High-affinity binding of glycogen-synthase phosphatase to glycogen particles in the liver

Role of glycogen in the inhibition of synthase phosphatase by phosphorylase *a*

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1. Post-mitochondrial supernatants were prepared from the livers of 24 h-fasted rats. Upon centrifugation at high speed, the major part of the glycogen-synthase phosphatase activity sedimented with the microsomal fraction. However, two approaches showed that the enzyme was associated with residual glycogen rather than with vesicles of the endoplasmic reticulum. Indeed, the activity was entirely solubilized when the remaining glycogen was degraded either by glucagon treatment *in vivo* or by α-amylolysis *in vitro*. No evidence could be found for an association of glycogen-synthase phosphatase with the smooth endoplasmic reticulum, as isolated with the use of discontinuous sucrose gradients. 2. After solubilization by glucagon treatment *in vivo*, synthase phosphatase could be transferred to glycogen particles with very high affinity. Half-maximal binding occurred at a glycogen concentration of about 0.25 mg/ml, whereas glycogen synthase and phosphorylase required 1.5–2 mg/ml. 3. In gel-filtered extracts prepared from glycogen-depleted livers, the activation of glycogen synthase was not inhibited at all by phosphorylase *a*. The inhibition was restored when the liver homogenates were prepared in a glycogen-containing buffer. The effect was half-maximal at a glycogen concentration of about 0.25 mg/ml, and virtually complete at 1 mg/ml. These findings explain long-standing observations that in fasted animals the liver contains appreciable amounts of both synthase and phosphorylase in the active form.

INTRODUCTION

Previous data from our laboratory have indicated that the activity of glycogen-synthase phosphatase in the liver is shared by a glycogen-bound enzyme and a cytosolic fraction [1]. Negligible activity appeared to be associated with nuclei and mitochondria [2] and with the microsomal fraction [1]. Synthase phosphatase G (i.e. the glycogen-associated enzyme, previously referred to as the G-component of synthase phosphatase) is tightly regulated. It is inhibited by phosphorylase *a*, and is therefore responsible for the glucose-induced activation of glycogen synthase [3]. Its inhibition by Ca^{2+} in the micromolar range may contribute to the overall mechanism by which Ca^{2+}-mobilizing glycogenolytic agents achieve the inactivation of glycogen synthase [4]. The loss of functional synthase phosphatase G limits glycogen synthesis in chronically diabetic rats [5] as well as in adrenalectomized starved rats [6]. The induction of synthase phosphatase G by glucocorticoid hormones accounts for the onset of glycogen synthesis in the liver of the developing fetal rat [7].

Yet several research groups have argued that the major synthase phosphatase activity in the liver is associated with the microsomal fraction [8–10], particularly with that corresponding to the smooth endoplasmic reticulum [10]. Hence the name 'SER-synthase-phosphatase' was coined [11]. The activity of the latter enzyme was substantially decreased in the livers of adrenalectomized starved rats [11,12] and of diabetic rats [13,14]. SER-synthase-phosphatase behaves therefore functionally like an almost exact copy of synthase phosphatase G [5,6]. In the present study we have investigated their relationship.

Appreciable quantities of phosphorylase *a* and of synthase *a* coexist in the liver of the fasted animal [15,16]. These observations indicate that the activity of synthase phosphatase is not adequately inhibited by phosphorylase *a* in the fasted state. The present paper shows that the regulatory effect of phosphorylase *a* requires a minimal concentration of glycogen to which the interacting enzymes must be bound.

Some of the present results have been published as symposium proceedings [17].

MATERIALS AND METHODS

Materials

The α-glucosidase inhibitor 1-deoxynojirimycin and the α-amylase inhibitor BAY e4609 were obtained from Bayer A. G. The source of other relevant biochemicals has been previously reported [1].

Handling of animals and livers

Male Wistar rats weighing 250–300 g were either fed *ad libitum* or fasted for 24 h. When specified, the latter animals were given 0.35 mg of glucagon intraperitoneally 30 min before death, to achieve a maximal glycogen depletion. All animals were killed by decapitation between 09:00 and 10:00 h.

Livers were rinsed in ice-cold 0.15 M-NaCl and homogenized in a Potter–Elvehjem tube in 1.5–4 vol. of standard buffer (0.25 M-sucrose/0.5 mM-dithiothreitol/50 mM-imidazole, adjusted at room temperature to pH 7.4). Where indicated, the buffer contained also a mixture of proteinase inhibitors [1 mM-benzamidine, 1 mM-phenylmethanesulphonyl fluoride, 0.1 mM-tosyl-

phenylalanylchloromethane ('TPCK') and 3 mM-EGTA] and/or a mixture of inhibitors of glycogen hydrolases (0.1 mg each of 1-deoxynojirimycin and BAY e4609/ml). The homogenates were centrifuged for 10 min at 8000 *g* in the cold. The post-mitochondrial supernatants (extracts) were further treated as indicated below.

Gel-filtered extracts. Portions (1 ml) of extracts prepared from 40% (w/v) homogenates were filtered through a column of Sephadex G-25 (5 cm × 1.5 cm) equilibrated in the homogenization buffer. After addition of 5 mM-$(NH_4)_2SO_4$, the protein fraction was incubated at 25 °C at a final concentration of about 20% with respect to the liver homogenate from which it was derived. Samples were periodically withdrawn and immediately assayed for phosphorylase and glycogen synthase.

Subcellular fractions. The extracts prepared from 33% (w/v) homogenates were centrifuged at 220000 *g* for 35 min. The high-speed pellet, consisting mainly of microsomal fraction and a variable amount of glycogen, was resuspended in standard buffer to the volume of the initial extract, and re-centrifuged at high speed. The supernatant (wash fluid) was combined with the first supernatant (cytosol). The washed pellet was resuspended in standard buffer.

Discontinuous gradient centrifugation

According to Dallner [18], the smooth and rough microsomal fractions can be separated by the selective aggregation of the latter in the presence of Cs^+, after which they can be sedimented through a layer of 1.3 M-sucrose. We have adopted the procedure described by Graham [19], which has been used since 1982 by Margolis and co-workers (R. Margolis, personal communication). Livers were homogenized in 4 vol. of 0.25 M-sucrose, and the homogenates were centrifuged for 20 min at 10000 *g* [10]. Of each post-mitochondrial supernatant, 8.5 ml was layered on top of two sucrose layers (2 ml of 1.3 M and 1 ml of 0.6 M), each containing 15 mM-CsCl. The tubes were centrifuged for 1.5 h at 200000 *g* (40000 rev./min) in a Beckman SW41 rotor. From top to bottom were then successively recovered: the clear supernatant (cytosol); the 'light microsomal fraction', i.e. a double band near the 0.6 M-/1.3 M-sucrose interface, which should correspond to the smooth endoplasmic reticulum; the bulk of the 1.3 M-sucrose layer, which contained various sedimenting material; and the pellet ('heavy microsomal fraction'), which should correspond to the rough endoplasmic reticulum, but contained also most of the glycogen when present. Before the assays each fraction was adjusted to 8.5 ml with 0.25 M-sucrose, except for the 1.3 M-sucrose layer, which was diluted with water. It was checked that none of the assayed enzymes was affected by the presence of 15 mM-CsCl.

Assays

The activity of glycogen-synthase phosphatase was determined from the rate of activation of purified hepatic glycogen synthase in the presence of AMP and Mg^{2+} [4]. The final concentration of the homogenates was 2% (w/v), and subcellular fractions were used at an equivalent dilution with respect to the liver homogenates from which they were derived. During incubation of the mixture for 1 h at 25 °C, samples were withdrawn at regular intervals for the assay of synthase *a*. The substrate was a preparation of synthase *b* that was activated to a measurable extent by the glycogen-bound as well as by the cytosolic phosphatase, as present in subcellular fractions at 2% [20]. One unit of synthase phosphatase converts 1 unit of synthase *b* into *a* per min.

The assays of glycogen synthase *a* and synthase $a+b$ [1], phosphorylase *a* and phosphorylase $a+b$ [21] have been described. Lactate dehydrogenase was measured spectrophotometrically [22]. Glucose-6-phosphatase was assayed in detergent-treated preparations as described by Goldsmith & Stetten [23], except that 0.1 M-histidine was used to buffer the assay mixture at pH 6.5. The difference between total and non-specific phosphohydrolase activities was taken as the true glucose-6-phosphatase activity [23]. However, the contribution of non-specific phosphatases was negligible in the present work. One unit of these enzymes converts 1 μmol of substrate into product per min under the specified assay conditions. Glycogen and protein were assayed as previously described [21].

Table 1. Solubilization of glycogen-synthase phosphatase activity by glucagon treatment of fasted rats

Liver homogenates (20%, w/v) were prepared from two groups (untreated and glucagon-treated) of two fasted rats. The corresponding post-mitochondrial supernatants were fractionated as described in the Materials and methods section. Glycogen was measured in the freshly prepared homogenates. Synthase phosphatase activity is expressed as % of that measured when the two fractions were recombined before the assay. The latter values were 0.59 and 0.52 unit/g of liver in the untreated and glucagon-treated animals respectively. Data are shown from a single experiment. The distribution of the enzyme at other glycogen concentrations is illustrated in Fig. 1.

Experimental condition	Glycogen (mg/g of liver)	Synthase phosphatase activity (%) recovered in:	
		Pellet	Supernatant
Fasted	2.3	80	18
Fasted, glucagon-treated	0.1	7	86

RESULTS

Subcellular distribution in fasted-rat liver

Synthase phosphatase G sediments when liver extracts from fed rats are centrifuged at high speed [5]. However, it is not possible to separate in this way the glycogen particles from the microsomal fraction without considerable cross-contamination. Therefore we turned to livers from 24 h-fasted rats. As shown in Table 1, even in this condition the vast majority of the synthase phosphatase activity present in the post-mitochondrial supernatant sedimented with the microsomal fraction. The glycogen content of these livers was barely 4% of that encountered in the fed state (cf. Table 2), but in a parallel lot of glucagon-treated rats it was further lowered by an order of magnitude. In the latter liver extracts the activity of synthase phosphatase was almost entirely soluble (Table 1).

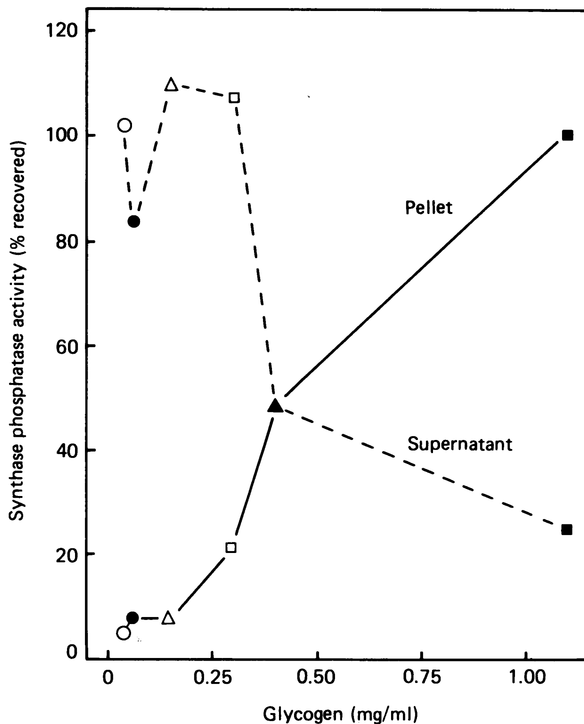

Fig. 1. Solubilization of synthase phosphatase by α-amylolytic degradation of the residual glycogen in liver extracts from fasted rats

Homogenates (25%, w/v) were prepared from three individual rat livers (identified by distinct symbol types). The corresponding post-mitochondrial supernatants were incubated for 30 min at 25 °C in the presence of either BAY e4609 (0.1 mg/ml) (black symbols) or standard buffer (corresponding white symbols). The preparations were then chilled, and a sample was removed for the determination of the glycogen concentration at the onset of the high-speed centrifugation. The synthase phosphatase activity measured in the pellet and in the corresponding supernatant is expressed as % of the activity recorded when the two fractions were recombined before the assay. The latter values were 0.59±0.01 and 0.60±0.06 unit/g of liver in the absence and presence of BAY e4609 respectively (means±S.E.M.; $n = 3$).

A subsequent series of experiments aimed at solving the question whether glucagon had solubilized the enzyme by decreasing glycogen or by enhancing the phosphorylation of some protein. For this purpose, liver homogenates were prepared from three individual fasted rats without hormone treatment. The corresponding extracts were then incubated at 25 °C before further fractionation. During this incubation period there is a substantial degradation of glycogen, which can be almost entirely blocked by the addition of a specific α-amylase inhibitor. Incubation with and without the inhibitor thus provides a means to vary the glycogen concentration *in vitro*. The results (Fig. 1) show a good correlation between the amount of sedimentable synthase phosphatase activity and the glycogen concentration in the extract at the onset of the high-speed centrifugation.

Binding of synthase phosphatase to added glycogen

The experiments illustrated in Table 1 and Fig. 1 imply that synthase phosphatase G binds to glycogen with an

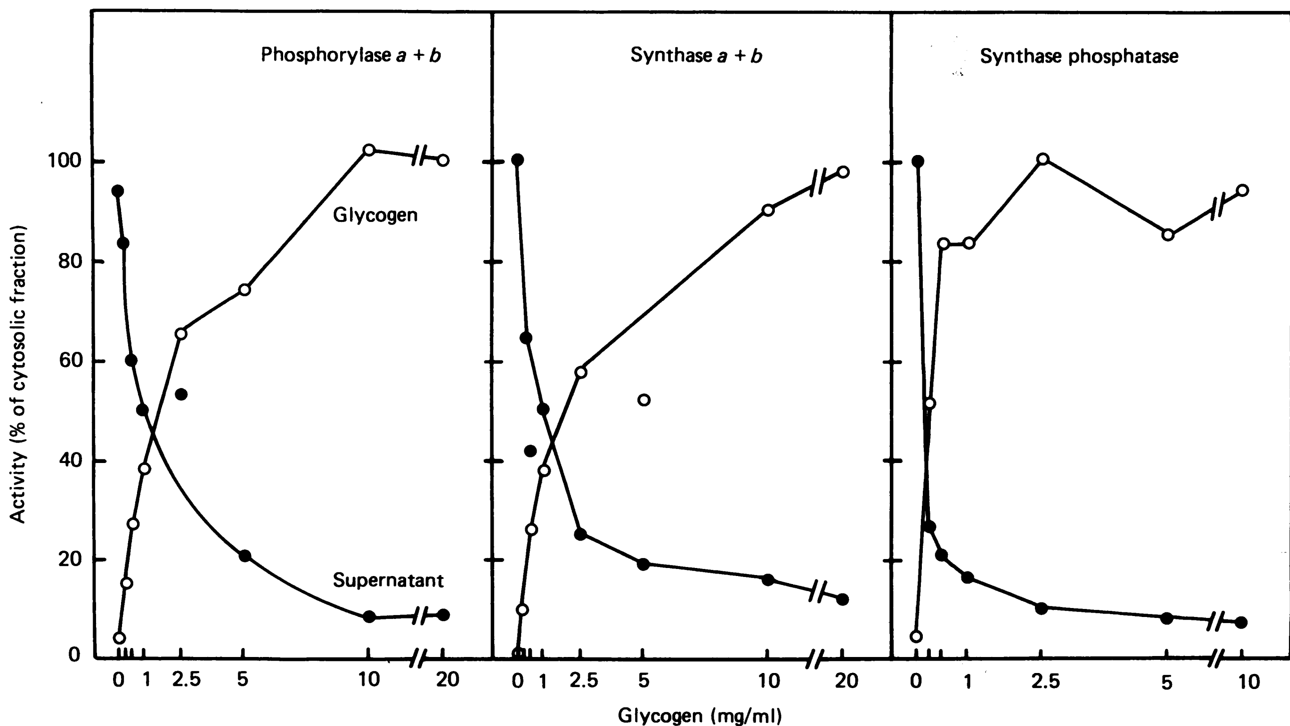

Fig. 2. Binding of solubilized enzymes to added particulate glycogen

A 20% (w/v) homogenate was prepared from the livers of two fasted glucagon-treated rats. High-speed centrifugation yielded the cytosolic fraction. Various glycogen concentrations (0–20 mg/ml) were added to portions of the cytosol, which were then re-centrifuged at high speed. Enzyme activities measured in each resuspended pellet and in the corresponding supernatant are expressed as % of the activity measured in the parent fraction. Data from a representative experiment are shown.

Table 2. Centrifugation of post-mitochondrial supernatants layered on a discontinuous sucrose gradient

Post-mitochondrial supernatants were prepared from the livers of either fed or fasted glucagon-treated rats and centrifuged as described in the Materials and methods section. The data are means ± S.E.M. ($n = 3$), except for one set of determinations (means of two experiments). A dash indicates that a reliable measurement could not be obtained. Values on the first two lines are in mg/g of liver (glycogen) or in units/g of liver (enzyme activities); all others are % recovered of the value measured in the post-mitochondrial supernatant.

Nutritional state	Fraction	Glycogen (mg/g of liver)	Lactate dehydrogenase	Glucose-6-phosphatase	Phosphorylase $a+b$	Synthase $a+b$	Synthase phosphatase
Glycogen-depleted	Post-mitochondrial supernatant	0.3±0.1	415±1	15.3±0.9	30.9±5.1	1.2±0.1	0.48±0.10
Fed		54.1±7.5	392±1	11.3	29.0±1.1	1.5±0.3	0.52±0.10
Glycogen-depleted	Supernatant	–	92.7±2.7	3.7±1.9	83.9±0.5	80.1±6.9	88.2±4.6
	Light microsomal fraction	–	10.4±3.2	65.5±8.4	8.1±1.5	15.1±0.5	9.4±3.6
	1.3 M-sucrose	–	2.1±0.5	12.9±7.8	4.9±2.2	2.5±1.0	0.8±0.1
	Heavy microsomal fraction	–	1.1±0.5	22.5±3.8	1.3±0.6	1.5±0.9	1.6±0.6
	Sum of fractions	–	106.3±1.7	104.6±5.6	98.2±1.2	99.2±2.3	100.0±2.2
Fed	Supernatant	1.7±0.1	94.4±1.0	2.8	64.9±1.8	24.9±0.9	17.2±1.4
	Light microsomal fraction	3.5±0.4	5.6±0.7	64.7	21.4±2.2	14.2±1.1	9.1±1.2
	1.3 M-sucrose	5.3±1.0	2.8±0.9	17.4	7.1±1.7	4.8±1.3	6.4±2.7
	Heavy microsomal fraction	87.9±6.5	0.9±0.1	20.1	6.3±1.6	2.8±0.8	26.9±3.3
	Sum of fractions	98.4±2.0	103.7±0.7	105.0	99.7±1.8	46.7±1.1	59.6±2.2

Table 3. Dissociation of the enzyme–glycogen complex by centrifugation through a sucrose barrier

A 20% (w/v) liver homogenate was prepared from a fasted glucagon-treated donor rat and centrifuged at high speed. After the addition of particulate glycogen to a final concentration of 10 mg/ml, the cytosolic fraction was re-centrifuged at high speed either as such or on top of a discontinuous sucrose gradient. At variance with the procedure as described in the Materials and methods section, all sucrose solutions contained 5 mM-EDTA, 2 mM-dithiothreitol and 50 mM-glycerol 2-phosphate (sodium salt) at pH 7.4, to enhance the stability of glycogen synthase.

		Percentage recovery of the value measured in the cytosolic fraction		
Type of centrifugation	Fraction	Glycogen	Phosphorylase $a+b$	Synthase $a+b$
No gradient	Supernatant	0.2	17.0	15.1
	Pellet	97.8	79.0	83.2
	Sum of fractions	98.0	96.0	98.3
Discontinuous sucrose gradient	Supernatant	0.3	63.3	67.7
	Dense sucrose	20.8	30.0	29.4
	Pellet	70.0	6.0	1.3
	Sum of fractions	91.1	99.3	98.4

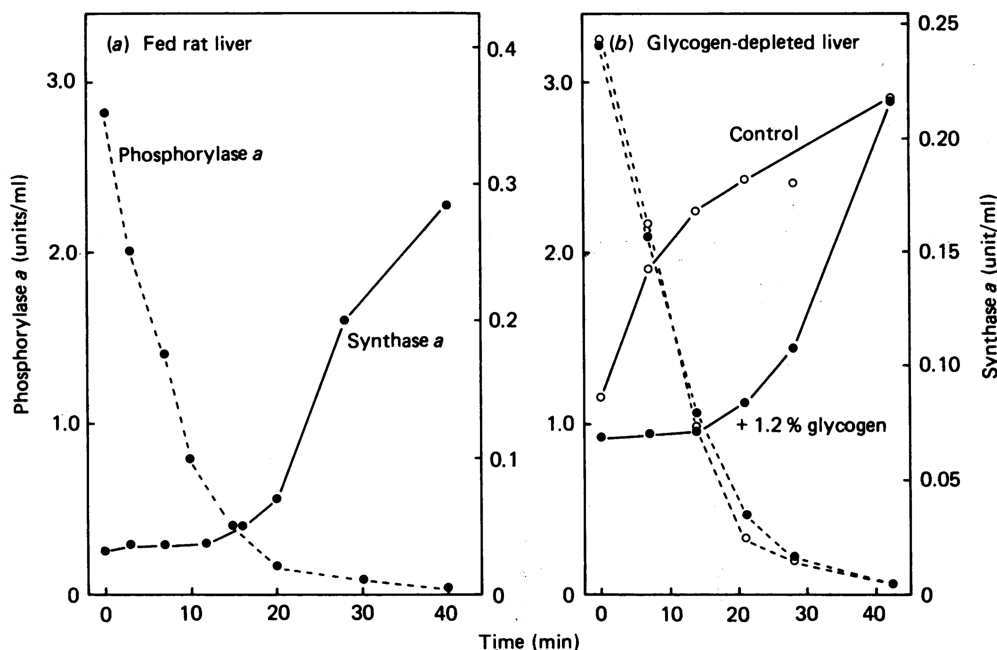

Fig. 3. Effect of the nutritional state on the inhibition of synthase activation by phosphorylase *a*

Gel-filtered liver extracts were prepared and incubated as described in the Materials and methods section. In (*b*) one liver lobe from a fasted glucagon-treated rat was homogenized in standard buffer only (○), and another lobe (●) was homogenized in a glycogen-containing buffer, to yield a final glycogen concentration (12 mg/ml) similar to that present in the gel-filtered extract prepared with standard buffer from the liver of a fed rat (*a*). Data from a representative experiment are shown.

unusually high affinity. This property has been directly and quantitatively explored in an approach where the enzyme was first solubilized by glucagon treatment *in vivo*. Various glycogen concentrations were then added to the cytosolic fraction, which contained also essentially all the phosphorylase and glycogen synthase activities. After high-speed centrifugation, the enzyme activities were determined in the supernatant and in the resuspended glycogen pellets. As shown in Fig. 2, each enzyme was progressively displaced from the supernatant to the pellet as the glycogen concentration was raised. Half-maximal binding of both phosphorylase and glycogen synthase occurred at a glycogen concentration of 1.5–2 mg/ml. However, at least half of the synthase phosphatase activity had already been displaced from the soluble fraction by the lowest glycogen concentration (0.25 mg/ml).

Use of sucrose density barriers

In order to investigate the association of synthase phosphatase with the smooth microsomal fraction, we have adopted the methodology used by Margolis and co-workers [10–14]. In view of the tight binding of synthase phosphatase G to glycogen, these experiments

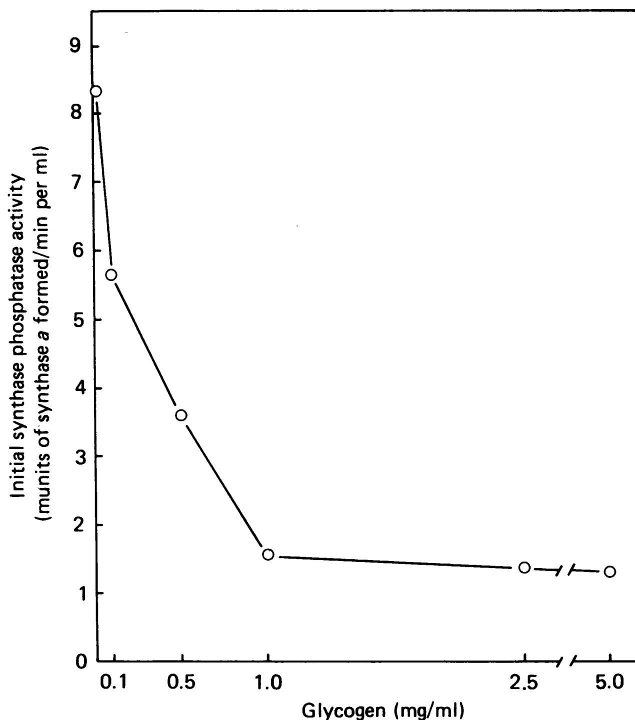

Fig. 4. Effect of the glycogen concentration on the inhibition of synthase phosphatase by phosphorylase *a*

Glycogen-depleted liver samples (0.1 mg of glycogen/g) were homogenized in standard buffer supplemented with proteinase inhibitors and glycogen-hydrolase inhibitors, and various concentrations of particulate liver glycogen. Gel-filtered extracts were prepared and incubated as described in the Materials and methods section. The initial activity of synthase phosphatase was determined from the increase in synthase *a* concentration, measured at 4 min intervals during the initial 16 min of incubation (cf. Fig. 3). The rate of phosphorylase inactivation was virtually identical at glycogen concentrations of 0.02 and 5 mg/ml. Data from a representative experiment are shown.

were performed with liver extracts from fed as well as fasted glucagon-treated rats (Table 2). In the latter case, 80–88% of phosphorylase, synthase and synthase phosphatase did not enter the sucrose gradient, and most of the remainder was recovered in the light microsomal fraction. These values are close to the distribution of a typical cytosolic enzyme such as lactate dehydrogenase. In glycogen-rich livers, it is clear that the vast majority of the synthase phosphatase indeed entered the gradient (17% soluble, versus 88% in the fasted condition). Although the low recovery of the enzyme activity within the gradient (42%) precludes a straightforward conclusion, there are two important features. First, about two-thirds of the recovered enzyme activity was associated with the heavy microsomal fraction, which contained 88% of the glycogen. Second, the light microsomal fraction contained 9% of the synthase phosphatase activity, irrespective of the nutritional state of the animal. In view of the distribution of lactate dehydrogenase, we have to conclude that we cannot find evidence for an association of synthase phosphatase with the smooth endoplasmic reticulum.

A remarkable feature was that insignificant amounts of typical glycogen-associated enzymes (phosphorylase, synthase) were recovered in the glycogen fraction (Table 2). The results suggested that the major part of these enzymes was dissociated from the glycogen particles at or shortly after contact with the dense sucrose medium. This hypothesis was investigated and confirmed by centrifuging an enzyme–glycogen complex either in an iso-osmotic medium or through a sucrose barrier (Table 3).

Synthase activation in glycogen-depleted liver extracts

Fig. 3(*a*) illustrates the typical activation pattern of glycogen synthase in a gel-filtered extract prepared from the liver of a fed rat. The activation of glycogen synthase is preceded by a lag, the length of which corresponds to the time required for the inactivation of phosphorylase. The lag is caused by the strong inhibition that phosphorylase *a* exerts on the activity of synthase phosphatase G ([3,24]; see ref. [25] for a recent review). In contrast, Fig. 3(*b*) shows the events during incubation of a similarly prepared extract from a fasted glucagon-treated rat. In this glycogen-depleted preparation the activation of glycogen synthase started at once, without any latency. Another liver lobe from the same animal was homogenized in a glycogen-containing buffer, to ensure a final glycogen concentration of 12 mg/ml during the incubation of the gel-filtered extract. The added glycogen was able to induce the classical lag period. At such concentrations, glycogen does not affect the activity of phosphorylase phosphatase (Fig. 3*b*).

We have subsequently investigated which concentrations of glycogen are required to restore the inhibition of synthase phosphatase by phosphorylase *a* in glycogen-depleted liver extracts. As illustrated in Fig. 4, the initial rate of activation of glycogen synthase *in vitro* decreased 6-fold when the glycogen concentration was raised from 0.02 to 1 mg/ml. Little additional effect was observed when the glycogen concentration was further increased to 5 mg/ml.

DISCUSSION

Subcellular distribution of synthase phosphatase

The present experiments confirm that the activity of glycogen synthase phosphatase, as present in normal rat liver extracts, is essentially partitioned into the cytosolic compartment and the glycogen particles. Synthase phosphatase G has an extremely high affinity for glycogen, as compared with glycogen synthase and phosphorylase. This observation implies that the phosphatase binds directly to glycogen rather than through an association with either synthase or phosphorylase. The glycogen-bound protein phosphatase from skeletal muscle contains a specific glycogen-binding subunit [26]. It is highly probable that a similar subunit is part of the hepatic enzyme. Synthase phosphatase G and the cytosolic synthase phosphatase contain the same catalytic subunit [27], and they should therefore be classified as type-1 [28] or ATP, Mg-dependent [29] protein phosphatases. Yet the two enzymes have quite distinct regulatory properties (see the Introduction). The present data suggest a structural basis for the failure of the cytosolic synthase phosphatase to bind to glycogen.

Our observations are consonant with those by Tsuiki and co-workers [30], who reported that, upon separation

of the glycogen and the microsomal fraction by differential centrifugation, the bulk of the synthase phosphatase activity was associated with the glycogen. However, those authors were puzzled by their observation that, even in livers from fasted rats, part of the activity was still associated with the particulate fraction. As in our experiments, these observations are probably explained by the high-affinity binding of synthase phosphatase G to residual glycogen.

Moreover, at closer inspection two previous reports [8,9] on the association of glycogen synthase phosphatase with microsomal fractions are equivocal. By ultrasonic treatment of a resuspended glycogen fraction, Hizukuri & Larner [8] were able to isolate a slowly sedimenting fraction that contained synthase phosphatase activity. The latter fraction was rich in smooth-surfaced vesicles and in glucose-6-phosphatase activity. This was suggestive evidence in favour of an association of synthase phosphatase with the smooth endoplasmic reticulum rather than with glycogen. However, the preparation used by Hizukuri & Larner [8] contained 2.5 mg of glycogen/ml, which should be sufficient to bind essentially all the synthase phosphatase G. Tan & Nuttall [9] found that the vast majority of the synthase phosphatase activity present in a post-mitochondrial supernatant was recovered in the microsomal fraction rather than in the glycogen fraction. However, when the organelles in the microsomal fraction were aggregated with $CaCl_2$ and eliminated by low-speed centrifugation, more than half of the synthase phosphatase activity was recovered in the supernatant (which should contain the residual glycogen). In view of these data, it seems to us that the reference to synthase phosphatase as a microsomal enzyme [9] was not appropriate. However, the observations by Tan & Nuttall [9] are important, because they indicate strongly that synthase phosphatase G binds preferentially to the smaller glycogen particles, which are most likely to contaminate the microsomal fraction.

Microsomes devoid of glycogen contain about 25% of the phosphorylase phosphatase activity present in a post-mitochondrial supernatant [27]. This enzyme is a type-1 protein phosphatase [27,31]. It has negligible synthase phosphatase activity in terms of activation of liver synthase *b* (the present paper; [27]). That other groups [10–14,31] found substantial synthase phosphatase activity associated with the microsomal fractions could possibly be explained by their type of assay, which is based on the activation [10–14] or dephosphorylation [31] of glycogen synthase from skeletal muscle. However, the possibility that residual glycogen contaminates the microsomal fraction, or is actually associated with the endoplasmic reticulum, has not adequately been answered to date. The technique of sucrose density barriers [10–14] presents an unexpected side problem, because at least phosphorylase and glycogen synthase dissociate largely from the sedimenting glycogen particles. Similar observations have been made previously on the protein–glycogen complex isolated from skeletal muscle. Wanson & Drochmans [32] reported that the glycogen particles could be stripped from adhering protein by sucrose gradient centrifugation almost as effectively as by treatment with 1% deoxycholate, and Meyer *et al.* [33] observed that phosphorylase, phosphorylase kinase and phosphorylase phosphatase were almost entirely released from the particles during sedimentation in a continuous sucrose gradient.

The absence of glycogen triggers a futile cycle

Glycogen metabolism in the liver of the normal fasted rat has been an intriguing anomaly for many years. There is an unusually high concentration of synthase *a* [15,16,34,35], in spite of the conspicuous absence of glycogen synthesis. Since fairly high concentrations of phosphorylase *a* are also present [16,34,35], it is probable (though not proven to date) that glycogen is continuously being synthesized and degraded in the liver of the fasted animal, without net deposition. It has been proposed that such a 'futile' cycle could be useful in preventing a complete disappearance of glycogen molecules during starvation [36]. Upon glucose administration the prominent change is an inactivation of phosphorylase, with [34–36] or without [16] a further activation of glycogen synthase, and glycogen deposition starts abruptly.

The coexistence of appreciable amounts of phosphorylase *a* and synthase *a* has also been noted in perfused livers and isolated hepatocytes from fasted donor rats [36], and emerges therefore as an intrinsic property of the liver in the fasting state. Hutson *et al.* [37] incubated hepatocytes from a fed rat in the absence of glucose, and resuspended the cells periodically in fresh medium to remove the glucose produced by glycogen breakdown. They observed that glycogen synthase *a* was formed as glycogen became depleted. These results point to a direct role of glycogen in preventing a futile cycle.

The coexistence of phosphorylase *a* and synthase *a* indicates that synthase phosphatase is not adequately inhibited by phosphorylase *a* in the fasted state. It was previously proposed that the full expression of the inhibitory effect might require a tight association of the interacting enzymes on to glycogen particles [34]. However, the demonstration of the latter point *in vitro* proved technically difficult, mainly because of a sluggish activation of glycogen synthase when glycogen-depleted liver extracts were incubated (results not shown). The inclusion of iso-osmotic sucrose [1] and of proteinase inhibitors (the present work) solved this problem satisfactorily. However, it should be noted that the induction of a latency by added glycogen (Figs. 3 and 4) was only obtained in a reproducible way when the livers were homogenized in a glycogen-containing buffer. Addition of glycogen to the gel-filtered extracts was less efficient, and sometimes failed to produce any effect. These observations indicate that discrete changes, possibly of proteolytic nature, occur rapidly in the absence of glycogen. Since the inhibition by phosphorylase *a* appears to require a specific subunit of synthase phosphatase G [24], there is an obvious target for such a putative proteinase.

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