

Notes & Tips

Colorimetric determination of pure Mg^{2+} -dependent phosphatidate phosphatase activity

Tara Havriluk^{a,1}, Fred Lozy^{a,1}, Symeon Sinioglou^b, George M. Carman^{a,*}

^a Department of Food Science and Rutgers Center for Lipid Research, Rutgers University, New Brunswick, NJ 08901, U.S.A.

^b Cambridge Institute for Medical Research, University of Cambridge, CB2 2XY Cambridge, U.K.

Received 3 August 2007

Available online 1 September 2007

Abstract

The malachite green-molybdate reagent was used for a colorimetric assay of pure Mg^{2+} -dependent phosphatidate phosphatase activity. This enzyme plays a major role in fat metabolism. Enzyme activity was linear with time and protein concentration, and with the concentration of water-soluble dioctanoyl phosphatidate. The colorimetric assay was used to examine enzyme inhibition by phenylglyoxal, propranolol, and dimethyl sulfoxide. Pure enzyme and a water-soluble phosphatidate substrate were required for the assay, which should be applicable to a well-defined large-scale screen of Mg^{2+} -dependent phosphatidate phosphatase inhibitors (or activators). © 2007 Elsevier Inc. All rights reserved.

Mg^{2+} -dependent PA^2 phosphatase (PAP1, 3-*sn*-phosphatidate phosphohydrolase; EC 3.1.3.4) catalyzes the dephosphorylation of PA yielding diacylglycerol and P_i [1–5]. PAP1 generates the diacylglycerol used for the synthesis of triacylglycerol and the diacylglycerol used for the synthesis of phosphatidylethanolamine and phosphatidylcholine via the Kennedy pathway [4,5]. Recent studies have identified human lipin 1 as a PAP1 enzyme [6]. In a mouse model, lipin 1 deficiency prevents normal adipose tissue development that results in lipodystrophy (i.e., loss of body fat) and insulin resistance, whereas excess lipin 1 promotes obesity and insulin sensitivity [7,8]. That human lipin 1 is a PAP1, the penultimate enzyme in the pathway to synthesize triacylglycerol from PA, provides a mechanistic basis for how lipin 1 regulates fat metabolism in mammalian cells. Accordingly, PAP1 activity may represent an important pharmaceutical target for the control of body fat in humans.

A large-scale search of inhibitors (or activators) of PAP1 activity requires a sensitive and convenient enzymatic assay. The radioactive assays [9,10] currently used to measure PAP1 activity are not conducive to a high-throughput screen of potential drugs to control enzyme activity. Aside from the radioactive nature of these assays, they require a chloroform–methanol–water phase partition to separate water-soluble $^{32}P_i$ from chloroform-soluble [^{32}P]PA or the separation (e.g., thin-layer chromatography) of [3H]diacylglycerol from [3H]PA [9,10]. We have developed a nonradioactive colorimetric PAP1 assay based on orthophosphate analysis using the malachite green–molybdate reagent [11,12]. The reagent forms a colored complex with orthophosphate that can be measured spectrophotometrically. This colorimetric assay is not suitable for use with cell extracts or with crude PAP1 preparations because of a high phosphate background, hence the reason for using the radioactive assays [9,10]. However, the colorimetric assay was suitable for measuring the activity of pure PAP1, and the assay was amenable to screening enzyme inhibitors.

Saccharomyces cerevisiae PAH1-encoded PAP1 [6] was expressed and purified to homogeneity as described by O'Hara *et al.* [13]. The yeast enzyme was used as a model PAP1 to develop the colorimetric assay. Pure PAP1 (12 ng) was used in a standard reaction mixture that

* Corresponding author. Fax: +1 (732) 932 6776.

E-mail address: carman@aesop.rutgers.edu (G.M. Carman).

¹ These authors contributed equally to this work.

² Abbreviations used: PA, phosphatidate; PAP1, Mg^{2+} -dependent PA phosphatase; DiC8 PA, dioctanoyl phosphatidate; DiC18 PA, dioleoyl phosphatidate; DMSO, dimethyl sulfoxide.

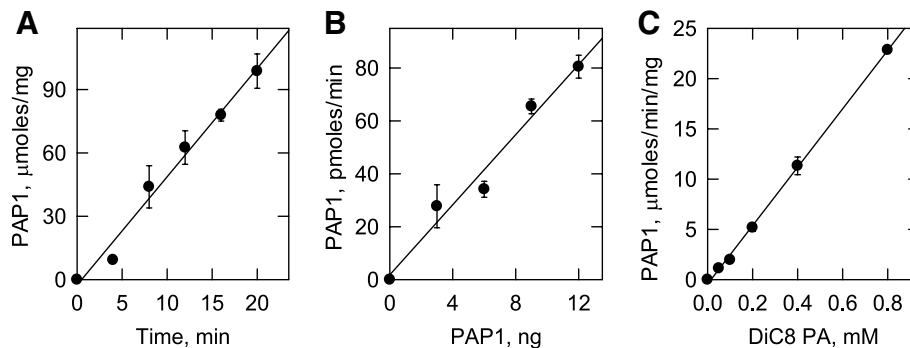


Fig. 1. Time course, enzyme concentration, and substrate concentration dependencies of the colorimetric assay on pure PAP1 activity. (A) PAP1 activity was measured with 0.2 mM DiC8 PA and 12 ng of pure enzyme for the indicated time intervals. (B) PAP1 activity was measured for 20 min with 0.2 mM DiC8 PA and the indicated amounts of pure enzyme. (C), PAP1 activity was measured for 20 min with 12 ng of pure enzyme and the indicated amounts of DiC8 PA. The lines were the result of a least-squares analysis of the data. The data shown were derived from triplicate determinations \pm SD.

contained 50 mM Tris–HCl buffer (pH 7.5), 1 mM $MgCl_2$, and 0.2 mM DiC8 PA (Avanti Polar Lipids) in a total volume of 0.1 ml. Unless otherwise indicated, all enzyme assays were conducted in triplicate for 20 min at 30 °C. The malachite green–molybdate reagent [11,12] was prepared as described by Mahuren *et al.* [14]³. The reaction was terminated by the addition of 200 μ l of the malachite green–molybdate reagent; 30 μ l of 1% polyvinyl alcohol was then added to the reaction to stabilize the color complex [14]. The reaction mixture was vortexed briefly and the absorbance of the solution was measured with a spectrophotometer at 660 nm. The color was stable for at least 1 h. The amount of orthophosphate formed was quantified from a standard curve using 0.5–4 nmol of potassium phosphate. The enzyme reactions and standard curve were performed in new plastic test tubes. This obviated the concern of interfering phosphates from tubes that have been washed with detergent [14]. Statistical analyses were performed with SigmaPlot software.

The PAP1 colorimetric assay was linear with respect to time (Fig. 1A) and enzyme concentration (Fig. 1B), indicating that the enzyme followed zero order kinetics under these reaction conditions. In addition, PAP1 activity was linear with respect to the DiC8 PA substrate at concentrations of 0.05–0.8 mM (Fig. 1C). Indeed, the analysis of potential inhibitors would be best carried out at a low substrate concentration at or below (e.g., <1 mM) the K_m value for the substrate.

For comparison, PAP1 activity was measured by following the release of $^{32}P_i$ from chloroform-soluble [^{32}P]DiC18 PA (10,000 cpm/nmol) as described by Carman and Lin [9]. In this assay, 0.2 mM [^{32}P]DiC18PA was solubilized with 2 mM Triton X-100 to give a surface concentration of 9 mol % [9]. The specific activity (5.6 ± 0.6 μ mol/min/mg) of the pure PAP1 enzyme determined with the colorimetric assay was in good agreement with the specific activity (5.2 ± 0.1 μ mol/min/mg) determined with the radioactive

assay. Whereas these values were close, it is difficult to make a comparison of an enzyme activity measured with a water-soluble substrate with that measured with a detergent-solubilized substrate [15].

The suitability of the colorimetric assay for screening for PAP1 inhibitors was tested with two known inhibitors of the enzyme, phenylglyoxal and propranolol [16,17]. Phenylglyoxal is an arginine reactive compound [18], whereas propranolol is thought to interact with the Mg^{2+} binding site of the enzyme [19]. Phenylglyoxal (Fig. 2A) and propranolol (Fig. 2B) inhibited PAP1 activity in dose-dependent manners with IC_{50} values of 1.3 and 0.2 mM, respectively. These values were in the same range determined for PAP1 activity measured by the radioactive assay with [^{32}P]DiC18PA [17].

Some enzyme inhibitors are not soluble in aqueous buffers and are commonly solubilized in DMSO. Accordingly, the effect of DMSO on PAP1 activity was tested using the colorimetric assay. The addition of DMSO to the reaction mixture resulted in a dose-dependent inhibition of PAP1 activity (Fig. 2C). A 1% concentration of DMSO is commonly used for screens of water-insoluble inhibitors, and only 25% of PAP1 activity was lost using that concentration (Fig. 2C). Thus, a significant amount of PAP1 activity would still be present in a control reaction when potential inhibitors were solubilized in 1% DMSO.

Detergents (e.g., Triton X-100 and Tween 20) that were used to solubilize water-insoluble DiC18 PA [9] caused a high background color. This problem was solved by using water-soluble DiC8 PA as substrate. That pure PAP1 was a requirement for the colorimetric assay might be considered a major limitation. However, this limitation is also a major benefit because the screen for inhibitors (or activators) should be carried out under well-defined conditions that are free from other reactions that might generate orthophosphate and interfere with the interpretation of results. Obtaining large quantities of pure PAP1 enzyme is facilitated by the overexpression and purification of yeast [13] and human proteins [6]. As advertised by commercial vendors of the malachite green–molybdate reagent, the PAP1 colorimetric assay was applicability to a 96-well format

³ The malachite green–molybdate reagent (PiBlue) commercially prepared by BioAssay Systems worked equally as well as the reagent prepared in the laboratory.

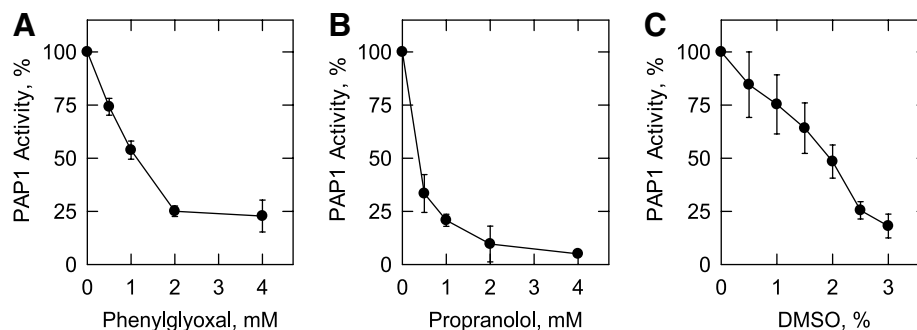


Fig. 2. Effects of phenylglyoxal, propranolol, and DMSO on PAPI activity measured with the colorimetric assay. PAPI activity was measured under standard conditions in the presence of the indicated concentrations of phenylglyoxal (A), propranolol (B), and DMSO (C). The data shown were derived from triplicate determinations \pm SD.

(data not shown), which should facilitate a large-scale screen of PAPI inhibitors (or activators).

Acknowledgments

This work was supported in part by United States Public Health Service, National Institutes of Health Grant GM-28140 (to G.M.C.) and by a Wellcome Trust Career Development Fellowship in Basic Biomedical Science (to S.S.). We thank Gil-Soo Han for helpful discussions during the course of this work.

References

- [1] S.W. Smith, S.B. Weiss, E.P. Kennedy, The enzymatic dephosphorylation of phosphatidic acids, *J. Biol. Chem.* 228 (1957) 915–922.
- [2] D.N. Brindley, Intracellular translocation of phosphatidate phosphohydrolase and its possible role in the control of glycerolipid synthesis, *Prog. Lipid Res.* 23 (1984) 115–133.
- [3] G.M. Carman, Phosphatidate phosphatases and diacylglycerol pyrophosphate phosphatases in *Saccharomyces cerevisiae* and *Escherichia coli*, *Biochim. Biophys. Acta* 1348 (1997) 45–55.
- [4] M.G. Kocsis, R.J. Weselake, Phosphatidate phosphatases of mammals, yeast, and higher plants, *Lipids* 31 (1996) 785–802.
- [5] M. Nanjundan, F. Possmayer, Pulmonary phosphatidic acid phosphatase and lipid phosphate phosphohydrolase, *Am. J. Physiol. Lung Cell Mol. Physiol.* 284 (2003) L1–L23.
- [6] G.-S. Han, W.-I. Wu, G.M. Carman, The *Saccharomyces cerevisiae* lipin homolog is a Mg^{2+} -dependent phosphatidate phosphatase enzyme, *J. Biol. Chem.* 281 (2006) 9210–9218.
- [7] M. Peterfy, J. Phan, P. Xu, K. Reue, Lipodystrophy in the fld mouse results from mutation of a new gene encoding a nuclear protein, lipin, *Nat. Genet.* 27 (2001) 121–124.
- [8] J. Phan, K. Reue, Lipin, a lipodystrophy and obesity gene, *Cell Metab* 1 (2005) 73–83.
- [9] G.M. Carman, Y.-P. Lin, Phosphatidate phosphatase from yeast, *Methods Enzymol.* 197 (1991) 548–553.
- [10] A. Martin, A. Gomez-Munoz, Z. Jamal, D.N. Brindley, Characterization and assay of phosphatidate phosphatase, *Methods Enzymol.* 197 (1991) 553–563.
- [11] K. Itaya, M. Ui, A new micromethod for the colorimetric determination of inorganic phosphate, *Clin. Chim. Acta* 14 (1966) 361–366.
- [12] P.P. van Veldhoven, G.P. Mannaerts, Inorganic and organic phosphate measurements in the nanomolar range, *Anal. Biochem.* 161 (1987) 45–48.
- [13] L. O'Hara, G.S. Han, S. Peak-Chew, N. Grimsey, G.M. Carman, S. Siniosoglou, Control of Phospholipid Synthesis by Phosphorylation of the Yeast Lipin Pah1p/Smp2p Mg^{2+} dependent Phosphatidate Phosphatase, *J. Biol. Chem.* 281 (2006) 34537–34548.
- [14] J.D. Mahuren, S.P. Coburn, A. Slominski, J. Wortsman, Microassay of phosphate provides a general method for measuring the activity of phosphatases using physiological, nonchromogenic substrates such as lysophosphatidic acid, *Anal. Biochem.* 298 (2001) 241–245.
- [15] G.M. Carman, R.A. Deems, E.A. Dennis, Lipid signaling enzymes and surface dilution kinetics, *J. Biol. Chem.* 270 (1995) 18711–18714.
- [16] Z. Jamal, A. Martin, A. Gomez-Munoz, D.N. Brindley, Plasma membrane fractions from rat liver contain a phosphatidate phosphohydrolase distinct from that in the endoplasmic reticulum and cytosol, *J. Biol. Chem.* 266 (1991) 2988–2996.
- [17] K.R. Morlock, J.J. McLaughlin, Y.-P. Lin, G.M. Carman, Phosphatidate phosphatase from *Saccharomyces cerevisiae*. Isolation of 45-kDa and 104-kDa forms of the enzyme that are differentially regulated by inositol, *J. Biol. Chem.* 266 (1991) 3586–3593.
- [18] R.M.C. Dawson, D.C. Elliott, W.H. Elliott, K.M. Jones, *Biochemical Reagents. B. Reagents for Protein Modification*, Oxford, 1986 (pp. 384–397).
- [19] A.A. Abdel-Latif, J.P. Smith, Studies on the effects of Mg^{2+} ion and propranolol on iris muscle phosphatidate phosphatase, *Can. J. Biochem. Cell. Biol.* 62 (1984) 170–177.