Available online at www.sciencedirect.com

SCIENCE DIRECT®

Analytical Biochemistry 352 (2006) 145–147

ANALYTICAL BIOCHEMISTRY

www.elsevier.com/locate/yabio

Notes & Tips

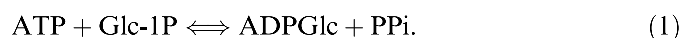
A colorimetric method for the assay of ADP-glucose pyrophosphorylase

Corina Fusari [1], Ana M. Demonte, Carlos M. Figueroa, Mabel Aleanzi, Alberto A. Iglesias *

Laboratorio de Enzimología Molecular, Facultad de Bioquímica y Ciencias Biológicas, Universidad Nacional del Litoral, Paraje "El Pozo" CC 242, S3000ZAA Santa Fe, Argentina

Received 16 November 2005
Available online 2 February 2006

ADP-glucose pyrophosphorylase (ADPGlcPPase,[2] ATP:α-D-glucose-1-P adenylyltransferase, EC 2.7.7.27) is the regulatory enzyme for the synthesis of glycogen and starch in bacteria and plants, respectively [1,2]. Most of the ADPGlcPPases so far characterized are allosterically regulated by key metabolites of the main route of carbon use in the corresponding organism [3]. They were grouped into nine classes based on the specificity for allosteric effector [1,2]. With the requirement of a divalent metal ion (Mg^{2+}), the enzyme catalyzes the reaction:



$$\text{ATP} + \text{Glc-1P} \Longleftrightarrow \text{ADPGlc} + \text{PPi}. \quad (1)$$

Although freely reversible in vitro, the reaction occurs irreversibly toward synthesis of ADPGlc in vivo after the hydrolysis of PPi by inorganic pyrophosphatase and the sugar nucleotide use for polysaccharide synthesis [1–3]. The reaction was first identified in soybean [4] and later found in different bacteria, green algae, and higher plants [1–3]. The dissimilar (source-dependent) allosteric properties exhibited by ADPGlcPPase, and its metabolic relevance, make the characterization of the enzyme an important issue to understand carbohydrate metabolism and to establish structure–function relationships in a protein. The accurate measurement of the enzyme activity in both forward and reverse directions is a critical issue in performing these studies.

Methods with different sensitivity, experimental difficulty, and cost have been developed for assay of the ADPGlcPPase activity [5–13]. A continuous pH-metric method assaying starch phosphorylase and ADPGlcPPase was reported as sensitive and easy [7], but this technique requires big reaction volumes and can be used only with low concentration of buffers. Also continuous is the spectrophotometric assay sensing Glc-1P production after coupling phosphoglucomutase and Glc-6P dehydrogenase and measuring NAD^+ reduction [12]. This assay has low sensitivity and is useful only for determining ADPGlc pyrophosphorolysis. A variant bioluminescent method, quantifying ATP, is more sensitive but still has the problem that it detects only the reverse reaction [5]. Methods using chromatographic (HPLC) and capillary electrophoresis techniques were developed for assay of both the reaction directions [8,10]. Although these methods are highly sensitive and accurate, both require expensive dedicated instruments and the use of HPLC implies the problem of handling organic waste disposal. Also awkward due to the equipment sophistication is the general assay for sugar nucleotidyltransferases using electrospray ionization mass spectrometry (ESI–MS) [13].

The most used methods for assaying ADPGlcPPase are those radiometric ones due to their sensitivity and accuracy. For ADPGlc pyrophosphorolysis, $[^{32}P]$PPi is used to measure radioactivity incorporation into ATP [6,9]. Indeed, $[^{32}P]$ATP is adsorbed on activated charcoal and, after washing and acid hydrolysis, radioactivity release is quantified. In the forward direction, the assay is based on the binding of the product $[^{14}C]$ADPGlc to DEAE–cellulose paper after an extensive enzymatic hydrolysis of the unreacted substrate $[^{14}C]$Glc-1P with alkaline phosphatase [6]. This latter assay was improved by coupling to the ADPGlcPPase reaction to that of glycogen synthase to specifically incorporate radioactive glucosyl residues from $[^{14}C]$ADPGlc into glycogen, which conveniently decreases the radioactivity background and increases sensitivity [11]. Beyond the pros and feasibilities, these radiometric assays have as disadvantages the cost, safety considerations, and hazardous waste generation as well as inclusion of time-consuming steps and a general difficulty in automating them.

The purpose of the current work was to develop a relatively simple method to assay ADPGlcPPase activity

* Corresponding author. Fax: +54 342 457 5216x217.
E-mail address: iglesias@fbcb.unl.edu.ar (A.A. Iglesias).
[1] Present address: INTA, 1712 Castelar, Argentina.
[2] *Abbreviations used:* ADPGlcPPase, ADP-glucose pyrophosphorylase; ESI–MS, electrospray ionization mass spectrometry; DTT, dithiothreitol; MG–am, Malachite Green–ammonium molybdate; AU, absorbance units.

0003-2697/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.ab.2006.01.024

having high sensitivity, accuracy, and reliability. We sought a procedure allowing the assay in both of the reaction directions, based on a technique suitable for the screening of numerous samples. The method quantifies inorganic orthophosphate released from the specific hydrolysis of the enzyme activity products. For ADPGlc synthesis, the method measures Pi after hydrolysis of PPi by inorganic pyrophosphatase. Pyrophosphorolysis is assayed by determining Pi derived from CF_1-ATPase-mediated hydrolysis of ATP. Pi dosage is performed by the technique based in the formation of a phosphomolybdate–Malachite Green complex [14,15]. We optimized the procedure to a microscale grade, reaching convenient sensitivity and the possibility of automation, by using a microplate (multiwell) absorbance reader. A similar approach has been briefly described previously for assay GDP-mannose pyrophosphorylase from fungi [16], although it gave no specific details for optimal conditions or for assaying both of the reaction directions.

Recombinant ADPGlcPPases from *Escherichia coli* and *Agrobacterium tumefaciens* were expressed in *E. coli* and purified as done previously [17–19]. Native PAGE was performed on slabs as described elsewhere [20]. Inorganic pyrophosphatase was obtained from Sigma (St. Louis, MO, USA). CF_1-ATPase was partially purified from spinach leaves after Strotmann and coworkers [21]. The clear supernatant containing the enzyme detached from chloroplast membranes was concentrated by ultrafiltration, divided into aliquots, and stored at −80 °C. Before use, unfrosted CF_1 was activated by 2 h treatment at 25 °C in a medium containing 40 mM Tris–HCl buffer (pH 8.0), 2 mM EDTA, and 55 mM dithiothreitol (DTT) [22]. All plastic and glassware used for Pi measurement were thoroughly washed with 50% (v/v) sulfuric acid. The Malachite Green–ammonium molybdate (MG–am) solution was prepared by combining 3 volumes of 0.5 mM aqueous solution of Malachite Green (oxalate salt from Sigma, cat. no. M6880) and 1 volume of 0.034 M ammonium molybdate in 4 M HCl. The mixture was stirred (20–30 min) and filtered through Whatman no. 4 paper. This solution was stable for at least 1 month at 4 °C. The color reagent was freshly prepared by adding 1 volume of 2% (v/v) Tween 20 to 50 volumes of the former solution. The color reaction was developed by mixing samples (1.0 volume) with the color reagent (6.7 volumes). After 1 min, the addition of 1:10 total volume of 34% (w/v) sodium citrate stabilized the color during approximately 1 h. Photometry was performed at 650 nm in polystyrene flat-bottom microplates with a background of less than 0.04 absorbance units (AU).

The standard assay medium in the synthesis direction contained 80 mM Mops–NaOH buffer (pH 8.0), 7.0 mM $MgCl_2$, 1.0 mM Glc-1P, 1.5 mM ATP, 0.2 mg/ml BSA, and 1.5 U/ml inorganic pyrophosphatase. The medium for the pyrophosphorolysis direction contained 80 mM Mops–NaOH (pH 8.0), 5.0 mM $MgCl_2$, 100 mM NaF, 1.0 mM ADPGlc, and 1.0 mM PPi. For colorimetric assays, reactions were stopped by the addition of the color reagent or by 45 s incubation in a boiling water bath for synthesis or pyrophosphorolysis, respectively. For the latter, samples were cooled on ice, and after the addition of 1 U of activated CF_1-ATPase incubated for 30 min at 30 °C, this was followed by toting up with the MG–am reagent. The compositions of media for radiometric measurements in both reaction directions were similar except that the respective radiolabeled substrates ([^{14}C]Glc-1P, ~1000 cpm/nmol; [^{32}P]PPi, ~2000 cpm/nmol) were used as described elsewhere [6,11,23]. The kinetic data were plotted as initial velocity (v, nmol min^{-1}) versus ligand concentration using a computer program [24]. The kinetic parameters $S_{0.5}$ and $A_{0.5}$ are the amount of substrate and activator giving 50% of the V_{max} and activation, respectively. The Hill number is expressed as n_H.

Considering that in the assay for activity of ADPGlcPPase the highly sensitive radiometric methods detect product formation less than 10 nmol, we optimized the conditions to measure Pi by using the MG–am reagent in such a range. Standard curves were made with increasing amounts (0–15 nmol) of Pi (standard solution from the Wiener Laboratory [Rosario, Argentina] or alternatively prepared with KH_2PO_4 dried 4 h at 110 °C [15]) contained in three different volumes (0.075, 0.15, and 0.30 ml) with the addition of proportional volumes (0.5, 1.0, and 2.0 ml) of MG–am. All curves were linear, and the lower volume condition was the most profitable, obtaining a slope of 0.15 AU/nmol and an R coefficient of 0.99 ($P < 0.0001$). Identical results were obtained when calibration curves were performed in standard media for the assay of the enzyme, thereby reinforcing the usefulness of the MG–am reagent to measure ADPGlcPPase.

To validate the dosage of Pi with MG–am to measure ADPGlcPPase activity, we performed assays with the recombinant enzyme from *E. coli* in both of the reaction directions in parallel with the respective radiometric method taken as a reference. In these studies, conditions for the colorimetric assay were fixed by performing the respective ADPGlcPPase reaction in a volume of 0.075 ml and then developing the color by the addition of 0.5 ml MG–am. Fig. 1 shows that the relationship between the activity measured with the colorimetric method and that measured with the radiometric method was linear for up to 10 nmol of product formed; with slopes calculated in 1.05 and R coefficients of 0.988 ($P < 0.0001$) (synthesis direction) and 0.991 ($P < 0.0001$) (pyrophosphorolysis direction). The limit of detection for the colorimetric assay was 0.30 ± 0.05 nmol of product ($P = 0.05$, considered as Pi). The mean coefficient of variation was $7 \pm 3\%$ ($P = 0.05$), determined by a quintuplicate of 10 samples covering the full linear range of the assay (0.3–18.0 nmol of Pi).

Further evidence on the accuracy of the MG–am colorimetric assay was obtained by kinetic characterization of the recombinant enzymes from *E. coli* and *A. tumefaciens*. As shown in Supplementary table 1, the kinetic parameters for all of the substrates (with or without the respective activator) determined with the colorimetric method were in very good agreement with those values obtained with the radioactive technique performed in parallel. Also similar were the $A_{0.5}$ values measured for the respective activators of the

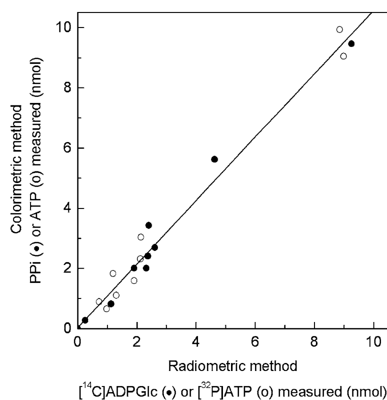


Fig. 1. Correlation between ADPGlcPPase activity measured with the colorimetric method and that measured with the radiometric method for the directions of synthesis and pyrophosphorolysis of ADPGlc. Values correspond to measurements of enzyme activity between 0 and 20 min under experimental linear reaction conditions (substrate consumption <20%).

enzyme from the two bacterial sources: Fru-1,6-bisP (*E. coli*) and Fru-6P and pyruvate (*A. tumefaciens*) [1,2]. The good correlation of the kinetic parameters strongly supports the validity and accuracy of the MG–am colorimetric assay.

The method was also shown to be useful for detecting ADPGlcPPase activity in native PAGE. Thus, after electrophoresis run at 4 °C of extracts containing the recombinant enzymes from *E. coli* and *A. tumefaciens*, gels where soaked with the mixture for the assay in the ADPGlc synthesis direction during 30 min at room temperature, followed by 15 min incubation with MG–am reagent. Washing the gels with 5 N HCl revealed a green band corresponding to the enzyme within 3 to 10 min. It was necessary to evaluate incubation time with the MG–am reagent depending on the presence of contaminants in the sample, but the ADPGlcPPase band appeared first and gave the staining good specificity.

Acknowledgment

This work was supported by grants from UNL (CAI + D 2002), CONICET (PIP 6358), and ANPCyT (PICTO'03 01-13241, PICT'03 01-14723, and PAV'03 137). C. M. Figueroa is a fellow, and A. A. Iglesias is a research member, of CONICET.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ab.2006.01.024.

References

[1] M.A. Ballicora, A.A. Iglesias, J. Preiss, ADP-glucose pyrophosphorylase: a regulatory enzyme for plant starch synthesis, Photosynth. Res. 79 (2004) 1–24.

[2] M.A. Ballicora, A.A. Iglesias, J. Preiss, ADP-glucose pyrophosphorylase: a regulatory enzyme for bacterial glycogen synthesis, Microbiol. Mol. Biol. Rev. 67 (2003) 213–225.

[3] M.N. Sivak, J. Preiss, Starch: Basic Science to Biotechnology, Academic Press, San Diego, 1998.

[4] J. Espada, Enzymic synthesis of adenosine diphosphate glucose from glucose-1-phosphate and adenosine triphosphate, J. Biol. Chem. 237 (1962) 3577–3581.

[5] T.M. Ching, A rapid and sensitive assay of ADP glucose pyrophosphorylase using luciferase, Anal. Biochem. 111 (1981) 327–330.

[6] H.P. Ghosh, J. Preiss, Adenosine diphosphate glucose pyrophosphorylase: a regulatory enzyme in the biosynthesis of starch in spinach leaf chloroplasts, J. Biol. Chem. 241 (1966) 4491–4504.

[7] D.A. McCracken, W.M. Rutherford, A rapid, sensitive assay for starch phosphorylase and ADPglucose pyrophosphorylase, Anal. Biochem. 101 (1980) 275–277.

[8] M.W. Roberts, J. Preiss, T.W. Okita, A capillary zone electrophoresis assay for the nucleoside transfer enzyme adenosine diphosphate-glucose pyrophosphorylase, Anal. Biochem. 225 (1995) 121–125.

[9] L. Shen, J. Preiss, The activation and inhibition of bacterial adenosine-diphosphoglucose pyrophosphorylase, Biochem. Biophys. Res. Commun. 17 (1964) 424–429.

[10] D.M. Stark, K.P. Timmerman, G.F. Barry, J. Preiss, G.M. Kishore, Role of ADPglucose pyrophosphorylase in regulating starch levels in plant tissues, Science 258 (1992) 287–292.

[11] A. Yep, C.M. Bejar, M.A. Ballicora, J.R. Dubay, A.A. Iglesias, J. Preiss, An assay for adenosine 5′-diphosphate (ADP)-glucose pyrophosphorylase that measures the synthesis of radioactive ADP-glucose with glycogen synthase, Anal. Biochem. 324 (2004) 52–59.

[12] W.C. Plaxton, J. Preiss, Purification and properties of non-proteolytically degraded ADPglucose pyrophosphorylase from maize endosperm, Plant Physiol. 83 (1987) 105–112.

[13] C.J. Zea, N.L. Pohl, General assay for sugar nucleotidyltransferases using electrospray ionization mass spectrometry, Anal. Biochem. 328 (2004) 196–202.

[14] P.A. Lanzetta, L.J. Alvarez, P.S. Reinach, O.A. Candia, An improved assay for nanomole amounts of inorganic phosphate, Anal. Biochem. 100 (1979) 95–97.

[15] H.H. Hess, J.E. Derr, Assay of inorganic and organic phosphorus in the 0.1–5 nanomole range, Anal. Biochem. 63 (1975) 607–613.

[16] A. Ohta, H. Chibana, M. Arisawa, M. Sudoh, The *VIG9* gene products from the human pathogenic fungi *Candida albicans* and *Candida glabrata* encode GDP-mannose pyrophosphorylase, Biochim. Biophys. Acta 1475 (2000) 265–272.

[17] A.D. Uttaro, R.A. Ugalde, J. Preiss, A.A. Iglesias, Cloning and expression of the *glgC* gene from *Agrobacterium tumefaciens*: purification and characterization of the ADPglucose synthetase, Arch. Biochem. Biophys. 357 (1998) 13–21.

[18] D.F. Gomez-Casati, R.Y. Igarashi, C.N. Berger, M.E. Brandt, A.A. Iglesias, C.R. Meyer, Identification of functionally important amino-terminal arginines of *Agrobacterium tumefaciens* ADP-glucose pyrophosphorylase by alanine scanning mutagenesis, Biochemistry 40 (2001) 10169–10178.

[19] M.A. Ballicora, J.I. Sesma, A.A. Iglesias, J. Preiss, Characterization of chimeric ADPglucose pyrophosphorylases of *Escherichia coli* and *Agrobacterium tumefaciens*: importance of the C-terminus on the selectivity for allosteric regulators, Biochemistry 41 (2002) 9431–9437.

[20] B.D. Hames, One-dimensional polyacrylamide gel electrophoresis, in: B.D. Hames, D. Rickwood (Eds.), Gel Electrophoresis of Proteins: A Practical Approach, Oxford University Press, Oxford, UK, 1994.

[21] H. Strotmann, H. Hesse, K. Edelmann, Quantitative determination of coupling factor CF_1 of chloroplasts, Biochim. Biophys. Acta 314 (1973) 202–210.

[22] R.E. McCarty, E. Racker, Partial resolution of the enzymes catalyzing photophosphorylation: III. Activation of adenosine triphosphatase and ^{32}P-labeled orthophosphate-adenosine triphosphate exchange in chloroplasts, J. Biol. Chem. 243 (1968) 129–137.

[23] M.K. Morell, M. Bloom, V. Knowles, J. Preiss, Subunit structure of spinach leaf ADPglucose pyrophosphorylase, Plant Physiol. 85 (1987) 182–187.

[24] S.P. Brooks, A simple computer program with statistical tests for the analysis of enzyme kinetics, Bio. Techniques 13 (1992) 906–911.