A Colorimetric Determination of Inositol Monophosphates as an Assay for D-Glucose 6-Phosphate-1L-*myo*Inositol 1-Phosphate Cyclase

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A rapid and convenient chemical assay for the enzyme D-glucose 6-phosphate-1L-*myo*inositol 1-phosphate cyclase is described. The 1L-*myo*inositol 1-phosphate formed enzymically was oxidized with periodic acid liberating inorganic phosphate, which was assayed. *myo*Inositol 2-phosphate can be assayed in the same way. Glucose 6-phosphate and other primary phosphate esters gave only very small quantities of inorganic phosphate under the conditions described. The K_m of the enzyme for D-glucose 6-phosphate, $7.5 \pm 2.5 \times 10^{-4}$ M, was identical with that measured by the radiochemical method. 2-Deoxy-D-glucose 6-phosphate was a powerful competitive inhibitor, K_i $2.0 \pm 0.5 \times 10^{-5}$ M, but was not a substrate for the enzyme.

The enzyme D-glucose 6-phosphate-1L-*myo*inositol 1-phosphate cyclase has been partially purified from yeast (Chen & Charalampous, 1966; Charalampous & Chen, 1966), *Neurospora* (Pina & Tatum, 1967), higher plants (Von Ruis, Molinari & Hoffmann-Ostenhof, 1967) and animal tissues (Eisenberg, 1967) and some aspects of its mechanism of action have been investigated (Barnett & Corina, 1968; Sherman, Stewart & Zinbo, 1969). However, further purification and investigation of the kinetic properties of the enzyme have been severely hampered by the length and difficulty of the radiochemical assay for the enzyme, in which radioactive D-glucose 6-phosphate is enzymically converted into 1L-*myo*inositol 1-phosphate, which must then be converted into inositol and separated from contaminating glucose before counting of radioactivity, preferably as the recrystallized hexa-acetate.

An alternative assay has been used (Eisenberg, 1967) in which a specific phosphatase, inactive towards D-glucose 6-phosphate, is extracted from rat testes and used to release inorganic phosphate enzymically from the 1L-*myo*inositol 1-phosphate. The inorganic phosphate is then measured.

This paper describes a rapid and simple chemical assay for the inositol phosphate produced by the cyclase. The method can be used to assay other inositol monophosphates.

MATERIALS AND METHODS

Chemicals. D-Glucose 6-phosphate disodium salt, trichloroacetic acid (AnalaR), sodium sulphite (AnalaR), ammonium molybdate (AnalaR), sulphuric acid (AnalaR)

and ascorbic acid were obtained from BDH Chemicals Ltd. (Poole, Dorset, U.K.), NAD⁺ was obtained from Boehringer Corp. (London W.5, U.K.) and *myo*inositol 2-phosphate dicyclohexylamine salt from Calbiochem (London W.1, U.K.). 2-Deoxy-D-glucose 6-phosphate was obtained from Sigma (London S.W.6, U.K.). 1L-*myo*inositol 1-phosphate dicyclohexylamine salt was made as previously described (Mercier, Barnett & Géro, 1969). D-[6-³H]Glucose 6-phosphate and [³H]H₂O were obtained from The Radiochemical Centre (Amersham, Bucks., U.K.).

2-Deoxy-D-[2-³H]glucose (2-deoxy-D-[2-³H]arabino-hexose). D-Glucal (100 mg) was dissolved in tritiated water (1 ml, 50 mCi) and conc. H₂SO₄ (0.05 ml) was carefully added at 0°C. The well-stoppered solution was left for 5 h at 0°C and diluted with water (4 ml), the solution was neutralized with Amberlite MB 1 resin and filtered. The filtrate was freeze-dried and the tritiated water recovered in a liquid-N₂ trap. The sugar was chromatographed on Whatman 3MM paper with butan-1-ol-ethanol-water (4:1:5, by vol.; upper layer) as solvent, eluted and evaporated to dryness *in vacuo* at 35°C; yield 20 mg, 24 μ Ci/mmol. The sugar was converted into the 6-phosphate as described by Barnett & Corina (1966) with a buffer containing 5 mM-MgCl₂.

Enzyme preparation. A partially purified enzyme preparation was obtained by slight modification of the procedure previously described (Barnett & Corina, 1968). The testes (about 20 g) of six rats were homogenized in 2 vol. of ice-cold 0.154 M-KCl, pH 7.4, and centrifuged at 160 000 g at 2°C for 80 min. The supernatant was heated at 60°C for exactly 2 min to destroy phosphatases and then fractionated with (NH₄)₂SO₄. The 30–40%-saturated fraction, which was precipitated at 3°C by (NH₄)₂SO₄ at concentrations between 165.5 g/l and 222.2 g/l was redissolved in 5 ml of 50 mM-tris-acetate buffer, pH 7.4, containing GSH (1 mM) and EDTA (1 mM) and dialysed against the same buffer overnight. The preparation had an activity of about 0.5 unit/ml, where one unit is defined

as $1\mu\text{mol}$ of inositol 1-phosphate formed/h at 37°C in a 1ml incubation mixture containing 2mM-glucose 6-phosphate and the usual additions. The protein concentration was 1–1.5mg of protein/ml as measured by the method of Lowry, Rosebrough, Farr & Randall (1951) with bovine serum albumin as the protein standard. For most experiments this preparation was diluted twofold with buffer. It could be stabilized by addition of dithiothreitol (final concentration 0.5mM).

Assay of inositol monophosphates. The inositol phosphate ($0\text{--}0.5\mu\text{mol}$, 0.5ml) was incubated at 37°C for 1h (1.5h for inositol 2-phosphate) with 0.2M- NaIO_4 (0.5ml). Then 1M- Na_2SO_3 (1ml) was added to destroy excess of NaIO_4 and the liberated P_i was measured by the method of Chen, Toribara & Warner (1956). A reagent (2ml), prepared by adding 1 vol. of 3M- H_2SO_4 , 1 vol. of 2.5% (w/v) ammonium molybdate and 2 vol. of water followed by 1 vol. of 10% (w/v) ascorbic acid, was then added and the solution (total volume 4ml) reincubated at 37°C for 1.5h. The colour was read at 820nm (range $0\text{--}0.1\mu\text{mol}$) or 660nm (range $0\text{--}0.5\mu\text{mol}$) in a Unicam SP. 600 spectrophotometer, light-path 1cm. Phosphate release was quantitative and an inorganic phosphate standard curve may be used. A blank untreated with NaIO_4 or Na_2SO_3 gives the amount of inorganic or acid-labile phosphate present. Trichloroacetic acid may be used as a protein precipitant. Use of HClO_4 gives a faint precipitate of (presumably) KClO_4 even with AnalaR reagents. Only 2mmol of P_i are released/mol of glucose 6-phosphate under these conditions. HIO_4 may be used in place of NaIO_4 , allowing the volume of the periodate (1M) added to be 0.1ml, but the more acid conditions cause slightly higher phosphate release from glucose 6-phosphate.

D-Glucose 6-phosphate-1L-myoinositol 1-phosphate cyclase assay. The enzyme (0.2ml) was added to 5mM-D-glucose 6-phosphate (0.2ml) and 5mM-NAD⁺ (0.1ml). All solutions were made up in 50mM-tris-acetate buffer, pH7.4, containing EDTA (0.1ml) and ammonium acetate (1.0mM). Tubes were incubated at 37°C for the required time (usually 1h) and 20% (w/v) trichloroacetic acid (0.2ml) was added to terminate the reaction. The precipitated protein was centrifuged off and the supernatant (0.5ml) was incubated with 0.2M- NaIO_4 . The periodate was destroyed and the inorganic phosphate measured as described for the assay of inositol 1-phosphate. The glucose 6-phosphate used contained inorganic phosphate and therefore a blank, in which trichloroacetic acid was added before the enzyme, was used. This blank also corrected for the slight hydrolysis of phosphate from glucose 6-phosphate on periodate oxidation. Phosphatases active in the enzyme preparation, which might give an erroneous assay, may be tested for by the assay of free phosphate only. In this case the HIO_4 and Na_2SO_3 are omitted. However, with the Mg^{2+} -free, EDTA-containing buffer, no phosphatase activity could be detected, although phosphatases were known to be present.

For K_m determinations, the glucose 6-phosphate concentrations were 0.4–8.0mM and a blank was used at each concentration.

Radiochemical assay of the cyclase. The enzyme was assayed as previously described, with $[6\text{-}^3\text{H}]\text{glucose 6-phosphate}$ as substrate (Barnett & Corina, 1968). When 2-deoxy-D- $[2\text{-}^3\text{H}]\text{glucose}$ was tested as substrate, carrier

inositol was not added, but otherwise the same procedure was followed. The area of the chromatogram corresponding to deoxyinositols was eluted and its radioactivity counted.

RESULTS AND DISCUSSION

Conditions for the assay of 1L-myoinositol 1-phosphate in the presence of D-glucose 6-phosphate. In initial experiments to determine the optimum conditions for the periodate oxidation of inositol phosphates, the time-course of the oxidation was followed by assay of inorganic phosphate released for both 1L-myoinositol 1-phosphate and myo-inositol 2-phosphate, different concentrations of periodate being used. Some of the results are shown in Fig. 1. After 1h at 37°C with 0.1M-sodium periodate (final concn.), oxidation of 1L-myo-inositol 1-phosphate was complete and the glucose 6-phosphate gave only 2mmol of phosphate/mol. When these conditions were used for the assay of 1L-myoinositol 1-phosphate as inorganic phosphate released, by the phosphate method of Chen *et al.* (1956), a standard curve was linear over the range $0\text{--}0.5\mu\text{mol}$. A similar curve for myo-inositol 2-phosphate, with a 90min incubation at 37°C with 0.1M-sodium periodate, was also linear.

Conditions for the colorimetric assay of D-glucose 6-phosphate-1L-myoinositol 1-phosphate cyclase. Since the K_m of D-glucose 6-phosphate for the enzyme is low, a concentration of 2mM is convenient

Fig. 1. Time-course of inorganic phosphate released as a percentage of total phosphate after incubation of inositol phosphates with periodate at 37°C . ○, 0.2mM-1L-myo-inositol 1-phosphate with 0.1M- NaIO_4 ; ▲, 1.0mM-myo-inositol 2-phosphate with 0.1M- NaIO_4 ; ●, 0.4mM-1L-myo-inositol 1-phosphate with 0.05M- NaIO_4 ; ■, 8.0mM-D-glucose 6-phosphate ($\% \times 100$) with 0.1M- NaIO_4 .

for the standard assay system. The final conditions chosen are described in the Materials and Methods section. Since commercial samples of glucose 6-phosphate contain inorganic phosphate, and there is a slight release of inorganic phosphate from glucose 6-phosphate during periodate oxidation (Fig. 1), it is essential to use adequate controls. The most convenient is one in which trichloroacetic acid is added before the enzyme. Mg^{2+} is excluded from the assay buffer, which also contains EDTA to chelate traces of Mg^{2+} which may be present in the extract. With this buffer phosphatase activity, which can be detected by using a control that is incubated with enzyme but not treated with $NaIO_4$ and Na_2SO_3 , was not observed. The assay can detect 0.005 units of enzyme ($0.005 \mu\text{mol/h}$).

Determination of K_m for D-glucose 6-phosphate and K_i for 2-deoxy-D-glucose 6-phosphate. The K_m for glucose 6-phosphate was determined by using the concentration range 0.4–8 mM by both the colorimetric and radiochemical methods. The K_m was $7.5 \pm 2.5 \times 10^{-4} M$ by either method. The K_i for 2-deoxy-D-glucose 6-phosphate was $2.0 \pm 0.5 \times$

$10^{-5} M$ (Fig. 2) and was determined by using both 1 mM and 0.1 mM concentrations of inhibitor. Any substrate activity of the deoxy sugar would not be detected by the colorimetric assay, since the deoxyinositol phosphate that would be formed is not oxidized by periodate with release of phosphate, therefore the ability of the deoxy sugar to act as a substrate was tested by the radiochemical method. It was not a detectable substrate.

General discussion. Assay of D-glucose 6-phosphate–1L-myoinositol phosphate cyclase has hitherto been very tedious. Most workers (Chen & Charalampous, 1966) have followed the enzyme activity by the incorporation of radioactivity from D-glucose 6-phosphate into inositol, after suitable hydrolysis of the initially formed 1L-myoinositol 1-phosphate. This method requires considerable skill and even in experienced hands takes several days.

An alternative method involves the use of a specific inositol phosphatase isolated from testes (Eisenberg, 1967) which specifically cleaves myo-inositol 1-phosphate in the presence of glucose 6-phosphate, giving inorganic phosphate, which may then be assayed.

Recent work on the periodate oxidation of monomethyl ethers of inositol (Girma, Rokicka & Szabo, 1969) indicated that methoxymalondialdehyde was first formed and spontaneously hydrolysed to methanol and hydroxymalondialdehyde, which was then further oxidized. By analogy inositol monophosphates might be expected to be oxidized by periodate as shown in Scheme 1 leading to the quantitative formation of inorganic phosphate. In contrast glucose 6-phosphate will give the stable glyceraldehyde phosphate. Since inorganic phosphate is readily assayed in the presence of organic phosphates a rapid and purely chemical assay of the cyclase is possible.

Preliminary tests showed that simple conditions could be obtained to give a quantitative release of inorganic phosphate from inositol phosphates in the presence of glucose 6-phosphate (Fig. 1). Destruction of periodate with sodium sulphite allowed analysis of

Fig. 2. Lineweaver-Burk plots of $1/v$ against $1/s$ for D-glucose 6-phosphate (0.4–8 mM) in the presence (□) and absence (●) of 1 mM-2-deoxy-D-glucose 6-phosphate.

Scheme 1.

inorganic phosphate colorimetrically. NAD^+ , which is added as a cofactor in the enzyme assay, did not interfere when the periodate oxidation was carried out in acid solution.

The final assay proved to be very reproducible and relatively rapid. It was used to determine the K_m for glucose 6-phosphate and also the K_i for the inhibitor 2-deoxy-D-glucose 6-phosphate which was shown to be competitive.

The mechanism of action of the enzyme generally accepted (Barnett & Corina, 1968; Sherman *et al.* 1969) involves the NAD^+ oxidation of the C-5 hydroxyl group to an oxo group, an internal aldol condensation to give inosose 1-phosphate which is then re-reduced by the NADH formed in the first step. Retention of the hydrogen atom at C-2 was observed during the cyclization. The low K_i of 2-deoxy-D-glucose 6-phosphate for the enzyme, $2.0 \pm 0.5 \times 10^{-5} \text{ M}$, is therefore noteworthy since it was not a substrate. The high binding capacity of the deoxy sugar, and yet its inability to act as a substrate, is difficult to account for by electronic effects and suggests that the C-2 hydroxyl group is important for catalytic activity, perhaps being involved in the conformational stability of the active site.

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