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An enzymatic fluorimetric assay for glucose-6-phosphate: application in an *in vitro* Warburg-like effect

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Abstract

Recently, there has been a resurgence of interest in the regulatory role of cell metabolism in tumor biology and immunology. To assess changes in metabolite levels in cell populations and tissues, especially from small clinical samples, highly sensitive assays are required. Based upon glucose 6-phosphate's reaction and the diaphorase-resazurin amplifying system, we have developed a fluorescence methodology to measure glucose 6-phosphate concentrations in cell extracts. In this approach, glucose 6-phosphate is oxidized by glucose-6-phosphate dehydrogenase in the presence of NADP⁺, and the stoichiometrically generated NADPH is then amplified by the diaphorase-cycling system to produce a highly fluorescent molecule - resorufin. The limit of detection (LOD) of the assay is 10 pmol. The assay has a Z' factor of 0.81. Its usefulness is demonstrated by experiments in which the pyruvate kinase inhibitor, phenylalanine, is added to cells. After 2 hours incubation at 37° C, glucose-6-phosphate levels rose by 20%, thus illustrating an *in vitro* Warburg-like effect on cell metabolism.

Keywords

Leukocyte; Metabolism; Glucose 6-phosphate; Sugar phosphate; L-phenylalanine

Introduction

Upon entry into cells, glucose is converted by hexokinase (or glucokinase) into glucose 6-phosphate (G6P). G6P has three principal intracellular fates [1]. It can enter glycolysis via phosphoglucose isomerase to provide cellular energy or carbon skeletons for biosynthesis. G6P may also be metabolized by G6P dehydrogenase thereby entering the hexose monophosphate shunt to provide cells with reducing power and nucleic acid precursors. Finally, G6P may be converted to G1P by phosphoglucomutase, the first step in glycogen synthesis. Hence, G6P is

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a key metabolic substrate because it lies at the intersection of several major metabolic pathways.

G6P measurement techniques include chromatographic [2–5] and enzymatic procedures [6, 7]. Chromatographic methods require an expensive apparatus. Enzymatic methods are specific, rapid and convenient to perform. During enzymatic detection, G6P is oxidized to gluconolactone 6-phosphate by G6P dehydrogenase (G6PD); NADP⁺ is reduced simultaneously to NADPH. Therefore, G6P can be measured by the absorbance at 339 nm or fluorescence at 470 nm of NADPH [6,7]. Due to the weak fluorescence of NADPH and interference from other autofluorescent biological molecules, this method's detection limit is ~ 3.0 μ M [6].

Methods for increasing the sensitivity of enzyme immunoassays have been proposed [8,9]. In these methods amplification is achieved by employing an enzyme to produce a catalytic activator for a secondary system, which amplifies the detectable change. An example of this is the diaphorase-resazurin system, which provides rapid signal amplification [10]. It has been successfully applied for cell quantitation using G6PD [11] and in 2-deoxyglucose measurements [12]. In these assays, NADPH reduces the weakly fluorescent dye resazurin to the highly fluorescent molecule resorufin in the presence of diaphorase. As the emission peak of resorufin is found at 587 nm, autofluorescence from biological samples is negligible.

In this paper, we report a sensitive enzyme amplification assay for the quantitative measurement of intracellular G6P in a 96-well microplate. We also demonstrate the perturbation of metabolism caused by the addition of phenylalanine to Jurkat cells.

Materials and Methods

Chemicals

Resazurin was purchased from Molecular Probes, Inc. (Eugene, OR). G6P, G6PD, diaphorase, tricine and nicotinamide adenine dinucleotide phosphate (NADP⁺) were obtained from Sigma (St. Louis, MO).

Cell culture

Jurkat cells (ATCC, Manassas, VA) were maintained in RPMI-1640 medium (Invitrogen, Carlsbad, CA) containing 10% FCS and 1% antibiotics. For phenylalanine experiments, cells in medium were placed into sterile tubes at a total volume of 1 ml. To one tube, L-phenylalanine was added to yield a final concentration of 6 mM. Cells were incubated for 2 hr at 37°C and then metabolites were extracted as described below.

Sample extraction

The extraction procedure was adapted from Antonio et al. [5] and Ritter et al. [13] with some modifications. In brief, 5×10^6 cells were washed twice with 1 ml of PBS, then 1 ml of ice-cold MeOH/CHCl₃ (2:1) was added, mixed with a vortexer, then stored at –20° C for 2 h. 500 μ l of 50% MeOH in 4mM tricine solution (pH 5.4) was then added, mixed and centrifuged for 10 min at 18,000 $\times$ g (4°C). The upper phase was transferred to a sample tube and kept at 4°C. The chloroform phase was extracted again with 1ml of 50% MeOH in tricine. Aqueous phases from both extractions were combined, centrifuged for 5min at 18,000 $\times$ g (4°C). The upper clear solution was then dried with a Savant SpeedVac system. The extracts were stored at –70°C.

Microplate assay

Just prior to the assay, the extracted samples were dissolved in 50–100 μ l of millipore water. The assay procedure was as follows: a 10 μ l volume of G6P standards (0, 0.1, 0.2, 0.3, 0.4,

0.6, 0.8, 1.0, 2.0, 3.0, 4.0, 6.0, 8.0, 10.0 μM) and extraction samples were pipetted to a 96-well plate followed by the addition of 90 μl of an assay cocktail containing 50 mM triethanolamine (TEA) (pH 7.6), 1.0 mM MgCl_2 , 100 μM NADP^+ , 10 μM resazurin, 0.1 U/ml G6PDH, and 0.2 U/ml diaphorase. These mixtures were incubated for 30 minutes at room temperature. Fluorescence at 590 nm was measured using excitation at 530 nm. Background fluorescence was corrected by subtracting the value of the no-G6P control from all sample readings. Fluorescence was measured using a FlexStation II plate reader (Molecular Devices, Sunnyvale, CA) in a 96-well black assay plate (Costar 3603, Corning).

Results and discussion

Optimization of the assay

G6PD is a key reagent in this G6P assay (Fig. 1). Of the three types of commercially available G6PD enzymes (from baker's yeast, torula yeast, and the bacterium *Leuconostoc mesenteroides*), the leuconostoc [14] and baker's yeast enzymes [15] were recommended by previous investigators. To select the most suitable enzyme, we compared the activities of G6PD from *Leuconostoc mesenteroides* (G8529, lyophilized powder) and from baker's yeast (*S. cerevisiae*) (Type VII G7877, ammonium sulfate suspension) in the same experiment. The results show that the signal-to-noise ratio for leuconostoc G6PD is 1.6-fold better than that of baker's yeast, primarily due to its lower background intensity (Fig. 2A). Therefore, we used G6PD from *Leuconostoc mesenteroides* in subsequent experiments.

The measured fluorescence is not highly sensitive to G6PD concentration, as illustrated for 100 and 1000 pmol G6P in the presence of 0–0.4 U/ml G6PD in Fig. 2B. This is especially true at low levels of G6P (100 pmol/well). According to the unit definition, 0.1 U G6PD is enough to convert 100 nmol of G6P into 6-phosphogluconate per minute. Hence, we used 0.1 U/ml G6PD in the reaction mixture, which is both economical and highly sensitive.

As Fig. 2A and Fig. 2C show, the measured fluorescence intensity is weakly dependent upon the concentration of diaphorase above 0.1 U/ml. For a G6P concentration of 100 pmol, 0.2 U/well yields an acceptable signal-to-noise ratio. As 0.1 U diaphorase catalyzes the oxidation of 100 nmol of NADPH to NADP^+ per minute, 100 nmol of resazurin would optimally be reduced to resorufin. As high concentrations of diaphorase yield little improvement in detection capability, we adopted a concentration of 0.2 U/ml diaphorase for the reaction mixture.

We next assessed the effect of pH on the fluorescence signal produced by the assay. Taking the optimal pH ranges of G6PD and diaphorase into consideration, we performed the assay in TEA buffer at pH 7.0, 7.4, 7.6, 7.8 and 8.1. Fig. 3A shows calibration curves at different pHs. The buffer solution's pH has no significant effect on the fluorescence intensity measured in the assay. As the recommended pH range for resorufin is between 7.5 and 8.5, we performed the assays at pH of 7.6 unless otherwise noted.

To ascertain the optimal incubation time, kinetic experiments were performed. As illustrated in Fig. 3B, the fluorescence intensities generated by 100 to 1000 pmol G6P are stable at times greater than 10 min. A 30-minute incubation at room temperature was used in the assay to insure that the reaction went to completion.

As indicated in Fig. 3C, the fluorescent signal is maximal at a concentration of 15 μM resazurin at 1 nmol G6P. As the resazurin concentration was increased above 15 μM , no further increase in fluorescence was observed. However, the signal-to-noise ratio decreases as the resazurin concentration is increased (Fig. 3D). To avoid this reduction in signal-to-noise ratio, the dye concentration should be lower than 15 μM . As the upper quantitation limit of the assay is equivalent to the amount of resazurin in each well, we used 10 μM of resazurin in the reaction

mixture to optimize the signal-to-noise ratio while maintaining an acceptable quantitation limit. At this concentration the maximum quantitation limit is 1 nmol/well of G6P.

Validation of the assay

The reaction specificity was assessed by dropout experiments in which each important reagent was sequentially omitted from the reaction mixture. The results show that only a complete reaction mixture demonstrates a significant increase in fluorescence and that the fluorescence intensity is G6P concentration-dependent (Fig. 4A). It is important to note that G6P determination is specific; for example, glucose and fructose-6-phosphate do not interfere with the assay [6].

Fig. 4B shows a plot of the measured fluorescence intensity as a function of G6P concentration. Data from two experiments are plotted together in this figure. This standard curve covers a G6P concentration range from 10 pmol to 1 nmol/well. The data points can be fitted to a line with a high correlation coefficient.

The limit of detection (LOD) and limit of quantitation (LOQ) were calculated [16,17] as: $LOD = 3\sigma/S$ and $LOQ = 10\sigma/S$, where σ = standard deviation of the background and S = the slope of the calibration curve. σ was obtained by measuring the fluorescence intensity of nine blank (or background) samples in which all reagents except G6P was added. The measured standard deviation of the fluorescence was $\sigma = 337.5$ fluorescence intensity units. S was calculated from the standard curve of Fig. 4B ($S = 114.2$ fluorescence intensity units/pmol G6P). According to these data, $LOD = 10$ pmol and $LOQ = 30$ pmol.

The intra- run precision was assessed by five replicate measurements of three samples containing lowest (30 pmol), midpoint (500 pmol) and highest concentrations (1000 pmol) of G6P. The inter-run precision was calculated by analyzing the same samples on five consecutive days. The intra and inter-run accuracy ($n = 5$) were determined by calculating the percent errors. The calibration curves were run in parallel with each set of control samples. The results are shown in Table 1. The accuracy and precision of the assay are acceptable. As might be expected, the precision and accuracy are best at the highest G6P concentrations tested.

Zhang et al. [18] have suggested that the Z' factor should be utilized to assess the quality of an assay. The Z' factor is defined as:

$$Z' = 1 - \frac{(3\sigma_{c+} + 3\sigma_{c-})}{|\mu_{c+} - \mu_{c-}|}$$

where μ_{c+} and μ_{c-} are the means of positive control and negative control signals and σ_{c+} and σ_{c-} are the signals' standard deviations. The means and standard deviations were calculated from twelve separate wells with buffer alone and 1000 pmol of G6P, respectively. The Z' factor was 0.81, which indicates that this is excellent assay for measurement of G6P in the range of 30 – 1000 pmol/well.

Application of the assay

We next sought to test this G6P assay using a cellular system. To account for potential losses due to the extraction protocol, we first tested the extraction yield of G6P. In this case a G6P standard solution (500 pmol) was subjected to the same extraction procedures used with cells. We found an extraction yield of 93% (measured G6P 465.7 ± 16.0), indicating that the procedure was very efficient. To assess G6P in cell extracts, we used the assay described above to measure the G6P concentration in Jurkat cells under control conditions or after treatment with 6 mM L-phenylalanine. L-phenylalanine inhibits the activity of pyruvate kinase by 50%

at 6 mM [19]. Cells were treated with phenylalanine for 2 hr at 37°C. As a reduction in pyruvate kinase activity leads to a decrease in glycolytic flux, an increase in the upstream metabolite G6P would be expected. As shown in Table 2, after treatment of Jurkat cells with L-phenylalanine for 2 hr at 37°C, the G6P concentration is about 20% higher than untreated cells.

These Jurkat cell extracts showed that the concentration of G6P was about 100 pmol/10⁶ cells. Assuming a cell diameter of 6 µm, an intracellular concentration of 100 µM is estimated. This number seems reasonable. For example, the concentration of G6P has been reported to be 300 µmol/gram of liver tissue [20], which is approximately 300 µM. This somewhat larger value for the liver is expected because of its role in glycogen synthesis and extracellular G6P may also contribute to the measured concentration. Just as reduced pyruvate kinase activity in tumor cells induces the Warburg effect and an increase in upstream metabolite levels, the reduction in pyruvate kinase activity mediated by phenylalanine treatment promotes an increase in G6P levels. As Jurkat cells are transformed, it may be difficult to stimulate a large increase in upstream metabolites. These findings illustrate the usefulness of this assay.

In summary, we developed a simple, rapid and sensitive fluorimetric assay for direct quantitation of glucose-6-phosphate in cell extracts. We also optimized the source and concentration of G6PD, the pH, the concentrations of resazurin and diphorase, and incubation time. A panel of dropout experiments confirmed the validity of the assay. Moreover, the calibration curves were found to be linear with G6P at 30–1000 pmol/well. The LOD of this assay is 10 pmol, which is about 30-fold more sensitive than the established enzyme assay [6].

This methodology will find many applications in biochemistry and cell biology. For example, it will be useful in monitoring the Warburg effect in tumor cells as well as metabolic changes during immune cell activation and differentiation. It will also be useful in the analysis of G6P levels in small clinical samples. Moreover, it will provide the quantitative data necessary for computational modeling of biochemical reactions.

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Fig. 1.
The mechanism of the G6P assay.

Fig. 2.

Optimization of the G6P assay. Unless otherwise noted, each point is a mean of three individual wells under the same conditions; error bars represent the standard deviations. A. Effect of G6PD source on the fluorescence signals. In these experiments G6PD-A is the G6PD from baker's yeast whereas G6PD-B is the G6PD from *Leuconostoc mesenteroides*. A concentration of 0.1 U/ml was employed. G6P was used at 1 nmol/well. The coupled enzyme diaphorase was used at 0, 0.2 and 0.4 U/ml. The enzyme from *L. mesenteroides* was employed as it was found to have the highest signal-to-noise ratio. B. Effect of G6PD concentration on the fluorescence measurements. G6PD from *Leuconostoc mesenteroides* was used in these experiments. Experiments were conducted with 100 pmol (open bars) and 1000 pmol (cross-hatched bars) of G6P. The fluorescence intensity is plotted versus the G6PD concentration in units/ml. The measured fluorescence intensity is not sensitive to the G6PD concentration under these conditions. C. Effect of diaphorase concentration in this assay. Experiments were conducted with 100 pmol (open bars) and 1000 pmol (cross-hatched bars) of G6P. The fluorescence intensity is plotted versus the diaphorase added in U/ml. A concentration of 0.2 U/ml diaphorase was chosen for the assay.

Fig. 3.

Characterization of the G6P assay. A. The effect of pH on the fluorescence. The assays were performed in triethanolamine (TEA) buffer at pH 7.0, 7.2, 7.4, 7.6, 7.8 and pH 8.1. The amount of G6P was varied from 0 to 1000 pmol. No significant effect of pH on the measured fluorescence intensity was found. The pH did not affect the fluorescence emission intensity of assay. B. The time course of fluorescence development at G6P concentrations of 100, 400, and 1000 pmol. These findings confirm that fluorescence development is rapid, stable, and G6P concentration-dependent. C. The effect of resazurin concentration on the measurement of G6P at 1 nmol/well. The fluorescence intensity is plotted at the ordinate whereas the resazurin concentration is given on the abscissa. Panel D shows the signal-to-noise ratio at the ordinate and the resazurin concentration at the abscissa. As expected due to its weak fluorescence, the background intensity increases as the resazurin concentration increases whereas the signal-to-noise ratio decreases as the resazurin concentration increases. For detection of lower G6P concentrations, a resazurin concentration of 10 μ M is recommended.

Fig. 4.

Validation of the G6P assay. Each datum point is a mean of triplicate measurements. A. Dropout experiments for the G6P assay performed at G6P amounts of 1000 pmol, 500 pmol, and 100 pmol. Results are listed for the following experiments: 1. Complete, 2. No G6P, 3. No resazurin, 4. No NADP+, 5. No G6PD, and 6. No diaphorase. Only the complete reaction medium exhibits a relevant signal. Note that the weak fluorescence of resazurin accounts for the background fluorescence. B. A typical calibration curve for G6P assay. The background has been subtracted from these data points. The data are fit by the linear equation: $y = 114.2x$, where $n = 16$.

Table 1

Intra- and inter-run accuracy and precision of the assay for measurement of G6P (n = 5)

Added	Amount of G6P (pmol/well)		Accuracy (%)	
	Found		Intra-run	Inter-run
	Intra-run	Inter-run		
30	33.6 * (6.6% **)	32.4 (10.5%)	12 ***	8.1
500	538.3 (2.3%)	519.6 (5.7%)	7.7	3.9
1000	966.4 (1.2%)	970.4 (2.6%)	-3.4	-3.0

* Mean of five measurements for each sample

** Coefficient of variation (CV)

*** Percent error

Table 2

Calculated content of G6P in Jurkat cells and extraction yield measurement

Control and Cells	Content of G6P (pmol/10 ⁶ cells)
Untreated cells	84.8 [*] ± 3.3 ^{**}
L-phenyalanine treated cells	100.9 ± 11.7

^{*} Mean and

^{**} standard deviations were calculated from three measurements for each sample

All samples were subjected to the extraction procedure described in the Materials and Methods.