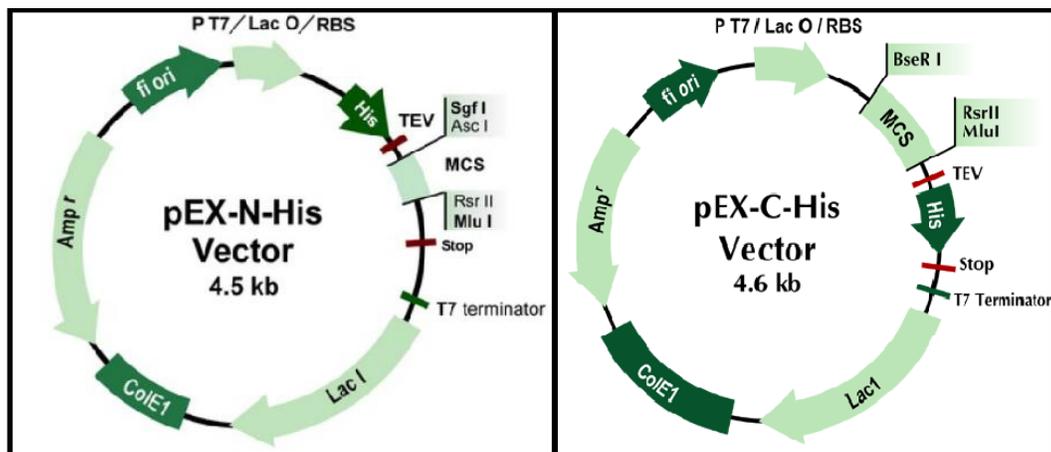
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SUPPLEMENTARY INFORMATION



Supplementary Figure 1. Activity analysis of both N-terminally and C-terminally tagged Isoform A and control Isoform B.



Supplementary Figure 2. Schematics of expression vectors with N-terminal and C-terminal polyhistidine tags.