Hexose Phosphate Synthase in Trimethylamine-Grown Bacterium 2B2, a Facultative Methyilotroph

By ROGER B. COX and L. J. ZATMAN

Department of Microbiology, University of Reading, Reading RG1 5AQ, U.K.

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Hexose phosphate synthase and hexulose phosphate isomerase activities were found in trimethylamine-grown bacterium 2B2, a facultative methyilotroph. The activities were separated by column chromatography of cell extracts on DEAE-cellulose. Hexulose phosphate isomerase activity was measured spectrophotometrically by using the product of the hexose phosphate synthase reaction as substrate.

Hexose phosphate synthase activity (see Quayle, 1972) has been demonstrated in some obligate methyilotrophs which grow on methane (Lawrence & Quayle, 1970) and in the non-methane-utilizing obligate methyilotrophs bacterium 4B6 (Colby & Zatman, 1972) and organism W1 (Dahl *et al.*, 1972). *Pseudomonas* C (Stieglitz & Mateles, 1973) is the only known example of a facultatively methyilotrophic bacterium in which hexose phosphate synthase activity has been demonstrated. The present paper provides evidence supporting the conclusion that bacterium 2B2 (Colby & Zatman, 1973) is a second example of a facultatively methyilotrophic bacterium which contains hexose phosphate synthase activity when grown on a C_1 substrate.

Materials and methods

The chelated mineral base E medium of Owens & Keddie (1969) fortified with vitamins as described by Colby & Zatman (1973) was used as the growth medium. Filter-sterilized trimethylamine hydrochloride was added to a final concentration of 0.2%. Solidified medium was prepared by the addition of Oxoid Ionagar no. 2 (12 g/litre) to the liquid medium. Organisms for the preparation of cell-free extracts were grown in 2-litre conical flasks containing 1 litre of medium and incubated on an orbital shaker at 30°C. Cells were harvested in mid-exponential phase and were washed twice with ice-cold 50 mM-sodium phosphate buffer, pH 7.5, containing 5 mM-MgCl₂. Cell pellets for the preparation of cell-free extracts were either used immediately or stored at -70°C until required. Thick suspensions of cells in this phosphate-MgCl₂ buffer were disrupted by passing twice through a French pressure cell, pre-cooled to 0°C, at a pressure of 15000 lb/in² (103 MPa); whole cells and debris were removed by centrifugation at 35000g for 30 min and the supernatant was used as crude extract. Protein concentrations were determined with the Folin phenol reagent by the method of Kennedy & Fewson (1968) with crystalline bovine plasma albumin as the standard.

The following enzyme activities were assayed at 30°C by the published methods quoted: hydroxypyruvate reductase (EN 1972 1.1.1.29*), Lawrence *et al.* (1970); ATP malate lyase, Cox & Zatman (1973); malyl-CoA lyase (EN 1972 4.1.3.24), Salem *et al.* (1973) [(S)-(β -hydroxysuccinyl)-N-octanoyl-cysteamine for the synthesis of malyl-CoA was a generous gift from Professor J. R. Quayle, Department of Microbiology, University of Sheffield, Sheffield, U.K.]; transaldolase (EN 1972 2.2.1.2), Tchola & Horecker (1966); transketolase (EN 1972 2.2.1.1), De la Haba & Racker (1955), in the presence of added ribulose phosphate 3-epimerase (EN 1972 5.1.3.1); ribulose phosphate 3-epimerase was measured in the transketolase assay mixture in the presence of added transketolase. The spectrophotometric assay of hexose phosphate synthase was done by using the assay mixture of Dahl *et al.* (1973) supplemented with 1.5 units of ribose phosphate isomerase (EN 1972 5.3.1.6); in this assay, the addition of glucose phosphate isomerase (EN 1972 5.3.1.9) and glucose 6-phosphate dehydrogenase (EN 1972 1.1.1.49) links the production of fructose 6-phosphate with NADP⁺ reduction (see Scheme 1). Reaction mixture A, for hexose phosphate synthase, assay by measurement of formaldehyde disappearance, contained in 0.8 ml: 50 μ mol of sodium phosphate buffer, pH 7.0, 5.0 μ mol of MgCl₂, 5.0 μ mol of ribose 5-phosphate, 1.5 units of ribose phosphate isomerase and 5.0 μ mol of formaldehyde. After 2 min pre-incubation at 30°C, 0.2 ml of enzyme preparation was added; at suitable time-intervals 0.1 ml volumes were transferred into 1 ml of 10% (w/v) trichloroacetic acid and the concentration of formaldehyde was determined by the method of Nash (1953).

Ribose phosphate isomerase, ribulose phosphate 3-epimerase, transketolase (type IV) and glucose phosphate isomerase were purchased from the Sigma (London) Chemical Co. Ltd., Kingston-upon-Thames, Surrey KT2 7BH, U.K., and glucose

* From the 1972 edition of Enzyme Nomenclature; see *Comprehensive Biochemistry* (1973) vol. 13 (Florkin, M. & Stotz, E. N., eds.), Elsevier, Amsterdam and New York.

6-phosphate dehydrogenase (grade I) was from Boehringer Corp. (London) Ltd., Ealing, London W5 2TZ, U.K.

One enzyme unit is the amount of enzyme that catalyses the transformation of $1\mu\text{mol}$ of substrate/min (or the formation of $1\mu\text{mol}$ of product/min) at 30°C under the assay conditions described.

Experimental and results

Crude extracts of trimethylamine-grown bacterium 2B2 were examined for hydroxypyruvate reductase, ATP malate lyase and malyl-CoA lyase activities; none of these enzyme activities was detectable. Attempts to determine hexose phosphate synthase activity in crude extracts by the spectrophotometric method gave equivocal results; the rate of NADP^+ reduction was rapid in the absence of formaldehyde and this rate was not increased by the addition of formaldehyde. Nevertheless, formaldehyde disappearance from reaction mixture A with crude extract was rapid (initial rate $0.48\mu\text{mol/min}$ per mg of protein) and was dependent on the presence of ribose 5-phosphate (see Scheme 1), suggesting the presence of hexose phosphate synthase.

Measurement of the specific activities of transketolase, transaldolase and ribulose phosphate 3-epimerase in crude extracts gave values of 410, 690 and 170 units/mg of protein respectively, results which suggest that the NADP^+ reduction observed in the spectrophotometric hexose phosphate synthase assay probably occurs via the route shown within the broken lines of Scheme 1. This suggestion is supported by the observation that crude extracts catalyse the formation of hexulose in reaction mixture A, as detected by the method of Dische & Devi (1960), from ribose 5-phosphate in the presence or absence of form-

aldehyde. In this respect bacterium 2B2 differs from pseudomonad C of Stieglitz & Mateles (1973) in that crude extracts of the latter organism formed only an insignificant amount of hexulose phosphate from ribose 5-phosphate in the absence of formaldehyde (and ribose 5-phosphate disappearance was similarly formaldehyde-dependent).

There are, however, at least two possible routes which could account for the ribose 5-phosphate-dependent disappearance of formaldehyde (see Scheme 1): one route would be mediated by hexose phosphate synthase, whereas the other would be mediated by transketolase, where the latter enzyme uses formaldehyde as the acceptor molecule (Dickens & Williamson, 1958), yielding dihydroxyacetone and glyceraldehyde 3-phosphate. These two routes are not necessarily mutually exclusive. Evidence for the presence of hexose phosphate synthase and hexulose phosphate isomerase in trimethylamine-grown bacterium 2B2 was obtained by column chromatography of crude extract on DEAE-cellulose as described below.

Nucleic acids (and about 7% of the total protein) were removed from the crude extract (6ml, 27.0mg of protein/ml) by adding 1.5ml of a protamine sulphate solution (15mg/ml) at 0°C followed by centrifugation at $10000g$ for 5min. The pellet was discarded and the supernatant (100mg of protein, 5ml total vol.) was applied to the top of a column ($18\text{cm} \times 1.5\text{cm}$) of DEAE-cellulose (Cellex D) equilibrated at 3°C with 20mM-sodium phosphate buffer, pH 7.0, containing 5mM- MgCl_2 . The column was eluted with approx. 50ml of the latter buffer and then with a linear gradient of NaCl (0–0.5M) in this same buffer, and fractions (2.7ml) were collected. The results of a typical experiment are shown in Fig. 1.

Scheme 1. Pathways of formaldehyde and ribose 5-phosphate metabolism considered during the study of C_1 metabolism with preparations from trimethylamine-grown bacterium 2B2

Abbreviations: HuPS, hexose phosphate synthase (hexulose phosphate synthase); HuPI, hexulose phosphate isomerase; RPI, ribose phosphate isomerase; RuPE, ribulose phosphate 3-epimerase; TK, transketolase; TA, transaldolase; R5P, ribose 5-phosphate; Ru5P, ribulose 5-phosphate; Xu5P, xylulose 5-phosphate; G3P, glyceraldehyde 3-phosphate; S7P, sedoheptulose 7-phosphate; E4P, erythrose 4-phosphate; F6P, fructose 6-phosphate; G6P, glucose 6-phosphate; 6PG, 6-phosphogluconate; DHA, dihydroxyacetone; Hu6P, D-arabino-3-hexulose 6-phosphate; HCHO, formaldehyde.

Fig. 1. DEAE-cellulose chromatography of protamine sulphate-treated crude extract

Hexose phosphate synthase (●) and hexulose phosphate isomerase (▲) were measured spectrophotometrically as described in the text. E_{254} (—) of the column eluate was measured with an Isco u.v. monitor (light-path 10mm). Δ, Chloride gradient. The arrow shows where the chloride gradient was started.

Hexose phosphate synthase activity was found in column fractions by two methods using reaction mixture A: (1) by detection of the ribose 5-phosphate and ribose phosphate isomerase-dependent disappearance of formaldehyde, and (2) by detection of the formation of hexulose by the method of Dische & Devi (1960). Hexulose formation only occurred in those fractions that catalysed the disappearance of formaldehyde, i.e. fractions 38 to 60.

Hexulose phosphate isomerase activity (see Scheme 1) was assayed in column fractions with hexulose phosphate as substrate and this was prepared by the action of the hexose phosphate synthase fraction on formaldehyde and ribose 5-phosphate as follows. Hexose phosphate synthase (3 units, from fractions 44 and 45), 250 μmol of sodium phosphate buffer, pH 7.0, 25 μmol of $MgCl_2$, 100 μmol of ribose 5-phosphate, 20 units of ribose phosphate isomerase and 100 μmol of formaldehyde were incubated in a total volume of 5 ml for 30 min at 30°C. The reaction mixture was then cooled in an ice-water bath, deproteinized by passage at 0°C through a UM2 ultrafilter (1000 mol.wt. cut-off; Amicon Ltd., High Wycombe, Bucks., U.K.) and, when necessary, stored at -20°C. Hexulose phosphate isomerase was assayed by determining the rate of formation of fructose 6-phosphate, measured as the rate of $NADP^+$ reduction in the presence of glucose phosphate isomerase and glucose 6-phosphate dehydrogenase. The reaction mixture contained in 1 ml total volume: 50 μmol of sodium phosphate buffer, pH 7.0, 5.0 μmol of $MgCl_2$, 0.5 μmol of $NADP^+$, 1 unit of glucose phosphate isomerase, 1 unit of glucose 6-phosphate dehydrogenase and the hexulose phosphate isomerase substrate, nominally 0.5 μmol on the basis of the extent of $NADP^+$ reduction in this assay. The reaction mixture was incubated at 30°C and the reaction started by the addition of the hexulose phosphate isomerase enzyme preparation. After a lag of about 1 min the rate of increase in E_{340}

became linear. This rate was used to calculate enzyme activity in fractions suitably diluted such that activity was proportional to enzyme concentration.

Once a purified hexulose phosphate isomerase preparation had been obtained, quantitative determinations of hexose phosphate synthase activities could be done spectrophotometrically by using the reaction mixture described under 'Materials and methods' supplemented with excess (300 munits) of hexulose phosphate isomerase (fractions 13 and 14); the results are shown in Fig. 1. Controls without enzyme (hexose phosphate synthase), with boiled enzyme or without hexulose phosphate isomerase showed no activity.

In the experiment of Fig. 1, total yields of hexose phosphate synthase and hexulose phosphate isomerase were 30 and 35 units respectively, which correspond to specific activities in the crude extract of 270 munits/mg of protein for hexose phosphate synthase and 315 munits/mg of protein for hexulose phosphate isomerase. Fractions 13 and 14, and 42-50 (the fractions containing the highest specific activities of hexulose phosphate isomerase and hexose phosphate synthase respectively), were each pooled and assayed for transketolase, transaldolase and ribulose phosphate 3-epimerase activities. No transketolase was detected in the pooled isomerase fractions, but the pooled synthase fractions showed a low activity of this enzyme, amounting to a total of about 1 unit. No transaldolase or ribulose phosphate 3-epimerase activities were detected in either pool. The absence of ribulose phosphate 3-epimerase from the synthase pool eliminates the possibility that formaldehyde disappearance and hexulose production are mediated by routes involving transketolase, and the dependence of $NADP^+$ reduction on added hexulose phosphate isomerase in the spectrophotometric assay indicates that this reduction is mediated by hexulose phosphate synthase.

Discussion

The identification by Kemp (1974) of the initial product of the hexose phosphate synthase reaction as D-arabino-3-hexulose phosphate suggests that the enzyme 'hexose phosphate synthase' should be renamed 'hexulose phosphate synthase' and the corresponding isomerase be called 'hexulose phosphate isomerase'.

The finding of a second example of a facultatively methylotrophic bacterium having hexulose phosphate synthase (and isomerase) provides additional evidence that this enzyme is not confined to the obligate methylotrophs.

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