Phosphorylase a is an allosteric inhibitor of the glycogen and microsomal forms of rat hepatic protein phosphatase-1

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The dephosphorylation of glycogen synthase by protein phosphatase-1 in hepatic glycogen and microsomes was inhibited by nanomolar concentrations of phosphorylase a. The I_{50} for phosphorylase a was 1000-fold lower than its K_m as a substrate, while tryptic digestion increased the I_{50} 1000-fold without affecting K_m. Protein phosphatase-1 from skeletal muscle and protein phosphatase-2A from liver were only inhibited at 1000-fold higher concentrations. Protein phosphatase-1 became desensitized to phosphorylase a when released from hepatic microsomes, but sensitivity was partially restored by readdition of the solubilized enzyme to the microsomes. The results demonstrate that phosphorylase a is a potent allosteric inhibitor of hepatic protein phosphatase-1 and suggest that inhibition may be conferred by a novel phosphorylase a-binding subunit.

Glycogen synthase   Protein phosphatase-1   (Liver)   Protein phosphorylation   Hormonal regulation
Microsome

1. INTRODUCTION

Phosphorylase a is an important regulator of glycogen synthase phosphatase activity in mammalian liver, that controls the level of glycogen synthase activation by extracellular signals such as glucose, insulin and glucagon [1–7]. However, the mechanism by which phosphorylase a inhibits glycogen synthase phosphatase is unresolved. Glycogen synthase phosphatase activity sensitive to inhibition by phosphorylase a was reported to be associated with hepatic microsomes [8] as well as hepatic glycogen [2,9], and to be distinct from phosphorylase phosphatase. Thus, glycogen synthase phosphatase and phosphorylase phosphatase activities could be partially resolved by chromatography on phosphocellulose [10] or by gel-filtration [8], while tryptic digestion increased

phosphorylase phosphatase several-fold, yet decreased glycogen synthase phosphatase activity [10].

Recently, we confirmed these observations, but were able to explain them in a quite different way [11]. Glycogen synthase phosphatase and phosphorylase phosphatase in the glycogen or microsomal fractions of either liver or muscle could be inhibited almost completely by inhibitor-1 or inhibitor-2 [11,12] and the concentrations required for 50% inhibition of both activities were very similar [11]. These, and other [11], observations indicated that the major protein phosphatase associated with the glycogen and microsomes of liver or muscle was a form of protein phosphatase-1 [13,14] and that this enzyme was responsible for the dephosphorylation of both phosphorylase a and glycogen synthase. The activation of phosphorylase phosphatase and inhibition of glycogen synthase phosphatase activity produced by tryptic digestion of hepatic glycogen and microsomes were shown to be intrinsic properties of protein phosphatase-1, since similar observa-

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tions were made following trypsinisation of protein phosphatase-1 that had been purified to homogeneity from the glycogen fraction of rabbit skeletal muscle [11]. The apparent resolution of phosphorylase phosphatase and glycogen synthase phosphatase activities by ion-exchange chromatography [10] or gel-filtration [8] could therefore be explained by separation of proteolytically modified and unmodified forms of protein phosphatase-1 with quite different phosphorylase phosphatase/glycogen synthase phosphatase activity ratios [11].

Here, we demonstrate that inhibition of glycogen synthase phosphatase by phosphorylase a is explained by an extremely potent and specific allosteric inhibition of hepatic protein phosphatase-1.

2. MATERIALS AND METHODS

2.1. Materials

Procedures for purifying proteins and sources of other materials are referenced in [11,15,16].

2.2. ^{32}P-labelled protein substrates (10^6 cpm/nmol)

Phosphorylase a (glycogen phosphorylase, EC 2.4.1.11; 1.0 mol phosphate/mol 97 kDa subunit) and glycogen synthase (EC 2.4.1.11) labelled in site-2 (0.5 mol phosphate/mol 86 kDa subunit) were phosphorylated using phosphorylase kinase (EC 2.7.1.38). ^{32}P-glycogen synthase labelled in sites-3 (1.3–1.4 mol phosphate/mol subunit) was phosphorylated with glycogen synthase kinase-3 (EC 2.7.1.37). ^{32}P-phosphorylase kinase (1.5–1.8 mol phosphate/$\alpha\beta\gamma\delta$ unit) containing equal amounts of radioactivity in the α- and β-subunits was labelled with cyclic AMP-dependent protein kinase (EC 2.7.1.37). Procedures for phosphorylating each protein and for freeing ^{32}P-labelled substrates from [γ-^{32}P]ATP were described in [17]. Unlabelled phosphorylase a was prepared in an identical manner to ^{32}P-labelled phosphorylase a, except that unlabelled ATP replaced [γ-^{32}P]ATP.

2.3. Preparation of glycogen and microsomal fractions

These were prepared from livers of normally fed rats in the presence of the proteinase inhibitors

phenylmethanesulphonylfluoride (PMSF), benzamidine and leupeptin, as in [11]. The glycogen + microsomal fraction was also isolated from rabbit skeletal muscle [11]. Each fraction was resuspended at 0°C in 50 mM Tris-Cl pH 7.0, 0.1 mM EGTA, 0.1% (v/v) 2-mercaptoethanol (buffer A) containing PMSF (0.1 mM), benzamidine (1 mM) and leupeptin (4 μg/ml) to give final protein concentrations of 20 ± 5 mg/ml [11]. The glycogen and microsomal fractions were prepared within 3 h and used immediately.

2.4. Treatment of the glycogen and microsomal fractions with trypsin

Aliquots of each fraction (0.3 ml) were incubated for 10 min at 30°C with 0.1 ml trypsin (2 mg/ml, EC 3.4.21.4). Digestions were terminated by addition of 0.08 ml of trypsin inhibitor (5 mg/ml), transferred to ice and used within 30 min.

2.5. Assay of protein phosphatases

Incubations (0.03 ml) were performed at 30°C in buffer A containing 1.0 mg bovine serum albumin/ml, 3% (v/v) glycerol, 5 mM caffeine, ^{32}P-labelled substrate and protein phosphatase. Reactions were initiated with phosphatase, after preincubating the other components for 2 min at 30°C. Assays were carried out for 5 min and terminated and analysed as in [18]. Substrate concentrations were 10.3 μM (1 mg/ml) phosphorylase a, 1.0 μM (0.086 mg/ml) glycogen synthase, and 0.7 μM (0.25 mg/ml) phosphorylase kinase. Release of ^{32}P-radioactivity was restricted to <30% (phosphorylase a), <25% (glycogen synthase) and <15% (phosphorylase kinase), to ensure that rates of dephosphorylation were linear with respect to time. Assays were performed in duplicate, and control incubations carried out in which protein phosphatase was replaced by buffer. These values were subtracted from those obtained in the presence of phosphatase.

The resuspended glycogen and microsomal fractions were assayed at a final dilution of 15-fold (glycogen synthase) or 30-fold (phosphorylase a and phosphorylase kinase). The glycogen + microsomal fraction from skeletal muscle was assayed at a 10-fold higher dilution.

3. RESULTS

3.1. *Influence of phosphorylase a on dephosphorylation of glycogen synthase and phosphorylase kinase by protein phosphatase-1 in the hepatic glycogen fraction*

The dephosphorylation of glycogen synthase labelled in sites-3 was potently inhibited by phosphorylase *a*, and half-maximal inhibition was observed at only 2–3 nM (fig.1). This was only slightly greater than the concentration of protein phosphatase-1 in the assay, which was estimated to be 0.5–1.0 nM, assuming that its specific activity was identical to that of the glycogen-bound form of protein phosphatase-1 from skeletal muscle [23]. The I_{50} for phosphorylase *a* was 1000-fold lower than the apparent K_m for phosphorylase *a* as a substrate, which was $\approx 20 \mu M$ (not shown). Phosphorylase *b* was 10000-fold less effective than phosphorylase *a* as an inhibitor (fig.1).

Incubation of the glycogen fraction with trypsin increased phosphorylase phosphatase activity 3–4-fold and decreased glycogen synthase (sites-3) phosphatase activity by 50% (in the absence of Mg^{2+}), as reported previously [11]. However, despite a large increase in the phosphorylase phosphatase/glycogen synthase phosphatase activity ratio, the concentration of phosphorylase *a* required to inhibit dephosphorylation of glycogen synthase by 50% increased 1000-fold (fig.1). The K_m for phosphorylase *a* as a substrate was not changed significantly by trypsin (not shown).

Phosphorylase *a* was also able to inhibit the dephosphorylation of glycogen synthase labelled in site-2. However, the I_{50} was ≈ 10 nM (fig.1), 3–5-fold higher than that required to inhibit the dephosphorylation of sites-3. This difference was obtained with several preparations of the hepatic glycogen fraction, in which experiments with glycogen synthase labelled in site-2 or sites-3 were performed in parallel. As observed with glycogen synthase labelled in sites-3, tryptic digestion inhibited the dephosphorylation of glycogen synthase labelled in site-2 by 46 ± 8% (± SE for 6 preparations), and increased the I_{50} for phosphorylase *a* 1000-fold (fig.1).

The dephosphorylation of phosphorylase kinase was also inhibited by phosphorylase *a*, but the I_{50} was 50–100 nM (fig.1), even higher than that observed for the dephosphorylation of glycogen synthase labelled in site-2. Tryptic digestion increased phosphorylase kinase phosphatase activity by 75 ± 5% (± SE for 6 preparations) and also abolished inhibition by phosphorylase *a* (fig.1).

3.2. *Influence of phosphorylase a on dephosphorylation of glycogen synthase and phosphorylase kinase by protein phosphatase-1 in the muscle glycogen fraction*

Mvumbi et al. [10] have reported that the dephosphorylation of glycogen synthase in skeletal muscle extracts is unaffected by phosphorylase *a*. The experiments described in the preceding section were therefore repeated using the glycogen + microsomal fraction from rabbit skeletal muscle. Phosphorylase *a* was found to be a very weak inhibitor of the dephosphorylation of glycogen synthase (labelled in sites-3 or site-2) and phosphorylase kinase. The I_{50} was $>10 \mu M$, 1000–5000-fold higher than the I_{50} for dephosphorylation of glycogen synthase in the hepatic glycogen fraction. Incubation of the muscle glycogen fraction with trypsin increased phosphorylase phosphatase activity about 2-fold and decreased glycogen synthase (sites-3) phosphatase activity by about 50% in the absence of Mg^{2+}, as reported previously [11]. Glycogen synthase (site-2) phosphatase activity was also decreased by 49 ± 3% (± SE for 3 preparations), while phosphorylase kinase phosphatase activity was increased by 35 ± 6% (± SE for 3 preparations). Tryptic digestion had almost no effect on the I_{50} for phosphorylase *a* (fig.1).

3.3. *Influence of phosphorylase a on dephosphorylation of glycogen synthase and phosphorylase kinase by the hepatic microsomal fraction*

Phosphorylase *a* was a potent inhibitor of glycogen synthase phosphatase activity in liver microsomes (fig.2). The I_{50} values of 8 nM or 20 nM using glycogen synthase phosphorylated in sites-3 or site-2, respectively, were slightly higher than those observed in the hepatic glycogen fraction. The I_{50} values were 500–1000-fold lower than the apparent K_m for phosphorylase *a* as a substrate, which was $20 \mu M$ (not shown). Phosphorylase *b* was over 1000-fold less effective than phosphorylase *a* as an inhibitor (fig.2).
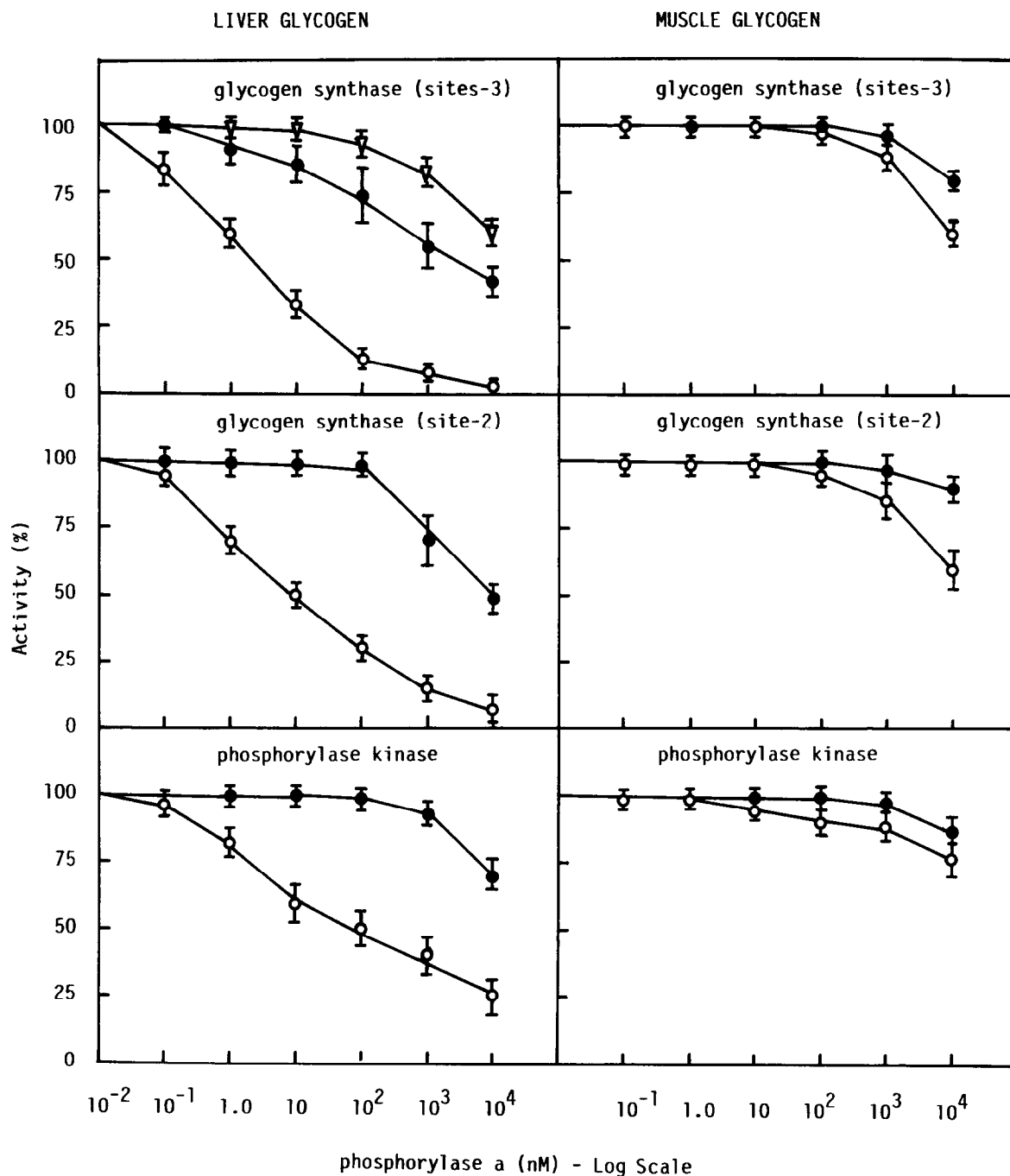
Fig.1. Influence of unlabelled phosphorylase a on dephosphorylation of ^{32}P-glycogen synthase and ^{32}P-phosphorylase kinase by the glycogen fractions from rat liver and rabbit skeletal muscle. Experiments were carried out before (○) or after (●) tryptic digestion, and activities are given relative to control incubations in which phosphorylase a was omitted. (∇) Effect of replacing phosphorylase a with phosphorylase b. Results are expressed as ± SE for 6 (liver) or 3 (muscle) different preparations of the glycogen fraction.
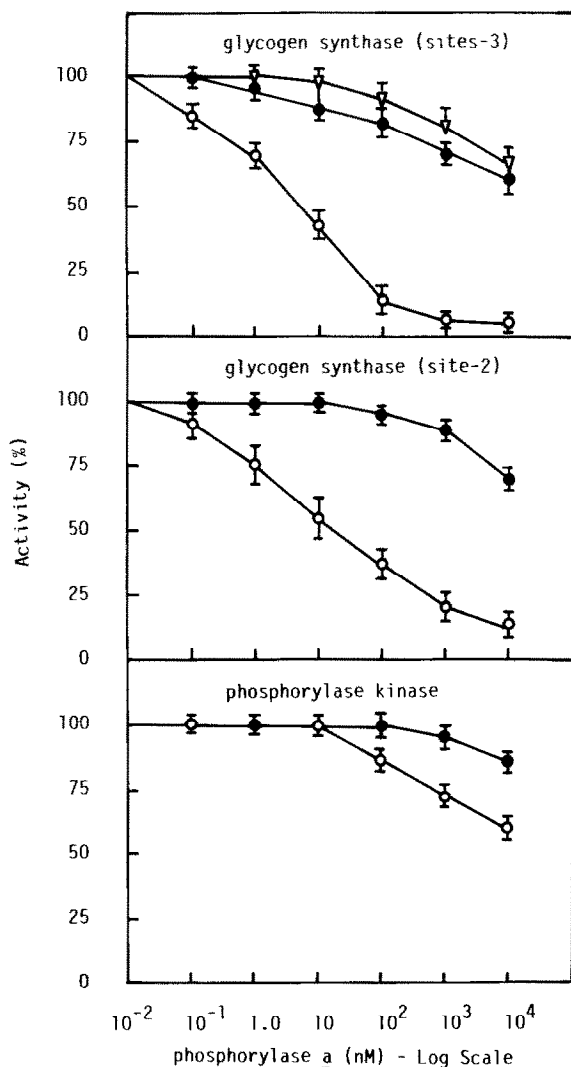
Fig.2. Influence of unlabelled phosphorylase a on dephosphorylation of ^{32}P-glycogen synthase and ^{32}P-phosphorylase kinase by the hepatic microsomal fraction. Experiments were carried out before (○) or after (●) tryptic digestion, and activities are given as ± SE (6 microsomal preparations) relative to control incubations in which phosphorylase a was omitted. (▽) Experiment in which phosphorylase a was replaced by phosphorylase b.

Tryptic digestion of the microsomal fraction increased phosphorylase phosphatase activity 3–4-fold and decreased glycogen synthase (sites-3) phosphatase activity by 40% (in the absence of Mg^{2+}), as reported previously [11]. Tryptic digestion decreased glycogen synthase (site-2) phosphatase activity by 33 ± 6% (± SE for 6

preparations). As observed with the glycogen fraction, tryptic digestion of the microsomes increased the I_{50} for phosphorylase a about 1000-fold (fig.2), without changing the K_m for phosphorylase a as a substrate (not shown).

The dephosphorylation of phosphorylase kinase was very poorly inhibited by phosphorylase a in the microsomal fraction, the I_{50} being $>10 \mu M$, even before tryptic digestion (fig.2). Tryptic digestion increased phosphorylase kinase phosphatase activity by 68 ± 5% (± SE for 6 preparations).

3.4. Influence of phosphorylase a on the dephosphorylation of glycogen synthase in rat liver extracts

Approximately half of the glycogen synthase phosphatase activity in liver extracts is catalysed by protein phosphatase-1 and half by protein phosphatase-2A, when assays are performed in the absence of divalent cations [12,15]. To compare the effectiveness of phosphorylase a as an inhibitor of protein phosphatases-1 and -2A, its ability to inhibit the dephosphorylation of glycogen synthase (labelled in sites-3) was therefore examined in liver extracts in the presence and absence of inhibitor-2. The experiments demonstrated that the inhibitor-2-sensitive glycogen synthase phosphatase activity (i.e. protein phosphatase-1) was potently inhibited by phosphorylase a, whereas the inhibitor-2-insensitive activity (i.e. protein phosphatase-2A) was unaffected by phosphorylase a (fig.3).

3.5. Desensitization and resensitization of hepatic protein phosphatase-1 to inhibition by phosphorylase a

Microsomes were incubated for 30 min at 0°C in the presence of 0.5 M NaCl as described in the legend to fig.4. This treatment released 97% of the protein phosphatase-1 from the microsomal membranes and desensitised the enzyme to inhibition by phosphorylase a (fig.4). However, when the solubilised enzyme was mixed with the salt-extracted microsomes sensitivity to inhibition by phosphorylase a was partially recovered (fig.4). About 30% of the solubilised phosphatase rebound to the microsomal membranes and this fraction ('reconstituted microsomes') was only $\simeq 5$-fold less sensitive to phosphorylase a than the freshly isolated microsomes (fig.4).
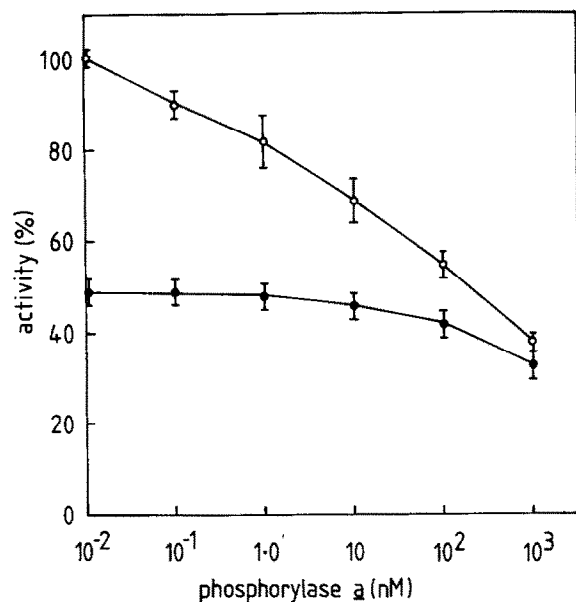
Fig.3. Influence of unlabelled phosphorylase a on dephosphorylation of ^{32}P-glycogen synthase (labelled in sites-3) by rat liver extracts. Extracts obtained by centrifugation of rat liver homogenates for 15 min at $10000 \times g$ [11] were diluted 8-fold in buffer A containing PMSF (0.1 mM), benzamidine (1 mM) and leupeptin (4 μg/ml) and incubated for 25 min at 30°C in the presence (●) and absence (○) of inhibitor-2 (100 nM). The long preincubation with inhibitor-2 is essential to inhibit protein phosphatase-1 activity completely [11]. An aliquot of this solution (0.01 ml) was then added to 0.02 ml of ^{32}P-labelled glycogen synthase (1.33 μM) containing different concentrations of unlabelled phosphorylase a, to start the reaction. After 5 min at 30°C, the assays were terminated and analysed as in [18]. 100% activity corresponds to that measured in the absence of either inhibitor-2 or phosphorylase a. This experiment was repeated using 3 different extracts, with very similar results.
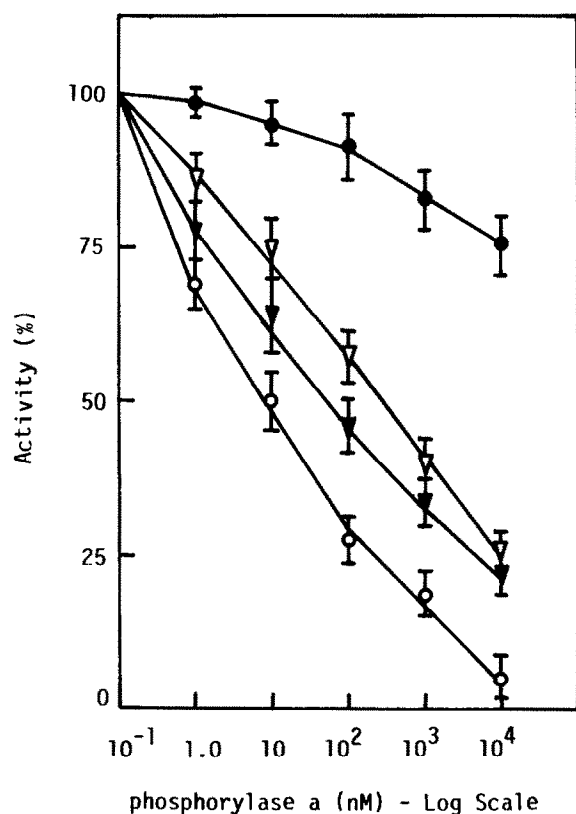

Fig.4. Desensitisation and resensitisation of hepatic microsomal glycogen synthase phosphatase activity to inhibition by phosphorylase a. Resuspended liver microsomes (1.0 ml) were incubated for 30 min at 0°C with 0.1 ml of 5 M NaCl and centrifuged for 30 min at $180000 \times g$. The supernatant, containing 97% of the protein phosphatase activity, was removed and the pellet resuspended in 10 ml of buffer A containing PMSF (0.1 mM), benzamidine (1 mM) and leupeptin (4 μg/ml). After recentrifugation for 30 min at $180000 \times g$, the supernatant was discarded and the pellet resuspended in 1.0 ml of buffer A + proteinase inhibitors ('salt-extracted microsomes'). The first supernatant, containing nearly all the protein phosphatase activity, was dialysed for 90 min at 0°C against buffer A + proteinase inhibitors ('dialysed microsomal supernatant') and then mixed with the salt-extracted microsomes. After 60 min at 0°C, the suspension was recentrifuged for 30 min at $180000 \times g$ and the pellet resuspended in 1.0 ml of buffer A + proteinase inhibitors to yield reconstituted microsomes. The graph shows the influence of unlabelled phosphorylase a on rate of dephosphorylation of ^{32}P-glycogen synthase (labelled in sites-3) using untreated microsomes (○), the dialysed microsomal supernatant (●), the dialysed microsomal supernatant mixed with salt-extracted microsomes (▽), and reconstituted microsomes (▼). The results are expressed as ± SE for 3 different preparations.
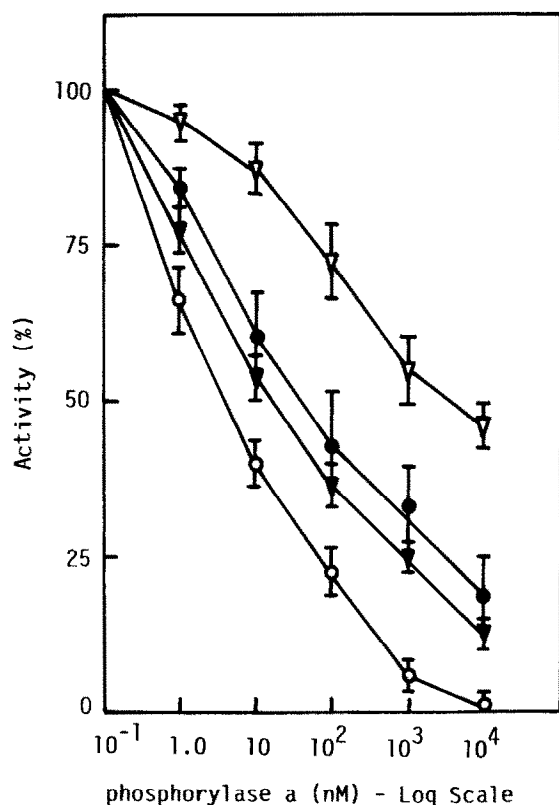
Fig.5. Effect of solubilisation on sensitivity of glycogen synthase (sites-3) phosphatase activity to phosphorylase a in the hepatic glycogen fraction. The resuspended glycogen fraction (1.0 ml) was incubated for 30 min at $0°C$ with 0.1 ml of 5 M NaCl and centrifuged for 30 min at $180000 \times g$. The supernatant was dialysed against buffer A containing PMSF (0.1 mM), benzamidine (1 mM) and leupeptin (4 μg/ml) and termed 'dialysed supernatant'. The glycogen pellet was resuspended in 1.0 ml of buffer A + proteinase inhibitors and termed 'salt-extracted glycogen'. A further aliquot of the untreated glycogen fraction was incubated for 3 h at $0°C$ with α-amylase (0.1 mg/ml), and after centrifugation for 30 min at $180000 \times g$, the 'amylase supernatant' was decanted. A third aliquot of the untreated glycogen fraction was digested with trypsin as described under section 2. The graph shows the effect of unlabelled phosphorylase a on rate of dephosphorylation of glycogen synthase (labelled in sites-3) using the dialysed supernatant (●), salt-extracted glycogen (○), amylase supernatant (▼) and trypsin-treated glycogen (▽). The results are expressed as ± SE for experiments performed on 3 different preparations of the hepatic glycogen fraction.

The hepatic glycogen fraction was also extracted with 0.5 M NaCl, but only 30–50% of the activity was solubilised by this treatment. Furthermore, the solubilised enzyme retained appreciable sensitivity to phosphorylase a, although it was not nearly as sensitive as the activity which remained bound to glycogen (fig.5). Sensitivity of the salt-extracted enzyme to phosphorylase a was variable from experiment to experiment (large standard error bars in fig.5). The glycogen-bound form of protein phosphatase-1 could be solubilised completely by digestion with α-amylase and this material retained considerable sensitivity to phosphorylase a (fig.5), although the I_{50} was still about 10-fold higher than in the freshly prepared glycogen pellets (fig.1).

4. DISCUSSION

Four lines of evidence demonstrate that phosphorylase a inhibits the dephosphorylation of glycogen synthase, catalysed by hepatic protein phosphatase-1, through an allosteric mechanism.

(i)   The I_{50} for phosphorylase a was in the nanomolar range, and over 1000-fold lower than its K_m as a substrate.

(ii)   Tryptic digestion increased the I_{50} for phosphorylase a 1000-fold without affecting the K_m for phosphorylase a as a substrate.

(iii)   Phosphorylase a did not inhibit the glycogen synthase phosphatase activity of protein phosphatase-1 in the muscle glycogen fraction (fig.1).

(iv)   Phosphorylase a did not inhibit the dephosphorylation of glycogen synthase catalysed by hepatic protein phosphatase-2A (fig.3), which is also a very active phosphorylase phosphatase [12,13,15,19].

The molecular mechanism by which phosphorylase a inhibits hepatic protein phosphatase-1 is unknown. Our working hypothesis is that the enzyme is composed of three components, a catalytic C-subunit, a G- or M-component responsible for anchoring the enzyme to either glycogen or microsomes, and a P-subunit which confers sensitivity to inhibition by phosphorylase a. These ideas are based on the results presented in fig.4, and on the structure of the glycogen-bound form of the

skeletal muscle enzyme which is composed of a 37 kDa C-subunit and a 103 kDa glycogen-binding G-subunit [16]. The association of hepatic protein phosphatase-1 with glycogen and its many similarities with the muscle enzyme [11] suggest that it may contain analogous G- and C-subunits. The properties of the hepatic microsomal enzyme are also very similar, although not identical ([11] and figs 1 and 2). This enzyme may therefore have the structure MC, where the M-subunit is responsible for anchoring the C-subunit to the microsomal membranes. Protein phosphatase-1 released from hepatic microsomes was insensitive to inhibition by phosphorylase a, but sensitivity could be partially recovered by mixing the solubilized enzyme with the microsomes (fig.4). This suggests that a P-component responsible for conferring sensitivity to phosphorylase a remained associated with the microsomal membranes, even after extraction with salt. However, the apparent molecular mass of the solubilised enzyme is >100 kDa [11] and it is capable of rebinding to the microsomes (see section 3). Thus extraction with NaCl would appear to release the M-subunit, as well as the C-subunit. The simplest structure for the hepatic microsomal enzyme would therefore be MCP. By analogy, the hepatic glycogen-bound enzyme would have the structure GCP, although reversible dissociation of a P-subunit has yet to be demonstrated (fig.5). The glycogen-bound form of protein phosphatase-1 from muscle may be insensitive to phosphorylase a because it lacks a P-subunit, while trypsin may abolish sensitivity of the hepatic enzyme to phosphorylase a either by destroying the P-subunit or the binding site for the P-subunit on the phosphatase. Tryptic digestion also degrades the G-subunit and converts the 37 kDa C-subunit to an active 33 kDa C'-fragment (discussed in [11,16]).

The inhibition of hepatic protein phosphatase-1 by phosphorylase a is complex, because the I_{50} varies considerably, from substrate to substrate (figs 1 and 2). Phosphorylase a inhibits the dephosphorylation of glycogen synthase more potently if it is labelled in sites-3 rather than in site-2, while dephosphorylation of glycogen synthase is inhibited more potently than dephosphorylation of phosphorylase kinase (fig.1). Dephosphorylation of phosphorylase a is presumably not inhibited at all because:

(i) Substrate inhibition was not detectable from 2–30 μM phosphorylase a.

(ii) Phosphorylase a was dephosphorylated very efficiently by protein phosphatase-1 in the liver microsomes, and no increase in phosphorylase phosphatase activity occurred when the enzyme was solubilised with salt, a treatment which abolished allosteric inhibition of the glycogen synthase phosphatase activity by phosphorylase a.

Differential sensitivity to inhibition by phosphorylase a may be of physiological importance. The very low I_{50} for inhibition of glycogen synthase phosphatase is consistent with the observation that activation of glycogen synthase is completely suppressed in the livers of normally fed animals, when the proportion of phosphorylase in the a-form exceeds 10% [2]. It may also explain the finding that incubation of rat hepatocytes with glucagon increases the phosphate content of glycogen synthase primarily in a cyanogen bromide fragment that is not phosphorylated by cyclic AMP-dependent protein kinase [20]. This mechanism would be similar to the way in which adrenaline is believed to increase the phosphorylation of glycogen synthase at sites-3 in skeletal muscle [21], with phosphorylase a playing an analogous role to inhibitor-1. A separate role for inhibitor-1 in the regulation of hepatic glycogen metabolism is not excluded, however.

Although the concentration of phosphorylase a required to inhibit dephosphorylation of phosphorylase kinase is high by comparison with glycogen synthase, the effect is specific for the hepatic glycogen-bound form of protein phosphatase-1 (figs 1 and 2) and the I_{50} of $\simeq 0.1$ μM is still 100-fold lower than the average intracellular concentration of phosphorylase in liver calculated to be $\simeq$ 15 μM from the data in [22]). Inhibition of phosphorylase kinase phosphatase by phosphorylase a could represent a mechanism for amplifying the effects of hormones that stimulate glycogenolysis.

Protein phosphatase-1 has a broad substrate specificity in vitro, and is likely to participate in the regulation of cellular processes other than glycogen metabolism [13,14]. More protein phosphatase-1 activity is associated with microsomes than with glycogen [11,15], where it is

presumably involved in the dephosphorylation of microsomal proteins, rather than the enzymes of glycogen metabolism. The retention by the microsomal enzyme of allosteric regulation by phosphorylase *a* is therefore intriguing because it raises the possibility that phosphorylase *a* may play a more general role in the regulation of liver metabolism.

ACKNOWLEDGEMENTS

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