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Comparison of the Substrate Specificities of Protein Phosphatases Involved in the Regulation of Glycogen Metabolism in Rabbit Skeletal Muscle

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Muscle extracts were subjected to fractionation with ethanol, chromatography on DEAE-cellulose, precipitation with $(NH_4)_2SO_4$ and gel filtration on Sephadex G-200. These fractions were assayed for protein phosphatase activities by using the following seven phosphoprotein substrates: phosphorylase *a*, glycogen synthase b_1, glycogen synthase b_2, phosphorylase kinase (phosphorylated in either the α-subunit or the β-subunit), histone H1 and histone H2B. Three protein phosphatases with distinctive specificities were resolved by the final gel-filtration step and were termed I, II and III. Protein phosphatase-I, apparent mol.wt. 300000, was an active histone phosphatase, but it accounted for only 10–15% of the glycogen synthase phosphatase-1 and glycogen synthase phosphatase-2 activities and 2–3% of the phosphorylase kinase phosphatase and phosphorylase phosphatase activity recovered from the Sephadex G-200 column. Protein phosphatase-II, apparent mol.wt. 170000, possessed histone phosphatase activity similar to that of protein phosphatase-I. It possessed more than 95% of the activity towards the α-subunit of phosphorylase kinase that was recovered from Sephadex G-200. It accounted for 10–15% of the glycogen synthase phosphatase-1 and glycogen synthase phosphatase-2 activity, but less than 5% of the activity against the β-subunit of phosphorylase kinase and 1–2% of the phosphorylase phosphatase activity recovered from Sephadex G-200. Protein phosphatase-III was the most active histone phosphatase. It possessed 95% of the phosphorylase phosphatase and β-phosphorylase kinase phosphatase activities, and 75% of the glycogen synthase phosphatase-1 and glycogen synthase phosphatase-2 activities recovered from Sephadex G-200. It accounted for less than 5% of the α-phosphorylase kinase phosphatase activity. Protein phosphatase-III was sometimes eluted from Sephadex G-200 as a species of apparent mol.wt. 75000 (termed IIIA), sometimes as a species of mol.wt. 46000 (termed IIIB) and sometimes as a mixture of both components. The substrate specificities of protein phosphatases-IIIA and -IIIB were identical. These findings, taken with the observation that phosphorylase phosphatase, β-phosphorylase kinase phosphatase, glycogen synthase phosphatase-1 and glycogen synthase phosphatase-2 activities co-purified up to the Sephadex G-200 step, suggest that a single protein phosphatase (protein phosphatase-III) catalyses each of the dephosphorylation reactions that inhibit glycogenolysis or stimulate glycogen synthesis. This contention is further supported by results presented in the following paper [Cohen, P., Nimmo, G. A. & Antoniw, J. F. (1977) *Biochem. J.* **162**, 435–444] which describes a heat-stable protein that is a specific inhibitor of protein phosphatase-III.

Ever since the discovery that cyclic AMP-dependent protein kinase (EC2.7.1.37) activates skeletal-muscle phosphorylase kinase (EC 2.7.1.37) and inactivates skeletal-muscle glycogen synthase (EC 2.4.1.11) (Soderling *et al.*, 1970), there has been considerable interest in whether the two protein phosphatase activities that reverse these reactions are also catalysed by a single enzyme. However, a major experimental problem in the investigation of this question has been the preparation of phosphorylated phosphorylase kinase and glycogen synthase substrates that are suitable for the assay of

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phosphorylase kinase phosphatase (EC 3.1.3.–) and glycogen synthase phosphatase (EC 3.1.3.–). Thus recent work has shown that phosphorylase kinase and glycogen synthase are both subject to phosphorylation by two different protein kinases, and that these phosphorylations may involve different sites on the enzymes (Wang *et al.*, 1976; Nimmo *et al.*, 1976*b*).

Phosphorylase kinase can be activated *in vitro* by two distinct phosphorylation mechanisms (Walsh *et al.*, 1971): (a) phosphorylation catalysed by cyclic AMP-dependent protein kinase; (b) phosphorylation catalysed by phosphorylase kinase itself (termed autophosphorylation).

However, phosphorylase kinase activated by cyclic AMP-dependent protein kinase alone is still phosphorylated at two specific serine residues, one on the α-subunit and one on the β-subunit of the enzyme (Cohen *et al.*, 1975*a*). It has been established that the activity of phosphorylase kinase is determined by the extent of phosphorylation of a unique serine resiude on the β-subunit, whereas the phosphorylation of the α-subunit appears to control the rate at which the β-subunit can be dephosphorylated (Cohen & Antoniw, 1973).

Similarly, glycogen synthase can be inactivated through two distinct phosphorylation mechanisms (Nimmo & Cohen, 1974; Nimmo *et al.*, 1976*b*): (a) phosphorylation catalysed by cyclic AMP-dependent protein kinase; (b) phosphorylation catalysed by glycogen synthase kinase-2, traces of which may contaminate purified glycogen synthase (Nimmo & Cohen, 1974).

Cyclic AMP-dependent protein kinase and glycogen synthase kinase-2 appear to phosphorylate different sites on glycogen synthase and give rise to forms of the enzyme (termed b_1 and b_2 respectively) that are kinetically distinct. Glycogen synthases b_1 and b_2 can therefore be used to test for the possible existence of two phosphatase activities, which have been termed glycogen synthase phosphatase-1 and glycogen synthase phosphatase-2 respectively (Nimmo *et al.*, 1976*a*).

We have found (Antoniw & Cohen, 1975, 1976) that phosphatase activity in muscle extracts towards phosphorylase kinase could be fractionated into two components specific for the α- and β-subunits of the enzyme. In the present paper, we use the same tissue extracts and ^{32}P-labelled phosphoprotein substrates in defined states of phosphorylation to examine the specificities of each of the protein phosphatases involved in the regulation of glycogen metabolism in rabbit skeletal muscle. Preliminary accounts of part of this work have been presented at the 4th International Interconvertible Enzyme Symposium at Arad, Israel, in April 1975 (Cohen *et al.*, 1976*b*), at a CIBA Foundation Symposium at London in July 1975 (Cohen *et al.*, 1976*a*) and at a British Biochemical Society Meeting at Edinburgh in September 1975 (Cohen *et al.*, 1975*b*).

Experimental

Materials

Histone H1 and histone H2B preparations [see Bradbury (1975) for nomenclature] were generous gifts from Professor T. A. Langan, University of Denver, Denver, CO, U.S.A., and Dr. E. W. Johns, Chester Beatty Research Institute, London S.W.3, U.K., respectively. The sources of other materials have been given previously (Antoniw & Cohen, 1976).

Buffer solutions

The following two solutions were used repeatedly in this work. Solution A contained 0.05*I* Tris/HCl (pH7.0, 25°C), 1.0mM-EDTA and 50mM-mercaptoethanol; solution B contained 50mM-glycerol 2-phosphate (sodium salt), 2.0mM-EDTA (sodium salt), 50mM-mercaptoethanol, and was adjusted to pH7.0 with HCl.

Enzyme preparations

All enzymes were prepared from rabbit skeletal muscle.

Phosphorylase kinase was prepared as described previously and was more than 95% pure as judged by polyacrylamide-gel electrophoresis (Cohen, 1973). The enzyme was stored in solution B at 15mg/ml at 0–4°C. The A_{280} of a 1% solution of phosphorylase kinase was taken as 12.4 and the minimal binding weight ($\alpha\beta\gamma$) as 318000g. The specific activity was 9 units (μmol/min)/mg (Cohen, 1973).

Phosphorylase *b* was prepared by the method of Fischer & Krebs (1958) and recrystallized three times. The crystals were collected by centrifugation, at 10000*g* for 10min freeze-dried immediately and stored at −15°C. AMP was removed by dissolving the freeze-dried crystals in solution B and passing this solution through Norit A/cellulose (Cohen, 1973). The absorbance ratio 260/280 nm of the enzyme freed from AMP was 0.53–0.54. The A_{280} of a 1% solution of phosphorylase was taken as 13.1, the subunit mol.wt. as 100000 and the specific activity was 80 units/mg (Cohen *et al.*, 1971). The enzyme was homogeneous as judged by polyacrylamide-gel electrophoresis.

Glycogen synthase was prepared and assayed as described by Nimmo *et al.* (1976*a*). It was at least 90% homogeneous as judged by polyacrylamide-gel electrophoresis. It was devoid of phosphorylase activity, but its contamination with phosphorylase kinase was about 0.5% by weight, as judged by enzymic analysis. The enzyme was stored at 5mg/ml in solution B containing 10% glycerol at 0°C. The

A_{280} of a 1% solution of glycogen synthase was taken as 13.4, the subunit mol.wt. as 88000 and the specific activity was 15–20 units/mg (Nimmo *et al.*, 1976*a*).

Glycogen synthase kinase-2 was purified from the pH6.1 supernatant of a glycogen synthase preparation up to and including the DEAE-cellulose step (Nimmo *et al.*, 1976*b*). The preparation was free of cyclic AMP-dependent protein kinase activity, as judged by assays with histone H1 as substrate. It was also devoid of casein kinase and histone kinase activities.

The peak-I isoenzyme of cyclic AMP-dependent kinase was partially purified (Cohen, 1973), separated from phosphorylase and glycogen synthase kinase-2 (Nimmo *et al.*, 1976*b*) and stored at −15°C in solution B.

The specific inhibitor protein of cyclic AMP-dependent protein kinase was partially purified from the pH6.1 supernatant of a glycogen synthase preparation by 90°C heat-treatment, precipitation by trichloroacetic acid, chromatography on DEAE-cellulose at pH5.0 and gel filtration on Sephadex G-75 (superfine) (Ashby *et al.*, 1972). The last step resolved the inhibitor of cyclic AMP-dependent protein kinase from a specific protein phosphatase inhibitor (Cohen *et al.*, 1977).

α-Phosphorylase kinase phosphatase and β-phosphorylase kinase phosphatase were partially purified as described previously (Antoniw & Cohen, 1976).

Protein phosphorylation

All determinations were carried out in microcentrifuge tubes. Protein samples (0.05ml) were added to 1.0ml of 5% (w/v) trichloroacetic acid (25% for histones H1 and H2B), and 0.1ml of bovine serum albumin was added as carrier. The concentration of the carrier protein was 5mg/ml (15mg/ml for histones H1 and H2B). The protein precipitates were collected, redissolved, reprecipitated and washed as described by Walsh *et al.* (1971). Finally the protein was redissolved in 0.2ml of 90% (w/v) formic acid. Then 1.0ml of the dioxan-based scintillant of Bray (1960) was added and the solution was counted for radioactivity in a Beckman LS-300 liquid-scintillation spectrometer.

Preparation of ^{32}P-labelled phosphoprotein substrates

[γ-^{32}P]ATP (5×10^7c.p.m./μmol) was used in all experiments.

Phosphorylase kinase. Phosphorylase kinase has a much higher K_m for ATP than has cyclic AMP-dependent protein kinase and its activity is dependent on Ca^{2+}. Therefore, by using low ATP concentrations and by including EGTA in the incubations, preparations of ^{32}P-labelled phosphorylase kinase can be obtained in which the contribution of autophosphorylation (see the introduction) is less than 5% of the total phosphorylation (Walsh *et al.*, 1971; Cohen, 1973).

^{32}P-labelled substrates, phosphorylated by the action of cyclic AMP-dependent protein kinase and labelled specifically in either the α-subunit or the β-subunit, were prepared as described previously (Antoniw & Cohen, 1976). The preparations were stored at 10mg/ml at 4°C in solution A containing 50mM-NaF. The NaF prevented a slow release of ^{32}P radioactivity as P_i catalysed by trace endogenous phosphorylase kinase phosphatase activities.

Phosphorylase a. The incubation comprised phosphorylase *b* (5mg/ml), phosphorylase kinase (0.03 mg/ml), 50mM-Tris, 50mM-glycerol 2-phosphate, 1.0 mM-ATP and 10mM-magnesium acetate, pH8.2. After incubation for 60min at 25°C, the reaction was terminated by the addition of an equal volume of 90%-satd. $(NH_4)_2SO_4$, pH7.0. The precipitate was collected by low-speed centrifugation, washed with solution A containing 45%-satd. $(NH_4)_2SO_4$ and redissolved in 1.0ml of solution A. The substrate was dialysed against 500vol. of solution A for 16h at 4°C with one change of dialysis buffer. The crystals of phosphorylase *a* which form during the dialysis were collected by centrifugation at 10000*g* for 10min and the supernatant containing traces of remaining phosphorylase *b* and phosphorylase kinase was discarded. The crystals were redissolved in solution A containing 0.25M-NaCl at 25°C. The substrate contained 1.03 ± 0.04 mol of phosphate per subunit. The phosphate is located on a specific serine residue 14 amino acids from the *N*-terminus of the protein (Titani *et al.*, 1975). The phosphorylase *a*, stored at 15mg/ml at 4°C in solution A containing 0.25M-NaCl, lost less than 1% ^{32}P radioactivity as inorganic phosphate per month under these conditions.

Phosphorylation of glycogen synthase. Purified glycogen synthase is contaminated with trace glycogen synthase kinase-2 and trace cyclic AMP-dependent protein kinase activities. Glycogen synthase kinase-2 has a much higher K_m for ATP than has cyclic AMP-dependent protein kinase, and the activity is unaffected by cyclic AMP or the heat-stable protein that inhibits cyclic AMP-dependent protein kinase (Nimmo & Cohen, 1974). Therefore, by using a low ATP concentration in the incubation, ^{32}P-labelled glycogen synthase preparations can be obtained in which the contribution of glycogen synthase kinase-2 is only 5–10% of the total phosphorylation. Similarly, by omitting cyclic AMP and including the inhibitor protein, ^{32}P-labelled glycogen synthase preparations can be obtained in which the contribution of cyclic AMP-dependent protein kinase to the total phosphorylation is negligible.

Glycogen synthase b_1. The incubation (3.0ml) comprised glycogen synthase (0.4mg/ml), glycerol 2-phosphate (10mM), pH7.0, EGTA (0.2mM),

EDTA (0.4mM), cyclic AMP (0.01mM), ATP (0.2mM), magnesium acetate (2.0mM) and cyclic AMP-dependent protein kinase. The concentration of the last component was such that the half-time for phosphorylation was less than 2min. The phosphorylation reached a plateau after about 20min, when 1.1mol of phosphate had been incorporated per subunit. The activity ratio of the enzyme, defined as the activity in the absence of glucose 6-phosphate relative to the activity in the presence of glucose 6-phosphate, declined from 0.80 to 0.18 during this incubation (Nimmo *et al.*, 1976*b*). Control experiments suggested that phosphorylation catalysed by glycogen synthase kinase-2, which is an endogenous contaminant in glycogen synthase preparations, was limited to 0.05–0.10mol of phosphate per subunit under these conditions. At the end of the reaction, the solution was made 10mM in EDTA (sodium salt, pH7) and passed through a column (20cm × 1cm) of Sephadex G-25 equilibrated in solution A containing 50mM-NaF to remove excess of ATP. The remaining traces of ATP were then removed by overnight dialysis against solution A containing 50mM-NaF. The ^{32}P-labelled glycogen synthase b_1 lost no ^{32}P radioactivity as P_i when stored for 2 weeks at 4°C.

Glycogen synthase b_2. The incubation comprised glycogen synthase (1.0mg/ml), glycerol 2-phosphate (25mM), EDTA (1.0mM), EGTA (0.2mM), inhibitor protein, ATP (1.0mM), magnesium acetate (10mM) and glycogen synthase kinase-2, pH7.0. The concentration of the inhibitor protein (0.15mg/ml) was sufficient to inactivate all traces of endogenous cyclic AMP-dependent protein kinase in the glycogen synthase preparations. The concentration of glycogen synthase kinase-2 was such that the half-time for phosphorylation was about 5min and the reaction was complete within 45min. The reaction reached a plateau near 0.9mol of phosphate incorporated per subunit, and the activity ratio in the absence and presence of glucose 6-phosphate declined from 0.80 to 0.08 (Nimmo *et al.*, 1976*b*). The reaction was then terminated and treated as described for the preparation of glycogen synthase b_1 above. No ^{32}P radioactivity was released from the substrate during storage over a 2-week period.

^{32}P-labelled histone H1 and histone H2B. The incubation (5ml) was identical with that for the preparation of glycogen synthase b_1, except that histones (0.2mg/ml) replaced the glycogen synthase. On the basis of a mol.wt. of 21000 for histone H1 and 15000 for histone H2B (DeLange & Smith, 1971) and protein concentrations determined from amino acid analysis, histone H1 phosphorylation reached a plateau at 0.7mol/mol and histone H2B at 1.85mol/mol. This is consistent with the finding that cyclic AMP-dependent protein kinase phosphorylates a single serine residue (serine-37) on histone H1 (Langan, 1971) and two serine residues (serine-32 and serine-36) on histone H2B (Hashimoto *et al.*, 1975; Yeaman *et al.*, 1976, 1977).

The reactions were terminated by the addition of 0.1vol. of 50% (w/v) trichloroacetic acid. The solutions were concentrated by vacuum dialysis and then extensively dialysed against solution A. The preparations were centrifuged to remove denatured non-histone protein derived from the partially purified cyclic AMP-dependent protein kinase and stored at 0–4°C. No ^{32}P radioactivity was released from these proteins during storage for at least 1 month at 4°C.

Assay of protein phosphatase activities

A mixture comprising 0.02ml of solution A containing 100mM-NaF and 0.02ml of protein phosphatase (diluted in solution A containing 6mM-$MnCl_2$ and 1.0mg of bovine serum albumin/ml) was warmed at 25°C for 2min. The reaction was initiated by the addition of 0.02ml of ^{32}P-labelled protein in solution A containing 50mM-NaF. After 5min at 25°C the reactions were terminated by the addition of 0.1ml of ice-cold 17.5% trichloroacetic acid and 0.1ml of 6mg of bovine serum albumin/ml. The solution was kept on ice for 10min and then centrifuged at 15000*g* for 2min. A portion (0.2ml) of the supernatant was added to 1.0ml of scintillant and counted for radioactivity. Reaction blanks were prepared in an identical manner, except that the protein phosphatase was replaced by solution A containing 6mM-$MnCl_2$ and 1.0mg of bovine serum albumin/ml. This corrected for traces of ^{32}P radioactivity released from the substrate during storage and also for slight endogenous protein phosphatase activity in the ^{32}P-labelled substrates. Endogenous phosphatase activity released less than 2% of the total radioactivity during the period of the assay with all substrates. The assays were linear with time and enzyme concentration until at least 30% of the total radioactivity had been released in all cases. One unit of phosphatase activity is defined as the amount of enzyme which catalyses the release of 1.0nmol of phosphate/min under the standard assay conditions. As described previously (Antoniw & Cohen, 1976) the inclusion of free 1mM-$MnCl_2$ in the assay prevents the inhibition of phosphorylase kinase phosphatases and glycogen synthase phosphatases by NaF.

Since preparations of ^{32}P-labelled histones and phosphorylase *a* were completely devoid of endogenous phosphatase activities, these substrates could be stored and assayed in the absence of NaF. For the assay of histone phosphatases the trichloroacetic acid used to terminate the reaction was raised to 50%, the bovine serum albumin was raised to 30mg/ml, and a 0.2ml portion of the supernatant was added to 3.0ml of scintillant and counted for radioactivity.

The final substrate concentrations were: phosphorylase kinase (1.0mg/ml; 3×10^{-6}M in terms of $\alpha\beta\gamma$ units), phosphorylase *a* (1.0mg/ml; 1.0×10^{-5}M), glycogen synthase b_1 or b_2 (0.1mg/ml; 1.2×10^{-6}M), histone H1 (0.1mg/ml; 5×10^{-6}M), histone H2B (0.03mg/ml; 2×10^{-6}M).

Results

Co-purification of β-phosphorylase kinase phosphatase, phosphorylase phosphatase and glycogen synthase phosphatase-1

α-Phosphorylase kinase phosphatase and β-phosphorylase kinase phosphatase were partially purified from skeletal-muscle extracts by precipitation with ethanol, chromatography on DEAE-cellulose, fractionation with $(NH_4)_2SO_4$ and gel filtration on Sephadex G-200 (Antoniw & Cohen, 1976). These two activities co-purify up to and including the $(NH_4)_2SO_4$ step, but are resolved on Sephadex G-200 (Antoniw & Cohen, 1976). Fractions from the Sephadex G-200 column were also assayed for phosphorylase phosphatase and glycogen synthase phosphatase-1 activities and the results are shown in Fig. 1. The elution profiles for these two activities were almost identical with that of β-phosphorylase kinase phosphatase. The three activities, β-phosphorylase kinase phosphatase, phosphorylase phosphatase and glycogen synthase phosphatase-1, were then assayed at each step of the purification and the results are shown in Tables 1 and 2. All three activities co-purified through each step of the procedure, resulting in a product purified 300–400-fold from the ethanol precipitation step (600–700-fold in the peak fractions). A similar degree of purification for the pooled fractions (300–720-fold) was obtained in four different preparations. The α-phosphorylase kinase phosphatase was purified 500-fold (1000-fold in the peak fractions).

The gel-filtration behaviour of β-phosphorylase kinase phosphatase (but not α-phosphorylase kinase phosphatase) varied from preparation to preparation. In the experiment shown in Fig. 1, this enzyme behaved as a single component, termed protein phosphatase-IIIA for reasons described below; this component had mol.wt. 75000 (Fig. 2). However, in subsequent preparations variable amounts of a second component, termed protein phosphatase-IIIB, were observed. This component had apparent mol.wt. 46000 (Fig. 2). A preparation which contained almost equal amounts of protein phosphatase-IIIA and -IIIB on an activity basis is shown in Fig. 3, and one which contained almost exclusively protein phosphatase-IIIB is shown in Fig. 4. It can be seen that 95% of the phosphorylase phosphatase and 80% of the glycogen synthase phosphatase-1 activity were eluted with β-phosphorylase kinase phosphatase, whether this emerged as protein phosphatase-IIIA or -IIIB. Of ten different preparations, four were more than 80% protein phosphatase-IIIA, four were more than 80% protein phosphatase-IIIB, and two showed approximately equal amounts of the two components.

Histone H1 and histone H2B phosphatases

Fractions from the Sephadex G-200 column shown in Fig. 3 were assayed for histone H1 and histone H2B phosphatase activities. Four peaks were resolved (Fig. 5), which were termed protein phosphatases-I, -II, -IIIA and -IIIB in order of decreasing molecular size. Their molecular weights were estimated to be 300000, 170000, 75000 and 46000 respectively (Fig. 2). Protein phosphatase-II corresponds to the α-phosphorylase kinase phosphatase activity described previously (Antoniw & Cohen,

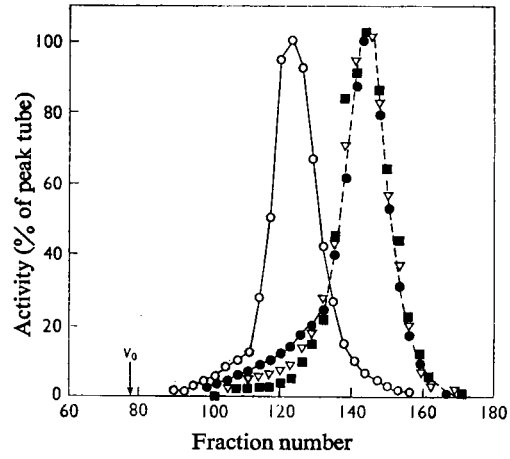

Fig. 1. *Gel filtration of protein phosphatases on a column (150cm×2.5cm) of Sephadex G-200*

A sample (2.4ml) of partially purified protein phosphatase obtained after step 5 (Table 1) was applied to the column, which was equilibrated with solution A containing 6mM-$MnCl_2$. The flow rate was 8ml/h and fractions of volume 2.6ml were collected. The excluded volume (V_0) was 201ml. The fractions were assayed with the substrates described in Table 1 and glycogen synthase b_1 (0.95mol of phosphate per subunit). ○, α-Phosphorylase kinase phosphatase; ●, β-phosphorylase kinase phosphatase; ■, phosphorylase phosphatase; ▽, glycogen synthase phosphatase-1. Activities in the peak tubes were: phosphorylase phosphatase (31.5 units/ml), glycogen synthase phosphatase-1 (4.6 units/ml), β-phosphorylase kinase phosphatase (3.5 units/ml) and α-phosphorylase kinase phosphatase (4.6 units/ml).

Table 1. *Co-purification of β-phosphorylase kinase phosphatase and phosphorylase phosphatase activities with protein phosphatase-IIIA*

The substrates used to assay for these phosphoprotein phosphatase activities consisted of: *A*, phosphorylase kinase, $\alpha = 0.76$ mol of phosphate/$\alpha\beta\gamma$, $\beta = 0.06$ mol of phosphate/$\alpha\beta\gamma$; *B*, phosphorylase kinase, $\alpha = 0.07$ mol of phosphate/$\alpha\beta\gamma$, $\beta = 0.45$ mol of phosphate/$\alpha\beta\gamma$; *C*, phosphorylase *a*, 1.06 mol of phosphate/phosphorylase subunit. Protein was measured by the procedure of Lowry *et al.* (1951), with bovine serum albumin as standard; 800 g of muscle was used in this preparation.

		A α-Phosphorylase kinase phosphatase			*B* β-Phosphorylase kinase phosphatase			*C* Phosphorylase phosphatase		
Step	Protein (mg)	Specific activity (units/mg)	Purification (-fold)	Yield (%)	Specific activity (units/mg)	Purification (-fold)	Yield (%)	Specific activity (units/mg)	Purification (-fold)	Yield (%)
1. Extract	33900	(0.02)	(0.3)	(48)	(0.06)	(0.4)	(59)	(0.4)	(0.5)	(62)
2. 30% ethanol precipitation	25500	0.06	1.0	100	0.13	1.0	100	0.76	1.0	100
3. Redissolved ethanol supernatant	3630	0.24	4.2	60	0.46	3.7	52	2.7	3.6	51
4. DEAE-cellulose, pH 7.0	149	2.9	51	30	3.0	24	14	21	28	16
5. $(NH_4)_2SO_4$ precipitation	69.9	6.6	120	32	5.3	43	12	31	41	11
6a. Sephadex G-200, peak 1	6.81	29	510	14	3.8	30	0.8	20	26	0.7
6b. Sephadex G-200, peak 2	3.62	3.5	63	0.9	41	320	4.6	303	400	5.7

1976), and protein phosphatases-IIIA and -IIIB correspond to the two forms of the enzyme that catalyse the dephosphorylation of the β-subunit of phosphorylase kinase, phosphorylase *a* and glycogen synthase b_1 (Fig. 3). Protein phosphatase-I is an active histone phosphatase which has only slight activity against the phosphorylated enzymes of glycogen metabolism in the standard assay (Fig. 5).

Protein phosphatase-III dephosphorylates the two phosphorylation sites on histone H2B (serine-32 and serine-36) at the same rate, as do protein phosphatases-I and -II (not illustrated). The experiments were performed with analogous procedures to those used to measure the relative rates of phosphorylation of serine-32 and serine-36 by cyclic AMP-dependent protein kinase (Yeaman *et al.*, 1977).

Co-purification of glycogen synthase phosphatase-1 *and glycogen synthase phosphatase*-2

The fractions from the Sephadex G-200 column shown in Fig. 4 were assayed for glycogen synthase phosphatase-2 activity; the elution profile obtained for this activity was identical with that for glycogen synthase phosphatase-1 (Fig. 6). Most (75–80%) of the activity measured with glycogen synthase b_1 or b_2 as substrate was eluted in a position corresponding to protein phosphatases-IIIA and -IIIB. However, Figs. 3 and 6 also show that 10–15% of the glycogen synthase phosphatase activity eluted from Sephadex G-200 was associated with protein phosphatase-1 and a further 10–15% with protein phosphatase-II. In a separate experiment, glycogen synthase phosphatase-2 and phosphorylase phosphatase activities were assayed at each step of the purification. The ratios of the two activities were similar after each step (Table 2). No evidence was obtained for the existence of a separate enzyme that specifically dephosphorylated the site(s) on glycogen synthase phosphorylated by glycogen synthase kinase-2.

Conversion of glycogen synthases b_1 *and* b_2 *into glycogen synthase a*

The ability of protein phosphatase-IIIB to reactivate glycogen synthases b_1 and b_2 was investigated. The results of this experiment are shown in Figs. 7(*a*) and 7(*b*). The protein phosphatase was able to achieve almost complete dephosphorylation of both glycogen synthase *b* preparations. There was a good correlation between the extent of dephosphorylation and the increase in the activity ratio, defined as the activity in the absence of glucose 6-phosphate relative to the activity in the presence of glucose 6-phosphate. The final activity ratio attained was very similar to that of the glycogen synthase *a* preparation at the start of the experiment (Fig. 7).

Table 2. *Co-purification of β-phosphorylase kinase phosphatase, phosphorylase phosphatase, glycogen synthase phosphatase-1 and glycogen synthase phosphatase-2 activities*

Abbreviations: β-PhKP, β-phosphorylase kinase phosphatase; PhP, phosphorylase phosphatase; GSP-1, GSP-2, glycogen synthetase phosphatases-1 and -2. The substrates used to assay these protein phosphatase activities consisted of: phosphorylase kinase ($\alpha = 0.07$ mol of phosphate/$\alpha\beta\gamma$, $\beta = 0.45$ mol of phosphate/$\alpha\beta\gamma$); phosphorylase *a* (1.03 mol of phosphate/subunit); glycogen synthase b_1 (1.2 mol of phosphate/subunit); glycogen synthase b_2 (0.85 mol of phosphate/subunit). The values in the three columns were obtained from three different preparations.

Step	Ratio of phosphatase activities		
	β-PhKP/PhP	GSP-1/PhP	GSP-2/PhP
1. 30% ethanol precipitation	0.17	0.12	0.06
2. Redissolved ethanol supernatants	0.17	0.11	0.05
3. DEAE-cellulose, pH7.0	0.15	0.12	0.04
4. 45% $(NH_4)_2SO_4$ precipitation	0.17	0.145	0.045
5. Sephadex G-200 (protein phosphatase-III)	0.13	0.09	—

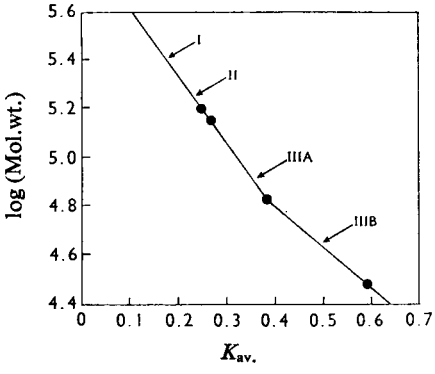

Fig. 2. *Estimation of the molecular weights of protein phosphatases by gel filtration on Sephadex G-200*

The column (150 cm × 2.5 cm), equilibrated in solution A containing 6 mM-$MnCl_2$, was calibrated with rabbit muscle aldolase (mol.wt. 158000), rabbit muscle lactate dehydrogenase (mol.wt. 138000), bovine serum albumin (mol.wt. 68000) and bovine carbonic anhydrase (mol.wt. 29500). The excluded volume (V_0) was determined with Blue Dextran.

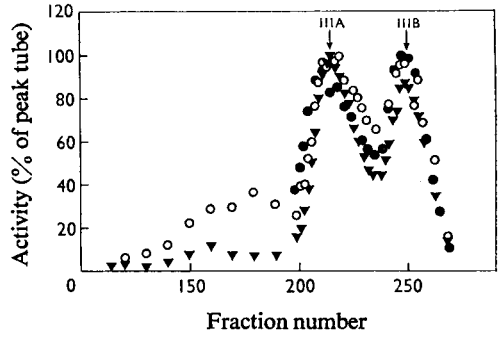

Fig. 3. *Elution profile from a column (150 cm × 2.5 cm) of Sephadex G-200 showing equal amounts of peaks IIIA and IIIB*

A sample (2.7 ml) of partially purified protein phosphatase obtained after step 5 (Table 1) was applied to the column, which was equilibrated with solution A containing 6 mM-$MnCl_2$. The flow rate was 9 ml/h and 2.3 ml fractions were collected. The excluded volume, V_0, was 280 ml. The fractions were assayed with the following substrates: ○, glycogen synthase b_1 containing 1.04 mol of phosphate per subunit; ▼, phosphorylase *a* containing 0.88 mol of phosphate per subunit; ●, phosphorylase kinase containing 1.15 mol of phosphate in the α-subunit and 1.1 mol in the β-subunit. Only β-phosphorylase kinase phosphatase activity is illustrated. Activities in the peak tubes were: phosphorylase phosphatase (5.9 units/ml); glycogen synthase phosphatase-1 (0.81 unit/ml) and β-phosphorylase kinase phosphatase (1.8 units/ml).

Discussion

Zieve & Glinsmann (1973) were the first to report that phosphorylase kinase phosphatase and glycogen synthase phosphatase activities co-purified through three steps of purification. They also reported that activated phosphorylase kinase was a competitive inhibitor of the glycogen synthase phosphatase reaction, and they therefore suggested that these two protein phosphatase reactions might be catalysed by the same enzyme. However, the method they used to prepare activated phosphorylase kinase (DeLange *et al.*, 1968) would result in extensive autophosphorylation as well as phosphorylation catalysed by cyclic AMP-dependent protein kinase. Equally, their preparation of glycogen synthase *b* involved phosphorylation by endogenous protein kinases at an early stage in the preparation (Brown & Larner, 1971) and would be expected to result in phosphorylation both by cyclic AMP-dependent protein kinase and by glycogen synthase kinase-2 (Nimmo & Cohen, 1977). As a consequence, it was not clear from their experiments which sites were dephosphorylated. Similar

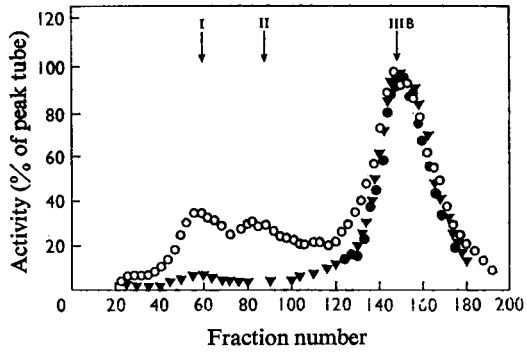

Fig. 4. *Elution profile from a column* (150 *cm* × 2.5 *cm*) *of Sephadex G-200 showing predominantly peak IIIB*
A sample (4.0ml) was applied to the column and 4.4ml fractions were collected. Other conditions are as in Fig. 3. The fractions were assayed with: ▼, phosphorylase *a* containing 0.91mol of phosphate per subunit; ○, glycogen synthase b_1 containing 1.09mol of phosphate per subunit; ●, phosphorylase kinase containing 0.7mol of phosphate in the α-subunit and 1.0mol in the β-subunit. Only β-phosphorylase kinase phosphatase activity is illustrated.

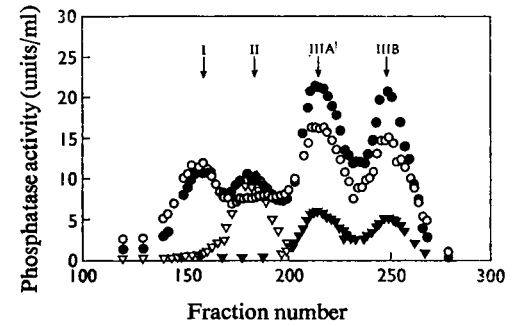

Fig. 5. *Elution profile of histone phosphatases from a column* (150 *cm* × 2.5 *cm*) *of Sephadex G-200*
Fractions from the column shown in Fig. 3 were analysed for histone phosphatase activity with the following substrates: ○, histone H1 containing 0.72 mol of phosphate per mol; ●, histone H2B containing 1.83mol of phosphate per mol. Phosphorylase phosphatase (▼) and α-phosphorylase kinase phosphatase (▽) activities are also shown. The substrates used to assay these activities are described in Fig. 3. β-Phosphorylase kinase phosphatase activity is omitted.

reservations apply to the work of Nakai & Thomas (1973, 1974) in heart muscle.

In this paper, we have re-examined the question of structural relationships between the protein phosphatases of glycogen metabolism using phospho-

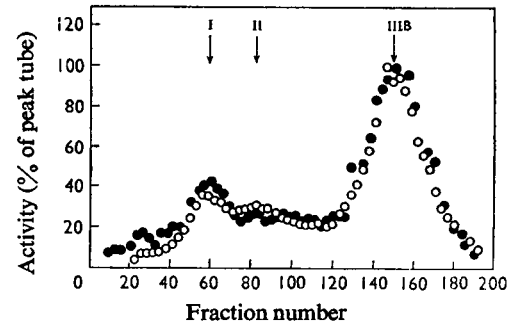

Fig. 6. *Elution of glycogen synthase phosphatase-1 and glycogen synthase phosphatase-2 from Sephadex G-200*
Fractions from the column shown in Fig. 4 were assayed for glycogen synthase phosphatases-1 and -2 with the following substrates: ○, glycogen synthase b_1 containing 1.09mol of phosphate per subunit; ●, glycogen synthase b_2 containing 0.82mol of phosphate per subunit.

protein substrates in defined states of phosphorylation. The results confirm and extend those of Zieve & Glinsmann (1973). Several lines of evidence suggest that, under our assay conditions, the dephosphorylation of the β-subunit of phosphorylase kinase, phosphorylase *a* and glycogen synthases b_1 and b_2 are catalysed by a single major activity in skeletal muscle, termed protein phosphatase-III. Firstly, these four activities co-purify through six steps of purification, up to and including the final gel filtration on Sephadex G-200 (Tables 1 and 2). Secondly, the four activities of protein phosphatase-III are not separated by gel filtration, whether this enzyme emerges as protein phosphatase-IIIA or -IIIB or a mixture of the two forms, and the ratios of the four activities are identical in both protein phosphatase-IIIA and -IIIB. This last result would appear to represent strong evidence that the four phosphatase activities share at least a common subunit. In the following paper (Cohen *et al.*, 1977) we present further evidence in support of this idea. The four activities of protein phosphatase-III are shown to be inhibited in an identical manner by a heat-stable protein in skeletal muscle, which is more than 200 times less effective in inhibiting protein phosphatases-I and -II (Cohen *et al.*, 1977). The protein inhibitor has also been used to show that 85–90% of the phosphorylase phosphatase and glycogen synthase phosphatase-1 and -2 activities in skeletal-muscle extracts are catalysed by protein phosphatase-III (Cohen *et al.*, 1977).

Figs. 3 and 5 show that protein phosphatases-I and -II each contain 10–15% of the glycogen synthase phosphatase activity that is recovered from Sephadex G-200, and these fractions also have slight phos-

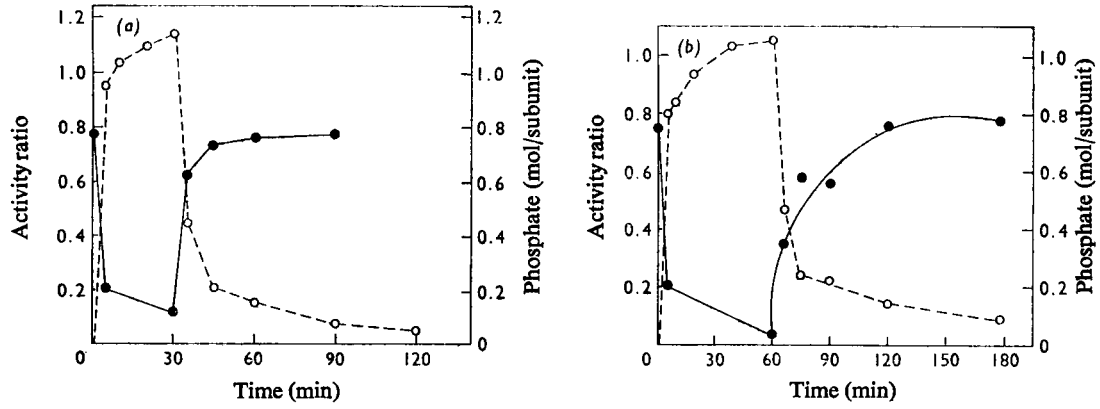

Fig. 7. *Interconversion of glycogen synthases a and b_1 (a) and a and b_2 (b)*
(*a*) Glycogen synthase *a* was incubated with cyclic AMP-dependent protein kinase, cyclic AMP and MgATP as described in the Experimental section. After 30min the reaction mixture was rapidly filtered through Sephadex G-25, and 1vol. of protein phosphatase-IIIB in solution A containing 6mM-$MnCl_2$ was then added to 2vol. of the glycogen synthase b_1. ○, Phosphate/subunit of glycogen synthase b_1; ●, activity ratio (defined in the text). (*b*) Glycogen synthase *a* was incubated with glycogen synthase kinase-2, the inhibitor protein of cyclic AMP-dependent protein kinase and MgATP. After 30min, the reaction mixture (glycogen synthase b_2) was treated as in (*a*). ○, Phosphate/subunit of glycogen synthase b_2; ●, activity ratio.

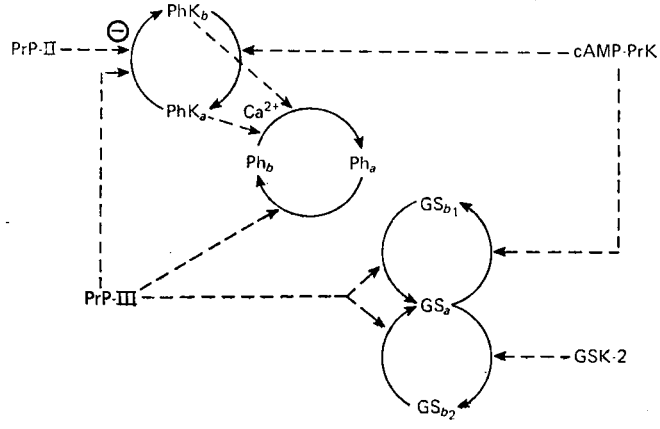

Fig. 8. *Relationship between the protein kinase and protein phosphatase activities of glycogen metabolism*
Abbreviations: Ph, phosphorylase; PhK, phosphorylase kinase; GS, glycogen synthase; GSK-2, glycogen synthase kinase-2; cAMP-PrK, cyclic AMP-dependent protein kinase; PrP, protein phosphatase; *b*, inactive or less active form; *a*, activated form.

phorylase phosphatase activity. These activities are not the result of contamination with aggregated forms of protein phosphatase-III. The evidence for this statement and implications of this finding are considered in the following paper (Cohen *et al.*, 1977).

The reason for the elution of protein phosphatase-III from Sephadex G-200 sometimes as protein phosphatase-IIIA and sometimes as protein phosphatase-IIIB in unknown. However, two possible explanations for this anomalous behaviour are that the two peaks result from limited proteolysis or from the dissociation of a regulatory subunit. The latter

possibility seems more likely in view of the work of Huang & Glinsmann (1975). These workers also resolved two forms of muscle phosphorylase phosphatase with mol.wts. about 70000 and 50000 by centrifugation in a sucrose density gradient. However, the latter species was only observed after incubation with cyclic AMP-dependent protein kinase, cyclic AMP and MgATP. They presented evidence which indicated that the two forms differed from one another by the presence or absence of a regulatory subunit, which could be phosphorylated. This phosphorylation decreased the phosphorylase phosphatase activity 4–5-fold. If their interpretation is correct, it seems possible that their species of mol.wt. 70000 and 50000 correspond to protein phosphatases-IIIA and -IIIB. However, a detailed analysis of the homogeneous proteins will be necessary to substantiate this.

The findings presented in the present paper are in conflict with two previous reports. Riley *et al.* (1968) and Gratecos *et al.* (1974) have both stated that highly purified muscle phosphorylase phosphatase showed no activity towards phosphorylase kinase. Gratecos *et al.* (1974) also reported that their preparation was unable to dephosphorylate histones or protamine. These workers did not, however, present experimental evidence in support of these statements and the reasons for the discrepancies with our findings are unclear.

The present data are, however, consistent with results obtained in other mammalian tissues. Nakai & Thomas (1973, 1974) partially purified a glycogen synthase phosphatase from bovine heart muscle by precipitation with ethanol and chromatography on DEAE-cellulose and selected a fraction eluted at 0.25M- and 0.30M-NaCl for further analysis. This fraction possessed phosphorylase phosphatase activity, as well as histone and casein phosphatase activities. These four activities were eluted together if the fraction was subjected to either gel filtration on Sephadex G-100 or isoelectric focusing. In addition, the heat-stability characteristics of the four activities were very similar. Brandt *et al.* (1975) purified liver phosphorylase phosphatase to homogeneity. This preparation had glycogen synthase phosphatase activity, and the phosphorylase phosphatase and glycogen synthase phosphatase activities co-purified throughout the isolation procedure with rabbit liver phosphorylase *a* and glycogen synthase *b* as substrates (Killilea *et al.*, 1976). They also stated, as a note added in proof, that their purified enzyme was able to dephosphorylate phosphorylase kinase from skeletal muscle and the phosphorylated regulatory subunit of cyclic AMP-dependent protein kinase from bovine cardiac muscle. Khandelwal *et al.* (1976) also isolated homogeneous phosphorylase phosphatase from rabbit liver. The purified enzyme catalysed the dephosphorylation of muscle glycogen synthase b_1, muscle phosphorylase kinase and phosphorylated histone and casein. The relative activities of the phosphatases with respect to phosphorylase *a*, glycogen synthase b_1, histone and casein remained constant through the purification. The activities with different substrates decreased in parallel when the phosphatase was heated, and activity towards a given substrate was inhibited competitively by each of the alternative substrates.

Our current ideas about the regulation of glycogen metabolism by phosphorylation–dephosphorylation are schematically represented in Fig. 8. Cyclic AMP-dependent protein kinase can both activate phosphorylase kinase and inhibit glycogen synthase (Soderling *et al.*, 1970). The latter enzyme can also be inactivated by glycogen synthase kinase-2, but this protein kinase does not affect phosphorylase kinase (Nimmo *et al.*, 1976*b*). Likewise phosphorylase kinase can activate phosphorylase, but it does not affect glycogen synthase (Nimmo *et al.*, 1976*b*). However, there appears to be a single major protein phosphatase which can reverse all of these phosphorylation reactions and so inhibit glycogenolysis or activate glycogen synthesis. In addition, there is another activity, termed α-phosphorylase kinase phosphatase or protein phosphatase-II, which specifically catalyses the dephosphorylation of the α-subunit of phosphorylase kinase. Since the phosphorylation of the α-subunit of phosphorylase kinase greatly increases the rate at which the β-subunit can be dephosphorylated by protein phosphatase-III (Cohen & Antoniw, 1973), α-phosphorylase kinase phosphatase is an activity which antagonizes the action of protein phosphatase-III and inhibits the reconversion of phosphorylase kinase *a* into *b* (Fig. 8). Protein phosphatase-II catalyses the dephosphorylation of the α-subunit of phosphorylase kinase at least 20-fold more rapidly than that of the β-subunit (Antoniw & Cohen, 1975, 1976). The existence of α-phosphorylase kinase phosphatase shows that there is not a single activity in mammalian tissues which reverses all the phosphorylations catalysed by cyclic AMP-dependent protein kinase.

Although protein phosphatase-III seems to catalyse four functionally related dephosphorylations, it is important to stress that each of these four activities can be and are regulated quite independently through selective changes in the conformation of each substrate (Cohen, 1976). Thus phosphorylase phosphatase activity is inhibited by AMP (Gratecos *et al.*, 1974), β-phosphorylase kinase phosphatase is activated by the phosphorylation of the α-subunit of phosphorylase kinase (Antoniw & Cohen, 1976), and glycogen synthase phosphatase is inhibited by glycogen (Larner *et al.*, 1968). The existence of a common phosphatase is therefore compatible with both synchronous and independent expressions of the various activities (Cohen, 1976).

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