Time-dependent pseudo-activation of hepatic glycogen synthase b by glucose 6-phosphate without involvement of protein phosphatases

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INTRODUCTION

Glycogen synthase is the rate-limiting enzyme of glycogen synthesis, catalysing the transfer of glucosyl units from UDP-glucose to glycogen [1]. Phosphorylation of multiple Ser residues in glycogen synthase by several protein kinases converts the enzyme from the active a-form into the inactive b-form [2,3]. Dephosphorylation (activation) is catalysed by the glycogen-bound type-1 protein phosphatase, of which liver- and muscle-specific isoforms exist [4–7]. Glycogen synthase b is routinely measured in the presence of 10 mM of the allosteric activator glucose 6-phosphate. The effect of glucose 6-phosphate on synthase a is antagonized by P_i [8]. The specific measurement of synthase a is classically performed in the presence of 10 mM sulphate, which mimics the effect of P_i without being a substrate for phosphorylase [8]. Intracellular P_i concentrations are sufficient to inhibit the allosteric activation of synthase b by physiological concentrations of glucose 6-phosphate, and hence only glycogen synthase a is thought to be responsible for glycogen synthesis in vivo. Indeed, under all metabolic conditions examined there was an excellent correlation between the activity of synthase a, measured in the presence of 10 mM sulphate, and the actual rate of glycogen synthesis in the liver and in isolated hepatocytes [1].

However, the effect of glucose 6-phosphate in vivo remains somewhat controversial, since, by binding to glycogen synthase, this compound might also affect kinases and phosphatases acting on glycogen synthase, and thus decrease the phosphorylation state of the enzyme. Glucose 6-phosphate enhances the dephosphorylation of purified glycogen synthase from skeletal muscle by synthase phosphatases [9], and opposes the activation in vitro of cAMP-dependent protein kinase by glycogen synthase [10].

The liver responds to an increase in the blood glucose level by an inactivation of phosphorylase and an activation of glycogen synthase. Until recently, the effects of a glucose load could be adequately described by a sequential dephosphorylation model [1], whereby glucose facilitates the dephosphorylation of glycogen phosphorylase through an allosteric mechanism. This dephosphorylation converts glycogen phosphorylase from the active a-form into the inactive b-form. Since phosphorylase a (but not the b-form) is a potent inhibitor of glycogen-synthase phosphatase activity, glycogen synthase activation starts after a lag period corresponding to the time needed for glycogen phosphorylase inactivation.

Recently, however, the use of synthetic glucose analogues led us and others [11,12] to the conclusion that glucose indeed causes the inactivation of phosphorylase, but that this is insufficient to trigger glycogen synthase activation. Phosphorylation of glucose and an increase in glucose 6-phosphate concentration appear essential for this activation. Since liver glycogen synthase lacks the principal phosphorylation sites for cAMP-dependent protein kinase [13], the latter kinase is unlikely to account for these effects. In a search for effects of glucose 6-phosphate on the glycogen-bound protein phosphatase from liver, we discovered that liver glycogen synthase is activated by mere incubation at 25 °C in the presence of glucose 6-phosphate. This process does not involve dephosphorylation of glycogen synthase, and is therefore referred to as ‘pseudo-activation’.

EXPERIMENTAL

Glycogen synthase was measured in the presence of 5 mM UDP-[U-14C]glucose (unless indicated otherwise), 1 % glycogen and either 10 mM Na_SO_4 for the assay of synthase a or 10 mM glucose 6-phosphate for the assay of total synthase activity (synthase a + b) [14]. One unit corresponds to the incorporation of 1 μmol of glucosyl units from UDP-glucose into glycogen/min.
at 25 °C. Results are expressed as means ± S.E.M. for the indicated number (n) of experiments.

Glucose 6-phosphate (monosodium salt) was purchased from Sigma, and Chelex-100 from Bio-Rad. The source of most other materials was as previously reported [4]. Glycogen synthase b was purified from dog liver by chromatography of the isolated protein–glycogen complex on DEAE-cellulose as described in [14]. The column was eluted with a linear salt gradient (0–1 M NaCl) containing 0.1 %, particulate glycogen, and the glycogen synthase–glycogen complex was re-sedimented by high-speed centrifugation. Incubations of glycogen synthase were routinely performed in buffer containing 50 mM glycylglycine, pH 7.4, and 0.5 mM dithiothreitol. The purified glycogen synthase migrated on SDS/PAGE (10 %, acrylamide) [15] as a 74.9 kDa protein and was essentially free of contaminating proteins, as judged by Coomassie Blue staining. The molecular mass of rabbit liver glycogen synthase has been reported as 85 kDa by SDS/PAGE [2], and the predicted mass of the rat liver enzyme is 80.5 kDa [13]. It is unknown whether glycogen synthase from dog liver has a lower molecular mass, or whether the enzyme underwent, during purification, a limited proteolysis at the C-terminus (see the Results section). It is also possible that the phosphorylation state of the enzyme and/or the electrophoretic technique adopted affected the apparent molecular mass. After transfer of the glycogen synthase from the polyacrylamide gel to a poly(vinylidene difluoride) membrane (Bio-Rad) by electroblotting [16], N-terminal micro-sequencing was performed on an ABI 473 protein sequencer operated in the pulsed-liquid mode as recommended by the manufacturer [17,18].

Casein kinases-1 and -2 were prepared from pig spleen [19]. Glycogen-synthase kinase-3 was purified from rabbit skeletal muscle [20]. Rabbit muscle glycogen synthase a was prepared as described by Takeda et al. [21], and inactivated in vitro to a synthase a/a+b ratio of about 0.5 through phosphorylation with the catalytic subunit of cAMP-dependent protein kinase (Sigma).

Subcellular fractions were prepared from rat liver as described in [4,14], except that 100 mM NaF was present throughout the preparation to inhibit all Ser/Thr-protein phosphatase activity. Essentially, overnight-fasted Wistar rats were injected intraperitoneally with 0.3 mg of glucagon 30 min before decapitation, to ensure maximal depletion of hepatic glycogen. The livers were removed, homogenized in buffer A (50 mM glycylglycine, 3 mM EGTA, 0.5 mM dithiothreitol, 5 % glycerol, 5 mM 2-mercaptoethanol, 100 mM NaF, 0.5 mM benzamidine and 0.3 mM PMSF, pH 7.4) plus 0.25 M sucrose, and centrifuged at 220000 g for 35 min to obtain a cytosolic fraction. Particulate liver glycogen (5 mg/ml) was added to the resulting liver cytosol, and a second high-speed centrifugation yielded a glycogen pellet with associated enzymes, which was washed once in buffer A and recentrifuged.

RESULTS

Pseudo-activation of glycogen synthase b without dephosphorylation

Homogeneously pure glycogen-bound synthase b from dog liver was incubated at 25 °C with or without glucose 6-phosphate, and at various time points samples were assayed (with 10-fold dilution) for synthase a activity in the presence of 10 mM sulphate. As shown in Figure 1, glycogen synthase remained inactive in the absence of glucose 6-phosphate, but incubation in the presence of glucose 6-phosphate resulted in a time-dependent increase in activity, suggesting a dephosphorylation of the enzyme. This increase in activity depended on the glucose 6-phosphate concentration in the range 2–10 mM. Concentrations above 10 mM could not be used, however, since the carry-over into the synthase assay (> 1 mM) then led to appreciable allosteric stimulation of synthase b (cf. the increase in activity at 0 min). The increase in glycogen synthase activity was not due to Ba2+, which may contaminate commercial glucose 6-phosphate preparations, or to other bivalent metal ions, since the effect remained unchanged after further purification of glucose 6-phosphate on a Chelex-100 cation exchanger.

Since glycogen synthase is normally activated by dephosphorylation, which, at least for the skeletal-muscle enzyme, is reportedly stimulated by glucose 6-phosphate [9], residual protein phosphatase activity present in the (apparently homogeneous) glycogen synthase preparations seemed an obvious explanation for the observed effects. To investigate this possibility, inhibitors of various protein phosphatases [5,22] were used, which, however, failed to affect the course of activation of purified glycogen synthase in the presence of 10 mM glucose 6-phosphate (results not shown). The latter inhibitors included 0.2 μM of modulator (inhibitor-2), a specific inhibitor of type-1 Ser/Thr protein phosphatases, 400 nM microcystin, a potent inhibitor of type-1 and type-2A Ser/Thr protein phosphatases, 1 mM EGTA plus 1 mM EDTA, which block the more specific type-2B (calcineurin, Ca2+-dependent) and type-2C (Mg2+-dependent) Ser/Thr-protein phosphatases, and 100 mM NaF, a general inhibitor of Ser/Thr protein phosphatases. Neither did 5 mM homoarginine, nor 5 mM phenylalanine, known as potent inhibitors of alkaline phosphatases [23], influence the activation reaction. We conclude that the observed activation of purified liver glycogen synthase by incubation in the presence of glucose 6-phosphate is not due to dephosphorylation, and should hence be termed ‘pseudo-activation’.

Two further lines of evidence ruled out the involvement of a protein-dephosphorylation mechanism in the pseudo-activation process. First, dog liver glycogen synthase b could be 32P-labelled by phosphorylation with casein kinase-2 and glycogen-synthase kinase-3, and this label was not removed during incubation in the presence of 10 mM glucose 6-phosphate (Figure 2). Second, prior incubation of synthase b for 1 h in the presence of 10 mM
glucose 6-phosphate did not significantly change the amount of phosphate (1.9 ± 0.3 mol/mol, versus 1.4 ± 0.3 mol/mol in the control; \( n = 3 \)) that could be incorporated by a mixture of three different kinases (glycogen-synthase kinase-3, casein kinase-1 and -2) in the presence of \( \gamma^{-32}P \)-labelled ATP (results not shown).

Incubation of glycogen synthase \( b \) from skeletal muscle in the presence of glucose 6-phosphate had no influence on its apparent activation state (results not shown), indicating that pseudo-activation is specific for the liver enzyme. Moreover, the activity ratio after incubation of a mixture of muscle and liver isoforms of glycogen synthase suggested that the muscle enzyme failed to activate, but did not influence the pseudo-activation of the liver enzyme.

Dephosphorylation of glycogen synthase by the hepatic glycogen-bound protein phosphatase is potently inhibited by phosphorylase \( a \) [1,4-6,22] and by thiophosphorylated phosphorylase \( a \) [4]. In keeping with the distinct nature of the pseudo-activation process, the latter was not affected by the addition of thiophosphorylated phosphorylase \( a \) (results not shown).

**Characteristics of the pseudo-activated enzyme**

The presence of some glucose 6-phosphate was essential for the expression of the activity of the pseudo-synthase \( a \). When the enzyme obtained by incubation for 30 min with 10 mM glucose 6-phosphate (Figure 1) was re-isolated as an enzyme–glycogen complex by high-speed centrifugation, the enzyme was again entirely inactive (results not shown); however, re-addition of 10 mM glucose 6-phosphate then resulted in an instantaneous re-activation.

Figure 3 illustrates the effect of the glucose 6-phosphate concentration on the generation of pseudo-synthase \( a \). After preincubation for 45 min at 25 °C, the enzyme was diluted 20-fold in an assay mixture containing 10 mM sulphate (Figure 3A) or without sulphate (Figure 3B). Non-preincubated synthase \( b \) (‘control’) was assayed similarly in the presence of the same final concentrations of glucose 6-phosphate. The data are means ± S.E.M. for three experiments. In the absence of sulphate, the \( K_s \) of synthase \( b \) for glucose 6-phosphate was 4.6 mM (B).

**Figure 2** Dephosphorylation or proteolysis are not involved in pseudo-activation

Purified liver glycogen synthase \( b \) was phosphorylated with 2 mM magnesium acetate, 100 \( \mu \)M \( \gamma^{-32}P \)ATP, casein kinase-2 and glycogen-synthase kinase-3. The labelled synthase was incubated at 25 °C in the presence of 10 \( \mu \)M microcystin and 10 mM glucose 6-phosphate. At the indicated time points, portions were boiled in sample buffer and analysed by SDS/PAGE (10% gels) and autoradiography. The kDa values are those of standard proteins.
Figure 4. Pseudo-activation increases the $V_{\text{max}}$ of glycogen synthase without change in the $K_m$ for UDP-glucose

Purified dog liver synthase $b$ (75 m-units/ml) was incubated during 30 min at 25 °C in the presence of 10 mM glucose 6-phosphate (‘pre-incubated’; ●), before a 10-fold dilution in assay mixtures containing 10 mM sulphate and the indicated concentrations of UDP-glucose. Non-incubated synthase $b$ (‘control’; ○) was similarly assayed in the presence of a matching concentration of glucose 6-phosphate (0.5 mM). The graph shows Lineweaver–Burk plots of the means ± S.E.M. for three experiments. Abbreviation: mU, m-unit.

Figure 5. Pseudo-activation of glycogen synthase in the cytosolic fraction from glycogen-depleted rat livers

A cytosolic fraction was prepared as described in the Experimental section from a 20% (w/v) liver homogenate in the presence of 100 mM NaF and incubated at 25 °C in the absence (○, △) or presence (●, ▲) of 5 mM glucose 6-phosphate (Glc-6-P). At the indicated time points, samples were assayed with 10-fold dilution for glycogen synthase activity in the presence of either 10 mM Na$_2$SO$_4$ (●, ○) or 10 mM glucose 6-phosphate (▲, △). Results are means ± S.E.M. for three experiments. Abbreviation: U, unit.

Mechanism of the pseudo-activation

It has been mentioned [24] that incubation of liver glycogen synthase at 0 °C led to an inactivation of the enzyme, which was reversed upon incubation at 20 °C. Pseudo-activation appeared to be an irreversible process, however, since the pseudo-activated glycogen synthase did not revert to its original state on incubation on ice for several hours or even at −20 °C for up to 72 h.

Camici et al. [25] reported that limited trypsinolysis increased 16-fold the $V_{\text{max}}$ of glycogen synthase from rabbit liver. Limited proteolysis of liver glycogen synthase by an endogenous co-purifying protease might thus explain our observations. However, inclusion of several protease inhibitors (0.5 mM tosylphenylalanilaylchloromethane, 0.5 mM tosyl-lysylchiloromethane, 0.3 mM PMSF, 0.5 mM benzamidine and 5 µM leupeptin) did not influence the pseudo-activation (results not shown). This result was further substantiated by the observation that the migration of glycogen synthase during SDS/PAGE was strictly unchanged after the pseudo-activation (Figure 2). Moreover, limited chymotrypsin treatment of glycogen synthase (known to digest the C-terminus [13,26]) led to a mobility shift (from 74.9 kDa to 70.5 kDa) on SDS/PAGE, but did not affect the activation state of the synthase nor the subsequent pseudo-activation (results not shown). Furthermore, the glycogen synthase appeared on the poly(vinylidene difluoride) membrane as a good Coomassie-Blue-stainable band (20–30 pmol of protein), and yet no phenylthiohydantoin (PTH)-amino acids would be detected upon N-terminal sequencing, both before and after pseudo-activation. This strongly suggests that the N-terminal amino acid was blocked and consequently not accessible for Edman degradation. However, if pseudo-activation was caused by N-terminal proteolysis, then new N-termini should have been released and PTH-amino acids detected during sequencing. All these results indicate that pseudo-activation is not caused by proteolysis, but is most likely due to a conformational change and/or subunit rearrangements.

Pseudo-activation of glycogen synthase in crude rat liver fractions

Since all the previous experiments had been performed with glycogen synthase $b$ purified from dog liver, the question arose as to whether pseudo-activation might occur in crude liver fractions and other species. Hence overnight-fastened rats were treated with glucagon, so as to deplete hepatic glycogen and to inactivate glycogen synthase. The liver cytosol, containing the solubilized glycogen synthase, was then incubated at 25 °C with or without 5 mM glucose 6-phosphate, while the inclusion of 100 mM NaF prevented dephosphorylation of the synthase. Nevertheless, a time-dependent increase in activity was observed during incubation in the presence of glucose 6-phosphate (Figure 5). This pseudo-activation reached a maximum after 30 min, when the activity measured in the presence of sulphate plus 0.5 mM glucose 6-phosphate roughly equaled the initial ‘total’ activity (measured in the presence of 10 mM glucose 6-phosphate). As with the purified enzyme (cf. Figure 3A), pseudo-activation also increased total glycogen synthase activity, by about 30%.

As expected, glycogen synthase $b$ as present in a crude enzyme–glycogen complex from rat liver also underwent pseudo-activation, although 200 nM microcystin was included to block the glycogen-bound protein phosphatase (results not shown). After incubation for 50 min in the presence of 5 mM glucose 6-phosphate, the activity of pseudo-synthase $a$ reached $84.5 \pm 0.5\%$ of the total synthase activity ($n = 3$). Under the latter conditions, the pseudo-activation was not inhibited by physiological $P_i$ concentrations (2 mM) and ionic strength (150 mM KCl).

DISCUSSION

Pseudo-activation of liver glycogen synthase and its mechanism

Glucose 6-phosphate can increase the activity of glycogen synthase $b$ in several ways. It is a well-known direct allosteric activator [24], and it may also indirectly favour the dephos-
phorylation of glycogen synthase b by altering the activities of protein kinases and protein phosphatases in a substrate-directed way [9,10]. In this work we describe an additional mechanism by which glucose 6-phosphate can affect glycogen synthesis. The compound promotes the transformation of liver glycogen synthase b into a pseudo-synthase a during incubation at 25 °C. Pseudo-synthase a differs from synthase a in that it is inactive in the presence of sulphate without glucose 6-phosphate, and it differs from synthase b in that the stimulatory effect of glucose 6-phosphate is not cancelled by sulphate.

The mechanism of this pseudo-activation remains open at present, but our data argue against a covalent modification. The failure of fluoride as well as of specific protein phosphatase inhibitors (microcystin, inhibitor-2 or modulator, EGTA, EDTA, homoarginine and phenylalanine) to interfere with pseudo-activation of glycogen synthase rules out the possibility of a dephosphorylation. This conclusion is furthermore corroborated by findings that 32P-labelled synthase b was not dephosphorylated during incubation with glucose 6-phosphate, and that such an incubation did not increase the amount of phosphate that could be incorporated by a host of different protein kinases.

Another possibility is limited proteolysis. Previous work [27–29] has shown that limited action of trypsin, chymotrypsin and subtilisin converts muscle glycogen synthase into a form with slightly lower subunit mass and altered kinetic properties. Of particular interest in the present context is the report by Takeda and Larner [27] that a Ca2+-dependent protease was detected in some highly purified preparations of muscle synthase b. However, in our study EDTA and EDTA remained without any effect on the pseudo-activation process. Limited action of trypsin also decreased the subunit mass of glycogen synthase b from rabbit liver by 7–10 kDa, either without change in Vmax [26] or with a near 20-fold increase in both Vmax and Km for UDP-glucose, and a 3.5-fold increase in affinity for glucose 6-phosphate [25]. The latter increase in Vmax fits into the picture of pseudo-activation, but the increase in Km does not (Figure 4), and several other observations argue against a limited proteolysis as the mechanism of pseudo-activation. First, we failed to detect any change in subunit mass when glycogen synthase was present as a single band on SDS/PAGE, allowing us to exclude a shift by as little as 2 kDa (Figure 2). Second, a series of protease inhibitors did not interfere with pseudo-activation. Third, chymotryptic shortening by 4.5 kDa at the C-terminus did not affect the subsequent pseudo-activation. Fourth, the N-terminus of our glycogen synthase preparation was blocked, as shown by its inaccessibility to Edman degradation. Although we cannot exclude the possibility that this blockade was artificially caused during purification, pseudo-activation failed to release new free amino acid termini which should have been detected as PTH-amino acids. Hence N-terminal proteolysis can be virtually excluded as a mechanism of pseudo-activation.

The irreversibility of the process clearly distinguishes pseudo-activation from recovery of glycogen synthase after cold inactivation [24]. Further, cold inactivation occurred only in the absence of glycogen, and recovery upon warming at 20 °C did not require glucose 6-phosphate [24].

By exclusion, our data suggest that a conformational change of glycogen synthase during incubation at 25 °C accounts for pseudo-activation. Native liver glycogen synthase exists most likely in a dimeric form, but various oligomeric forms can be observed under different conditions [2]. Possibly, the polymerization state of the enzyme might change upon incubation at 25 °C. Glucose 6-phosphate has actually been reported to induce aggregation of dimeric glycogen synthase from rat muscle [30]. However, since liver glycogen synthase is highly unstable in the absence of glucose, it is difficult to check this possibility with conventional biochemical techniques.

Relevance of pseudo-activation

Although pseudo-activation has only been observed in vitro, the facts that physiological concentrations of P, were unable to block the effect and that as little as 0.2–0.5 mM glucose 6-phosphate was sufficient to maintain substantial activity of pseudo-synthase a suggest a possible physiological importance. This is further suggested by our observation that pseudo-activation was not restricted to purified dog liver synthase b, but occurred readily in rat liver cytosol (Figure 5). According to the data in Figure 3, the presence of pseudo-synthase a in liver preparations should be revealed, at least qualitatively, by the difference between synthase activities recorded in the presence of 10 mM sulphate plus 0.5 mM glucose 6-phosphate and 10 mM sulphate only. However, these conditions may need optimization for species other than the dog.

It must be pointed out that our findings have important consequences for the assay method of glycogen-synthase phosphatase. Dephosphorylation of glycogen synthase can be measured either by the release of radioactive phosphate from (muscle) glycogen synthase labelled in vitro, or from activity measurements during the conversion of naturally phosphorylated liver glycogen synthase b into the a-form. As discussed previously [31], the latter approach is often essential to arrive at physiologically meaningful conclusions. However, the present work shows that extreme caution should be taken when interpreting the results obtained if glucose 6-phosphate is added to the latter assay. It is then essential to perform matched control incubations in the presence of phosphatase inhibitors such as fluoride or microcystin, to account for the pseudo-activation of glycogen synthase. In fact, the speed and extent of pseudo-activation of purified liver synthase b has prevented us from using this substrate to investigate the effect of glucose 6-phosphate on the synthase phosphatase reaction.

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