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Detection of specific glucose-3-phosphatase activity in rat liver

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Abstract

Sugar-3-phosphates are related to aspects of diabetes which depend on protein glycosylation events. Sorbitol-3-phosphate and fructose-3-phosphate occur in normal and diabetic individuals, and glucose-3-phosphate is a potential intermediate in their biosynthesis. Almost nothing is known about enzyme pathways for their metabolic turnover. We have found that part of the phosphohydrolytic activity on glucose-3-phosphate in rat liver supernatants corresponds to a specific, Mg^{2+}-dependent, glucose-3-phosphatase much less or not active on other phosphate esters, including glucose-1-phosphate, glucose-6-phosphate, fructose-1-phosphate, fructose-6-phosphate and *p*-nitrophenyl-phosphate. This finding opens a route to a better understanding of the metabolism and role of sugar-3-phosphates.

Key words: D-Glucose-3-phosphate; Glucose-3-phosphatase; Sugar-3-phosphate; Rat liver; Diabetes

1. Introduction

Sugars phosphorylated at C-3 have been detected by ^{31}P NMR in normal and diabetic erythrocytes and lenses [1–4]. Since sorbitol-3-phosphate and fructose-3-phosphate are more abundant in samples from diabetic than from normal individuals, animal or human, and fructose-3-phosphate is a potent glycosylating agent for proteins [2], sugar-3-phosphates may be relevant to the non-enzymic glycosylations related to the sequelae of diabetes, particularly to diabetic cataract [1,2,5]. Two kinds of mechanisms have been proposed to explain the synthesis of sugar-3-phosphates: in one of them glucose would be phosphorylated in C-3 and glucose-3-phosphate would be converted to sorbitol-3-phosphate and fructose-3- phosphate; the other would involve the direct phosphorylation of sorbitol and/or fructose [1,2,5]. There is little information on this matter, although NMR evidence obtained directly from human erythrocyte lysates or perfused, intact mammalian lenses points to the occurrence of fructose-3- and/or sorbitol-3-phosphokinase(s) which, nonetheless, remain otherwise unrecognized [6,7]. On the other hand, nothing is known about enzymes involved in the metabolic turnover of sugar-3-phosphates. Here we report that rat liver contains a specific glucose-3- phosphatase, a kind of enzymatic activity not previously described.

2. Materials and methods

2.1. Materials

D-Glucose-3-phosphate, synthesized by a multi-step procedure and characterized by ^{13}C NMR spectroscopy, was a gift from M. Avalos (Department of Organic Chemistry, University of Extremadura, Badajoz, Spain). In summary, the preparation consisted of the following successive steps: (a) blocking of vicinal 1,2- and 5,6-hydroxyl groups of D-glucose by reaction with acetone in the presence of a catalytic amount of H_2SO_4 to yield 1,2,5,6-di-*O*-isopropylidene-α-D-glucofuranose [8]; (b) phosphorylation of the latter at the free 3-hydroxyl group by reaction with a slight excess of diphenyl phosphorochloridate in pyridine; (c) removal of the phenyl groups by hydrogenolysis in ethyl acetate over platinum; (d) removal of blocking isopropylidene groups by hydrolysis in 90% (vol/vol) trifluoroacetic acid; (e) 100-fold dilution in water, adjustment at pH 5 with 3 M Tris and purification of glucose-3-phosphate by chromatography on a Dowex-1 (Cl) column, from where it was eluted with a linear, 0–0.4 M NaCl gradient in water; (f) desalting of pooled fractions containing D-glucose-3-phosphate on a Sephadex G-10 column equilibrated in water.

For routine quantitative assay of D-glucose-3-phosphate concentrations, chromatographic fractions or stock solutions were treated with alkaline phosphatase and, after enzyme inactivation by heating for 2 min at 95°C, the contents of P_i (section 2.4, method A) and glucose (enzyme assay with hexokinase and glucose-6-phosphate dehydrogenase [9]) were measured versus controls carried out omitting phosphatase treatment. Controls of stock solutions yielded less than 2% free glucose or 2% P_i (by mol); phosphatase-dependent glucose and P_i contents agreed to within 5%. Purity and identity of D-glucose-3-phosphate were confirmed by ^{13}C NMR spectroscopy, as all the resonance signals observed could be assigned to the expected mixture of D-glucopyranose-3-phosphate anomers (Fig. 1). The same material, after complete dephosphorylation with alkaline phosphatase, gave a clearcut spectrum coinciding with that of authentic D-glucose (data not shown) and with published data for α- and β-D-glucopyranose [10].

Other (bio)chemicals and chromatography media were obtained either from Sigma (D-fructose-1-phosphate, phenylmethylsulfonyl fluoride, platinum oxide and Dowex-1 resin), Aldrich (diphenyl phosphorochloridate, pyridine and ethyl acetate), Fluka (trifluoroacetic acid), Merck (D-glucose-1-phosphate, D-glucose-6-phosphate, Tris, EDTA, NaCl, $MgCl_2$ and H_2SO_4), Boehringer (D-fructose-6-phosphate, *p*-nitrophenyl-phosphate and grade-I calf-intestine alkaline phosphatase), Pharmacia (Sephadex and Q-Sepharose gels) or Whatman (DEAE-cellulose DE52).

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2.2. Preparation and processing of rat liver supernatants

Female Wistar rats weighing around 250 g, and about 4 months old, were killed by decapitation and livers were excised, chopped and washed with cold saline. Homogenization was accomplished on ice with 2 ml of 50 mM Tris-HCl, pH 7.5, 0.5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride per gram fresh liver (in order to prevent possible proteolysis). The supernatant was obtained after a two-step centrifugation: first for 15 min at 27,000 × *g*, after which the precipitate was discarded, and second for 60 min at 100,000 × *g*. The final supernatant was fractionated with ammonium sulfate and the fraction precipitating between 30–60% saturation was resuspended in homogenization buffer to 1/4 of the supernatant volume. That ammonium sulfate fraction was used for chromatographic experiments.

2.3. Chromatographic fractionation

The columns used were of Sephadex G-100 (2 × 100 cm; equilibrated in and eluted with 20 mM Tris-HCl, pH 7.5, 0.5 mM EDTA), DEAE-cellulose (1.6 × 37 cm; equilibrated in 20 mM Tris-HCl, pH 7.5, and eluted with 20 mM Tris-HCl, pH 7.5, 0.5 mM EDTA, followed by a linear 0–400 mM KCl gradient in the same buffer) and Q-Sepharose (1.1 × 10 cm; equilibrated in 20 mM Tris-HCl, pH 7.5, and eluted with a linear 50–400 mM KCl gradient in the same buffer supplemented with 0.5 mM EDTA, applied immediately after loading the sample).

2.4. Assay of enzyme activities

All the phosphatase activities of glucose-3-phosphatase and alkaline phosphatase were assayed, unless otherwise stated, at 37°C in reaction mixtures containing: 50 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$ and 1 mM sugar-phosphate or *p*-nitrophenyl-phosphate ester. Blank mixtures, without substrate and/or enzyme, were incubated and processed in parallel to complete reaction mixtures. Incubations were carried out in a 0.1-ml or 0.2-ml volume and reactions were stopped and P_i assayed, by comparison to P_i standards, following one of two different methods. In method A, the enzyme incubations were finished by the addition of 1 ml of a reagent containing 0.38 M H_2SO_4, 2.5 mM ammonium heptamolybdate, 16 mM sodium dodecylsulfate and 70 mM ascorbic acid; A_{820} was measured after color developing at 45°C for 20 min. In method B, the composition of the reagent was the same except that ascorbic acid concentration was 4.7 mM; the reactions were stopped by the addition of 1 ml of this reagent, and A_{750} was measured after 10 min at room temperature. Methods A and B were interchangeable except that method A was 6-fold more sensitive and that, when glucose-1-phosphate was the substrate, method B had to be used to reduce its lability. All enzyme measurements were carried out under conditions of linearity with incubation time and amount of enzyme. One enzyme activity unit (U) is the amount transforming 1 μmol substrate/min.

3. Results and discussion

Under the seemingly (near-)optimal conditions used for the assay of the glucose-3-phosphatase studied in this paper (pH 7.5, 1 mM substrate, 5 mM $MgCl_2$), rat liver supernatants contained 100–200 mU of glucose-3-phosphatase activity per gram of fresh tissue, the major part of which precipitated with the 30–60% ammonium-sulfate saturation fraction. The question addressed in this study was whether (part of) that hydrolytic activity could be due to a specific phosphohydrolase. Gel filtration chromatography of the ammonium sulfate fraction yielded elution profiles of glucose-3-phosphatase showing two peaks of activity overlapped with a broad profile of activity on *p*-nitrophenyl-phosphate that was used as a probe for non-specific phosphatases (Fig. 2). The more abundant of those peaks was then submitted to two ion-exchange steps which allowed the recovery of a well shaped glucose-3-phosphatase peak. A formal purification protocol has not yet been developed, but after the steps shown in Fig. 2, and with respect to liver supernatants, glucose-3-phosphatase activity was enriched about

Table 1
Substrate specificity of rat liver glucose-3-phosphatase compared to calf intestine alkaline phosphatase

Substrate	Initial rate of P_i liberation (%)	
	Glucose-3-phosphatase	Alkaline phosphatase
Glucose-3-phosphate	100 ± 4	100 ± 8
Glucose-1-phosphate	15 ± 4	150 ± 10
Glucose-6-phosphate	< 2	125 ± 10
Fructose-1-phosphate	< 2	155 ± 10
Fructose-6-phosphate	< 2	130 ± 2
p-Nitrophenyl-phosphate	3 ± 0.5	210 ± 13

Activities on 1 mM sugar-phosphate or nitrophenyl-phosphate ester were all assayed measuring the released P_i by method B (section 2.4). The low activity of glucose-3-phosphatase on *p*-nitrophenyl-phosphate was also confirmed by measurements of released *p*-nitrophenol by recording the A_{405} increase. Glucose-3-phosphatase was the preparation obtained after the Q-Sepharose step (Fig. 2C). Alkaline phosphatase was a commercial preparation from calf intestine (Boehringer). Results are shown as means ± range of two measurements, calculated as percentages of the mean value obtained for the rate of hydrolysis of glucose-3-phosphate with each enzyme.

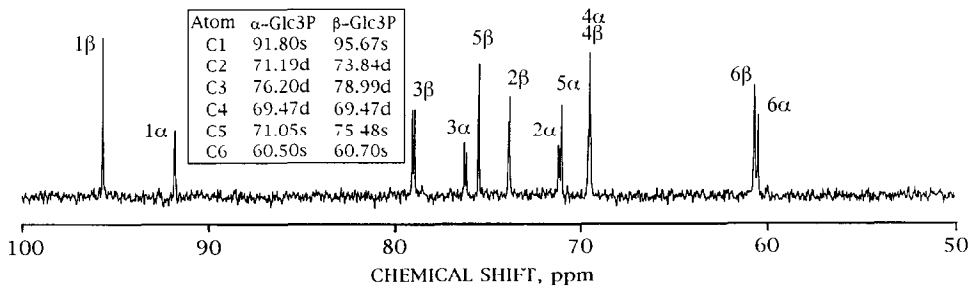

Fig. 1. Identification and purity of D-glucose-3-phosphate by ^{13}C NMR spectroscopy. A 200 MHz, proton-decoupled spectrum was recorded in 98% D_2O, with dioxane as external standard, from 20 μmol of sugar-phosphate prepared as described in section 2.1. Chemical shifts in ppm (δ) together with an indication of signal multiplicity (s, singlet; d, doublet) are summarized in the inset table. The assignment of signals to α- or β-D-glucopyranose-3-phosphate carbons was made by comparison to D-glucopyranose data [10] and considering the downfield shifts and peak splittings expected (and observed) for C3 upon esterification of the 3-hydroxyl group.

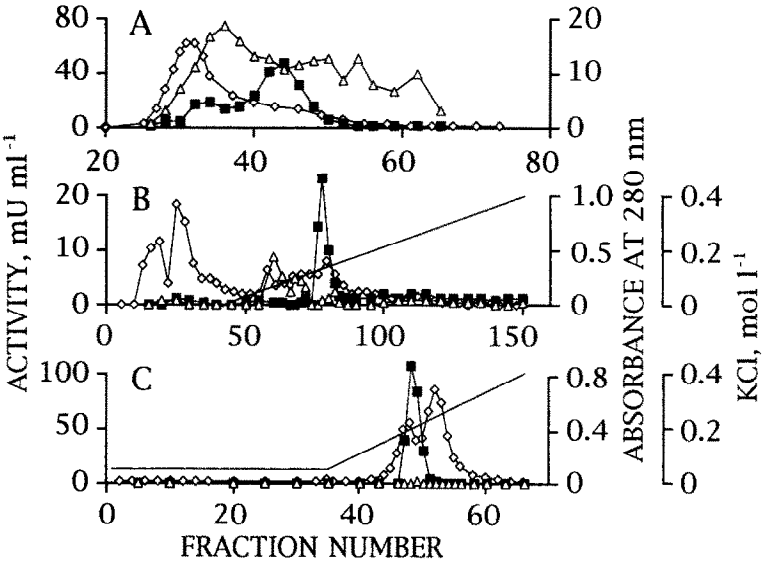

Fig. 2. Chromatographic evidence for the occurrence of specific glucose-3-phosphatase. (A) Gel filtration of an ammonium sulfate fraction (8 ml) on a Sephadex G-100 column; fraction vol. 4 ml. (B) Ion-exchange chromatography on DEAE-cellulose of the pooled fractions 40–48 from the gel-filtration experiment; fraction vol. 4 ml. (C) Ion-exchange chromatography on Q-Sepharose of the pooled fractions 75–83 which contained all the glucose-3-phosphatase activity from the DEAE-cellulose experiment; fraction vol. 2.2 ml. Further experimental details in Section 2.3. (■) Glucose-3-phosphatase; (△) *p*-nitrophenyl-phosphatase; (◇) A_{280}.

20-fold, with a final specific activity of 50–100 mU/mg, and a 10–20% recovery. That preparation was devoid or showed very low activity on *p*-nitrophenyl-phosphate, glucose-6-phosphate, fructose-1-phosphate or fructose-6-phosphate, whereas a minor activity on glucose-1-phosphate was measured (Table 1).

Characterization of rat liver glucose-3-phosphatase indicated linearity of activity versus incubation time

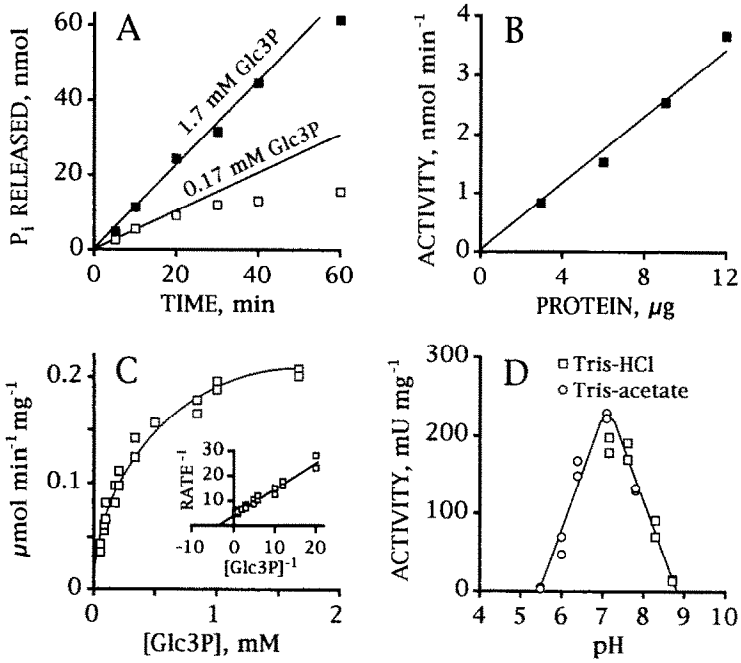

Fig. 3. Kinetics of glucose-3-phosphate hydrolysis by glucose-3-phosphatase. Enzyme from the Q-Sepharose step (Fig. 2C) was used and assayed by method A (section 2.4) with the variables indicated in each panel. (A) Time course of reaction with two initial concentrations of glucose-3-phosphate and 6 μg of protein: the straight lines represent initial rates. (B) Proportionality of initial rate with the amount of enzyme at 1.7 mM substrate. (C) Saturation kinetics. Data are a combination of two independent experiments yielding similar results. Saturation parameters for that combined set were estimated with the aid of Basic versions of computer programs fitting data by least squares to an hyperbola [11] or to a straight line [12]; respectively: K_m ± S.E.M., 243 ± 17 μM and 242 ± 28 μM; V_{max} ± S.E.M., 232 ± 6 mU/mg and 231 ± 19 mU/mg. (D) Response of activity to pH in incubation mixtures with two different buffers.

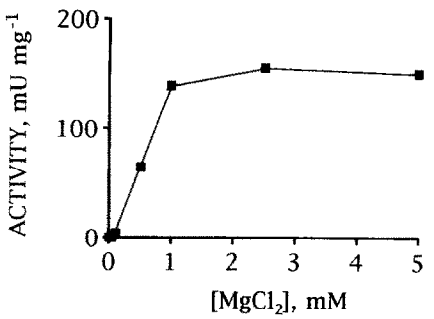

Fig. 4. Mg^{2+} requirement for the hydrolysis of glucose-3-phosphate by glucose-3-phosphatase. Enzyme from the Q-Sepharose step (Fig. 2C) was used after dialyzing it six times, each one for at least 4 h against 130 vols. of fresh buffer (20 mM Tris-HCl, pH 7.5). Activity was assayed by method A (section 2.4) with the indicated $MgCl_2$ concentrations.

(Fig. 3A) and amount of protein (Fig. 3B), a K_m about 250 μM (Fig. 3C), a pH–activity profile showing a maximum around pH 7 (Fig. 3D), a requirement for Mg^{2+} (Fig. 4) not fulfilled by $MnCl_2$, and an M_r about 50,000–60,000 estimated by gel-filtration (Fig. 5). Optimal assay conditions for the activity on glucose-1-phosphate, concerning pH, substrate and $MgCl_2$ concentration (not shown), were similar to those for the activity on glucose-3-phosphate.

The activity detected in this work may be either a new enzyme or a new activity of an enzyme whose principal substrate was not among those assayed by us. However, the preference for glucose-3-phosphate over other sugar phosphates and the very low activity on *p*-nitrophenyl-

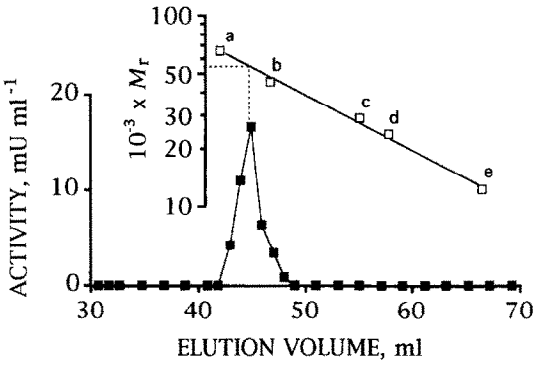

Fig. 5. Estimation of M_r of glucose-3-phosphatase by gel filtration chromatography. A Sephadex G-75SF column (0.9 × 135 cm) equilibrated and eluted at 1 ml/h with 20 mM Tris-HCl, pH 7.5, 0.5 mM EDTA, 0.1 M NaCl was used. 1-ml samples were chromatographed containing either glucose-3-phosphatase from the Q-Sepharose step (Fig. 2C) or one of the following protein markers: a, bovine serum albumin (66,000); b, ovalbumin (45,000); c, carbonic anhydrase (29,000); d, trypsinogen (24,000); e, cytochrome *c* (12,400). Elution profiles were obtained by measurements of glucose-3-phosphatase activity (section 2.4, method A) or, in the case of M_r markers, A_{280}. The upper panel shows the calibration graph with interpolation of glucose-3-phosphatase M_r.

phosphate makes it appear as a rather specific enzyme. For instance, calf intestine alkaline phosphatase (EC 3.1.3.1), under the same experimental conditions, attacked at similar rates all the compounds tested as substrates of glucose-3-phosphatase (Table 1). It remains to be established whether other sugar-3-phosphates, as fructose-3-phosphate or sorbitol-3-phosphate, are or not substrates of glucose-3-phosphatase.

Purification and detailed studies of enzymes participating in the synthesis and degradation of sugar-3-phosphates are very much needed for the understanding of the biological and pathological role(s) of sugar-3-phosphates, and because they may serve either as tools for the preparation of those compounds, which are not yet available in the market, or for their assay in biological samples. For instance, the glucose-3-phosphatase here reported could be the basis for an enzymic glucose-3-phosphate end-point assay coupled to a glucose-measuring system, after removal of glucose and, perhaps, glucose-1-phosphate from analytical samples. It would be extremely interesting if similar assays could be developed for sorbitol-3-phosphate and fructose-3-phosphate.

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