Chapter 7

Assessing the Biological Activity of the Glucan Phosphatase Laforin

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Abstract

Glucan phosphatases are a recently discovered family of enzymes that dephosphorylate either starch or glycogen and are essential for proper starch metabolism in plants and glycogen metabolism in humans. Mutations in the gene encoding the only human glucan phosphatase, laforin, result in the fatal, neurodegenerative, epilepsy known as Lafora disease. Here, we describe phosphatase assays to assess both generic laforin phosphatase activity and laforin's unique glycogen phosphatase activity.

Key words Glycogen, Lafora disease, Dual-specificity phosphatase

1 Introduction

Lafora progressive myoclonus epilepsy (LD, OMIM 254780) is a rare, fatal, neurological disorder characterized by neurodegeneration, epilepsy, and the accumulation of glycogen-like (polyglucosan) inclusions (named Lafora bodies, LBs) in neurons and other cell types in peripheral tissues. Two main causative genes have been identified, *EPM2A* that encodes the glucan phosphatase laforin and *EPM2B* that encodes the E3-ubiquitin ligase malin. It has been recently described that laforin and malin regulate glycogen biosynthesis [1–4], explaining why defects in any of these two proteins lead to the accumulation of LBs.

Laforin is the only human phosphatase with a carbohydrate-binding domain (CBM; belonging to the CBM20 family) in the same polypeptide [5, 6]. Laforin also contains a C-terminal dual-specificity phosphatase (DSP) domain, and utilizes a strictly conserved cysteine residue (Cys266) at the base of the active site to perform nucleophilic attack on the phosphorus atom of the substrate. The DSPs are a diverse clade of phosphatases within the larger protein tyrosine phosphatase superfamily that includes

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members able to dephosphorylate pSer, pThr, and pTyr as well as members that dephosphorylate non-proteinaceous substrates such as phosphoinositols, nucleic acids, and glycogen or starch [7]. Accordingly, recombinant laforin is able to dephosphorylate in vitro artificial phosphatase substrates such as 4-*para*-nitrophenylphosphate (pNPP) and 3-*O*-methyl fluorescein phosphate (OMFP; a more sensitive substrate of DSPs) [8–10]. Laforin is the founding member of the glucan phosphatase family, phosphatases that dephosphorylate either glycogen or starch [1]. Biochemical data, glycogen analysis from mouse models, and the laforin crystal structure all demonstrate that laforin dephosphorylates glycogen in vivo [11–13].

More than 33 Lafora disease point mutations in laforin have been described to date [14]. These mutations are scattered between laforin's CBM and DSP domains and they affect the phosphatase activity of laforin to different extents [13]. In order to correlate the phosphatase activity found in the mutated forms of laforin with their pathological output, we describe methods to quantify both the generic phosphatase activity of laforin and its specific glycogen phosphatase activity.

2 Materials

Prepare all solutions using double-distilled, deionized MilliQ filtered water. Sterile conditions are not required for in vitro phosphatase assays. All manipulations can be performed at room temperature unless otherwise specified.

2.1 pNPP/OMFP Phosphatase Assay

1. Phosphatase buffer 1: 100 mM Tris–HCl pH: 8, 150 mM NaCl, 10 mM DTT, 0.5 mM OMFP.
2. Phosphatase buffer 2: 0.1 M sodium acetate, 0.05 M Tris, 0.05 M Bis-Tris, pH: 8, 10 mM DTT, 10 mM pNPP.
3. Recombinant protein: At least 0.5 μg of purified phosphatase enzyme.
4. Sodium vanadate (Na_3VO_4) and carbohydrate reagents (i.e., glycogen).
5. 96-Well ELISA plates and multi-plate spectrophotometer reader.

2.2 Glycogen Phosphatase Assay

1. Phosphatase buffer 3 (5× stock): 0.5 M Sodium acetate, 0.25 M Bis, 0.25 M Tris. Adjust to pH 6.5 with HCl.
2. Glycogen purified from rabbit skeletal muscle.
3. Recombinant laforin: 100–500 ng per reaction.

4. Malachite green reagent: Ammonium molybdate tetrahydrate and malachite green carbinol hydrochloride.
5. Tween 20, *N*-ethylmaleimide (NEM), dithiothreitol (DTT).
6. Spectrophotometer.

3 Methods

pNPP is a classical colorimetric substrate used in enzymatic activity assays. Dephosphorylation of pNPP produces an increase in absorbance at the wavelength of 405–410 nm, proportional to pNP formation by hydrolysis. pNPP is highly specific for the active-site pocket of tyrosine protein phosphatases (PTPs); however, it is less efficient in the subfamily of dual-specificity phosphatases (DSPs). DSPs exhibit a shallower active-site pocket in which the long and narrow structure of pNPP, resembling tyrosine-phosphorylated residues, is not as easily accommodated in the DSP phosphatase active site. The OMFP structure comprises a series of aromatic rings that confer a planar structure more appropriate for the active-site cleft in DSPs, which are able to dephosphorylate both pTyr and pSer/Thr residues, as well as other phosphorylated substrates ([15]; Fig. 1). The hydrolysis of OMFP to OMF produces a similar increment in absorbance as that observed in pNP, but in this case at a higher wavelength (around 490 nm).

Either substrate can be added to a phosphatase buffer reaction that includes a specific amount of the purified phosphatase, producing an increment in absorbance proportional to both phosphatase and substrate concentrations. The reaction can be analyzed by a unique measurement after a defined incubation time. However, in the present protocol we describe a method for obtaining enzymatic activity curves of phosphatase activity, by measuring long-term kinetics of increases in absorbance via multiple and consecutive measurements. Thus, the protocol avoids having to stop the reaction (classically with a NaOH solution which abolishes further

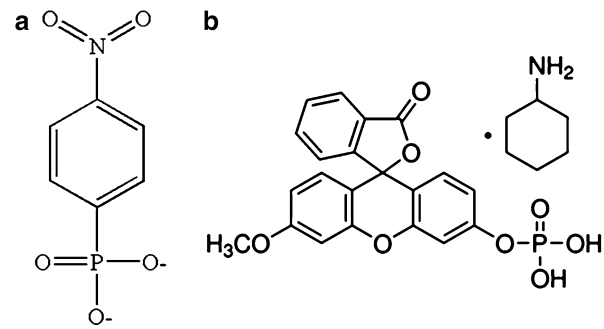

Fig. 1 pNPP (**a**) and OMFP (**b**) structures

dephosphorylation) and provides a first-glance overview of the kinetic features of the analyzed phosphatase. This method allows the evaluation of the effect of different compounds on the phosphatase activity of laforin, as in the case of the inhibitory action of glycogen, described below.

pNPP and OMFP assays (Subheading 3.1) are optimized to define the generic phosphatase activity of a specific phosphatase, i.e., laforin. These assays provide a fast, reproducible, colorimetric measurement of laforin's generic phosphatase activity. However, they do not utilize the biologically relevant substrate glycogen. The third assay in this chapter (Subheading 3.2) is specifically developed and optimized to detect glucan phosphatase activity of laforin using glycogen.

Malachite green-molybdate-based assays have been commonly used in the past for quantifying picomolar amounts of inorganic free phosphate [16–18]. Unlike OMFP and pNPP, malachite green reagent is not an artificial substrate. Instead, it forms a green color complex with free ortho phosphate in aqueous solutions under acidic conditions (*see* Fig. 4a). The malachite green phosphomolybdate complex is stable and provides a fast, nonradioactive way of detecting picomolar amounts of inorganic free phosphate at 620 nm. A recently developed malachite green-based assay is able to determine specific glucan phosphatase activity of laforin [19]. Also, a similar assay has been employed to detect phosphate release from laforin's physiological substrate, glycogen [11, 13]. Subheading 3.2 describes the methodology for quantifying phosphate release of rabbit skeletal muscle glycogen by laforin.

3.1 pNPP/OMFP Phosphatase Assay

1. *Preparation of phosphatase buffer*. First, determine the optimum pH for each phosphatase to be assayed (*see* **Note 1**). In the case of laforin the optimal pH is 8 [20]. DTT should be added just before use (in the case of laforin at 10 mM). A stock buffer solution (phosphatase buffers 1 and 2; *see* Subheading 2.1) should be prepared to use it as reaction buffer and to dilute the substrate of the reaction (pNPP/OMFP; *see* **Note 2** for details on solubility) and to prepare enzyme dilutions if needed. Once prepared, the buffer should be kept at 4 °C or in ice prior to use. The buffer should be freshly prepared, and DTT added immediately before the reaction starts.
2. *Preparation of protein dilutions*. Protein preparations should be in the range of 0.5–5 μg/μl (*see* **Notes 3** and **4**). In a standard phosphatase reaction, a minimum amount of 1 μg of protein will be required to obtain a relevant phosphatase activity. In the case of laforin we follow the reaction for 1 h for a good measurement of phosphatase activity under standard conditions.
3. *Preparation of the plates*. The final volume for the reaction is 200 μl. Each well will contain 100 μl of phosphatase buffer with purified protein (plus any other substance to be assayed,

e.g., vanadate or carbohydrates). One well should be left for a blank reaction containing only phosphatase buffer (*see* **Note 5**). The plate can be maintained on ice or at 4 °C.

4. *Preparation of the plate reader*. An automated program should be prepared to produce a series of consecutive measurements. The measuring parameters (interval between measurements; total duration of the assay) are dependent on the intrinsic phosphatase activity of the phosphatase used, the concentration of substrate, and the assayed conditions (i.e., presence of inhibiting compounds). These variables should be empirically evaluated in order to define the time needed to reach saturation, thus establishing the linear range of the reaction (*see* Fig. 2a for an example). The temperature should be set to the optimal for each phosphatase (37 °C for laforin and most human PTPs). Make sure that the reader has reached the desired temperature before starting the assay. For pNPP assays, absorbance should be measured at 405 nm; for OMFP assays, the absorbance measured is 490 nm.

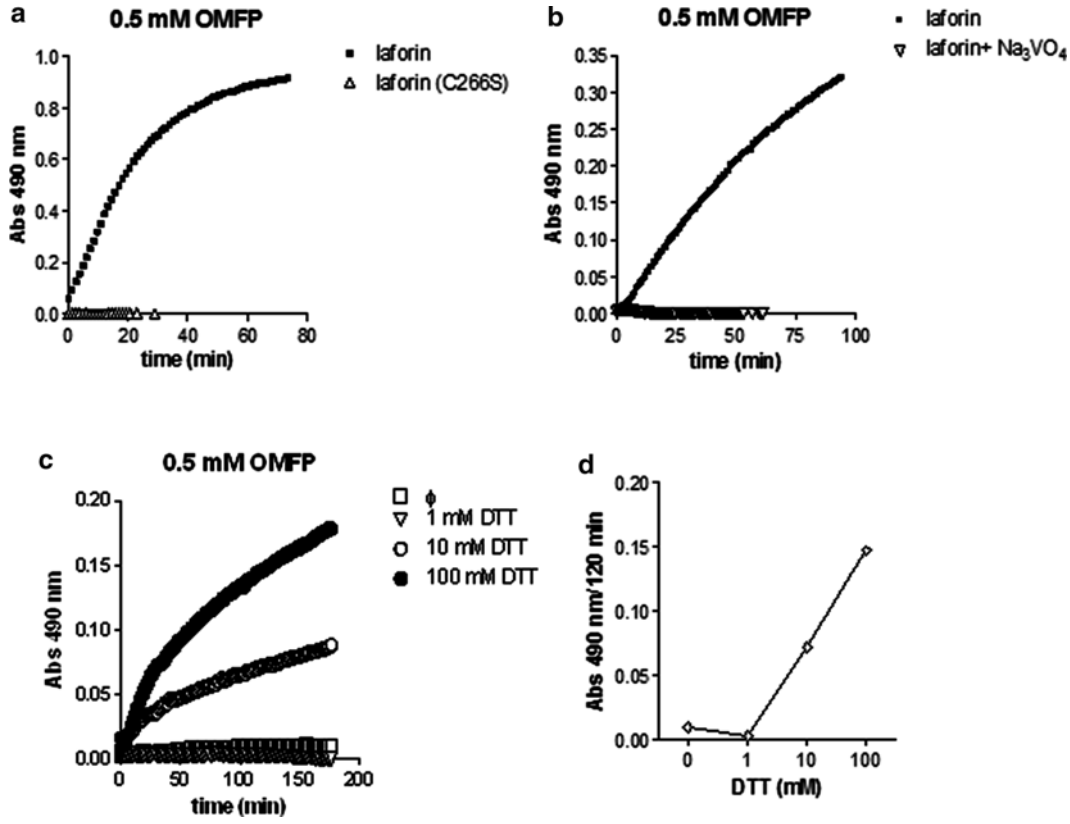

Fig. 2 Laforin phosphatase shows a typical enzymatic kinetics for OMFP dephosphorylation and a cysteine-dependent catalytic mechanism (**a**). Plots are shown for wild-type laforin and for the C226S laforin mutation, targeting the catalytic cysteine of the enzyme. (**b**) Vanadate inhibition. (**c**) Reducing environment dependence. (**d**) Plot of the maximal absorbance reached at different DTT concentrations

5. *Starting the reaction*. Since both pNPP and OMFP suffer slow but significant spontaneous dephosphorylation, all reactions including the blank should be started at the same time (*see* **Note 3**). Use a multichannel pipet to add 100 μl of reaction buffer containing the corresponding chromogenic substrate (*see* **Note 2**) quickly and equally to all the wells. Immediately after adding the substrate, initiate measuring via the plate reader.
6. *Displaying the results*. Absorbance values should be displayed in columns next to the corresponding time interval. If the reader software does not automatically normalize values, the absorbance value obtained for the blank well in any given time should be subtracted from the corresponding time interval in the rest of wells. Representing absorbance values in the Y-axis and time interval values in the X-axis will produce the curve for phosphatase activity (Fig. 2). Obtaining completely saturated curves is convenient for the determination of kinetic parameters (*see* **Note 6**). To compare the global dephosphorylation capacity of the enzyme, regardless of kinetics, maximum absorbance values can be displayed (*see* Fig. 2d). In order to compare different proteins or substrates, the absorbance values obtained with this method should be normalized and transformed adequately, converting absorbance values into amount of substrate hydrolyzed (the use of a pNP or OMF standard curve would be necessary) per protein amount and time unit; this final value is usually expressed as specific activity of the enzyme. The glucan phosphatase laforin displays a measurable phosphatase activity using pNPP or OMFP as a substrate, although OMFP is a more efficient substrate.

   Sodium vanadate is a specific inhibitor of cysteine-based PTPs that mimics the phosphate binding to the catalytic pocket of the enzyme, completely abolishing phosphatase activity [21]. Hence, addition of a final concentration of 1 mM sodium vanadate to the buffer solution provides a valuable tool as a negative control to distinguish artificial substrate dephosphorylation or contamination of the purified protein with non-PTP phosphatases (Fig. 2b).

   It has been described that glycogen decreases laforin activity against pNPP [9]. This effect could be explained by the binding of laforin to glycogen, which could interfere with the entry of the substrate to the catalytic pocket. The protocol described above can be used to assess the effect of any substance on the phosphatase reaction, just by including the compound in the phosphatase reaction buffer. As an example, we describe the use of the method to test the decrease in laforin OMFP activity by glycogen (Fig. 3). In these experiments, increasing concentrations of glycogen were used in the phosphatase reaction together with OMFP.

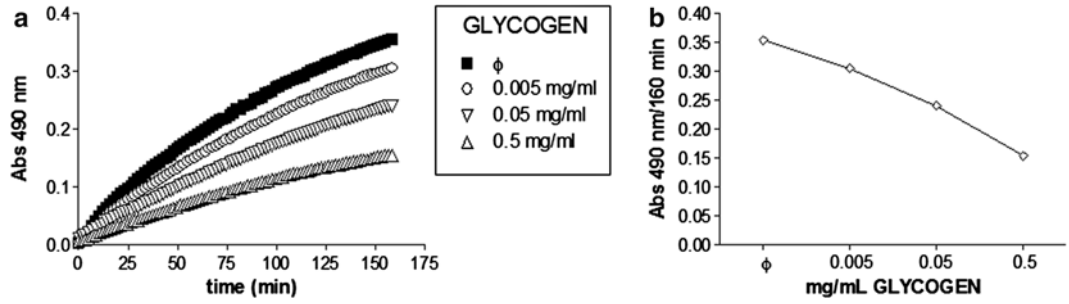

Fig. 3 Glycogen inhibition of the glucan phosphatase laforin

3.2 Glycogen Phosphatase Assay

1. *Preparation of assay buffer*. Similar to Subheading 3.1, it is important to determine the optimum pH for glycogen phosphatase activity. Laforin is active within a pH range of 6.5–8.0. The optimum pH for laforin's glycogen phosphatase activity is pH 6.5. Make a 5× assay buffer stock (phosphatase buffer 3; *see* Subheading 2.2) at the desired pH and store at 4 °C. Prior to the experiment, make a fresh 1× assay buffer solution with 10 mM DTT to prepare the necessary protein dilutions or substrate solutions (*see* **Note 7**).
2. *Standard curve for quantifying glycogen phosphatase activity*. Make a 100 mM stock solution of KH_2PO_4 using ultrapure H_2O (*see* **Notes 7** and **8**). Make serial dilutions to obtain solutions containing 100 pmol to 1 nmol of KH_2PO_4. Mix 40 µl of these solutions with 80 µl of malachite green reagent (with 0.01% Tween 20). Tween 20 stabilizes the malachite green phosphomolybdate complex for up to 48 h [22]. Incubate reactions for 30 min and measure absorbance at 620 nm using a spectrophotometer.
3. *Preparation of protein dilutions*. Protein dilutions should be in the range of 0.1–1.0 µg/µl. For example, laforin has a higher specific phosphatase activity compared to the plant glucan phosphatases (SEX4 and LSF2); therefore, the amount of protein must be appropriately calibrated to be within the linear range [2, 23, 24]. In a standard glucan phosphatase assay with amylopectin, a minimum amount of 100 ng of protein incubated for 10 min is sufficient to obtain absorbance values within the linear range [25]. However, rabbit skeletal muscle glycogen contains lower amounts of phosphate than potato amylopectin and the assay's color development depends on the amount of free phosphate present in the reaction (*see* **Note 9**). Therefore, begin the assay initially with 100 ng of protein and incubate for 30 min with 40–100 µg of glycogen to see whether there is detectable phosphatase activity under standard conditions. However, longer incubation times and higher substrate concentrations may be necessary depending on the amount of phosphate groups present in the rabbit skeletal muscle glycogen (*see* **Note 10**).

4. *Preparation of malachite green reagent.* Prepare malachite green reagent by making 1 volume of 4.2 % (w/v) ammonium molybdate tetrahydrate in 4 M HCl. Add 3 volumes of 0.045 % (w/v) malachite green carbinol hydrochloride (*see* **Note 7**). Stir the contents for 30 min at room temperature and filter with Whatman filter paper. Malachite green reagent can be stored at 4 °C away from light for a few weeks (*see* **Note 8**). Just before the experiment is initiated, add 10 % (v/v) Tween 20 to an aliquot of malachite green reagent to make the final concentration of Tween 20 to 0.01 % (v/v).
5. *Preparation of reaction tubes.* Each reaction is performed in a final volume of 20 µl, with six replicates for each enzyme to be tested. First, calculate the number of reactions to be performed and make a master mixture with all the assay components except the enzyme, as it will be added to start the reaction. For one reaction, mix 4 µl of 5× assay buffer of the desired pH, 9 µl of 5 mg/ml rabbit skeletal muscle glycogen, and 2 µl of 100 mM DTT in a 0.5 ml Eppendorf tube. These components will give a final concentration of 100 mM sodium acetate, 50 mM Bis-Tris, 50 mM Tris–HCl, 5 mM DTT, and 45 µg of rabbit skeletal muscle glycogen per reaction.
6. *Starting the assay.* The reaction starts when 5 µl of diluted enzyme (100–500 ng) are mixed with 15 µl of the assay components in the 0.5 ml Eppendorf tube. Timing is critical for the assay and a timer should be initiated once enzyme is added to the first reaction tube. Add enzyme to a tube in 30-s time intervals for subsequent reactions. Incubate all reactions for 30 min at 37 °C. Maintaining a constant and consistent incubation time for each tube is important in obtaining accurate and reproducible data (*see* **Note 11**).
7. *Terminating the assay.* The enzymatic activity of laforin can be stopped by adding 20 µl of 0.1 M NEM to the reaction mixture and vortexing the tube for 10 s. NEM modifies thiol groups and thereby irreversibly inhibits the phosphatase activity of all protein tyrosine phosphatases, including laforin [26]. Add 80 µl of malachite green reagent to each of the reaction tubes and vortex for 10 s. Incubate all reactions for 30 min at room temperature before measuring the absorbance at 620 nm using a spectrophotometer (*see* **Note 12**). The glucan phosphatase laforin has robust glycogen phosphatase activity while the human proteinaceous phosphatase VHR lacks this activity (Fig. 4b). Mutation of the laforin catalytic cysteine to serine (C266S) ablates the laforin glycogen phosphatase activity (Fig. 4b). Lafora disease patient mutations in the CBM (W32G) or DSP (T142A) domains dramatically decrease the laforin glycogen phosphatase activity (Fig. 4b) (*see* **Notes 13** and **14**).

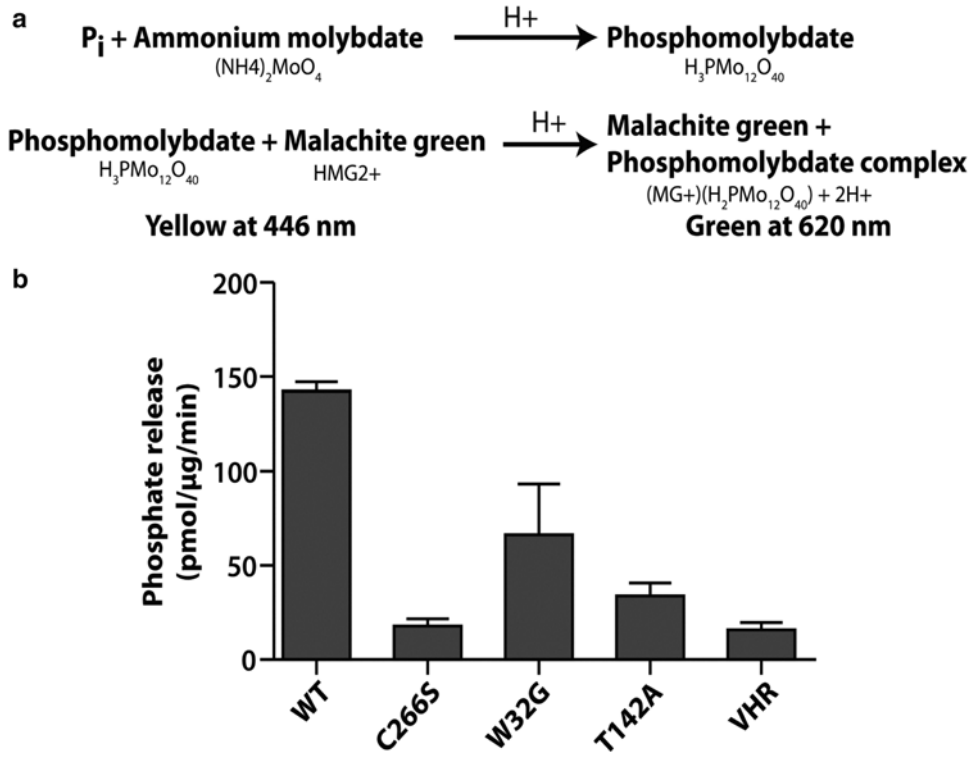

Fig. 4 Malachite green-based glycogen phosphatase assay. (**a**) When phosphate monoesters in glycogen are hydrolyzed, the free phosphate reacts with ammonium molybdate. At a low pH, the basic malachite green dye forms a complex with phosphomolybdate and shifts to its absorption maximum. This complex is stabilized by the addition of Tween 20, allowing for colorimetric detection at 620 nm that is linear with as little as 50–1000 pmol of P_i. (**b**) Specific activity of laforin wild type, laforin mutants, and human VHR against glycogen. A total of 100 ng of protein was incubated with 45 μg glycogen for 20 min. Each bar is the mean ± SEM of five replicates; $p < 0.05$

4 Notes

1. It is critical to assess the optimal pH for each phosphatase; the method presented in this chapter provides a straightforward way to assay different pH values in the phosphatase buffer, by comparing the enzyme kinetics in the same plate and experimental conditions.
2. The substrate for the phosphatase reaction should be prepared in a 2× concentration. Each substrate presents some particularities that should be considered. To prepare the *pNPP solution*, pNPP can be easily diluted in water, in concentrations ranging from 5 to 100 mM. For the reaction described here, a solution of 20 mM DTT in phosphatase reaction buffer should be prepared. When a volume of this solution is mixed with the same volume of phosphatase reaction buffer containing the

protein, a final concentration of 10 mM in the assay will be obtained. To prepare the *OMFP solution*, 1.05 mg OMFP should be first diluted in 1 ml methanol (other organic solvents can be assayed) and vortexed vigorously in order to obtain a homogeneous, milky-faint yellow 2 mM solution. Then, a 1:1 solution in phosphatase reaction buffer will produce a colorful, yellow 1 mM preparation that can be used as the 2× substrate solution. When a volume of this solution is mixed with the same volume of phosphatase reaction buffer containing the protein, a final concentration of 0.5 mM OMFP will be obtained.

3. The progress of the reaction must be observed to determine the proper measuring intervals. For very active phosphatases, a 10 mM pNPP solution can produce saturation within minutes; hence intervals in the range of 10–30 s should be sufficient to obtain a nice curve. In the case of laforin, the best curves are obtained using 0.5 mM OMFP as a substrate, and intervals in the minute range can lead to a nice curve in a period of ½ to 1 h.
4. Recombinant proteins often present either carboxy- or amino-terminal tags; it should be taken into account that these tags could affect phosphatase activity of the enzyme.
5. Given the catalytic requirements of the active site in cysteine-based PTPs, reducing conditions are required. Thus, reducing agents such as DTT, TCEP, or β-mercaptoethanol should be added to reaction mixtures. The dependence of each phosphatase to specific redox conditions should be assessed, since reducing agents can affect also the oligomerization state of proteins. In the example of the glucan phosphatase laforin, redox conditions affect oligomerization, and also affect phosphatase activity [20, 27] (Fig. 2c, d).
6. Performing phosphatase assays as those represented by Fig. 2 and using different substrate concentrations are useful to determine Michaelis-Menten constants. When using different substrate concentrations, a blank for each condition should be left without protein, since both pNPP and OMFP spontaneously dephosphorylate and dephosphorylation rate can be affected by concentration, as well as by any other substance added to the reaction buffer when comparing conditions and/or enzyme modifiers.
7. Malachite green reagent detects free phosphate in aqueous solutions. Prepare all solutions using ultrapure water that has the sensitivity of 18 MΩ cm at 25 °C. Diligently follow cleaning procedures as many soaps and dish detergents contain phosphate. All glassware should be rinsed extensively with ultrapure water before use to avoid any contaminations and to decrease the background absorbance of the assay.

8. Malachite green reagent is light sensitive and changes color from yellow to green/brown over time if it is exposed to light. Prepare new reagent in a timely manner before there is a color change, typically within weeks. Also, small divalent cations such as magnesium, calcium, and manganese have lower solubility in the presence of phosphate. Check water purity if precipitation occurs. A yellow color malachite green reagent with no precipitate gives reproducible and accurate measurements.
9. Mammalian glycogen contains small amounts of covalently attached phosphate groups. Use of commercially available glycogen as a substrate has not been successful. It may be due to the low availability/accessibility of phosphate groups in certain types of glycogen [1]. The Roach group reported that rabbit skeletal muscle glycogen is a substrate for malachite green-based glycogen dephosphorylating assays and they have published elegant methodology to purify glycogen from rabbit skeletal muscle [11, 28].
10. Glycogen consists of a series of concentric rings of glucose chains. This unique structure may hinder laforin's activity to remove phosphate groups in the inner regions of glycogen. Increased sensitivity may be achieved by adding alpha amylase and amyloglucosidase enzymes to the substrate to disrupt glycogen structure [11].
11. Low levels of color development may be due to inadequate amounts of substrate, enzyme, or incubation times. Optimize the assay with each preparation of rabbit skeletal muscle glycogen.
12. If a heavy precipitation is observed in the reaction with glycogen but not in the standard curve reactions, then there are two possibilities to consider. (a) The precipitation could be due to higher phosphate content on the rabbit skeletal muscle glycogen. If this is the case, then dilute the glycogen stock, using less glycogen per reaction. (b) The precipitation could be due to phosphate contamination in the enzyme or glycogen samples.
13. Kinetic studies are possible with the assay; however, interpreting kinetics of glycogen dephosphorylation by laforin is complex [11]. In addition to glycogen being polydispersed, laforin is a dimer containing two catalytic sites and two carbohydrate-binding modules. Unlike OMFP/pNPP assay described above, the progress of the color development cannot be assayed over time. Separate reactions are needed for each time point.
14. This method can be easily amenable to a 96-well plate or 396-well plate format if high-throughput detection is needed.

Acknowledgments

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