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An assay for adenosine 5′-diphosphate (ADP)-glucose pyrophosphorylase that measures the synthesis of radioactive ADP-glucose with glycogen synthase☆

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Abstract

Adenosine 5′-diphosphate (ADP)-glucose pyrophosphorylase (ADP-Glc PPase) catalyzes the conversion of glucose 1-phosphate and adenosine 5′-triphosphate to ADP-glucose and pyrophosphate. We present a radioactive assay of this enzyme with a higher signal/noise ratio. After stopping the reaction that uses [^{14}C]glucose 1-phosphate as a substrate, the ADP-[^{14}C]glucose formed as a product is converted to [^{14}C]glycogen by the addition of glycogen synthase and nonradioactive glycogen as primer. The final product is precipitated and washed, and the radioactivity is measured in a scintillation counter. The [^{14}C]glucose 1-phosphate that did not react is easily eliminated during the washes. We have found that this assay produces much lower blanks than previously described radioactive methods based on binding of ADP-[^{14}C]glucose to *O*-(diethylaminoethyl)-cellulose paper. In addition, we tested the kinetic parameters for the effectors of the *Escherichia coli* ADP-Glc PPase and both assays yielded identical results. The presented method is more suitable for K_m or $S_{0.5}$ determinations of ADP-Glc PPases having high apparent affinity for glucose 1-phosphate. It is possible to use a higher specific radioactivity to increase the sensitivity at lower concentrations of [^{14}C]glucose 1-phosphate without compromising the blanks obtained at higher concentrations.
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ADP-glucose pyrophosphorylase (ATP:α-D-glucose-1-P adenylyltransferase; EC 2.7.7.27; ADP-Glc PPase[1]) catalyzes the synthesis of ADP-glucose (ADP-Glc) from ATP and α-D-glucose 1-phosphate (Glc1P).

$$\mathrm{ATP} + \mathrm{Glc1P} \Longleftrightarrow \mathrm{ADP\text{-}Glc} + \mathrm{PP_i} \qquad (1)$$

The enzymatic reaction takes place in the presence of a divalent metal ion (Mg^{2+}), and it is freely reversible in vitro. However, in vivo, the hydrolysis of PP_i by inorganic pyrophosphatase and the use of the sugar nucleotide as a glucosyl donor for the elongation of α1, 4-glucosidic chains makes it practically irreversible in the direction of ADP-Glc synthesis [1]. This reaction was first described in soybean [2] and was subsequently found in many bacterial extracts and plant tissues [3,4]. It is the first committed and the main regulatory step in the synthesis of glycogen in bacteria and starch in plants [3,4].

Most of the ADP-Glc PPases are allosterically regulated by small effector molecules. ADP-Glc PPase activators are generally key metabolites that indicate the presence of high carbon and energy contents within the cell. On the other hand, inhibitors are intermediates that

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[1] *Abbreviations used:* ADP-Glc PPase, ADP-glucose pyrophosphorylase; Glc1P, α-D-glucose 1-phosphate; Glc6P, α-D-glucose 6-phosphate; ADP-Glc, ADP-glucose; Fru-1,6-P_2, fructose 1,6-bisphosphate; PEP, phospho*enol*pyruvate; 3-PGA, 3-phosphoglyceric acid; BSA, bovine serum albumin; TEA, triethanolamine.

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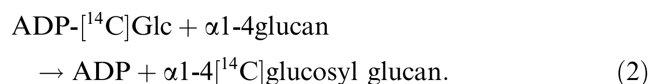
represent signals of low metabolic energy levels. Based on the specificity of activator and inhibitor, ADP-Glc PPases have been grouped into nine different classes [4]. For instance, the enzyme from *Escherichia coli* is in group I and is activated by fructose 1,6-bisphosphate (Fru-1,6-P_2) and inhibited by AMP [4]. Other major regulators of enzymes from other classes are fructose 6-phosphate (Fru6P), pyruvate, and 3-phosphoglycerate (3-PGA) as activators and ADP and P_i as inhibitors [4]. Some enzymes are partially activated by other metabolites such as phospho*enol*pyruvate (PEP), NAD derivatives, and other triose and sugar phosphates [5]. In all cases, the main activators are intermediates of the major carbon assimilatory pathway in the organism [4].

To understand the regulatory control of bacterial glycogen synthesis or starch synthesis in plants, ADP-Glc PPases have been the subject of numerous kinetic analyses [4,6]. Furthermore, it has been critical to accurately determine their kinetic properties for structure–function relationship studies [7–9]. Different methods for the assay of the ADP-Glc PPase in both synthesis (forward) and pyrophosphorolysis (reverse) directions have been described. Those comprise radiometric [10,11], pH metric [12], bioluminescent [13], chromatographic (HPLC) [14], capillary electrophoretic [15], and spectrophotometric techniques [16,17]. The synthesis of ADP-Glc is the direction that occurs in vivo and is the most important to be characterized. The pH metric technique can be used only with low concentrations of buffers and requires big reaction volumes. The HPLC and capillary electrophoretic techniques need expensive dedicated instruments. The spectrophotometric methods are easy to use but their sensitivity is lower, whereas the bioluminiscent procedure is sensitive but it measures the reverse direction. For those reasons, the most popular method to assay the enzyme in the direction of synthesis has been radiometric [11]. That assay is based on the binding of the product ADP-[^{14}C]Glc to DEAE-cellulose paper after an extensive enzymatic hydrolysis of the unreacted substrate [^{14}C]Glc1P with alkaline phosphatase [11]. That technique allows many samples to be processed at the same time, but in some situations the background obtained limits the sensitivity. In this work, we introduce a discontinous assay with a higher signal/noise ratio in which we use the ability of the glycogen synthase (ADP-glucose:1,4-α-D-glucan 4-α-D-glucosyltransferase; EC 2.4.2.21) from *E. coli* to specifically and quantitatively transfer the radioactive glucosyl unit of ADP-[^{14}C]Glc into glycogen (α1-4glucan):



$$\text{ADP-}[^{14}\text{C}]\text{Glc} + \alpha 1\text{-4glucan} \rightarrow \text{ADP} + \alpha 1\text{-4}[^{14}\text{C}]\text{glucosyl glucan}. \quad (2)$$

In this reaction, the formation of ADP is highly favored and its equilibrium is even more shifted when the pH increases. The concentration equilibrium constant $K' = [\text{ADP}]/[\text{ADP-Glc}]$ increases from 55 to 151 when the pH is increased from 5.3 to 6.8 [18]. After elongating the glucan chains with the product ADP-[^{14}C]Glc using glycogen synthase, we selectively precipitate the glycogen. The unreacted substrate of the ADP-Glc synthesis reaction, [^{14}C]Glc1P, remains in solution to be washed and discarded.

Materials and methods

Materials

α-D-[U-^{14}C]glucose 1-phosphate was purchased from Amersham Pharmacia Biotech (Piscataway, NJ). ADP-[U-^{14}C]glucose was synthesized as described previously [19]. Rabbit liver glycogen was purchased from Sigma (St. Louis, MO). The DEAE-cellulose paper used was DE81 anion exchanger from Whatman (Clifton, NJ). Scintillation cocktail for aqueous samples was Safety-solve from Research Products International Corp. (Mount Prospect, IL).

Enzymes

Alkaline phosphatase from *E. coli*, α-amylase from porcine pancreas, and inorganic pyrophosphatase from baker's yeast were purchased from Sigma. The recombinant ADP-Glc PPase from *E. coli* was expressed and purified as described previously [20]. One unit (U) of alkaline phosphatase is defined as 1 μmol of *p*-nitrophenyl phosphate hydrolyzed min^{-1} at 37 °C, pH 10.4. One unit (U) of ADP-Glc PPase is defined as 1 $\mu\text{mol min}^{-1}\text{ mg}^{-1}$.

The recombinant glycogen synthase from *E. coli* was obtained as follows. Glycogen synthase gene (*glgA*) was subcloned into the pET-24a vector (Novagen) in the *Nde*I-*Sac*I sites to obtain the plasmid pAY1. One liter of BL21(DE3) cells (Stratagene) transformed with pAY1 was grown at 37 °C until $OD_{600} = 0.6$ in a spectrophotometer (Beckman DU 650) (4.8×10^8 cfu/ml), and induced with 1 mM iospropylβ-D-thiogalactoside overnight at room temperature. The cells were centrifuged, resuspended in potassium phosphate buffer (pH 6.8), 5 mM dithiothreitol, and 0.1 M NaCl (∼3 ml/g cells), and sonicated. The suspension obtained was centrifuged at 12,000*g* for 15 min, and α-amylase was added to the supernatant in a ratio of 2 μg/ml and left overnight at 4 °C with stirring. This extract was ultracentrifuged at 100,000*g* for 90 min and the supernatant was precipitated with ammonium sulfate (0–25% cut) followed by centrifugation at 12,000*g* for 20 min. The precipitate was resuspended in 20 mM triethanolamine-HCl (TEA-HCl) (pH 7.5) and desalted on Econo-Pac Bio-Rad 10 DG disposable chromatography columns equilibrated with the same buffer. The desalted sample was applied to a

Mono-Q HR 10/10 (FPLC; Pharmacia) and eluted with a linear KCl gradient (20 column volumes, 0–0.6 M) in buffer 20 mM TEA-HCl (pH 7.5). The active fractions were monitored by SDS–PAGE and the purest fractions were pooled, concentrated, and brought to 20 mM TEA-HCl (pH 7.5) using Ultrafree-15 centrifugal filter device (Millipore) with a Biomax-10 membrane. The concentrated sample (2 mg/ml) was stored at −80 °C and remained stable for at least 1 year.

The glycogen synthase obtained in this preparation was homogeneous on SDS–PAGE and the total was 1.55 mg with a specific activity of 505 μmol min^{-1} mg^{-1}. Contaminations with α-amylase activity or endogenous ADP-Glc PPase were negligible, <0.0005 and <0.001 μmol min^{-1} mg^{-1}, respectively. One unit (U) of glycogen synthase is defined as 1 μmol of glucose transferred min^{-1} mg^{-1}. One liter of culture yields enough purified glycogen synthase to perform ~7000 ADP-Glc PPase assays as described in this paper.

[^{14}C]glycogen

Rabbit liver glycogen (2.5 mg) was incubated for 1 h at 37 °C in a reaction tube containing 200 nmol ADP-[^{14}C]Glc (8.3 Bq/nmol), 10 μmol bicine-NaOH (pH 8.0), 100 μg bovine serum albumin (BSA), 100 nmol magnesium acetate, and 0.1 unit (2 ng) of purified *E. coli* glycogen synthase in a final volume of 200 μl. The reaction was stopped with 2 ml of 75% methanol/1% KCl, incubated on ice for 10 min, and centrifuged in a tabletop centrifuge for 3 min at 4000 rpm. The precipitate was drained, washed twice with the same solution, and finally dissolved in double-deionized water.

ADP-Glc PPase reaction

The reaction was conducted as described previously [20] in presence of 100 mM Hepes (pH 8.0), 0.2 mg/ml BSA, 7 mM $MgCl_2$, 1.5 mM ATP, 2 mM fructose 1,6-bisphosphate, 1.5 U/ml inorganic pyrophosphatase, and 0.5 mM [^{14}C]Glc1P (10.7 Bq/nmol), unless otherwise indicated, in a total volume of 200 μl. For the Glc1P saturation curves, the specific activity of [^{14}C]Glc1P was 85.8 Bq/nmol. When indicated, 34 ng of purified *E. coli* ADP-Glc PPase was added and after incubation for 10 min at 37 °C the reaction was terminated by heating in a water-boiling bath for 1 min. For the Glc1P saturation curves, the amount of *E. coli* ADP-Glc PPase used was 0.7 ng. The product ADP-[^{14}C]Glc was determined as indicated.

DEAE paper method

The method for determination of ADP-[^{14}C]Glc synthesis in presence of the substrate [^{14}C]Glc1P was as described previously with slight modifications [11]. After the reaction was stopped, 20 μl of buffer 50 mM Hepes (pH 8.0) containing 0.03 mg (0.4 U) of alkaline phosphatase was added and incubated at 37 °C for 16 h to ensure a complete hydrolysis of the [^{14}C]Glc1P. Half of the mixture (0.11 ml) was adsorbed onto individual DEAE-cellulose paper disks of 2.5 cm diameter. The disks were washed twice, 12 at the same time, with 2 liters of deionized water on a 3.0-liter Buchner glass filter connected to a water vacuum pump. The disks were dried with a heat lamp and the radioactivity was counted as previously described [11].

Calculation of kinetic constants

The corrected radioactivity used to calculate the initial velocity was obtained after subtracting the radioactivity of the sample and the control (no enzyme). When an effector curve was performed, the rest of the components of the reaction mixture were kept at the concentrations stated above. The kinetic data were plotted as initial velocity in nmol min^{-1} (v) versus effector concentration in μM ([S]). This plot was used to fit the parameters of a modified Hill equation [21], $v = V_{max} \cdot [S]^n/(S_{0.5}^n + [S]^n)$, by the Levenberg–Marquardt nonlinear least squares algorithm provided by the computer program Origin 5.0 [22]. The constant $S_{0.5}$ is the amount of substrate or cofactor (Mg^{2+}) needed to obtain 50% of the maximum activity (V_{max}). When the Hill coefficient (n) was not significantly different from 1.0, the Hill equation reduced to a hyperbolic equation. In that case, the amount of substrate needed to obtain 50% of the maximum activity was expressed as K_m. The activation curves were fitted with the same equation but the constant $S_{0.5}$ was replaced by $A_{0.5}$, which is the amount of activator needed to obtain 50% of the V_{max}. Inhibition curves were fitted to the equation $v = V_o \cdot I_{0.5}^n/(I_{0.5}^n + [I]^n)$. In that case, [I] is the concentration of inhibitor, V_o is the velocity in absence of inhibitor, and $I_{0.5}$ is the amount of inhibitor needed to obtain 50% of V_o. The deviations shown are the standard deviations obtained by the Levenberg–Marquardt method [22].

Results

Determination of the ADP-Glc product by the glycogen synthesis procedure

The method described in this work deals with a different way of analyzing the ADP-[^{14}C]Glc formed as a product. It takes advantage of the glycogen synthase specificity to transfer the radioactive glucosyl moiety to a precipitable compound. After the enzymatic reactions were run and terminated as indicated under Materials and methods, 50 μl of a solution containing 0.2 μg

(0.1 U) of glycogen synthase, 0.5 mg of glycogen, and 50 mM Hepes (pH 8.0) was added and the mixture was incubated for 1 h at 37 °C. The [^{14}C]glucose incorporated into the methanol-insoluble polysaccharide was determined by a modified procedure [23]. [^{14}C]glycogen was precipitated, after the addition of 0.1 ml of 10 mg/ml glycogen as carrier, with 2 ml of 75% methanol/1% KCl. Samples were incubated 10 min on ice and centrifuged 3 min at ~2000*g* in a tabletop centrifuge; the supernatants were discarded. The tubes were inverted and left upside down on paper towels for at least 0.5 min. Afterward, they were tapped four times to efficiently remove the remaining drops that contain the unreacted [^{14}C]Glc1P. The pellet was dissolved in 0.2 ml of water and precipitated again in the same fashion with 2 ml of 75% methanol/1% KCl. As the last step, the precipitate was further washed with another 2 ml of 75% methanol/1% KCl. The samples were dissolved in 1 ml of water, an aliquot of 0.5 ml was withdrawn and mixed with 4 ml of scintillation cocktail, and the radioactivity was counted for 1 min. Counting for 10 min to decrease the variability was generally useful only when the radioactivity measured was less than ~400 cpm.

The parameters that affect the conversion of ADP-Glc into the glycogen precipitate are (i) the equilibrium constant of reaction 2, (ii) the efficiency of the glycogen precipitation, (iii) the time of incubation with glycogen synthase, and (iv) the initial concentration of ADP-Glc. Those were analyzed separately to evaluate the general efficiency of the procedure.

Equilibrium of the glycogen synthesis reaction. Reaction 2, catalyzed by glycogen synthase, has an equilibrium constant that highly favors the incorporation of glucose into glycogen in a broad range of pH [18]. To verify that this reaction is quantitative under the conditions of the assay, 200 μM ADP-[^{14}C]Glc (8.3 Bq/nmol) was incubated at 37 °C with glycogen synthase in 100 mM Hepes (pH 8.0), 0.2 mg/ml BSA, 7 mM $MgCl_2$ in a total volume of 200 μl. After 1 h, an aliquot was separated by paper chromatography in ethanol/ammonium acetate, pH 7.5, as described previously [19]. More than 99.5% of the radioactivity was converted to a slow-moving peak that corresponded to glycogen (data not shown).

Efficiency of [^{14}C]glycogen precipitation. To measure the efficiency of this step, a 0.20-ml aliquot of [^{14}C]glycogen (2.5 mg/ml, 133 Bq/mg) was precipitated, washed, dissolved, and counted as described. This was compared to a control (100%) in which the radioactivity was measured directly. The efficiencies obtained were between 85 and 93% depending on the number and type of washes (data not shown). We found that the best compromise between yield and background was to dissolve the glycogen precipitated in the first, but not in the second, round of washes. In this way, the blanks were kept low and the efficiency of precipitation was 90% and very reproducible.

Time curve of conversion from ADP-[^{14}C]Glc to [^{14}C]glycogen. To ensure that reaction 2 proceeds to completion, we ran a curve to find the optimum time to incubate the product of the ADP-Glc PPase reaction with glycogen synthase. After 2 min of reaction with 0.1 U of glycogen synthase, the glucose transferred from ADP-Glc to glycogen reached a plateau (Fig. 1). An incubation of 1 h guaranteed that the reaction was complete and we decided to take it as the standard procedure.

Linearity. The conversion of ADP-Glc into glycogen was linear in a very broad range of concentrations, from 0.1 to 1000 nmol of ADP-Glc (Fig. 2). The curve was a straight line with a slope of 0.90, indicating that the efficiency of the method was consistently 90% in all the points assayed.

Comparison with the method based on binding to anion exchange paper

The glycogen synthesis method and the one based on the binding of ADP-Glc to DEAE-cellulose were compared kinetically. The parameters for the effectors of the *E. coli* ADP-Glc PPase were determined with both methods in parallel (Table 1). There was no significant difference between the constant values from both procedures (Table 1). In addition, the sigmoidicity of the curves were indistinguishable and the Hill coefficients (*n*) were not appreciably different (Table 1). The method based on the glycogen synthesis generated smoother

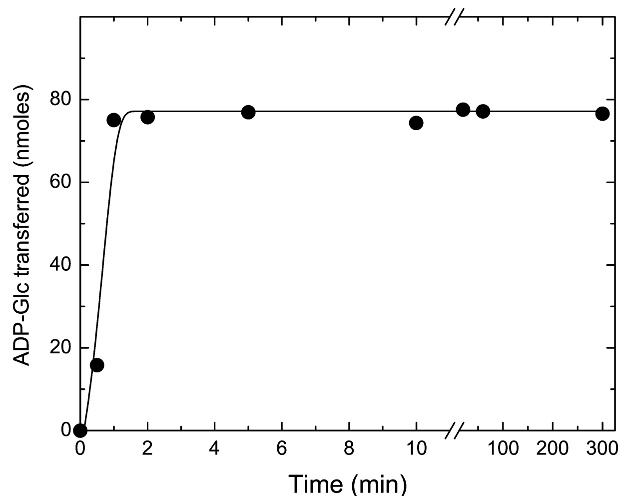


Fig. 1. Time course of the glycogen synthase reaction. The reaction mixture contained 80 nmol of ADP-[^{14}C]Glc (8.3 Bq/nmol) in 200 μl of a solution of 0.2 mg/ml BSA, 7 mM $MgCl_2$, and 100 mM Hepes (pH 8.0). The reaction was started by adding 50 μl of a mixture containing 0.1 U of glycogen synthase and 0.4 mg of rabbit liver glycogen in Hepes 100 mM (pH 8.0). The reaction was incubated in a water bath at 37 °C and stopped at different time points by boiling for 1 min. The [^{14}C]glucose incorporated into the methanol-insoluble polysaccharide was determined as described in the text.

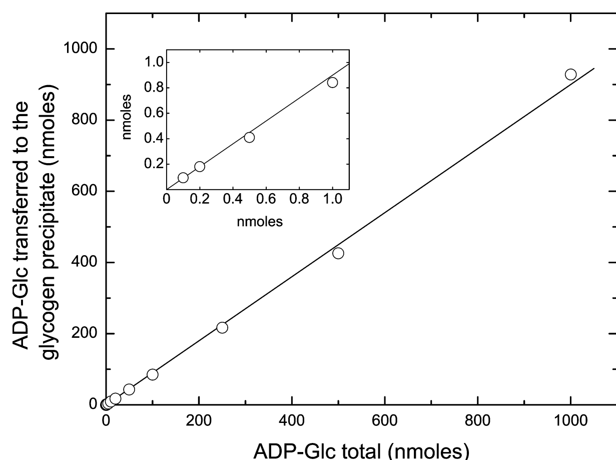


Fig. 2. Linearity of the glycogen synthase reaction. The reaction was conducted for 60 min as described in the legend of Fig. 1 with varying amounts of ADP-[^{14}C]Glc (8.3 Bq/nmol).

curves shown by the lower standard deviations of the fitted parameters (Table 1). The raw data from the Glc1P saturation curve show that the blanks obtained with the glycogen synthesis procedure were much lower (Fig. 3). With the DEAE-cellulose paper binding method, at higher concentrations of Glc1P, the blanks grew to be even higher than the corrected radioactivity, which is the difference between the sample and the control curves

We studied whether there are common effectors of the ADP-Glc PPases that interfere in the glycogen synthesis step. After 1 h of incubation with glycogen synthase, ADP was the only tested metabolite that seems to partially interfere. The transferences to [^{14}C]glycogen were 84 and 78% when 0.5 and 1000 nmol of ADP-[^{14}C]Glc were incubated, respectively. This was expected because ADP is a product of reaction 2 and, as such, is a glycogen synthase inhibitor. The addition of alkaline phosphatase to the mixture easily overcame this problem. The alkaline phosphatase removed the ADP and the efficiency of transference was restored to ~100% (Table 2). The rest of the effectors assayed did not seem to substantially inhibit the glycogen synthesis (Table 2).

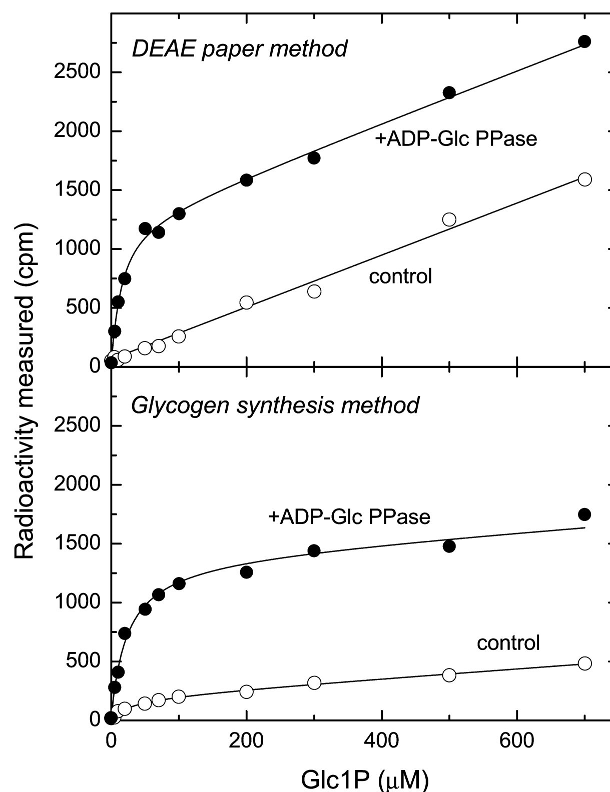


Fig. 3. Comparison of the Glc1P curves for the ADP-Glc PPase from *E.coli* by different assay methods. Each reaction was performed as described under Materials and methods using varying concentrations of Glc1P. A control with no ADP-Glc PPase added was included for each concentration of Glc1P. The DEAE-cellulose paper method was performed as described under Materials and methods and the glycogen synthesis method as described in the text.

Discussion

In this paper, we introduce a new radiometric assay for ADP-Glc PPase with a higher signal/noise ratio. The rationale of the procedure is to convert the product ADP-[^{14}C]Glc into [^{14}C]glycogen to isolate it from the substrate [^{14}C]Glc1P by precipitation. Polysaccharides can be isolated from aqueous solutions by the addition of a water-miscible solvent such as acetone or lower

Table 1
Comparison of kinetic constants of the *E. coli* ADP-Glc PPase obtained by two different methods

Effector (constant)	DEAE paper method		Glycogen synthesis method	
	Constant value[a] (μM)	n	Constant value[a] (μM)	n
Glc1P (K_m)	17 ± 3	1[b]	15 ± 3	1[b]
ATP ($S_{0.5}$)	258 ± 35	1.4 ± 0.2	216 ± 22	1.5 ± 0.2
$MgCl_2$ ($S_{0.5}$)	1770 ± 110	4.4 ± 1.2	1800 ± 55	4.9 ± 0.7
Fru-1,6-P_2 ($A_{0.5}$)	51 ± 11	1.7 ± 0.5	58 ± 4	1.7 ± 0.2
AMP($I_{0.5}$)	16.5 ± 2.0	2.0 ± 0.4	18.5 ± 0.3	1.9 ± 0.05

[a] Kinetic constants were calculated as indicated under Materials and methods.
[b] Curves were hyperbolic.

Table 2
Influence of ADP-Glc PPase effectors on the glycogen synthesis step

Effector	Relative efficiency of [^{14}C]glucose incorporation to glycogen (%)	
	0.5 nmol ADP-[^{14}C]Glc[a]	1000 nmol ADP-[^{14}C]Glc[b]
Control[c]	100 ± 1	100 ± 1
Fru-1,6-P_2[c]	95 ± 3	100 ± 2
Fru6P	100 ± 1	97 ± 1
Pyruvate	98 ± 1	96 ± 1
3-PGA	96 ± 1	96 ± 1
Glc6P[d]	100 ± 1	99 ± 3
Glc1P	102 ± 2	97 ± 1
ATP	100 ± 1	98 ± 1
ADP[c]	84 ± 1	78 ± 1
ADP[c] + AP[e]	100 ± 1	103 ± 1
AP[e]	97 ± 2	102 ± 1
AMP	98 ± 2	95 ± 1
P_i	96 ± 1	97 ± 1
NADP	101 ± 1	94 ± 1
NAD	96 ± 1	95 ± 1
PEP[c]	97 ± 1	100 ± 5

The reaction mixture (200 μl) contained 7 mM $MgCl_2$, 100 mM Hepes (pH 8.0), 0.2 mg/ml BSA, 5 mM of the effector studied and the amount of ADP-[^{14}C]Glc indicated. No effector was added to the control. The reaction was started by adding 50 μl of a mixture containing 0.1 U of glycogen synthase and 0.4 mg of rabbit liver glycogen in Hepes 100 mM (pH 8.0) and was incubated in a water bath at 37 °C for 1 h. The reaction was stopped by boiling for 1 min and the [^{14}C]glucose incorporated into the methanol-insoluble polysaccharide was determined as described in the text. The incorporation was normalized to the controls, which yielded 0.48 and 950 nmol of [^{14}C]glucose when the reaction mixtures contained 0.5 and 1000 nmol of ADP-[^{14}C]Glc, respectively. Experiments were run in duplicates unless stated otherwise and the data shown are the average ± standard deviation.

[a] The specific activity was 83.3 Bq/nmol.

[b] The specific activity was 0.83 Bq/nmol.

[c] Samples were run in quadruplicates.

[d] Glc6P, α-D-glucose 6-phosphate.

[e] (AP), alkaline phosphatase (0.1 unit) was added to the sample.

alcohol [24]. Several protocols based on those properties were devised to isolate glycogen [25–30]. We chose 75% methanol/1% KCl because it has been proven effective in the assay of ADP-Glc-dependent glycogen/starch synthases [23,31] and because salts are important to induce the precipitation [28]. The technique presented in this work produces results similar to those of a previously described method [11], but the backgrounds are lower. Substrate curves performed with both the DEAE-cellulose paper binding method and the new glycogen synthesis procedure yielded kinetic constants that were not significantly different.

The lower blanks provided by this assay resulted in an advantage that was most evident for Glc1P curves. For many ADP-Glc PPases, the Glc1P curves display a problem observed in enzymes with high apparent affinity for the substrate. For an accurate measure of activity, the product ADP-Glc should be determined in the initial part of the time curve, where the velocity does not change significantly during the course of the reaction. At concentrations below the K_m, a pseudo first-order kinetic dominates and the velocity decreases proportionally to the consumption of substrate. For that reason, it is important to control how much substrate reacts, reducing either the amount of enzyme or the reaction time. Therefore, the method of detection should be sensitive enough for the limited amount of product obtained. High sensitivity is critical when the K_m is low because lower concentrations of substrate should be assayed and the tolerance for its consumption is more restricted. To increase the sensitivity of the assay, the specific radioactivity of the substrate [^{14}C]Glc1P can be raised, but it causes an increase of the background. This problem is observed in the upper part of the substrate curve and it could be severe when the product ADP-[^{14}C]Glc is analyzed by binding to anion exchange papers. The window of specific radioactivity combined with the amount of enzyme that should be used for the whole Glc1P curve is very narrow.

Adjusting the specific radioactivity of [^{14}C]Glc and the reaction time (or amount of enzyme) for each part of the substrate curve is an alternative to ensure enough sensitivity and linearity. However, this is tedious and inaccurate since minor experimental errors could lead to curves that appear to be disjointed. The presented procedure avoids these problems because it clearly yields lower blanks (Fig. 3), and the whole curve could be constructed with different concentrations of [^{14}C]Glc1P of constant specific radioactivity. One interesting aspect of the glycogen synthesis method is that the specificity of the detection is very high since only ADP-Glc reacts with glycogen synthase [18]. In the previous procedure, anything that transforms Glc1P into a compound with a negative charge resistant to alkaline phosphatase would bind to DEAE-cellulose paper. For instance, conversion to gluconate 6-phosphate by contaminating phosphoglucomutase and glucose-6-phosphate dehydrogenase, formation of 1,2-cyclic phosphates, or radiolysis can all generate byproducts that interfere. The only disadvantage of this method is that it cannot be used in substrate-specificity experiments. However, the general idea of this procedure could be potentially used to measure UDP-Glc PPase activity. It would require in the second step an UDP-Glc-specific glycogen synthase, which is commercially available.

Conversion of ADP-[^{14}C]Glc to [^{14}C]glycogen is a quantitative process. The only step in which there is a little loss of product is in the precipitation, but it is only ~10%. This is not a real problem for K_m determinations since the efficiency is constant and reproducible for a broad range of concentrations (Fig. 2) and the shape of the curve is not affected. For an even more accurate determination of V_{max}, the exact efficiency of precipitation in a given experiment can be measured by the inclusion of a [^{14}C]glycogen control sample. The previous procedure based on binding to DEAE-cellulose also needed a standard spot of

the radioactive substrate into a filter paper. The efficiency of the scintillation counting decreases up to ~20% when the radioactive material is dried into a paper. This self-absorption phenomenon has already been described [32]. Alternatively, [^{14}C]glycogen synthesized could be determined by paper chromatography [33]. However, we found that the precipitation procedure is more suitable for handling a large number of samples.

Lowering the blanks is extremely important because it is generally the main factor for improving the sensitivity of a radiometric method [32]. For assays based on the binding of product to membrane filters, it has been suggested that prefiltration of the radioactive substrate through the membrane would remove traces of potentially interfering radiochemical impurities [34]. However, in this particular case, that approach is not possible because the substrate Glc1P binds to the DEAE filters also. We reduced this problem with the technique presented in this paper. It is possible that the traces of radiochemical impurities do not interfere with the glycogen precipitation in the same extent as they do with the binding to DEAE paper. The glycogen synthesis procedure has been used successfully for 1 year by four different researchers from two different laboratories. The blanks have been consistently between 40 and 80 cpm under standard conditions (0.5 mM [^{14}C]Glc1P at 17 Bq/nmol; further details as described under Materials and methods), whereas the blanks with the DEAE paper have been typically ~300 cpm. A recent comparison by triplicates yielded 37 ± 4 and 295 ± 30 cpm for the glycogen synthesis and the DEAE paper method, respectively. Traces of contaminating activity of endogenous ADP-Glc PPase coming from the purification of the glycogen synthase could potentially increase the blanks. In case this problem is observed, we recommend including 20 mM EDTA in the incubation of the samples with glycogen synthase. The ADP-Glc PPase from *E. coli* is completely inactive in absence of Mg^{2+}, whereas the activity of the glycogen synthase from *E. coli* in presence of EDTA is 54% compared to the activity in presence of 10 mM $MgCl_2$ (data not shown).

In conclusion, we present a radiometric method for the assay of ADP-Glc PPases that is specific and provides a low background. It shows important advantages over the ones previously described in the literature, particularly, when accurate determination of K_m of ADP-Glc PPases with high affinity for the substrates is needed.

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