N-Acetyl- β -D-glucopyranosylamine 6-phosphate is a specific inhibitor of glycogen-bound protein phosphatase 1

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Previous work has shown that the C-1-substituted glucose-analogue *N*-acetyl- β -D-glucopyranosylamine (1-GlcNAc) is a competitive inhibitor of glycogen phosphorylase (GP) and stimulates the inactivation of this enzyme by GP phosphatase. In addition to its effects on GP, 1-GlcNAc also prevents the glucose-activated activation of glycogen synthase (GS) in whole hepatocytes. Such an effect on GS was thought to be due to the formation of 1-GlcNAc-6-P by the action of glucokinase within the hepatocyte [Board, Bollen, Stalmans, Kim, Fleet and Johnson (1995) *Biochem. J.* **311**, 845–852]. To investigate this possibility further, a pure preparation of 1-GlcNAc-6-P was synthesized. The effects of the phosphorylated glucose analogue on the activity of protein phosphatase 1 (PP1), the enzyme responsible for dephosphorylation and activation of GS, are reported. During the present study, 1-GlcNAc-6-P inhibited the activity of the glycogen-bound form of PP1, affecting both the GSb phosphatase

and GPa phosphatase activities. A level of 50% inhibition of GSb phosphatase activity was achieved with 85 μ M 1-GlcNAc-6-P in the absence of Glc-6-P and with 135 μ M in the presence of 10 mM Glc-6-P. At either Glc-6-P concentration, 500 μ M 1-GlcNAc-6-P completely inhibited activity. The Glc-6-P stimulation of the GPa phosphatase activity of PP1 was negated by 1-GlcNAc-6-P but there was no inhibition of the basal rate in the absence of Glc-6-P. 1-GlcNAc-6-P inhibition was specific for the glycogen-bound form of PP1 and did not inhibit the GSb phosphatase activity of the cytosolic form of the enzyme. The present work explains our previous observations on the inactivating effects on GS of incubating whole hepatocytes with 1-GlcNAc. These observations have their basis in the inhibition of glycogen-bound PP1 by 1-GlcNAc-6-P. A novel inhibitor of PP1, specific for the glycogen-bound form of the enzyme, is presented.

INTRODUCTION

Previous attempts to manipulate the glycogen metabolism of hepatocytes by inhibiting the activity of glycogen phosphorylase (GP) have shown that such inhibition may be readily achieved. Massillon et al. [1] showed that 2-deoxy-2-fluoro- α -D-glucopyranosyl fluoride (F_2 -Glc) was a potent inactivator of GP in hepatocytes. Another glucose analogue, *N*-acetyl- β -D-glucopyranosylamine (1-GlcNAc), was synthesized as one of a series of N-linked C-1 substituents of D-glucose [2]. This compound proved a potent competitive inhibitor of crude extracts of GP [3] and also caused rapid inactivation of the enzyme in whole hepatocytes as the result of a stimulatory effect on GP phosphatase activity. A rate of inactivation similar to that produced by 50 mM glucose was seen with 10 mM 1-GlcNAc [4].

Despite the ease with which GP may be inactivated within hepatocytes, the reciprocal activation of glycogen synthase (GS) has proved more elusive. F_2 -Glc fails to activate GS [1], and 1-GlcNAc not only fails to activate but also reverses the glucose-activated activation of GS [4]. The observations of Carabaza et al. [5] that only phosphorylatable analogues of D-glucose could achieve activation of GS in whole hepatocytes may account for the failure of F_2 -Glc to cause activation, since this compound is considered metabolically inert [1]. However, it has been shown that 1-GlcNAc is a substrate for hepatic glucokinase and that 1-GlcNAc-6-P accumulates inside hepatocytes on incubation with 1-GlcNAc [4]. Indeed, the presence of the phosphorylated species, 1-GlcNAc-6-P, seems to be necessary for the inactivation of GS. Under conditions in which 1-GlcNAc-6-P cannot be formed, such as in gel-filtered liver extracts in which a lack of ATP precludes phosphorylation of 1-GlcNAc, activation of GS is observed when 1-GlcNAc is present [4]. Recent studies have

emphasized the importance of glucose phosphorylation in the activation of GS, and Glc-6-P is now considered the dominant species in this role [1,5,6]. Thus a speculative basis for the failure of 1-GlcNAc to activate GS in hepatocytes, that 1-GlcNAc-6-P competes with the structurally similar Glc-6-P for a site on either the GS enzyme or one of the interconverting enzymes, has been considered [4]. GS may be phosphorylated at nine serine sites by at least six kinases [7,8], with phosphorylation resulting in inactivation. Corresponding activation is achieved largely through the action of protein phosphatase 1 (PP1) in either its glycogen-bound or cytosolic form.

The present work has focused on the action of 1-GlcNAc-6-P on GS and on its activation by PP1. A pure preparation of 1-GlcNAc-6-P, free from the non-phosphorylated 1-GlcNAc, has been synthesized, and the effects on the GS phosphatase activities of the glycogen-bound and cytosolic forms of PP1 and of the isolated catalytic subunit have been investigated. The potential for competition of 1-GlcNAc-6-P with Glc-6-P has been assessed. Effects on these phosphatase activities with other phosphorylated substrates both having (GPa) and lacking (myelin basic protein, casein and histone IIA) Glc-6-P binding sites are presented for comparative purposes. Such a study has allowed identification of the site of action of 1-GlcNAc-6-P in the inhibition of GS activation and further illuminates the role of Glc-6-P in this system.

MATERIALS AND METHODS

1-GlcNAc and [¹⁴C]1-GlcNAc were synthesized as reported previously [4]. Glc-6-P, microcystin-LR, histone IIA, myelin basic protein and the catalytic subunit of protein kinase A were purchased from Sigma (St. Louis, MO, U.S.A.). Casein was

Abbreviations used: F_2 -Glc, 2-deoxy-2-fluoro- α -D-glucopyranosyl fluoride; 1-GlcNAc(-6-P), *N*-acetyl- β -D-glucopyranosylamine (6-phosphate); Glc-6-P, glucose 6-phosphate; GP, glycogen phosphorylase; GS, glycogen synthase; PP1, protein phosphatase 1; DTT, dithiothreitol.

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prepared by the method of Mercier et al. [9]. Purified dog liver GSb and α -particulate glycogen were prepared as described elsewhere [10]. AG1-X8 resin (formate form; 200–400 mesh) was purchased from Bio-Rad Laboratories, Richmond, CA, U.S.A., and ion-exchange (DE81) paper discs were from Whatman International Ltd., Maidstone, Kent, U.K. Sources of all other materials, except those with preparations described below, are as reported previously [4,11].

Preparation of 1-GlcNAc-6-P

The method takes advantage of the fact that 1-GlcNAc is a substrate for rat liver glucokinase [4]. After phosphorylation, [^{14}C]-1-GlcNAc may be separated from [^{14}C]-1-GlcNAc-6-P by ion-exchange chromatography.

Glucokinase was partially purified and separated from hexokinase by ion-exchange chromatography [12]. An aliquot of the resulting preparation of glucokinase (0.8 unit) was dialysed against 1 litre of 25 mM Hepes/1 mM dithiothreitol (DTT)/100 mM KCl, pH 7.4, for 2 h at 4 °C. Enzyme, 60 mM MgCl_2 , 50 mM ATP and 150 mM 1-GlcNAc (containing 5 $\mu\text{Ci}/\text{mmol}$ [^{14}C]-1-GlcNAc) with the above buffer to a total volume of 5 ml, were incubated for 24 h at 37 °C. A column (approximate volume 10 ml) of AG1-X8 resin (formate form) was prepared by equilibrating the resin with water (resulting pH 5.2). The sample was diluted to 50 ml and applied to the column with collection of 5 ml fractions. The column was washed with 60 ml of water, followed by a linear gradient of 0.5 M formic acid and 1 M formic acid/0.3 M ammonium formate in 2 $\times$ 50 ml. To identify fractions containing 1-GlcNAc-6-P, aliquots were spotted on to ion-exchange discs, which bind 1-GlcNAc-6-P but not 1-GlcNAc, washed with water, dried and the radioactivity measured. 1-GlcNAc-6-P was eluted between fractions 21 and 24. Fractions containing 1-GlcNAc-6-P were pooled and the volume reduced by two cycles of lyophilization. Such a preparation of 1-GlcNAc-6-P contained non-detectable levels of ATP (measured as the increase in A_{340} when the sample was added to a mixture containing 70 mM Tris/HCl, pH 7.5, 1 mM NADP, 7 mM MgCl_2 , 1 unit/ml Glc-6-P dehydrogenase, 50 mM glucose and 2.5 units/ml hexokinase). During separate assays, 1-GlcNAc-6-P did not inhibit the activity of Glc-6-P dehydrogenase. Measurements of ADP, AMP (measured as described by Janorek [13]) and Glc-6-P (measured by the method of Bergmeyer et al. [14]) also revealed non-detectable levels.

Preparation of glycogen-bound PP1

Phosphatases were prepared from the glycogen (glycogen-bound PP1) and post-glycogen (cytosolic PP1) fractions of liver by an adaptation of the method of Wera et al. [15].

A 15 h-starved rat was injected intraperitoneally with 0.3 mg of glucagon 30 min before it was killed. The liver was removed, rinsed with 0.9% NaCl, snipped with scissors and a 33% homogenate made with buffer A (50 mM glycylglycine, 3 mM EGTA, 0.5 mM DTT, 5% glycerol, 5 mM 2-mercaptoethanol, 0.5 mM benzimidazole, 0.3 mM PMSF, pH 7.4) containing 250 mM sucrose. The homogenate was centrifuged at 220000 g for 35 min at 4 °C, and the resulting supernatant filtered through glass wool. An aliquot (2.5 ml) of the filtrate was applied to a column of Sephadex G-25 (approximate volume 5 ml), equilibrated with buffer A, and 3.5 ml of buffer A was used to elute it. The eluate was enriched with 0.5% particulate glycogen, mixed and centrifuged as above. The resulting supernatant (the second supernatant fraction) is considered to contain the cytosolic PP1 activity. The pellet was homogenized in 2 ml of buffer A, using a glass homogenizer on ice, followed by a third centrifugation as

above. The resulting pellet (the second glycogen pellet) is considered to contain the glycogen-bound PP1 activity. For the assay described below, the second glycogen pellet was re-suspended and homogenized in a sufficient volume of buffer A to achieve a 100% extract with respect to the original weight of liver (0.825 ml).

Assay of glycogen-bound and cytosolic PP1 activities with a GSb substrate

The GSb phosphatase activity of PP1 (in either its glycogen-bound or cytosolic forms) was measured as the increase in GSa activity with time when an aliquot of the PP1 preparation was incubated with GSb. Selective measurement of the resulting GSa activity was achieved by the inclusion of 10 mM Na_2SO_4 (which potentially inhibits GSb) in the assay medium.

A suspension of the second glycogen pellet was incubated at 30 °C in 10 vol. of a mixture containing 5 mg/ml BSA, 0.5 unit/ml purified dog liver GSb and buffer A with the addition of Glc-6-P, 1-GlcNAc-6-P or 1-GlcNAc as appropriate. At various time points, 10 μl aliquots of the mixture were removed, added to 3 μl of a 1:1 mixture of 1 mM 5'-iodotubercidin and 10 μM microcystin, to prevent further kinase and phosphatase activity, and cooled on ice. This suspension was assayed at 30 °C for the activity of GSa by the method described previously [4]. Assay of the cytosolic phosphatase activity was performed in a similar manner by incubating the second supernatant fraction in 4 vol. of the above mixture and taking samples for measurement as detailed above. For assay of GSa activity after incubations in the presence of Glc-6-P, the Glc-6-P was first removed by the addition of 5 μl of a mixture containing 80 units/ml Glc-6-P dehydrogenase, 25 mM NAD^+ , 33 mM Tris, 70 μM MgCl_2 , 33 μM DTT and 7 mM EDTA, pH 8.1, and the assay conducted as above. Removal of the Glc-6-P prevents overestimation of GSa activity as the result of stimulation of the Glc-6-P-dependent GSb or the pseudo-activation of GS [16].

One unit of GS activity is defined as the amount of enzyme required to catalyse the transfer of 1 μmol of glucose from UDP-glucose to glycogen during 1 min.

Assay of glycogen-bound PP1 activity with radiolabelled substrates

Myelin basic protein, casein and histone IIA were labelled by incubation with protein kinase A and [$\gamma^{32}\text{P}$]ATP, and phosphatase activity was measured by the release of [^{32}P]P_i into the supernatant after precipitation of the protein [11]. Radiolabelled GP_a (^{32}P -GP_a) was prepared [17] and its rate of dephosphorylation (at 30 °C) measured in a similar manner to that of the other radiolabelled substrates above, except that 5 mM caffeine was included in the assay of GP_a dephosphorylation.

Preparation and assay of the isolated catalytic subunit of PP1

The isolated catalytic subunit of PP1 was prepared [18] and its activity with a GSb and a GP_a substrate measured as detailed for the glycogen-bound enzyme above. For measurements of GSb dephosphorylation, the catalytic subunit was present at a concentration of 2 μM and for GP_a dephosphorylation at 50 pM. Assays were performed at 30 °C.

Treatment of results

Lines of regression were generated for the data presented in Figures 1 (over the period 0–10 min) and 4 (0–60 min) in order to calculate PP1 activities as the increase in GSa activity with time

(units of GSa activity min per ml). For Figure 5, a line of regression (not shown) was generated for the period 0–15 min. The gradients of the regression lines are considered to represent the rates of GSb phosphatase activity over the time courses measured where the relationship between the increase in GSa activity and time is linear. Activities of GSb phosphatase for each Figure, under each condition measured, are given in the legends.

RESULTS

The results presented in Figure 1 show that 1-GlcNAc-6-P is a potent inhibitor of the GSb phosphatase activity of glycogen-bound PP1. In the absence of Glc-6-P (Figure 1a), the activity of glycogen-bound PP1 over 10 min of the 20 min time course was 7.6×10^{-3} units/min per ml (from the gradient of the line of regression) in the absence of any addition. This rate decreased more than 3-fold to 2.3×10^{-3} units/min per ml with $100 \mu\text{M}$ 1-GlcNAc-6-P and to non-detectable rates with $500 \mu\text{M}$. In terms of the activation states of GS achieved under these conditions, after 20 min of incubation, GSa was present at only 5% of its control value if $500 \mu\text{M}$ 1-GlcNAc-6-P was present and at 26% of its control value if $100 \mu\text{M}$ 1-GlcNAc-6-P was present. This pattern of inhibition was repeated in the presence of 10 mM Glc-6-P (Figure 1b). Again, $500 \mu\text{M}$ 1-GlcNAc-6-P proved a potent inhibitor, reducing GSb phosphatase activity to non-detectable rates while $100 \mu\text{M}$ 1-GlcNAc-6-P reduced the rate 2-fold (from 2.1×10^{-2} to 1.08×10^{-2} units/min per ml). In terms of the activation state of GS achieved, after 20 min of incubation, this was reduced to 2% of control values by the presence of $500 \mu\text{M}$ 1-GlcNAc-6-P and to 50% by $100 \mu\text{M}$ 1-GlcNAc-6-P. The concentration-dependence of 1-GlcNAc-6-P inhibition may be seen in Figure 2. Concentrations required to elicit 50% inhibition

Figure 2 Percentage inhibition of GSb phosphatase activity with increasing concentration of 1-GlcNAc-6-P

The GSb phosphatase activity of glycogen-bound PP1 was measured over a range of 1-GlcNAc-6-P concentrations from 0 to $500 \mu\text{M}$, and percentage inhibition calculated relative to control values in the absence of 1-GlcNAc-6-P. □, No Glc-6-P; ■, 10 mM Glc-6-P. Means $\pm$ S.E.M. from five separate preparations are presented (the error bars are within the area of the symbol).

Figure 1 GSb phosphatase activity of glycogen-bound PP1: effects of 1-GlcNAc-6-P

Glycogen-bound PP1 was prepared and the GSb phosphatase activity measured as detailed in the Materials and methods section. (a) Activity in the absence of Glc-6-P; (b) activity in the presence of 10 mM Glc-6-P. □, No addition; △, $100 \mu\text{M}$ 1-GlcNAc-6-P; ▲, $500 \mu\text{M}$ 1-GlcNAc-6-P; ◆, 50 mM 1-GlcNAc. The rates of GS activation (from the gradients of the regression lines over 0–10 min) in units/min per ml for each condition are as follows. (a) No addition (□), 7.6×10^{-3} ; $100 \mu\text{M}$ 1-GlcNAc-6-P (△), 2.3×10^{-3} ***; $500 \mu\text{M}$ 1-GlcNAc-6-P (▲), non-detectable activity***; 50 mM 1-GlcNAc (◆), 6.9×10^{-3} . (b) No addition (□), 2.1×10^{-2} ; $100 \mu\text{M}$ 1-GlcNAc-6-P (△), 1.08×10^{-2} ***; $500 \mu\text{M}$ 1-GlcNAc-6-P (▲), non-detectable activity***; 50 mM 1-GlcNAc (◆), 2.2×10^{-2} . *** $P < 0.001$ (by Student's *t* test), compared with no addition. Unmarked differences are not significant. Means $\pm$ S.E.M. from at least five different phosphatase preparations are presented.

Figure 3 Dependence of the GSb phosphatase activity of glycogen-bound PP1 on the concentration of Glc-6-P: effects of 1-GlcNAc-6-P

Activity of GSa was measured after an 8 min incubation of GSb with glycogen-bound phosphatase (see the Materials and methods section for details). □, No 1-GlcNAc-6-P; △, 100 μ M 1-GlcNAc-6-P. Means $\pm$ S.E.M. from five separate preparations are presented (where not shown, the error bars are within the area of the symbol).

of activity are about 85 μ M in the absence of Glc-6-P and 135 μ M in the presence of 10 mM Glc-6-P. Over the 1-GlcNAc-6-P concentration range used, percentage inhibitions achieved are similar in the presence and absence of Glc-6-P despite the higher phosphatase activities attained in the former. Under similar conditions, the unphosphorylated 1-GlcNAc, up to 50 mM, failed to inhibit GSb phosphatase activity (Figures 1a and 1b). It should be noted that, under all conditions used for the assay of GSb phosphatase, 0.5 μ M microcystin abolished the observed activity. It is likely therefore that 1-GlcNAc-6-P may be accounted an inhibitor of glycogen-bound PP1 activity and not of the process of pseudo-activation of GS, sometimes observed at high concentrations of Glc-6-P [16]. GSb phosphatase activity was measured as the increase in GSa activity when GSb was incubated with a sample containing glycogen-bound PP1. During separate assays of GS activity alone, no inhibition of either GSa (prepared by the action of PP1 on purified GSb) or GSb activity was apparent in the presence of 1-GlcNAc-6-P up to 500 μ M (results not shown).

Glc-6-P is a potent stimulator of the GSb phosphatase activity of glycogen-bound PP1 [6] as can be seen by a comparison of Figures 1(a) and 1(b), in the absence of Glc-6-P and in the presence of 10 mM Glc-6-P respectively. The presence of 10 mM Glc-6-P stimulated the rate of conversion of GSb into GSa 2.8-fold. Effects of 100 μ M 1-GlcNAc-6-P on the Glc-6-P-dependence curve can be seen in Figure 3. Glycogen-bound PP1 activity was measured as the increase in GSa activity after an 8 min incubation of the phosphatase with GSb. In the presence of 1-GlcNAc-6-P, the slightly sigmoidal shape of the curve, apparent in the absence of the inhibitor, was retained. However, even very high concentrations of Glc-6-P (10 mM) failed to

Table 1 Rates of dephosphorylation of 32 P-GPa by glycogen-bound PP1 and by the isolated catalytic subunit: effects of Glc-6-P and 1-GlcNAc-6-P

Release of [32 P] P_i during incubation of GPa with either glycogen-bound PP1^a or its isolated catalytic subunit^b was measured as the c.p.m. of the supernatant after precipitation of the protein (see the Materials and methods). Incubation of phosphatase with GPa was for 2 min with glycogen-bound PP1 and for 8 min with the catalytic subunit. Means $\pm$ S.E.M. from three separate preparations are presented. ** 0.01 > P > 0.001, compared with value in the absence of 1-GlcNAc-6-P.

Concentration of Glc-6-P (mM)	10 ⁻³ × Release of [32 P] P_i (c.p.m. in supernatant)	
	No 1-GlcNAc-6-P	100 μ M 1-GlcNAc-6-P
0 ^a	4.08 $\pm$ 0.1	4.60 $\pm$ 0.2
0.5 ^a	6.11 $\pm$ 0.3	**3.30 $\pm$ 0.2
10 ^a	14.3 $\pm$ 0.5	**5.40 $\pm$ 0.2
0 ^b	3.55 $\pm$ 0.2	4.05 $\pm$ 0.2
0.5 ^b	6.85 $\pm$ 0.4	**4.63 $\pm$ 0.1
10 ^b	9.89 $\pm$ 0.9	**4.64 $\pm$ 0.1

overcome the inhibition. This suggests that inhibition is not the result of simple competition between Glc-6-P and 1-GlcNAc-6-P for the same site on the substrate or the phosphatase enzyme. It should be noted that, at all concentrations of Glc-6-P tested (0, 0.5 and 10 mM), 500 μ M 1-GlcNAc-6-P reduced phosphatase activity to non-detectable rates.

Another substrate of glycogen-bound PP1 is GPa, the dephosphorylation of which may be measured as the release of [32 P] P_i from radiolabelled GPa. Such inactivation of GP is strongly stimulated by Glc-6-P with rates increasing 1.5-fold above basal with 0.5 mM Glc-6-P and 3.5-fold above basal with 10 mM Glc-6-P (Table 1). Although 1-GlcNAc-6-P did not inhibit basal rates of dephosphorylation of GPa (in the absence of Glc-6-P), inhibition was increasingly severe with rising Glc-6-P concentration (Table 1). Percentage inhibitions of phosphatase activity were 46% at 0.5 mM and 64% at 10 mM Glc-6-P. Further studies of glycogen-bound PP1 activity with substrates unlikely to possess a Glc-6-P-binding site showed that rates of dephosphorylation are not inhibited by 1-GlcNAc-6-P. With histone IIA or casein (results not shown), the rate of dephosphorylation was unaffected by 100 μ M 1-GlcNAc-6-P whereas that of myelin basic protein was stimulated (50% at 100 μ M 1-GlcNAc-6-P). These data suggest that the presence of a Glc-6-P-binding site on the substrate may be necessary to achieve at least part of the inhibitory effect of 1-GlcNAc-6-P.

The behaviour of the GSb phosphatase activity of cytosolic PP1, prepared from the post-glycogen fraction of rat liver (see the Materials and methods section), in the presence of 1-GlcNAc-6-P, can be seen in Figure 4. No inhibition by 1-GlcNAc-6-P is apparent; indeed, there is slight stimulation, such that the rate of dephosphorylation is increased from 1.8 $\times$ 10⁻³ units/min per ml in the absence of any addition to 2.5 $\times$ 10⁻³ units/min per ml in the presence of 100 μ M 1-GlcNAc-6-P. This stimulation is similar to that elicited by 10 mM Glc-6-P for which the rate of dephosphorylation is 2.2 $\times$ 10⁻³ units/min per ml (Figure 4).

The catalytic subunit of PP1 may be isolated and assayed for its ability to activate GSb, although high concentrations of the subunit are required (2 μ M) compared with those for a GPa substrate (50 pM). The GSb phosphatase activity of this species was unaffected by Glc-6-P (Figure 5) but was potently inhibited by 100 μ M 1-GlcNAc-6-P. The rate of GSb dephosphorylation was reduced more than 4-fold from 1.9 $\times$ 10⁻² units/min per ml

Figure 4 GSb phosphatase activity of cytosolic PP1: effects of Glc-6-P, 1-GlcNAc-6-P and 1-GlcNAc

Cytosolic PP1 was prepared and the GSb phosphatase activity measured as detailed in the Materials and methods section. $\square$, No addition; $\blacksquare$, 10 mM Glc-6-P; $\triangle$, 100 μ M 1-GlcNAc-6-P; $\blacklozenge$, 50 mM 1-GlcNAc. The phosphatase activities (measured from the gradients of the regression lines between 0 and 60 min) in units/min per ml for each condition are: no addition ($\square$), 1.8×10^{-3} ; 10 mM Glc-6-P ($\blacksquare$), 2.2×10^{-3} ; 100 μ M 1-GlcNAc-6-P ($\triangle$), 2.5×10^{-3} ; 50 mM 1-GlcNAc ($\blacklozenge$), 1.6×10^{-3} . **0.01 > P > 0.001, compared with no addition (Student's t test). Unmarked differences are not significant. Means $\pm$ S.E.M. from at least five separate preparations are presented (where not shown error bars are within the area of the symbol).

in the absence of any addition to 4.6×10^{-3} units/min per ml in the presence of 100 μ M 1-GlcNAc-6-P. This degree of inhibition may be sufficient to account for that observed with the glycogen-bound form of the enzyme (Figure 1a). Measurements of the GPa phosphatase activity of the isolated catalytic subunit showed strong stimulation by Glc-6-P (Table 1), in contrast with the GSb phosphatase activity of the same species. Rates of dephosphorylation of GPa were stimulated almost 2-fold at 0.5 mM and almost 3-fold at 10 mM Glc-6-P (Table 1). A similar pattern of inhibition by 1-GlcNAc-6-P emerged to that observed with the GPa phosphatase activity of the glycogen-bound form of the enzyme (Table 1). Again, there was no inhibition of basal rates of dephosphorylation (in the absence of Glc-6-P), but the stimulatory effect of rising Glc-6-P concentration was negated, resulting in 48% inhibition at 0.5 mM and 53% at 10 mM Glc-6-P. Values for dephosphorylation in the presence of 0.5 or 10 mM Glc-6-P plus 100 μ M 1-GlcNAc-6-P were similar to those for basal rates.

DISCUSSION

The data presented in this paper establish 1-GlcNAc-6-P as a potent inhibitor of the GSb phosphatase activity of PP1 and indicate that inhibition is specific for the glycogen-bound form of the enzyme. Inhibition of the rate of dephosphorylation of GSb reached 70% at 100 μ M 1-GlcNAc-6-P in the absence of Glc-6-P and around 49% in the presence of 10 mM Glc-6-P. Whether Glc-6-P was present or not, 500 μ M 1-GlcNAc-6-P abolished

Figure 5 GSb phosphatase activity of the isolated catalytic subunit of PP1: effects of Glc-6-P and 1-GlcNAc-6-P

The GSb phosphatase activity of the isolated catalytic subunit was measured in the same way as that of glycogen-bound PP1 (see the Materials and methods section). $\square$, No addition; $\blacksquare$, 10 mM Glc-6-P; $\triangle$, 100 μ M 1-GlcNAc-6-P. Phosphatase activities were measured as the gradient of the regression line between 0 and 15 min of incubation (not shown). Activities, in units/min per ml, are: no addition ($\square$), 1.9×10^{-2} ; 10 mM Glc-6-P ($\blacksquare$), 2.0×10^{-2} ; 100 μ M 1-GlcNAc-6-P ($\triangle$), 4.6×10^{-3} . *** P < 0.001 compared with no addition. Unmarked differences are not significant. Means $\pm$ S.E.M. from three separate assays are presented (where not shown error bars are within the area of the symbol).

PP1 activity. This action of 1-GlcNAc-6-P explains our previous observations on the failure of 1-GlcNAc to activate or allow activation of GS by glucose in hepatocytes [4] under conditions in which the analogue may be phosphorylated. Inhibition is confined to 1-GlcNAc-6-P, since high concentration of the non-phosphorylated 1-GlcNAc have no effect on the GSb phosphatase activity of glycogen-bound PP1. The further observations that, in the presence of the adenosine analogue and glucokinase inhibitor, 5'-iodotubercidin or in gel-filtered extracts, GS activation is allowed [4], are also accounted for since the prevention of formation of 1-GlcNAc-6-P will relieve the inhibition of PP1. The potency of 1-GlcNAc-6-P inhibition suggests that, under conditions of hepatocyte incubation with high 1-GlcNAc concentrations (10 mM or above), the GSb phosphatase activity of glycogen-bound PP1 will be substantially inhibited. Previous work [4] has shown that 1-GlcNAc-6-P accumulates to concentrations of approx. 46 μ M (31 nmol/ 10^6 cells) when hepatocytes are incubated with 20 mM 1-GlcNAc for 20 min. Given the stimulatory effects of the native 1-GlcNAc on GPa phosphatase activities, both GS and GP are likely to be present largely in the b-forms when hepatocytes are incubated with 1-GlcNAc. This raises the possibility of using 1-GlcNAc/1-GlcNAc-6-P as a tool to maintain the enzymes in their less active forms, allowing investigation of the effects of other factors on the system.

That the phenomenon of 1-GlcNAc-6-P-inhibition of PP1 is related, at least in part, to the stimulatory effects of Glc-6-P on the enzyme's activity is illustrated by the foregoing data. Examination of the interplay between the two phosphorylated sugars

allows an approach to the mechanism underlying the inhibitory effect of 1-GlcNAc-6-P. Dephosphorylation of the substrates GSb and GPa was inhibited but that of myelin basic protein, histone IIA and casein was not. This suggests that a Glc-6-P-binding site may be prerequisite for inhibition. However, the different behaviours of the two substrates, GSb and GPa, indicate that an effect more complex than the occupation of the Glc-6-P-binding site by 1-GlcNAc-6-P may be involved, at least for the former substrate. In neither case is inhibition the result of simple competition. High Glc-6-P concentrations fail to overcome the inhibitory effect and this is the case for both the glycogen-bound form of PP1 and its isolated catalytic subunit. However, in the case of GPa substrate, inhibition appears to be mediated entirely through the effects of Glc-6-P on the activity of PP1. Only a negation of the usual stimulatory action of Glc-6-P is observed, returning values in the co-presence of Glc-6-P and 1-GlcNAc-6-P to basal rates. This contrasts with the situation of the GSb substrate in which percentage inhibitions by 1-GlcNAc-6-P are similar in the presence and absence of Glc-6-P. Differences between the two substrates are reinforced by the behaviour of the isolated catalytic subunit: its activity is stimulated by Glc-6-P when GPa is dephosphorylated but unaffected by this agent when GSb is the substrate, despite the fact that activity with either substrate is inhibited by 1-GlcNAc-6-P. These observations suggest that inhibitory effects of 1-GlcNAc-6-P may operate through different modes for GSb and for GPa. Restriction of effects to negation of the Glc-6-P stimulation with GPa indicates that the inhibitor may bind to the Glc-6-P-binding site, preventing the latter from binding. If this is the case, binding of 1-GlcNAc-6-P must be with sufficient affinity to avoid its displacement by Glc-6-P. By contrast, when a GSb substrate is involved, it may be that 1-GlcNAc-6-P binds to a site other than the Glc-6-P-binding site, in order to exert its inhibitory effects. This would explain why a similar pattern of inhibition is observed whether PP1 is present as its isolated catalytic subunit (the activity of which is unaffected by Glc-6-P) or as the glycogen-bound holoenzyme. Inhibition of GSb dephosphorylation by the isolated catalytic subunit (76%) may be sufficient to account for that by glycogen-bound PP1 (70%).

Effects of 1-GlcNAc-6-P on PP1 activity are specific for the glycogen-bound form of the enzyme, leaving the cytosolic form uninhibited. This distinction remains unexplained since it is considered that both forms incorporate similar catalytic subunits. It is possible that the subunit that confers cytosolic properties on the holoenzyme stabilizes the catalytic subunit sufficiently to overcome inhibition. Such an effect may be related to the weak stimulation of this isoenzyme by Glc-6-P (a 1.2-fold increase in activity at 10 mM Glc-6-P) compared with the strong stimulation of the glycogen-bound form (a 2.8-fold increase at 10 mM Glc-

6-P). The stimulation of cytosolic PP1 elicited by 10 mM Glc-6-P is mimicked by 100 μ M 1-GlcNAc-6-P, presumably as a result of the latter binding to the Glc-6-P-binding site on GS. Detailed structural studies of the PP1 subunits with appropriate ligands are expected to illuminate the situation further.

The present work reveals a novel and potent inhibitor of PP1 activity, which is specific for the glycogen-bound species of the enzyme. The existence of such an inhibitor has important consequences with regard to attempts to manipulate the glycogen metabolism of hepatocytes by means of glucose-analogue inhibitors of GP.

The work presented in this paper has been supported by European Community grant B102 CT94 3025 (Carbohydrate Bioengineering Initiative). M.B. is a long-term Fellow of the Federation of European Biochemical Societies. The contribution of Helen Ardron in synthesizing 1-GlcNAc in the laboratory of Dr. G. W. J. Fleet is gratefully acknowledged. Many thanks are due to Professor W. Stalmans for generous provision of facilities, helpful discussion and critical reading of the manuscript and to Dr. M. Bollen for invaluable practical advice.

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