A spectrophotometric assay for 6-phosphogluconolactonase involving the use of immobilized enzymes to prepare the labile 6-phosphoglucono- δ -lactone substrate

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We report the development of a new spectrophotometric assay for 6-phosphogluconolactonase. The labile substrate 6-phosphoglucono- δ -lactone is prepared from glucose 6-phosphate by enzymes co-immobilized on Sepharose beads. The assay has the advantages of high sensitivity for routine determination of enzyme activity and allows determination of both K_m and V_{max} from a single assay. A method for estimating the contribution of spontaneous hydrolysis to total hydrolysis is described. This assay overcomes the problems encountered with all previous assays.

INTRODUCTION

6-Phosphogluconolactonase (PGL, 6-phospho-D-glucose- δ -lactone hydrolase, EC 3.1.1.31) is a cytosolic enzyme that catalyses the conversion of 6-phosphoglucono- δ -lactone into 6-phosphogluconate. This reaction, together with the reactions catalysed by glucose-6-phosphate dehydrogenase (GPDh, D-glucose 6-phosphate:NADP oxidoreductase, EC 1.1.1.49) and 6-phosphogluconate dehydrogenase [PGDh, 6-phospho-D-gluconate:NADP oxidoreductase (decarboxylating), EC 1.1.1.44], constitute the hexose monophosphate shunt:

This pathway is present in all cells and is an important source of cellular NADPH. Abnormal levels of activity of the enzymes of this pathway have been implicated in several metabolic diseases [1,2].

The kinetics of PGL have not been rigorously studied. The major difficulties are the lability and commercial unavailability of the substrate. The half-life of 6-phosphoglucono- δ -lactone (6PGLac) is less than 7 min at pH 7 and decreases rapidly with increasing alkalinity [3]. Studies on the kinetics of PGL have been based on the more stable synthetic substrate 6-phosphoglucono- γ -lactone [3]. However, this synthetic isomer contains a five-membered heterocyclic ring whereas the natural δ -lactone contains a six-membered heterocyclic ring.

Schofield & Sols developed an assay method that employs the natural 6PGLac [4]. However, their assay is

not suitable for use in kinetic studies. The assay mixture contains yeast GPDh and PGDh in large excess. 6PGLac is synthesized *in situ* from glucose 6-phosphate (Glc6P) with the production of NADPH (eqn. 1). Hydrolysis is determined from NADPH production as 6-phosphogluconate (6PGLon) is converted into ribulose 5-phosphate (eqn. 3). But, under the conditions of the assay, significant amounts of Glc6P remain at equilibrium [4]. As the assay proceeds, the equilibrium is disturbed by the removal of NADP⁺ and 6PGLac. The GPDh-catalysed

reaction contributes to total NADPH production. Hence the stoichiometry of the reaction is indeterminate.

We report the development of a new assay method for PGL that uses 6PGLac prepared from Glc6P by enzymes co-immobilized on Sepharose beads. The assay has the advantages of high sensitivity for routine enzyme activity determination and allows determination of both K_m and V_{max} from a single assay.

MATERIALS AND METHODS

Enzymes and reagents

Yeast GPDh, yeast PGDh, yeast glutathione reductase (GSRd), NADP⁺ (monosodium salt) and GSSG were purchased from Boehringer Mannheim, Mannheim,

Abbreviations used: PGL, 6-phosphogluconolactonase; GPDh, glucose-6-phosphate dehydrogenase; PGDh, 6-phosphogluconate dehydrogenase; GSRd, glutathione reductase; 6PGLac, 6-phosphoglucono- δ -lactone; 6PGLon, 6-phosphogluconate; Glc6P, glucose 6-phosphate.

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Germany. Glc6P (monosodium salt, grade 1), 5,5'-dithiobis-(2-nitrobenzoic acid) and CNBr-activated Sepharose 6MB were from Sigma Chemical Co., St. Louis, MO, U.S.A.

Preparation of the 6PGLac-generating column

Preparation of the gel. GPDh and GSRd were co-immobilized on CNBr-activated Sepharose 6B, as described by Ford & DeLuca [5], except that 0.1 M-sodium pyrophosphate buffer, pH 8.0, was used instead of sodium bicarbonate buffer. After completion of the coupling and washing procedure, the gel was resuspended in 0.1 M-sodium phosphate buffer, pH 7, containing 0.2% bovine serum albumin, 0.02% NaN_3 and 2 mM-dithiothreitol. The gel was stored at 4 °C, and the storage buffer was replaced every week with solution containing fresh dithiothreitol.

Packing of the column. Gel containing immobilized enzymes (1.5 ml equivalent to 0.5 g of dry gel) was poured into a 0.5 cm-diameter plastic column. Solutions were pumped through the column with a Gilson Minpuls peristaltic pump. Before use the gel was routinely washed with 10 column volumes of 50 mM-imidazole buffer, pH 7.1.

Determination of free and immobilized enzyme activities

Free and immobilized GPDh and GSRd activities were measured by continuous spectrophotometric analysis of the supernatant from a stirred batch reactor as described by Mattiasson & Mosbach [6]. Imidazole (50 mM), pH 7.1, was used as buffer in all assays.

GPDh activity. Assay mixtures for determination of GPDh activity contained 1 mM-Glc6P and 1 mM-NADP⁺. GPDh activity was calculated from NADPH formation assuming ϵ_{339} 6220 M⁻¹·cm⁻¹, and is expressed in units of $\mu\text{mol}/\text{min}$.

GSRd activity. GSRd activity was determined in the presence of 1 mM-GSSG and 200 μM -NADPH. Activity was calculated from NADPH consumption, and is expressed in units of $\mu\text{mol}/\text{min}$.

Combined GPDh and GSRd activities. Assay mixtures for determination of combined activity contained 1 mM-Glc6P, 1 mM-GSSG and 100 μM -NADP⁺. The assay was started by the addition of Glc6P and the increase in absorbance was monitored at 339 nm. Initially the NADPH/NADP⁺ ratio increased, but approached a constant value as the assay proceeded and steady-state conditions were established. Rate was determined by measuring GSH production after the steady state had been attained. Samples (50 μl) were withdrawn from the stirred batch reactor at 1 min intervals and added to 0.95 ml of 2.5 mM-5,5'-dithiobis-(2-nitrobenzoic acid). After incubation for 10 min the absorbance was measured at 412 nm. GSH concentration was determined assuming ϵ_{412} 13600 M⁻¹·cm⁻¹ for the nitromercaptobenzoate anion formed from 5,5'-dithiobis-(2-nitrobenzoic acid) [7].

Determination of reagent concentrations in column effluent

Column effluents were assayed to determine Glc6P, 6PGLon, NADP⁺, GSSG and 6PGLac concentrations.

Glc6P concentration. To facilitate rapid hydrolysis of 6PGLac the column effluent was adjusted to pH 8.5 with 5 M-KOH and left to stand. Within 45 min all 6PGLac present in the effluent had been converted into 6PGLon. NADP⁺ was added to give a final concentration of 2.5 mM. Yeast GPDh (2 units) was added and the absorbance increase was monitored at 339 nm. All Glc6P present in the assay solution was converted into 6PGLac within 15 min. The Glc6P concentration was calculated from NADPH formation.

6PGLon concentration. NADP⁺ was added to the effluent to give a final concentration of 2.5 mM. The 6PGLon concentration of this solution was determined from the increase in NADPH concentration after the addition of 1.5 units of PGDh. Within 1 min the NADPH concentration in the solution reflected the 6PGLon concentration in the column effluent (see the Discussion section).

6PGLon was formed in the effluent from the spontaneous hydrolysis of 6PGLac. Hence the concentration of 6PGLon in the effluent increased with time. The 6PGLon concentration of fractions was determined 2 min after elution.

GSSG concentration. NADPH was added to the column effluent to give a final concentration of 1 mM. The sample was loaded into a 2 mm-pathlength cuvette and 2 units of yeast GSRd were added. The GSSG concentration was calculated from the decrease in absorbance at 339 nm.

NADPH and NADP⁺ concentrations. The NADPH concentration of column effluent was determined from the absorbance at 339 nm. The effluent NADP⁺ concentration was calculated from the difference between the effluent NADPH and pre-column NADP⁺ concentration.

6PGLac concentration. See the Discussion section.

Procedure for generating 6PGLac and assaying PGL

Preparation of 6PGLac. A solution of 50 mM-imidazole buffer, pH 7.1, containing 1.1 mM-Glc6P, 1.3 mM-GSSG and 0.1 mM-NADP⁺ was pumped through the 6PGLac-generating column (Fig. 1). The first 10 ml of effluent was discarded.

Preparation of assay solution. A 360 μl portion of column effluent was transferred into a 2 mm-pathlength cuvette containing 20 μl of 50 mM-NADP⁺. The assay mixture contained 50 mM-imidazole buffer, pH 7.1, 200 μM -GSSG, 2.6 mM-NADP⁺, 2 mM-GSH, 100–200 μM -6PGLon and 800–900 μM -6PGLac.

Initiation of PGL assay. The assay was initiated by the addition of 10 μl of yeast PGDh solution containing 1.5 units. Within 1 min the steady state was established (period A in Fig. 2). Under steady-state conditions the hydrolysis of 1 μmol of 6PGLac resulted in the formation of 1 μmol of NADPH (eqn. 3). The spontaneous hydrolysis of 6PGLac was monitored by measuring the increase in absorbance at 339 nm with time (period B in Fig. 2).

Determination of PGL activity. Precisely 3.5 min after commencement of the assay, 10 μl of purified PGL

Fig. 1. Arrangement of the immobilized enzyme column and auxiliary pump

Effluent Glc6P was converted into 6PGlac by GPDh immobilized on the column matrix. The GPDh-catalysed reaction was driven to completion by the oxidation of NADPH by GSRd in the presence of GSSG.

Fig. 2. Progress curve for PGL assay

Absorbance was measured at 399 nm in a cuvette of 2 mm pathlength. The progress curve has been divided into three periods: period A (0–1 min), the steady state is being established; period B (1–2.5 min), the steady state has been established and the slope reflects the rate of spontaneous hydrolysis of 6PGlac; period C, where PGL has been added and the slope now reflects spontaneous and enzyme-catalysed hydrolysis of 6PGlac.

solution was added to the assay solution. The increase in absorbance with time then reflected the rate of spontaneous hydrolysis plus the rate of enzyme-catalysed hydrolysis (period C in Fig. 2). The rate of enzyme-catalysed hydrolysis was determined by subtracting the rate of spontaneous hydrolysis from the combined hydrolysis rate. The assay was conducted in a total volume of 400 μ l and at a constant temperature of 25 $^{\circ}$ C.

RESULTS

Efficiency of immobilization

The efficiency of enzyme immobilization was determined from bound and free enzyme activities after

coupling to the CNBr-activated Sepharose. The proportions of GPDh and GSRd activities that were coupled to the Sepharose were 11.4% and 6.9% respectively. This corresponded to an activity of 8.0 units/g of dry gel for GPDh and 4.2 units/g of dry gel for GSRd.

6PGlac production by co-immobilized GPDh and GSRd

Efficiency of 6PGlac production. Co-immobilized GPDh and GSRd were 3-fold more efficient in 6PGlac production and reached steady-state conditions more rapidly than did a mixture of the free enzymes. Other workers have reported similar effects for regenerative systems using co-immobilized enzymes [8,9]. The complex kinetics of co-immobilized enzymes have been treated in detail by several workers (see ref. [10]). At the steady state 22.6 μ mol of 6PGlac was formed/min per g of dry gel.

Effect of flow rate on 6PGlac production. Flow rate through the 6PGlac-generating column was an important factor in determining the effluent composition. Glc6P should remain in contact with the column long enough for complete conversion into 6PGlac. However, with increasing time on the column the amount of 6PGlac in the effluent decreased as a result of spontaneous hydrolysis. Complete conversion of Glc6P was achieved with the column described here when the flow rate was less than 0.35 ml/min per cm^2 . The flow-rate range chosen was 0.25–0.35 ml/min per cm^2 (Fig. 3). Experimentally this was also a convenient flow rate, since enough effluent was produced to permit assays every 5 min.

Stability of immobilized enzymes. GPDh and GSRd are quite stable when immobilized. The activity of both enzymes was still greater than 85% of the original activity 5 months after regular use.

Kinetic parameters of PGDh

The kinetic parameters of yeast PGDh were determined according to published procedures (see ref. [10]). Under the conditions used in our assay yeast PGDh had a K_m of 43 μ M and a specific activity of 10 units/mg of protein.

Fig. 3. Reagent (or substrate) concentrations in the column effluent

All assays were performed in duplicate and represent reagent concentrations 2 min after the elution of a 1 ml fraction.

DISCUSSION

PGDh requirements

The amount of PGDh present in the PGL assay determines the lag period that precedes the establishment of the steady state and the concentration of 6PGlon at the steady state. Because of the unstable nature of 6PGlac, the steady state should be established as quickly as possible. A low steady-state concentration of 6PGlon is desirable, since this greatly simplifies calculations for determining 6PGlac concentrations and spontaneous hydrolysis rates. The presence of a large excess of PGDh ensures that both of these conditions are satisfied. However, the use of an unnecessarily large amount of PGDh is wasteful and expensive. To determine the most economical use of PGDh in this assay we applied the theoretical treatment for coupled enzyme systems developed by Storer & Cornish-Bowden [11] (see also ref. [12]). For the calculations, the $K_m(6PGlon)$ for PGDh determined under the conditions of our assay was employed (see the Results section) and it was assumed that the maximum rate of hydrolysis did not exceed 0.2 mm/min. Under these conditions 1.5 units of PGDh are sufficient to ensure that the steady state is established within 45 s of initiation of the assay and re-established within 3 s of the addition of PGL. Under these conditions the concentration of 6PGlon is insignificant at the steady state (less than 2.5 μM).

Calculation of 6PGlac concentrations and spontaneous hydrolysis rates

6PGlac concentration. For the determination of kinetic parameters of an enzyme it is necessary to know the substrate concentration in the assay solution. Because 6PGlac starts to hydrolyse as soon as it is formed, previous PGL assay methods could only estimate the substrate concentration during activity measurements.

The assay method that we have developed allows the accurate determination of 6PGlac concentration at any time after the establishment of steady-state conditions. Every 1 μmol of 6PGlac that is hydrolysed produces 1 μmol of 6PGlon (eqn. 2). All 6PGlon formed in the assay solution is converted into ribulose 5-phosphate with the concomitant production of NADPH (eqn. 3). Under steady-state conditions the amount of 6PGlon present is insignificant. Hence any time after the establishment of the steady state the amount of NADPH present is equivalent to the amount of 6PGlac that has been hydrolysed. If 6PGlac hydrolysis is allowed to go to completion, the amount of NADPH present in the assay solution is equal to the total amount of 6PGlac that was formed by the 6PGlac-generating column. After the steady state is established, the 6PGlac remaining in the assay solution can be calculated from the difference between the total amount of 6PGlac that was formed and the amount that has been hydrolysed. Hence:

$$M_t = M_T - M_H$$

where M_t is the amount of 6PGlac at time t after the establishment of the steady state, M_T is the amount of NADPH after all 6PGlac has been hydrolysed and M_H is the amount of NADPH present at time t .

NADPH absorbs at 339 nm and hence steady-state 6PGlac concentrations can be calculated from the progress curve (Fig. 2):

$$C_t = 1.244(A_T - A_t)$$

where C_t is the concentration of 6PGlac at time t , A_T is the total change in absorbance after all 6PGlac has been hydrolysed, A_t is the absorbance at time t and 1.244 is a constant incorporating pathlength (0.2 cm) and the molar absorption coefficient for NADPH ($6220 M^{-1} \cdot cm^{-1}$).

For these calculations it is necessary to determine the absorbance of the assay solution after all 6PGlac has been hydrolysed. This can be determined by monitoring the reaction until there is no further change in absorbance. Alternatively, the total absorbance change can be calculated from the concentration of Glc6P in the pre-column solution.

Hydrolysis rate after addition of PGL. For an accurate estimation of enzyme-catalysed hydrolysis, the rate of spontaneous hydrolysis should be assessed throughout the course of the assay. The spontaneous hydrolysis of 6PGlac follows a first-order reaction [4], and therefore the rate of spontaneous hydrolysis is proportional to the concentration of 6PGlac. After addition of enzyme to the assay solution, the concentration of 6PGlac falls rapidly as a result of PGL-catalysed hydrolysis. Hence the contribution of spontaneous hydrolysis to the overall rate of hydrolysis decreases as the assay proceeds. The rate of spontaneous hydrolysis at any point on the progress curve can be calculated from the concentration of 6PGlac remaining and the rate of hydrolysis determined before addition of PGL (eqn. 2):

$$R_t = R_H \left(\frac{C_t}{C_H} \right)$$

where R_t is the rate of spontaneous hydrolysis at time t , R_H is the rate of spontaneous hydrolysis measured at the steady state before the addition of PGL, C_H is the concentration of 6PGlac at which R_H was measured and C_t is the concentration of 6PGlac at time t .

Spectrophotometers with microprocessors, such as the Varian DMS-200 on which this assay method was developed, can be programmed to perform these calculations.

Determination of kinetic parameters of PGL

A major advantage offered by this assay method is the potential to use the entire progress curve (Fig. 2) from a single run to determine kinetic parameters. At the steady state the concentration of 6PGlon is maintained at insignificant levels by the addition of PGDh. Hence PGL catalyses the hydrolysis of its substrate under initial-rate conditions at all points along the progress curve. The concentration of 6PGlac and the contribution of spontaneous hydrolysis to overall hydrolysis can be readily calculated. Ionic strength, pH and temperature all remain constant during the course of the assay. Thus kinetic parameters can be determined from the results of a single assay. This method has the further advantage that it is readily amenable to direct on-line computer analysis. A number of published treatments for analysis of progress curves are applicable to this assay [13].

Summary

Studies of the enzyme PGL and the reaction that it catalyses have been hindered by preparation and storage problems associated with the chemically unstable substrate 6PGlac. The 6PGlac-generating column described in the present paper allows rapid and

convenient preparation of unlimited amounts of 6PGlac. Previous methods for the assay for PGL activity have been expensive, poorly defined and difficult to perform. The assay method that we have developed is economical, easily performed and reliable. Furthermore, our assay method is unusually well suited to kinetic studies of PGL.

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