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Distinct type-1 protein phosphatases are associated with hepatic glycogen and microsomes

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The type-1 protein phosphatase associated with hepatic microsomes has been distinguished from the glycogen-bound enzyme in five ways. (1) The phosphorylase phosphatase/synthase phosphatase activity ratio of the microsomal enzyme (measured using muscle phosphorylase *a* and glycogen synthase (labelled in sites-3) as substrates) was 50-fold higher than that of the glycogen-bound enzyme. (2) The microsomal enzyme had a greater sensitivity to inhibitors-1 and 2. (3) Release of the catalytic subunit from the microsomal type-1 phosphatase by tryptic digestion was accompanied by a 2-fold increase in synthase phosphatase activity, whereas release of the catalytic subunit from the glycogen-bound enzyme decreased synthase phsophatase activity by 60%. (4) 95% of the synthase phosphatase activity was released from the microsomes with 0.3 M NaCl, whereas little activity could be released from the glycogen fraction with salt. (5) The type-1 phosphatase separated from glycogen by anion-exchange chromatography could be rebound to glycogen, whereas the microsomal enzyme (separated from the microsomes by the same procedure, or by extraction with NaCl) could not. These findings indicate that the synthase phosphatase activity of the microsomal enzyme is not explained by contamination with glycogen-bound enzyme. The microsomal and glycogen-associated enzymes may contain a common catalytic subunit complexed to microsomal and glycogen-binding subunits, respectively. Thiophosphorylase *a* was a potent inhibitor of the dephosphorylation of ribosomal protein S6, HMG-CoA reductase and glycogen synthase, by the glycogen-associated type-1 protein phosphatase. By contrast, thiophosphorylase *a* did not inhibit the dephosphorylation of S6 or HMG-CoA reductase by the microsomal enzyme, although the dephosphorylation of glycogen synthase was inhibited. The I_{50} for inhibition of synthase phosphatase activity by thiophosphorylase *a* catalysed by either the glycogen-associated or microsomal type-1 phosphatases, or for inhibition of S6 phosphatase activity catalysed by the glycogen-associated enzyme, was decreased 20-fold to 5–10 nM in the presence of glycogen. The results suggest that the physiologically relevant inhibitor of the glycogen-associated type-1 phosphatase is the phosphorylase *a*-glycogen complex, and that inhibition of the microsomal type-1 phosphatase by phosphorylase *a* is unlikely to play a role in the hormonal control of cholesterol or protein synthesis. Protein phosphatase-1 appears to be the principal S6 phosphatase in mammalian liver acting on the serine residues phosphorylated by cyclic AMP-dependent protein kinase.

Abbreviations: HMG-CoA reductase, hydroxymethylglutaryl-CoA reductase (EC 1.1.1.88); SDS, sodium dodecyl sulfate.

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Introduction

Protein phosphatase 1, one of the four principal serine/threonine-specific protein phosphatase catalytic subunits in mammalian cells, has a broad substrate specificity in vitro, and is capable of dephosphorylating many proteins that regulate a variety of cellular processes. It is distinguished by its sensitivity of the thermostable proteins inhibitor-1 and inhibitor-2 and its specificity for the β-subunit of phosphorylase kinase, in contrast to the three type-2 protein phosphatases (2A, 2B and 2C) which are unaffected by inhibitors-1 and 2 and preferentially dephosphorylate the α-subunit of phosphorylase kinase [1,2].

The free catalytic subunit of protein phosphatase-1 may not exist in vivo, and evidence is accumulating that it is complexed to a variety of proteins that either have a regulatory function and/or target the phosphatase to particular intracellular locations. As a result, protein phosphatase-1 is enriched in a variety of organelles and subcellular fractions, whereas the type-2 phosphatases are largely cytosolic [3–5].

Protein phosphatase-1 is the major protein phosphatase associated with hepatic microsomes and glycogen [6], and the hepatic glycogen-bound enzyme has a property which distinguishes it from the type-1 phosphatases present in skeletal muscle and other mammalian tissues. Its ability to dephosphorylate (activate) glycogen synthase is inhibited by nanomolar concentrations of phosphorylase *a* [7], a regulatory device which allows signals such as glucagon, vasopressin, insulin and glucose, to control the rate of glycogen synthesis [8,9].

We have reported that the type-1 protein phosphatase associated with the microsomes of fed rats has similar (but not identical) properties to the glycogen-bound enzyme [6], including potent inhibition of its glycogen synthase phosphatase activity by phosphorylase *a* [7]. However, it has been suggested by Mvumbi and Stalmans [10] that the microsomes used in this study may have been significantly contaminated with glycogen, and these investigators reported that glycogen-free microsomes have negligible synthase phosphatase activity, as judged by their ability to reactivate liver glycogen synthase [10].

In this paper, we establish that the type-1 protein phosphatase which is bound to hepatic microsomes can be distinguished from the glycogen-associated enzyme in several ways. We also address the question of whether microsomal protein phosphorylation is regulated by phosphorylase *a* in vivo.

Materials and Methods

Protein preparations. Glycogen-protein particles [11], the catalytic subunits of protein phosphatases-1 and 2A [12], and inhibitor-1 and inhibitor-2 [13] were purified from rabbit skeletal muscle. α-Amylase was isolated from human saliva [14] and α-amylase inhibitor (type-III) was purchased from Sigma (product number A-3535).

^{32}P-labelled substrates (10^6 cpm/nmol). [^{32}P]Phosphorylase *a*, [^{32}P]glycogen synthase (labelled in sites-3 by glycogen synthase kinase-3) and [^{32}P]phosphorylase kinase (labelled by cyclic AMP-dependent protein kinase and containing 0.8 mol phosphate/$\alpha\beta\gamma\delta$ unit in each of the α- and β-subunits) were prepared and freed from [γ-^{32}P]ATP as described in Ref. 15. Unlabelled phosphorylase *a* and thiophosphorylase *a* were made in an identical manner except that [γ-^{32}P]ATP was replaced by ATP and ATPγS (adenosine 5'-[γ-thio]triphosphate), respectively.

Preparation of ^{32}P-labelled ribosomal protein S6. Ribosomes were isolated from rat liver and dissociated into their subunits as described in Ref. 16. The small (40 S) ribosomal subunits (5.0 A_{260} units/ml) were phosphorylated in ten separate 0.5-ml incubations containing 50 mM Mops (4-morpholinepropanesulphonic acid) (pH 7.5), 1.0 mM dithiothreitol, 4 mM $MgCl_2$, 0.01 mM [γ-^{32}P]ATP (10 μCi) and catalytic subunit of cyclic AMP-dependent protein kinase (5 U/ml see Ref. 12 for unit definition). After 2 h at 30 °C, the reactions were combined, dialysed against 50 mM Tris-HCl (pH 7.3), 5 mM $MgCl_2$ and 1 mM dithiothreitol and 2.5 ml portions were layered over 0.5 ml of 30% (w/v) sucrose in 50 mM Tris-HCl (pH 7.3) in 1 ml ultracentrifuge tubes. After centrifugation for 3 h at 370000 × *g* at 4 °C, the sedimented ribosomal subunits were resuspended in 0.1 ml of 50 mM Tris-HCl (pH 7.3) and stored at −70 °C. They were diluted to 0.6 μM

before assay. Under these conditions, ^{32}P incorporation into the 40 S ribosomal subunit is solely into S6 and results in monophosphorylated and diphosphorylated forms of the protein [17].

Preparation of hepatic-glycogen protein particles and hepatic microsomes. Glycogen protein particles were prepared from the livers of fed adult Wistar rats (250–300 g), as described in Ref. 6, except that an additional proteinase inhibitor tosylphenylchloromethylketone (0.5 mM) was included, and the liver homogenates were initially centrifuged for 15 min at $16000 \times g$ (rather than for 15 min at $10000 \times g$) to facilitate removal of lysosomes [18]. After discarding the upper layer of microsomes, the glycogen pellet from one rat was resuspended in 30 ml of 50 mM Tris-HCl (pH 7.0), 0.1 mM EGTA, 0.1% (v/v) 2-mercaptoethanol, 5% (v/v) glycerol, 0.5 mM phenylmethylsulphonyl fluoride, 0.5 mM tosylphenylchloromethylketone, 1 mM benzamidine, 4 μg/ml leupeptin (buffer A) containing 0.3 M NaCl and was recentrifuged for 90 min at $100000 \times g$. The supernatant was discarded and the glycogen pellet was resuspended in 10 ml of buffer A and used within 3 h. Extraction with 0.3 M NaCl was carried out to remove protein phosphatase-1 from microsomes contaminating the glycogen-protein particles (see Results).

Hepatic microsomes were prepared as described for glycogen-protein particles, except that rats were deprived of food for 24 h to deplete glycogen. Microsomes from one rat liver were resuspended in 10 ml of buffer A.

Purification and assay of hepatic glycogen. Resuspended glycogen-protein particles (5 ml) were added to 20 ml of 1% SDS at ambient temperature and centrifuged for 90 min at $100000 \times g$. The supernatant was discarded and the glycogen pellet was resuspended in 25 ml of 1% SDS and recentrifuged. The pellet was resuspended in 25 ml of water, recentrifuged and the pellet was redissolved in 5 ml of water. After heating at 100 °C for 10 min and treatment with mixed bed resin (Bio-Rad AG 501-X8), the solution (hereafter referred to as 'purified glycogen') was stored at −20°. The glycogen contents of samples were assayed by two methods [19,20], which agreed to within ±10%.

Separation of protein phosphatase-1 from hepatic glycogen and microsomes. All operations were carried out at 0–4 °C. Aliquots of the resuspended glycogen-protein particles (2 ml) were diluted to 20 ml in 20 mM triethanolamine hydrochloride (pH 7.5) (4 °C), 10% (v/v) glycerol, 0.1% (v/v) 2-mercaptoethanol, 1 mM benzamidine, 4 μg/ml leupeptin, 0.2% Triton X-100 (buffer B) and applied to a 5 ml column of DEAE-Sepharose (Pharmacia) equilibrated in buffer B. The column was washed with buffer until glycogen and protein could no longer be detected in the effluent, and protein phosphatase-1 was eluted with 5 ml of buffer B plus 0.5 M NaCl. Resuspended microsomes were made to 0.2% in Triton X-100 and then chromatographed on DEAE-Sepharose in an identical manner. Both preparations were dialysed against buffer A and stored for up to a week at −20 °C.

Binding of protein phosphatase-1 to purified glycogen. Purified glycogen was diluted to 8 mg/ml and protein phosphatase-1 to 2 mU/ml (phosphorylase phosphatase activity) in 20 mM Mes (4-morpholinethanesulphonic acid)/30 mM Tris-HCl buffers (pH 6–8 at 0 °C) containing 50 ml NaCl and 1.0 mg/ml bovine serum albumin. Diluted glycogen (0.1 ml) was added to diluted phosphatase (0.1 ml), and, after incubation for 10 min at 0 °C, 0.15 ml of suspension was centrifuged at ambient temperature for 20 min at $120000 \times g$ in a Beckman airfuge. The supernatants were removed, the pellets were resuspended in 0.15 ml of the same buffer and both fractions were assayed for phosphatase activity. Control incubations were performed from which glycogen was omitted.

Assay of protein phosphatase activities. The dephosphorylation of phosphorylase *a* (10 μM) and glycogen synthase (1 μM) were measured as described in Ref. 6, except that the resuspended glycogen and microsomal fractions were assayed at final dilutions of 50–150-fold. The dephosphorylation of phosphorylase kinase (1 μM) and ribosomal protein S6 (0.2 μM) were assayed in an identical manner. 1 unit of activity (U) was defined as that amount which catalysed the release of 1.0 μmol of phosphate in 1 min.

Preparation of phosphatase-free microsomes for studies of the reactivation of HMG-CoA reductase. These were prepared as described for normal hepatic microsomes, with the following modifica-

tions. Rats were killed (by cervical dislocation to induce full phosphorylation of HMG-CoA reductase [21]) in the middle of the dark phase of the light/dark cycle (in order to maximally induce HMG-CoA reductase). The microsomal pellet (prepared in the presence of 50 mM KF to prevent dephosphorylation) was resuspended in buffer A containing 500 mM KCl and 50 mM KF and after standing for 30 min at 0 °C, the microsomes were resedimented. They were finally resuspended in 3 ml of fluoride-free medium as described in Ref. 6.

Dephosphorylation of HMG-CoA reductase. This was measured by the rate of reactivation of reductase in phosphatase-free microsomes, following addition of glycogen and microsomal fractions isolated from the livers of anaesthetized rats [21] obtained in the middle of the light phase, where HMG-CoA reductase activity is minimal. The reductase activity introduced with the phosphatase-containing microsomes represented less than 10% of total reductase activity in the incubations, and did not increase during the incubations (since HMG-CoA reductase was completely dephosphorylated during preparation of microsomes in the absence of fluoride). The glycogen fraction was minimally contaminated with HMG-CoA reductase activity. For these experiments, the phosphatase-containing microsomal and glycogen fractions from one rat were resuspended in only 3 ml and 5 ml, respectively, and reactivation of HMG-CoA reductase initiated by addition of 1 volume of these suspensions to 2 volumes of phosphatase-free microsomes.

Other analytical procedures. In order to release the free catalytic subunits from the native enzymes, the glycogen and microsomal fractions were incubated for 10 min with trypsin and digestions were stopped with excess trypsin inhibitor as in Ref. 6. Protein was measured according to Bradford [22].

Results

Differences between the glycogen and microsomal forms of protein phosphatase-1

Microsomes prepared from 24-h starved rats contained only traces of glycogen (less than 0.1 mg/g liver), over 1000-fold less than the glycogen fraction from fed animals (Table I). Since the microsomal fraction contained 40% of the synthase phosphatase activity present in the glycogen fraction (towards muscle glycogen synthase phosphorylated in sites-3) this suggested that the activity associated with the microsomes could not be explained by contamination with the glycogen-bound enzyme. Five further lines of evidence established that the glycogen and microsomal enzymes are distinct type-1 protein phosphatases:

1. The phosphorylase phosphatase/synthase phosphatase activity ratio of the hepatic microsomal type-1 phosphatase was 5-fold higher than that of the hepatic glycogen-bound enzyme (Table I).
2. Tryptic digestion increased the phosphorylase phosphatase activity of both the glycogen-bound and microsomal enzymes 3–4-fold. However, while trypsinisation decreased the synthase phosphatase activity of the glycogen fraction by 60%, the microsomal activity was doubled (Fig. 1). As a result, the phosphorylase phosphatase/synthase phos-

TABLE I

ASSOCIATION OF PHOSPHORYLASE PHOSPHATASE AND GLYCOGEN SYNTHASE PHOSPHATASE ACTIVITIES WITH HEPATIC GLYCOGEN AND MICROSOMAL FRACTIONS AND SKELETAL MUSCLE GLYCOGEN FRACTION

Phosphorylase phosphatase (PhP) and glycogen synthase phosphatase (GSP) activities associated with the hepatic glycogen fractions from fed rats, the microsomal fraction from 24-h starved rats and the skeletal muscle glycogen fraction from fed rabbits. S.E. is shown for the number of preparations shown in parentheses.

Fraction	PhP activity (mU/g liver)	GSP activity (mU/g liver)	Activity ratio (PhP/GSP)	Glycogen content (mg/g liver)
Liver glycogen	5.3± 0.9(8)	1.20±0.10(8)	4.4	240±50(8)
Liver microsomes	7.3± 1.3(6)	0.30±0.10(20)	24	< 0.1(7)
Muscle glycogen	58 ±19(4)	0.28±0.9(4)	210	n.d.

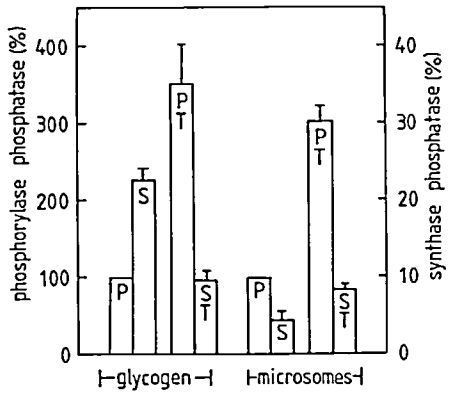

Fig. 1. Effect of trypsin on the phosphorylase phosphatase (P) and synthase phosphatase (S) activities of the glycogen and microsomal forms of protein phosphatase-1 from rat liver. The glycogen fraction from fed animals or the microsomes from 24-h starved animals was incubated in the presence (T) or absence of trypsin, as described in Ref. 6. Bars represent S.E. for five experiments.

phatase activity ratios of the glycogen and microsomal enzymes became very similar after tryptic digestion (Fig. 1). This would be expected if they shared a common catalytic subunit, because tryptic digestion is known to release the type-1 phosphatase catalytic subunit from higher molecular-mass complexes that are present in vivo [6]. The previous failure to observe activation of synthase phosphatase by trypsin in fed microsomes [6] may be explained by some contamination with the glycogen fraction.

3. It has been shown in Ref. 6 that inactivation of the native forms of hepatic type-1 protein phosphatases by inhibitors-1 and -2 is time-dependent, a 15–30 min preincubation with these proteins being required to observe maximal effects. By contrast, the action of these inhibitors on the free catalytic subunit was extremely rapid [6,11]. In the present work, the glycogen and microsomal fractions were preincubated for only 5 min with inhibitor-1 or inhibitor-2 to heighten differences between the native enzymes and catalytic subunits. Under these conditions, microsomal phosphatase-1 was inhibited to a greater extent than the glycogen-bound enzyme with either glycogen phosphorylase or glycogen synthase as substrate (Fig. 2). Although the differences were relatively small, they were obtained consistently. Following tryptic digestion both enzymes were inhibited in a similar manner to that of the catalytic subunit of protein phosphatase-1 from skeletal muscle (Fig. 2). This is as would be expected, since tryptic digestion releases the catalytic subunits from both enzymes [6].

4. When the microsomes were resuspended in buffer A and recentrifuged, about 50% of the synthase phosphatase activity was released into the supernatant, and the amount released in-

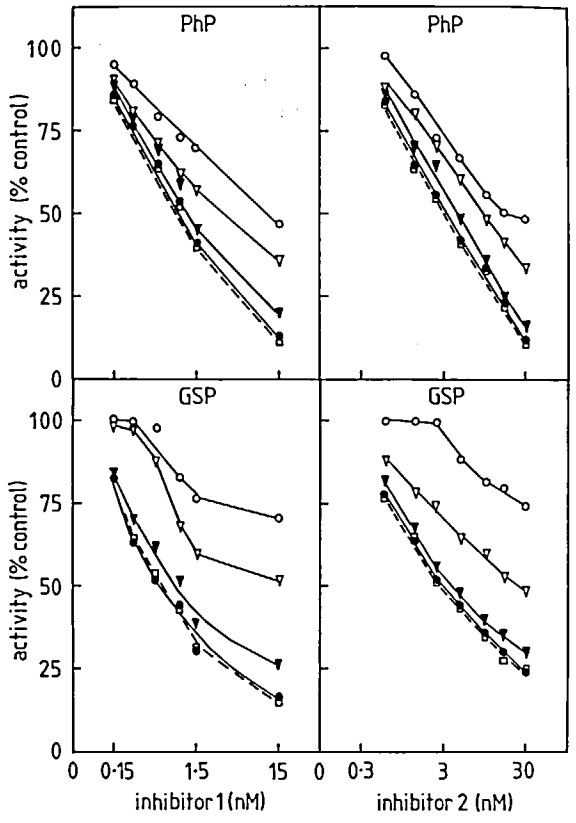

Fig. 2. Influence of inhibitor-1 and inhibitor-2 on phosphorylase phosphatase (PhP) and glycogen synthase phosphatase (GSP) activities associated with hepatic glycogen and microsomes. Fractions were assayed after a 5-min preincubation with inhibitor-1 or inhibitor-2. The circles show results obtained with the glycogen fraction, the triangles show those obtained with the microsomal fraction and the open squares represent data from experiments with the purified catalytic subunit of protein phosphatase-1 from skeletal muscle. The effect of inhibitors was studied before (open circles) and after (closed symbols) digestion with trypsin, as in Ref. 6. Assays were performed at an identical phosphatase dilution in all experiments. Similar results were obtained with three different preparations of both glycogen and microsomes.

creased to 95% if 0.3 M NaCl was included in the resuspension buffer (Fig. 3). Liberation of the phosphatase from the microsomes was associated with a modest (approx. 50%) increase in synthase phosphatase activity (Fig. 3). By contrast, little synthase phosphatase activity was released from the glycogen fraction, even after centrifugation in the presence of 0.3 M NaCl (Fig. 3). This demonstrates that synthase phosphatase activity in the microsomal fraction is not associated with contaminating glycogen particles.

5. Protein phosphatase-1 that had been freed from glycogen by chromatography on DEAE-Sepharose (see Materials and Methods) could bind to purified glycogen, whereas protein phosphatase-1 released from the microsomes in the same way (Fig. 4), or by extraction with 0.3 M NaCl (data not shown), could not.

Inhibition of the glycogen and microsomal type-1 protein phosphatases by phosphorylase a

A striking property of the hepatic glycogen-bound enzyme is the potent inhibition of its synthase phosphatase activity by phosphorylase *a* [7]. In the present work, thiophosphorylase *a* was found to be an equally effective inhibitor and was therefore employed for the studies described below because of its resistance to dephosphorylation.

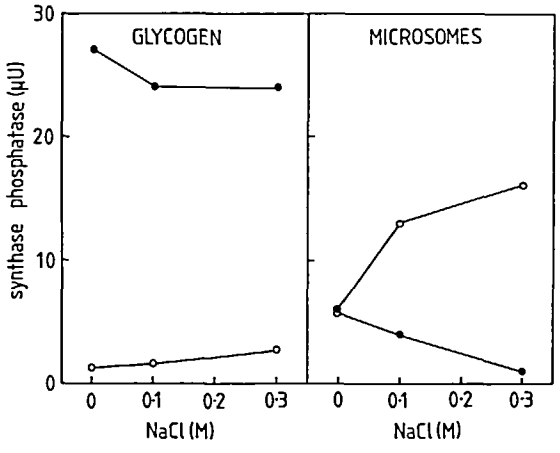

Fig. 3. Extraction of glycogen synthase phosphatase activity from the glycogen and microsomal fractions with sodium chloride. Resuspended glycogen or microsomes in buffer A (0.18 ml) were mixed with 0.02 ml of buffer A containing various concentrations of NaCl, to bring the salt concentrations to the values shown on the abscissa. After standing for 30 min at 0 °C, 0.15 ml of each suspension was centrifuged at ambient temperature in a Beckman airfuge for 15 min at 30 p.s.i. The supernatants were withdrawn and the pellets were resuspended in 0.15 ml of buffer A. Both the supernatants (o——o) and resuspended pellets (●——●) were then assayed for activity. Similar results were obtained with three preparations of the glycogen and microsomes.

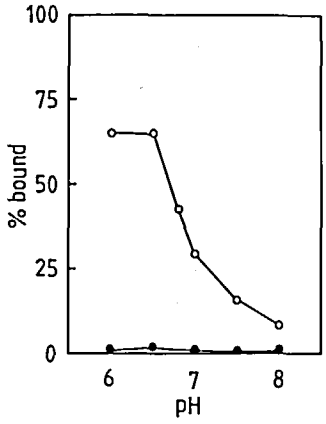

Fig. 4. Binding of the glycogen and microsomal derived type-1 protein phosphatases from rat liver to purified glycogen. The glycogen and microsomal fractions were chromatographed on DEAE-Sepharose to separate the phosphatase from glycogen and detergent-solubilized membranes, respectively, and their ability to bind to purified glycogen was examined as described under Materials and Methods. The graph shows the percent phosphorylase phosphatase activity recovered in the glycogen pellet, using protein phosphatase derived from the glycogen (o——o) and microsomal fractions (●——●). The recovery of activity was essentially quantitative at all pH values, and no phosphorylase phosphatase activity was sedimented in the absence of added glycogen. Very similar results were obtained with three preparations of the glycogen-derived enzyme and two preparations of the microsome-derived enzyme. Measurements of synthase phosphatase activity instead of phosphorylase phosphatase activity gave the same results.

The I_{50} for the glycogen-bound enzyme was approx. 5 nM, with approx. 90% inhibition occurring at 500 nM thiophosphorylase *a* (Fig. 5). By contrast, neither the free catalytic subunit released by tryptic digestion, nor the glycogen-bound enzyme from skeletal muscle, were inhibited significantly by 500 nM thiophosphorylase *a* (Fig. 5). Inhibition of the hepatic glycogen-bound enzyme by thiophosphorylase *a* was also dependent on the presence of glycogen. Digestion with α-amylase for 30 min at 30 °C increased the I_{50} for thiophosphorylase *a* to 200–300 nM, and following inclusion of an amylase inhibitor the readdition of purified glycogen decreased the I_{50} to approx. 20 nM. This was similar to the I_{50} of

control incubations (approx. 10 nM) from which α-amylase was omitted (Fig. 6). The effect of glycogen was half-maximal at about 1 mg/ml (data not shown).

The dephosphorylation of glycogen synthase by the hepatic microsomal type-1 protein phosphatase was inhibited by thiophosphorylase *a*, and sensitivity to this inhibitor was also greatly enhanced by glycogen. The I_{50} decreased from approx. 100 nM to 5–10 nM when glycogen (5 mg/ml) was included in the assays (Fig. 5), and the effect of glycogen was half-maximal at about 1 mg/ml (data not shown). Inhibition by thiophosphorylase *a* was abolished by tryptic digestion (Fig. 5).

The experiments described above demonstrated that the microsomal-form of protein phosphatase-1 has the potential to be regulated by thiophosphorylase *a*, and it was therefore of interest to examine whether the dephosphorylation of phosphoproteins present in microsomes, such as ribosomal protein S6 (the major phosphoprotein of the 40 S ribosomal subunit) and HMG-CoA reductase (which is inactivated by phosphorylation in vivo) would also be inhibited.

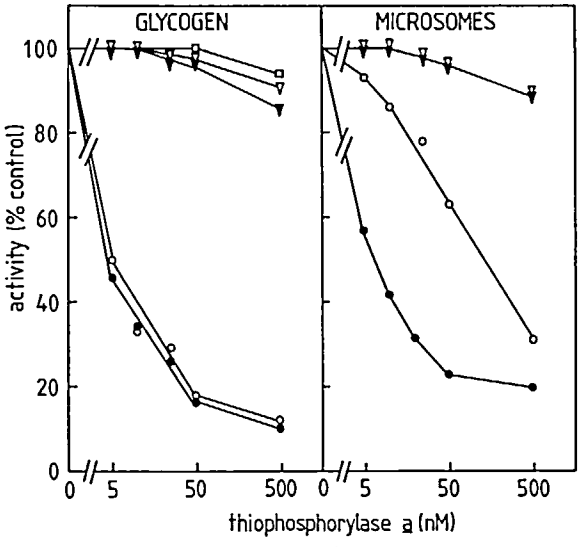

Fig. 5. Influence of thiophosphorylase *a* on the dephosphorylation of glycogen synthase. Experiments were carried out using the glycogen fraction from fed rats and the microsomal fraction from 24-h starved rats before (circles) and after (triangles) digestion with trypsin. Assays were performed in the presence (closed symbols) and absence (open symbols) of added purified glycogen (5 mg/ml). The effect of thiophosphorylase *a* on the synthase phosphatase activity of the rabbit skeletal muscle glycogen fraction (□———□), prepared as desribed in Ref. 11, is also shown. Similar results were obtained with three different phosphatase preparations.

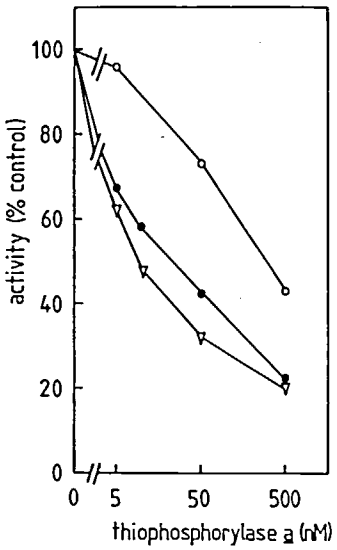

Fig. 6. Effect of glycogen removal and readdition on sensitivity to thiophosphorylase *a* of the hepatic glycogen-bound type-1 protein phosphatase. The resuspended glycogen fraction from rat liver (0.18 ml) was incubated with 0.02 ml of α-amylase (0.7 mg/ml) or water and after 30 min at 30 °C, 0.02 ml of α-amylase inhibitor was added, and the solutions were assayed for glycogen synthase phosphatase activity. Symbols: digested with α-amylase and assayed in the absence (○———○) or presence (●———●) of 5 mg/ml glycogen; sample incubated without amylase and assayed without glycogen (▽———▽). Similar results were obtained with three different phosphatase preparations.

Protein phosphatase-1 was found to be the major divalent cation-independent activity in liver that dephosphorylated S6. When rat liver homogenates (prepared as described in Ref. 6) were diluted 400-fold preincubated for 15 min in the presence or absence of inhibitor-2 (0.2 μM) and assayed. S6 phosphatase activity was inhibited 70% by inhibitor-2. Phosphorylase phosphatase activity in the same homogenates was inhibited by only 50%, as reported in Ref. 23. S6 was an excellent substrate for all forms of protein phosphatase-1, 10% dephosphorylation of this substrate occurring approx. twice as rapidly as the dephosphorylation of phosphorylase *a* in the standard assay (see Materials and Methods). By contrast, the time required for 10% dephosphory-

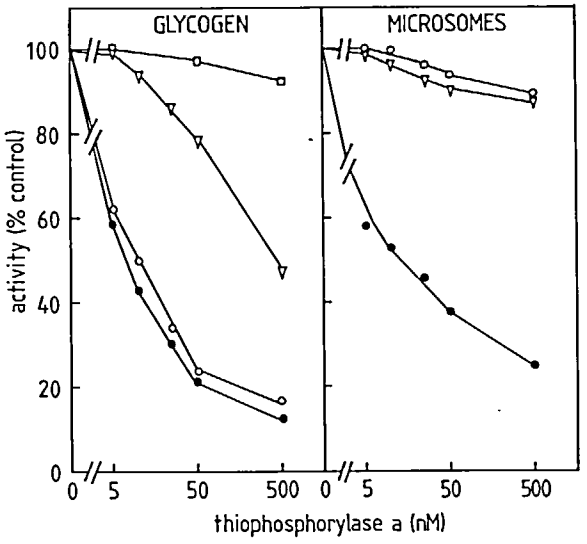

Fig. 7. Influence of thiophosphorylase *a* on the dephosphorylation of ribosomal protein S6 and other proteins by the glycogen and microsomal fractions from rat liver. The experiments were carried out as in Fig. 6 using ribosomal protein S6 (○———○), glycogen synthase (●———●), phosphorylase kinase (▽———▽) and phosphorylase *a* (□———□) as substrates. Assays with the microsomal fraction were carried out in the presence of 5 mg/ml glycogen. Similar results were obtained with three different preparations of the glycogen and microsomal fractions.

lation of S6 by the catalytic subunit of protein phosphatase 2A (the other major divalent cation-independent phosphatase) was 4-fold greater than for phosphorylase *a* (data not shown). The dephosphorylation of S6 in either the glycogen or microsomal fractions, could be inhibited 75–80% by inhibitor-2 (0.2 μM) provided that these fractions were preincubated with inhibitor for 15 min prior to assay. This degree of inhibition was similar to that observed under the same conditions using phosphorylase *a* or glycogen synthase as substrates [6].

The dephosphorylation of S6 by the hepatic glycogen-associated type-1 phosphatase was inhibited by thiophosphorylase *a* in an identical manner to that of the dephosphorylation of glycogen synthase (Fig. 7) and the sensitivity of S6 dephosphorylation to thiophosphorylase *a* was also dependent on the presence of glycogen (data not shown). By contrast, the dephosphorylation of S6 by the microsomal type-1 phosphatase was not inhibited significantly by thiophosphorylase *a*, even in the presence of glycogen (Fig. 7). These results are similar to those obtained using phosphorylase kinase as substrate ([7], Fig. 7). The dephosphorylation of phosphorylase kinase by the glycogen-associated type-1 phosphatase is inhibited by thiophosphorylase *a* (albeit with a much higher I_{50} value), whereas dephosphorylation by the microsomal enzyme is not. Thiophosphorylase *a* (500 nM) does not inhibit the dephosphorylation of phosphorylase *a* (1 μM) significantly ([7], Fig. 7), as discussed previously [7].

When HMG-CoA reductase was used as substrate, the reactivation (dephosphorylation) of this enzyme by either the glycogen or microsomal phosphatase was inhibited 70–75% after preincubation for 30 min with inhibitor-2 (0.2 μM), similar to the extent of inhibition of the phosphorylase phosphatase or synthase phosphatase

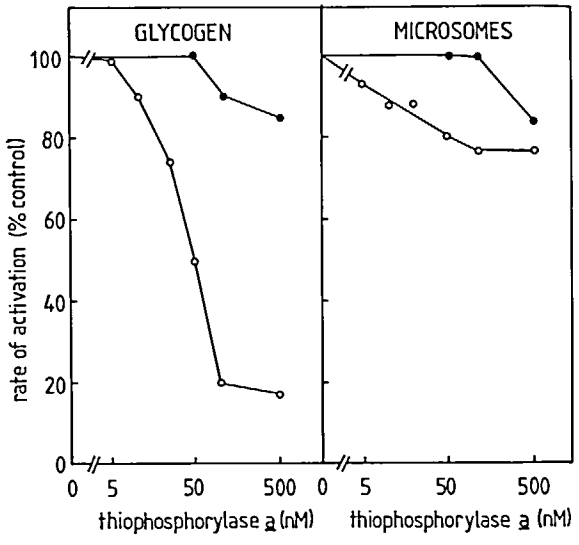

Fig. 8. Influence of thiophosphorylase *a* on the rate of reactivation of HMG-CoA reductase by the glycogen and microsomal fractions. Resuspended phosphatase-free microsomes prepared from rats killed in the middle of a dark cycle were incubated as in Fig. 6, with either the glycogen or the microsomal fraction prepared from rats killed mid-light cycle (see Materials and Methods for details). After 5 min at 30 °C, samples of the incubation mixture were taken for assay of HMG-CoA reductase activity. The extent of reductase activation was linear with time and represented 20–30% of total enzyme activity (measured in a parallel incubation after full dephosphorylation by purified protein phosphatase-1 catalytic subunit added exogenously). Symbols: ○———○, relative rate of reactivation before tryptic digestion; ●———●, relative rate of reactivation after tryptic digestion.

activity observed under the same conditions [6] The reactivation of HMG-CoA reductase by the glycogen-associated enzyme was inhibited by thiophosphorylase *a* before, but not after, digestion with trypsin (Fig. 8). By contrast, reactivation of HMG-CoA reductase by the microsomal enzyme was only inhibited slightly by thiophosphorylase *a* (Fig. 8), even in the presence of glycogen (not shown). The 20% inhibition is most likely explained by contamination with the glycogen fraction, since this experiment had to be performed with microsomes from fed animals, due to the precipitous drop in HMG-CoA reductase activity that occurs during starvation.

Discussion

Our current knowledge of type-1 protein phosphatases is largely derived from analysis of the rabbit skeletal muscle system. Three forms of protein phosphatase-1 have been identified in this tissue that are present in cytosol, glycogen and myofibrils. The cytosolic form is an inactive species, termed protein phosphatase-1_I, in which the catalytic (C) subunit is complexed to inhibitor-2 [24–26]. The glycogen-bound enzyme, termed protein phosphatase-1_G consists of the C-subunit (indistinguishable by peptide mapping from the C-subunit of protein phosphatas-1_I [11,26]) complexed to a glycogen-binding (G) subunit responsible for its association with this fraction [11,27]. The myofibrillar form is tightly bound to myosin and therefore termed protein phosphatase-1_M [28]. It has not yet been purified to homogeneity, but is likely to consist of the C-subunit complexed to an M-subunit responsible both for interaction with myosin [28] and for the enhanced myosin phosphatase activity of protein phosphatase-1_M [29].

By analogy with the situation in skeletal muscle, it is likely that the hepatic glycogen-bound type-1 protein phosphatase contains G- and C-subunits. However, it must clearly differ in some way from skeletal muscle protein phosphatase-1_G, since the synthase phosphatase activity of the latter is not inhibited by thiophosphorylase *a* (Fig. 5) and has a 50-fold higher phosphorylase phosphatase/synthase phosphatase activity ratio (Table I). The distinctive properties of the hepatic glycogen-bound enzyme could be conferred by a liver-specific G-subunit or by an additional subunit(s).

Several lines of evidence have established that the hepatic microsomal type-1 protein phosphatase(s) is distinct from the glycogen-bound enzyme, and our working hypothesis is that it is a complex between the C-subunit and another protein(s) responsible for association with microsomes. The different phosphorylase phosphatase/synthase phosphatase activity ratios of the hepatic glycogen and microsomal type-1 phosphatases (Table I) are explained by enhanced synthase phosphatase activity of the former and suppressed synthase phosphatase activity of the latter (Fig. 1). These results are strikingly similar to those obtained with protein phosphatase-1_M and -1_G from skeletal muscle [29], where the 3–4-fold higher myosin phosphatase/phosphorylase phosphatase activity ratio of protein phosphatase-1_M is explained by the enhanced myosin phosphatase activity of protein phosphatase-1_M and suppressed myosin phosphatase activity of protein phosphatase-1_G [29]. The observations strongly suggest that interaction of the type-1 protein phosphatase C subunit(s) with 'targetting' subunits not only directs the enzyme to particular intracellular locations, but also modifies the substrate specificity, so that it displays more (or less) activity towards certain substrates.

It has been known for several years that protein phosphatase-1 is the major phosphatase associated with rabbit reticulocyte ribosomes [30], and a further finding of the present work is that this enzyme is the major activity in rat liver that dephosphorylates the serine residues on ribosomal protein S6 phosphorylated by cyclic AMP-dependent protein kinase. This is consistent with the recent report that protein phosphatase-1 is the major activity in *Xenopus* oocytes that dephosphorylates other serine residues on S6 labelled by a mitogen-stimulated S6 kinase [31].

The finding that the I_{50} for phosphorylase *a* is greatly decreased in the presence of glycogen (Fig. 5), is in agreement with the recent work of Mvumbi and Stalmans [10], who reported that the activation of liver glycogen synthase was not inhibited by phosphorylase *a* in gel-filtered extracts prepared from rat livers depleted of glycogen by starvation and injection of glucagon. Inhibition

was restored if the livers were homogenised in a glycogen-containing buffer. The glycogen-bound form of protein phosphatase-1, glycogen synthase and phosphorylase *a* each interact with glycogen, so that the effect could be mediated by interaction of the polysaccharide with any or all three of these proteins. However, as the effect of glycogen is still observed with the microsomal type-1 phosphatase (which does not interact with glycogen, Fig. 4) or with the glycogen-bound phosphatase using ribosomal protein S6 as substrate (which is not known to interact with glycogen), it would appear that the effect of glycogen is mediated through interaction with phosphorylase *a*. The phosphorylase *a*-glycogen complex may, therefore, be the physiologically relevant inhibitor of hepatic protein phosphatase-1.

Although the hepatic microsomal type-1 phosphatase has the potential to be inhibited by phosphorylase *a* (Fig. 5), the dephosphorylation of two microsomal proteins, ribosomal protein S6 and HMG-CoA reductase was not inhibited, even in the presence of glycogen (Figs. 7 and 8). These observations, together with the finding that a low I_{50} for phosphorylase *a* requires the presence of glycogen, suggest that inhibition of protein phosphatase-1 by phosphorylase *a* does not underlie the increased phosphorylation of S6 [32] or HMG-CoA reductase (reviewed in Ref. 33) that occurs in rat liver in response to glucagon. Increased S6 phosphorylation can be explained by a direct phosphorylation of the protein catalysed by cyclic AMP-dependent protein kinase [34]. However, the mechanism by which glucagon increases the phosphorylation of HMG-CoA reductase is unclear, since HMG-CoA reductase kinase is not regulated by cyclic AMP, nor is it activated by cyclic AMP-dependent protein kinase (reviewed in Ref. 33). The present work suggests that the role of phosphorylase *a* inhibition of the hepatic type-1 protein phosphatases may be confined to the control of glycogen synthesis in the fed state.

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