Specificity of a Protein Phosphatase Inhibitor from Rabbit Skeletal Muscle

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A heat-stable protein, which is a specific inhibitor of protein phosphatase-III, was purified 700-fold from skeletal muscle by a procedure that involved heat-treatment at 95°C, chromatography on DEAE-cellulose and gel filtration on Sephadex G-100. The final step completely resolved the protein phosphatase inhibitor from the protein inhibitor of cyclic AMP-dependent protein kinase. The phosphorylase phosphatase, β -phosphorylase kinase phosphatase, glycogen synthase phosphatase-1 and glycogen synthase phosphatase-2 activities of protein phosphatase-III [Antoniw, J. F., Nimmo, H. G., Yeaman, S. J. & Cohen, P. (1977) *Biochem. J.* 162, 423–433] were inhibited in a very similar manner by the protein phosphatase inhibitor and at least 95% inhibition was observed at high concentrations of inhibitor. The two forms of protein phosphatase-III, termed IIIA and IIIB, were equally susceptible to the protein phosphatase inhibitor. The protein phosphatase inhibitor was at least 200 times less effective in inhibiting the activity of protein phosphatase-I and protein phosphatase-II. The high degree of specificity of the inhibitor for protein phosphatase-III was used to show that 90% of the phosphorylase phosphatase and glycogen synthase phosphatase activities measured in muscle extracts are catalysed by protein phosphatase-III. Protein phosphatase-III was tightly associated with the protein–glycogen complex that can be isolated from skeletal muscle, whereas the protein phosphatase inhibitor and protein phosphatase-II were not. The results provide further evidence that the enzyme that catalyses the dephosphorylation of the α -subunit of phosphorylase kinase (protein phosphatase-II) and the enzyme that catalyses the dephosphorylation of the β -subunit of phosphorylase kinase (protein phosphatase-III) are distinct. The results suggest that the protein phosphatase inhibitor may be a useful probe for differentiating different classes of protein phosphatases in mammalian cells.

Brandt *et al.* (1974, 1975) reported that more than 95% of the phosphorylase phosphatase in rat liver and rabbit liver extracts was present in an inhibited form. The enzyme could be activated 30–40-fold by precipitation with $(\text{NH}_4)_2\text{SO}_4$ and with ethanol at room temperature. They showed that the inhibition of the phosphatase resulted from its interaction with another protein(s). The protein inhibitor(s) was inactivated by precipitation with ethanol, or by incubation with trypsin, but it was stable to heating at 90°C. Brandt *et al.* (1974, 1975) also showed that a similar protein inhibitor was present in heart muscle and skeletal muscle. When heart muscle extracts were treated with ethanol, the phosphatase was activated 15–20-fold, which was only slightly less than the activation observed in liver extracts. However, the basal phosphorylase phosphatase activity was much higher in skeletal-muscle extracts, and only a fourfold activation was achieved with ethanol,

suggesting that the concentration of the protein inhibitor(s) was much lower in this tissue.

Huang & Glinsmann (1975, 1976*a,b*) demonstrated the presence of two heat-stable trypsin-labile inhibitors of phosphorylase phosphatase in partially purified preparations of the phosphatase from skeletal muscle. The inhibitor termed i_1 was only inhibitory when it was phosphorylated by the action of cyclic AMP-dependent protein kinase. In contrast, the inhibitor termed i_2 did not seem to be subject to regulation by phosphorylation. The i_1 and i_2 inhibitors could be separated by chromatography on DEAE-cellulose (Huang & Glinsmann, 1976*b*).

In the preceding paper (Antoniw *et al.*, 1977), it was shown that a single phosphatase, termed protein phosphatase-III, was the major enzyme that catalysed the dephosphorylation of phosphorylase a , the β -subunit of phosphorylase kinase, glycogen synthase b_1 and glycogen synthase b_2 in skeletal muscle. This activity, which existed in two forms termed IIIA and IIIB, could be resolved by gel filtration from the activity which catalysed the dephosphorylation of

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the α -subunit of phosphorylase kinase (protein phosphatase-II), and from an active histone phosphatase which had only slight activity against the phosphorylated enzymes of glycogen metabolism (protein phosphatase-I). In this paper, we describe the effect of a heat-stable protein phosphatase inhibitor, presumed to be i_2 , on the various activities of protein phosphatases-I, -II and -III.

Experimental

Materials

Trypsin (Worthington) treated with 1-chloro-4-phenyl-3-L-tosylamidobutan-2-one was purchased from Cambrian Chemicals, Croydon, Surrey, U.K. Soya-bean trypsin inhibitor and mixed histone type III were obtained from Sigma Chemical Co., London S.W.6, U.K. Histone H1 was a gift from Professor T. A. Langan, University of Denver, Denver, CO, U.S.A. 32 P-labelled phosphorylase a , phosphorylase kinase phosphorylated in both the α - and β -subunits or phosphorylated specifically in the α -subunit, glycogen synthase b_1 and phosphorylated histone H1 were prepared by using cyclic AMP-dependent protein kinase; glycogen synthase b_2 was prepared by using glycogen synthase kinase-2 (Antoniw *et al.*, 1977). Protein phosphatases-I, -II and -III were purified up to and including the gel filtration on Sephadex G-200 (Antoniw *et al.*, 1977). The sources of other materials have been given previously (Antoniw & Cohen, 1976; Nimmo *et al.*, 1976a).

Solution A. This solution was used repeatedly throughout this work and contained 0.05 *I* Tris/HCl (pH 7.0, 25°C) and 1.0 mM-EDTA.

Purification of the protein phosphatase inhibitor and the protein kinase inhibitor

A female New Zealand White rabbit was injected with a lethal dose of Nembutal (May and Baker, Dagenham, Essex, U.K.), and muscle from the hind limbs and back was excised and placed in ice. All subsequent steps were performed at 0–4°C unless otherwise specified. The muscle was minced, homogenized with 2.5 vol. of 4.0 mM-EDTA/14 mM-mercaptoethanol, pH 7.0, for 45 s in a Waring blender (low speed) and centrifuged at 6000 *g* for 45 min. The supernatant was decanted through glasswool (step 1) and adjusted to pH 6.1 with 1.0 *M*-acetic acid. The suspension was centrifuged at 6000 *g* for 45 min, and the pellet containing glycogen and associated enzymes was removed and used to prepare phosphorylase kinase (Cohen, 1973) and glycogen synthase (Nimmo *et al.*, 1976a). The pH 6.1 supernatant was adjusted to pH 7.0 with 5 *M*- NH_3 and 250 ml was transferred to a glass Pyrex flask and immersed in a boiling-water bath. The solution was stirred continuously until the temperature

had reached 95°C (10 min) and the heating was continued for a further 5 min. It was then cooled to 10°C by immersion in ice and passed through filter paper (step 2). The solution was dialysed against 5.0 mM-sodium acetate/1.0 mM-EDTA, pH 5.0, for 36 h with three changes of dialysis buffer and applied to a DEAE-cellulose column (8 cm $\times$ 3 cm) equilibrated in the same buffer. The column was washed with the same buffer until the A_{280} of the effluent has fallen to zero, and the protein phosphatase inhibitor and protein kinase inhibitor were eluted with 50 ml of 5.0 mM-sodium acetate/1.0 mM-EDTA/200 mM-NaCl, pH 5.0. The solution was concentrated to 0.6 ml by vacuum dialysis, dialysed against solution A for 18 h (step 3) and subjected to gel filtration on a column (150 cm $\times$ 1.5 cm) of Sephadex G-100 or G-75 (superfine grades). Fractions containing the protein phosphatase inhibitor (step 4A) and the protein kinase inhibitor (step 4B) were pooled and stored at 0–4°C.

Assay of the protein phosphatase inhibitor

Protein phosphatases-I, -II and -III were assayed with the 32 P-labelled phosphoprotein substrates listed under 'Materials', as described previously (Antoniw *et al.*, 1977). The inhibitor was assayed with the phosphorylase phosphatase activity of partially purified protein phosphatase-IIIB (Antoniw *et al.*, 1977). In preliminary experiments, the percentage inhibition observed was decreased with increasing concentrations of protein phosphatase-IIIB (Fig. 1).

Fig. 1. Effect of varying the concentration of protein phosphatase-III on its inhibition by the protein phosphatase inhibitor

The percentage inhibition of the enzyme is plotted as a function of enzyme concentration. The inhibition was calculated from assays carried out both in the presence of a fixed amount of protein phosphatase inhibitor (1.0 unit) and in the absence of inhibitor, at each protein phosphatase concentration. The protein phosphatase concentration refers to the units of enzyme present in the assay. Measurements were made with phosphorylase a as substrate.

Accordingly, protein phosphatase-IIIB was, as a routine, diluted to a concentration of 1.0 unit/ml in solution A, containing 6mM-MnCl₂, 1.0mg of bovine serum albumin/ml and 50mM-mercaptoethanol, where 1 unit of phosphatase activity is defined as that amount which releases 1.0nmol of phosphate/min from phosphorylase *a* in the standard assay (Antoniw *et al.*, 1977).

Solution A (0.02ml) containing 50mM-mercaptoethanol and the protein phosphatase inhibitor was mixed with 0.02ml of diluted protein phosphatase (0.02 unit) and preincubated at 25°C for 2min. The assay was initiated by the addition of 0.02ml of phosphorylase *a* (3.0mg/ml), and then carried out as described previously for the assay of protein phosphatases (Antoniw *et al.*, 1977). Reaction blanks were carried out in which the protein phosphatase was replaced by dilution buffer. All assays were performed in duplicate in the presence and absence of protein phosphatase inhibitor. In the standard assay in the absence of inhibitor, 15% of the phosphorylase *a* was converted into phosphorylase *b* in 5min. The degree of inhibition observed was independent of the preincubation time over the period 0.25–60min (not illustrated).

A plot of percentage activity against protein phosphatase inhibitor concentration was essentially linear over the range 0–50% inhibition (Fig. 2a), and quantitative assays were therefore carried out within these limits. One unit of protein phosphatase inhibitor is defined as that amount which inhibited the phosphorylase phosphatase activity of protein phosphatase-III by 50% under the standard assay conditions.

Assay of the protein kinase inhibitor

The assay was carried out by using the partially purified peak-I isoenzyme of cyclic AMP-dependent protein kinase (Nimmo *et al.*, 1976b) and a mixed

histone substrate. The assay mixture (0.1ml) contained the following components: glycerol 2-phosphate (sodium salt; 20mM), EDTA (0.8mM), histone (2.0mg/ml), cyclic AMP (0.01mM), cyclic AMP-dependent protein kinase (0.01 unit), [γ -³²P]ATP containing 10⁷ c.p.m./ μ mol (0.2mM), magnesium acetate (2.0mM) and 0.01ml of protein kinase inhibitor in solution A. The reaction was initiated by the addition of MgATP. After 10min at 30°C, a sample (0.05ml) was spotted on to cellulose phosphate paper (Witt & Roskoski, 1975) and immersed in water (50ml per paper) to stop the reaction. The papers were rapidly washed four times in tap water, dipped in acetone, dried and counted for radioactivity in a Beckman LS-330 scintillation counter.

The percentage inhibition observed at a fixed concentration of protein kinase inhibitor decreased with increasing cyclic AMP-dependent protein kinase concentrations (not illustrated). This was analogous to the results for the protein phosphatase inhibitor (Fig. 1). Therefore 0.01 unit of cyclic AMP-dependent protein kinase was used as routine in the inhibitor assay, where 1 unit of protein kinase activity is defined as that amount of enzyme which catalyses the incorporation of 1.0nmol of phosphate into 0.2mg of mixed histone/min in the standard assay. A plot of percentage activity against protein kinase inhibitor concentration was essentially linear up to 70% inhibition (Fig. 2b). One unit of protein kinase inhibitor is defined as that amount which inhibited cyclic AMP-dependent protein kinase by 50% under the standard conditions.

Results

Purification of the protein phosphatase inhibitor, and its separation from the protein inhibitor of cyclic AMP-dependent protein kinase

A summary of the purification of the protein phosphatase inhibitor is shown in Table 1. It was

Fig. 2. Standard graphs for the assay of the protein phosphatase inhibitor and protein kinase inhibitor (a) Effect of increasing protein phosphatase inhibitor on the phosphorylase phosphatase activity of protein phosphatase-IIIB. (b) Effect of increasing protein kinase inhibitor on the histone kinase activity of cyclic AMP-dependent protein kinase. Each enzyme was assayed as described in the Experimental section.

Table 1. *Purification of the protein phosphatase inhibitor (PrP-I) and protein kinase inhibitor (PrK-I)*

The inhibitor 'activities' in the extract (step 1) were measured after heating a 3ml sample in a boiling-water bath. The solution was cooled and centrifuged and the supernatant was dialysed exhaustively against solution A to remove low-mol.wt. inhibitors of protein phosphatase-III. Inhibitor 'activities' at steps 2 and 3 were measured after dialysis against solution A. Protein concentrations were measured by the procedure of Lowry *et al.* (1951), with bovine serum albumin as standard.

Step	Volume (ml)	Protein (mg)	Protein phosphatase inhibitor			Protein kinase inhibitor		
			Sp. activity (units/mg)	Purification (-fold)	Yield (%)	Sp. activity (units/mg)	Purification (-fold)	Yield (%)
1. Extract	294	5150	8.1	1	100	9.8	1	100
2. Boiled pH 6.1 supernatant	209	65	457	56	71	505	51	65
3. DEAE-cellulose, pH 5.0, eluate after concentration	0.6	6.6	2370	290	38	2690	270	35
4A. Sephadex G-100 (PrP-I fraction)	12.5	1.3	5720	706	18	—	—	—
4B. Sephadex G-100 (PrK-I fraction)	15.5	0.28	—	—	—	30300	3080	17

Fig. 3. Separation of the protein phosphatase inhibitor and the protein kinase inhibitor by gel filtration on a column (150cm x 1.5 cm) of Sephadex G-75

●, Protein phosphatase inhibitor; ○, protein kinase inhibitor; ----, A_{280} . Each fraction was 3.0ml.

Fig. 4. Estimation of the molecular weights of the protein phosphatase inhibitor (PrP-I) and the protein kinase inhibitor (PrK-I) on Sephadex G-100

The gel-filtration column was calibrated with the following marker proteins: bovine serum albumin (mol.wt. 68 000); ovalbumin (mol.wt. 43 000); bovine erythrocyte carbonic anhydrase (mol.wt. 29 500); myoglobin (mol.wt. 17 200).

purified 700-fold in an overall yield of 18%. The final gel filtration on Sephadex G-75 (Fig. 3) or G-100 (Table 1) completely separated the protein phosphatase inhibitor from the protein inhibitor of cyclic AMP-dependent protein kinase. The protein kinase inhibitor was purified 3000-fold with an overall yield of 17% (Table 1). The Sephadex columns were calibrated with marker proteins and the apparent molecular weights were found to be 65 000 for the protein phosphatase inhibitor and 27 000 for the protein kinase inhibitor (Fig. 4). The value for the protein kinase inhibitor is very similar to that obtained previously (Walsh *et al.*, 1971).

Some 20–30% of the protein phosphatase inhibitor 'activity' did not bind to DEAE-cellulose (although all the protein kinase inhibitor was retained by the ion-

exchanger) and this inhibitory material was not studied further. A variable amount of a second minor peak of protein phosphatase inhibitor was observed at the final gel-filtration step in three different preparations. This emerged just after the major protein phosphatase inhibitor but before the protein kinase inhibitor, and corresponded to a mol.wt. of 47 000. The elution profile in Fig. 3 shows a preparation having only a trace of this second component. All the experiments described below were carried out with the major protein phosphatase

inhibitor, apparent mol.wt. 65000 (Figs. 3 and 4). This inhibitor is tentatively assumed to be the i_2 protein described by Huang & Glinsmann (1976a,b), since it was an effective inhibitor without the need for prior incubation with cyclic AMP-dependent protein kinase, cyclic AMP and MgATP. However, the apparent mol.wt. of 65000 is higher than the value of 42000 reported by Huang & Glinsmann (1976a).

The purification method is similar to that described previously for the protein kinase inhibitor (Ashby & Walsh, 1972), except that the precipitation with 15% (w/v) trichloroacetic acid used between the heat-treatment and chromatography on DEAE-cellulose was omitted. This acid-precipitation step often gave low yields of protein phosphatase inhibitor 'activity', although the yield of protein kinase inhibitor was usually good. Many of the experiments described in this paper were, however, carried out with preparations in which the trichloroacetic acid-precipitation step was included, since this treatment did not appear to affect its behaviour on gel filtration or its inhibitory properties.

Fig. 5. *Reversal by dilution of the inhibition of protein phosphatase-IIIB by the protein phosphatase inhibitor* Two incubations were set up. In the first, protein phosphatase-III (5 units/ml) was incubated with protein phosphatase inhibitor (75 units/ml) for 15 min. In the second, the same concentration of enzyme was incubated in the absence of inhibitor. The two incubations were then subjected to successive dilutions, and the activities were measured at each dilution after 10 min as described in the Experimental section. The percentage inhibition of the enzyme is plotted against the units of protein phosphatase in the assay. The extent of inhibition decreases at the highest dilutions, although the protein phosphatase/protein phosphatase inhibitor ratio is identical in each assay. Very similar results were obtained in three different experiments with two preparations of protein phosphatase inhibitor.

Table 2. *Reversal by trypsin of the inhibition of protein phosphatase-IIIB by the protein phosphatase inhibitor*

The four incubations comprised the following: 1, protein phosphatase (1.0 unit/ml; 0.05 ml), solution A (0.05 ml), water (0.02 ml); 2, protein phosphatase (1.0 unit/ml; 0.05 ml), protein phosphatase inhibitor (50 units/ml; 0.05 ml), water (0.02 ml); 3, protein phosphatase (1.0 unit/ml; 0.05 ml), protein phosphatase inhibitor (50 units/ml; 0.05 ml), trypsin (0.2 mg/ml; 0.02 ml); 4, protein phosphatase (1.0 unit/ml; 0.05 ml), solution A (0.05 ml), trypsin (0.2 mg/ml; 0.02 ml). The incubations were left for 30 min at 20°C, and soya-bean trypsin inhibitor (1.0 mg/ml; 0.02 ml) was added. After standing for 10 min at room temperature (20°C), 0.04 ml samples were mixed with 0.02 ml of phosphorylase α (3.0 mg/ml) and assayed. The protein phosphatase inhibitor was in solution A and trypsin was dissolved in water. The soya-bean trypsin inhibitor added was sufficient to inhibit trypsin completely. The range of relative activities refer to three different experiments, with two preparations of protein phosphatase inhibitor and two preparations of protein phosphatase-III.

Incubation	Relative activity (%)
1. Phosphatase	100
2. Phosphatase+inhibitor	3-5
3. Phosphatase+inhibitor+trypsin	78-84
4. Phosphatase+trypsin	68-86

Reversibility of action of the protein phosphatase inhibitor

The inhibition of protein phosphatase-III by the i_2 protein was reversible. This was demonstrated by dilution of the complex formed between the i_2 protein and protein phosphatase-IIIB, and a typical experiment is shown in Fig. 5. It was also demonstrated by experiments involving incubation of the complex with trypsin. In agreement with the results of Brandt *et al.* (1975) and Huang & Glinsmann (1976a), trypsin had little effect on the activity of the phosphatase, but it destroyed the 'activity' of the i_2 protein (Table 2).

Inhibition of the various activities of protein phosphatase-III by the protein phosphatase inhibitor

The i_2 protein inhibited the phosphorylase phosphatase, the β -phosphorylase kinase phosphatase, the glycogen synthase phosphatase-1 and the glycogen synthase phosphatase-2 activities of protein phosphatase-IIIB in a very similar manner (Fig. 6), and at least 95% inhibition was observed at high concentrations of the i_2 protein. The phosphorylase phosphatase activities of protein phosphatases-IIIA and -IIIB were inhibited in an identical manner (not illustrated), indicating that the i_2 protein does not discriminate between the two forms of this enzyme.

Fig. 6. Influence of the protein phosphatase inhibitor on the various activities of protein phosphatase-III. ○, Phosphorylase phosphatase; ●, β-phosphorylase kinase phosphatase; ▼, glycogen synthase phosphatase-1; ▽, glycogen synthase phosphatase-2. All assays were carried out at a fixed concentration of protein phosphatase-III and various concentrations of the inhibitor. The substrates for the assays were prepared and characterized as described in the preceding paper (Antoniw *et al.*, 1977). The concentration of partially purified protein phosphatase inhibitor in the assay was 5 μg/ml. The final substrate concentrations were: phosphorylase α , 1.0 mg/ml; phosphorylase kinase, 1.0 mg/ml; glycogen synthase b_1 or b_2 , 0.1 mg/ml (Antoniw *et al.*, 1977).

Effect of the protein phosphatase inhibitor on the phosphorylase kinase phosphatase activities of protein phosphatases-II and -III

The effect of the i_2 protein on the activity of protein phosphatase-II against the α -subunit of phosphorylase kinase is compared in Fig. 7 with its effect on the activity of protein phosphatase-III against the β -subunit of phosphorylase kinase. The inhibition of protein phosphatase-II was only about 10% at an i_2 concentration 50 times that which inhibited protein phosphatase-III by 50%. The i_2 protein is therefore at least 200 times more effective in inhibiting protein phosphatase-III than in inhibiting protein phosphatase-II.

Since phosphorylase kinase fully phosphorylated in both the α - and β -subunits was used to assay both protein phosphatase-II and protein phosphatase-III, this is consistent with the results of Brandt *et al.* (1975), which showed that the protein phosphatase inhibitor in liver exerted its effect by binding to the phosphatase and not to the substrate. The same conclusion can be drawn from a consideration of the relative amounts of the i_2 protein and the protein substrate present in the assays. The partially purified i_2 protein inhibited the phosphorylase phosphatase activity of protein phosphatase-III by 50% at 5 μg/ml,

whereas the phosphorylase α concentration was 1.0 mg/ml in this assay (Fig. 6).

Effect of the protein phosphatase inhibitor on the various activities of protein phosphatases-I, -II and -III

In the preceding paper (Antoniw *et al.*, 1977) it was shown that trace phosphorylase phosphatase activity is associated with protein phosphatases-I and -II. It was not clear whether this represented an inherent activity of these two phosphatases or contamination with aggregated forms of protein phosphatase-III. Fig. 8 shows that the trace phosphorylase phosphatase activity of protein phosphatases-I and -II was only inhibited 5–10% by the i_2 protein, even at a concentration 50-fold in excess of that required to inhibit the phosphorylase activity of protein phosphatase-III by 50%. This indicates that protein phosphatases-I and -II do indeed have slight inherent phosphorylase phosphatase activity.

The effect of the i_2 protein on the various activities of protein phosphatases-I, -II and -III is summarized in Table 3. The results show that protein phosphatases-I and -II also have inherent glycogen synthase phosphatase-1 and glycogen synthase phosphatase-2 activities, and protein phosphatase-I has trace phosphorylase kinase phosphatase activity

Fig. 7. Effect of the protein phosphatase inhibitor on the phosphorylase kinase phosphatase activities of protein phosphatases-II and -III

○, α-Phosphorylase kinase phosphatase activity of protein phosphatase-II; ●, β-phosphorylase kinase phosphatase activity of protein phosphatase-III. Protein phosphatases-II and -III were each diluted to a concentration which dephosphorylated 10% of the phosphorylase kinase substrate in 10 min. The assays were carried out as described in the Experimental section with the same 32 P-labelled phosphorylase kinase substrate, which contained 1.05 mol of phosphate in the α -subunit and 1.05 mol of phosphate in the β -subunit (Antoniw & Cohen, 1976).

Fig. 8. Influence of the protein phosphatase inhibitor on the phosphorylase phosphatase activity of protein phosphatases-I, -II and -III

▼, Protein phosphatase-I; ○, protein phosphatase-II; ●, protein phosphatase-III. Each protein phosphatase was diluted to 1.0 unit/ml and assays were carried out with the same phosphorylase *a* substrate as described in the Experimental section.

Table 3. Effect of the protein phosphatase inhibitor (50 units) on the various activities of protein phosphatases-I, -II and -III

Peak fractions of protein phosphatases from Sephadex G-200 (Antoniw *et al.*, 1977) were assayed in the presence and absence of a fixed amount of the protein phosphatase inhibitor (50 units). The three phosphatases were diluted appropriately to give the same activity against any given substrate. Phosphorylase kinase was fully phosphorylated in both the α - and β -subunits. The final substrate concentrations were: phosphorylase *a*, 1.0mg/ml; phosphorylase kinase, 1.0mg/ml; glycogen synthase *b*₁ or *b*₂, 0.1mg/ml; histone H1, 0.1mg/ml (Antoniw *et al.*, 1977). Very similar results were obtained in experiments with two preparations of protein phosphatase inhibitor, protein phosphatase-III and phospho-protein substrate.

Phosphoprotein substrate	Percentage inhibition		
	Protein phosphatase-I	Protein phosphatase-II	Protein phosphatase-III
1. Phosphorylase <i>a</i>	15	11	96
2. Phosphorylase kinase	20	6	95
3. Glycogen synthase <i>b</i> ₁	13	11	99
4. Glycogen synthase <i>b</i> ₂	14	4	91
5. Histone H1	22	18	84

(against the α -subunit), since these activities are not sensitive to inhibition by the *i*₂ protein, whereas the corresponding activities of protein phosphatase-III

are inhibited by the *i*₂ protein under the same conditions.

When protein phosphatases-I and -II were incubated with trypsin, in the absence of the *i*₂ protein, according to the conditions described in Table 2, there was no detectable increase in the trace phosphorylase phosphatase activity of these enzymes (not illustrated). This shows that protein phosphatases-I and -II do not represent complexes formed between protein phosphatase-III and the protein phosphatase inhibitor.

Effect of the protein phosphatase inhibitor on muscle extracts

Since the *i*₂ protein appeared to be a specific inhibitor of protein phosphatase-III, it was decided to see whether it could be used to distinguish between different classes of protein phosphatases in muscle extracts. The results of this experiment are shown in Table 4. An *i*₂ protein concentration which inhibited the various activities of partially purified protein phosphatase-III B by 90% inhibited the phosphorylase phosphatase, glycogen synthase phosphatase-1 and glycogen synthase phosphatase-2 activities in muscle extracts by about 80%. This demonstrates that 90% of the phosphorylase phosphatase, glycogen synthase phosphatase-1 and -2 activities in muscle extracts are catalysed by protein phosphatase-III, when measured under the standard assay conditions. In contrast, the dephosphorylation of phosphorylase kinase phosphorylated in both the α - and β -subunits, catalysed by muscle extracts, was inhibited only by 35% (Table 4). This is consistent with previous findings (Antoniw & Cohen, 1976) that there is slightly more α -phosphorylase kinase phosphatase activity (catalysed by protein phosphat-

Table 4. Effect of the protein phosphatase inhibitor on protein phosphatase activities in muscle extracts

Muscle extracts were passed through Sephadex G-25 columns equilibrated in solution A+50mm-mercaptoethanol. They were then diluted to 1.0 unit/ml and assayed as described in the Experimental section, in the presence and absence of a fixed amount of protein phosphatase inhibitor. The same amount of protein phosphatase inhibitor inhibited partially purified protein phosphatase III B diluted to 1.0 unit/ml by 90%. Phosphorylase kinase was fully phosphorylated in both the α - and β -subunits. The final substrate concentrations in the assay are given in Table 3. Almost identical values were obtained with extracts prepared from two different animals.

Phosphoprotein substrate	Inhibition (%)
Phosphorylase <i>a</i>	81
Glycogen synthase <i>b</i> ₁	77
Glycogen synthase <i>b</i> ₂	82
Phosphorylase kinase	35

Table 5. *Typical values for the distribution of protein phosphatases, cyclic AMP-dependent protein kinase and their respective inhibitors during the isolation of the protein-glycogen complex*

Protein phosphatase-II was measured with phosphorylase kinase specifically phosphorylated in the α -subunit as substrate. Protein phosphatase-III was measured with phosphorylase *a* as substrate. For protein phosphatase inhibitor and protein kinase inhibitor, a 3.0ml sample of each fraction was heated for 3 min in a boiling-water bath. The solution was cooled and centrifuged and the supernatant dialysed exhaustively against solution A, to remove low-mol.wt. inhibitors, before each protein inhibitor was assayed. Values for the protein inhibitor 'activities' assume that no loss of inhibitory 'activity' occurs during boiling. The extract and pH6.1 supernatant were filtered through Sephadex G-25 equilibrated in solution A+50mM-mercaptoethanol before being assayed for protein phosphatases-II and -III. The final concentrations in protein phosphatase assays are given in Table 3. Very similar results were obtained for three separate preparations.

Step	Protein phosphatase-II	Protein phosphatase-III	Protein phosphatase inhibitor	Cyclic AMP-dependent protein kinase	Protein kinase inhibitor
1. Extract	100	100	100	100	100
2. pH6.1 supernatant	62	25	68	97	80
3. pH6.1 precipitate	7	58	6	2	9
4. 78000g supernatant	4	1	3	—	2
5. 78000g precipitate	4	47	0	—	8

ase-II and insensitive to the i_2 protein) than β -phosphorylase kinase phosphatase (catalysed by protein phosphatase-III and sensitive to the i_2 protein).

Distribution of protein phosphatases-II and -III, cyclic AMP-dependent protein kinase, the protein kinase inhibitor and the protein phosphatase inhibitor during the isolation of the protein-glycogen complex

Muscle extracts were fractionated to step 2 of the procedure for the purification of the protein phosphatase inhibitor described in the Experimental section. The protein-glycogen pellet, which was separated at this stage, was then resuspended at neutral pH and re-centrifuged at 78000g for 100 min. The various fractions were assayed for the following activities: protein phosphatase-III, with phosphorylase *a* as substrate; protein phosphatase-II, with phosphorylase kinase specifically phosphorylated in the α -subunit as substrate; the protein phosphatase inhibitor; cyclic AMP-dependent protein kinase; the protein kinase inhibitor. The results are shown in Table 5. Protein phosphatase-III is bound very tightly to the complex. Approx. 50% of the activity in the muscle extract was recovered in the final 78000g pellet. This is a little higher than the value reported by Meyer *et al.* (1970). It is comparable with the proportion of glycogen synthase recovered in the complex and considerably higher than the recovery of other enzymes of glycogen metabolism, such as phosphorylase kinase, phosphorylase and debranching enzyme (Meyer *et al.*, 1970; Taylor *et al.*, 1975). In contrast, protein phosphatase-II, the protein phosphatase inhibitor, cyclic AMP-dependent protein kinase and the protein kinase inhibitor were not precipitated with the complex, and these activities were largely recovered in the pH6.1 supernatant (Table 5).

Discussion

A heat-stable protein, distinct from the protein inhibitor of cyclic AMP-dependent protein kinase, has been purified 700-fold from skeletal muscle and shown to be a specific inhibitor of protein phosphatase-III. The protein is tentatively assumed to be the i_2 protein described by Huang & Glimsman (1976a,b), although further experiments will be necessary to establish whether or not this is the case.

The protein phosphatase inhibitor inhibited each of the four activities of protein phosphatase-III [phosphorylase phosphatase, β -phosphorylase kinase phosphatase, glycogen synthase phosphatase-1 and glycogen synthase phosphatase-2 (Antoniw *et al.*, 1977)] in a similar manner, but was at least 200 times less effective as an inhibitor of protein phosphatases-I and -II (Figs. 6, 7 and 8 and Table 3). These properties were used to demonstrate that protein phosphatase-III accounts for virtually all of the phosphorylase phosphatase and glycogen synthase phosphatase activity in muscle extracts as measured under the standard assay conditions (Table 4). The results strongly suggest that there is a single major activity (protein phosphatase-III) that catalyses each of the dephosphorylations that inhibit glycogenolysis or activate glycogen synthesis, as was proposed in the preceding paper (Antoniw *et al.*, 1977). Proteinphosphatase-III is specifically located in the protein-glycogen complex (Table 5) and this emphasizes its importance in the regulation of glycogen metabolism.

In the preceding paper, Antoniw *et al.* (1977) suggested that the enzyme that catalyses the dephosphorylation of the α -subunit of phosphorylase kinase (protein phosphatase-II) is distinct from that which catalyses the dephosphorylation of the β -subunit of

phosphorylase kinase (protein phosphatase-III), since the two enzymes have quite different specificities for phosphoprotein substrates and different behaviour on gel filtration. This is supported by two further pieces of evidence reported in this paper. (1) Protein phosphatase-III is inhibited by the protein phosphatase inhibitor, whereas protein phosphatase-II is not. (2) Protein phosphatase-III is precipitated with the protein-glycogen complex, whereas protein phosphatase-II does not.

Since these two enzymes each dephosphorylate just one of the two sites on phosphorylase kinase that are phosphorylated by cyclic AMP-dependent protein kinase (Antoniw & Cohen, 1976), there is not a single enzyme in mammalian cells which dephosphorylates all the serine residues that are phosphorylated by cyclic AMP-dependent protein kinase. It is therefore interesting to consider what features of phosphorylase kinase determine the specificities of these two phosphatases.

In the first paper of this series (Yeaman *et al.*, 1977), the amino acid sequences at these two sites were presented, and each was shown to contain two adjacent basic amino acids just *N*-terminal to the phosphorylatable serine residue. The presence of two such basic amino acids appears to be a feature common to the best substrates for cyclic AMP-dependent protein kinase (Yeaman *et al.*, 1977; Nimmo & Cohen, 1977), and the use of synthetic peptide substrates has indicated that this may be particularly important in the recognition of substrates (Zetterqvist *et al.*, 1976; Kemp *et al.*, 1976). The existence of separate enzymes for the dephosphorylation of the α - and β -subunits of phosphorylase kinase suggests that these two phosphatases and cyclic AMP-dependent protein kinase do not recognize the same structural features of phosphorylase kinase.

The protein phosphatase inhibitor did not inhibit the glycogen synthase phosphatase activity or the slight phosphorylase phosphatase activity associated with protein phosphatases-I and -II (Fig. 8 and Table 3). This suggests that the activities are not derived from contamination with aggregated forms of protein phosphatase-III, and that they may be inherent activities of these two phosphatases. Although these activities account for no more than about 10% of the total glycogen synthase phosphatase and phosphorylase phosphatase activities when muscle extracts are assayed under a standard set of conditions (Table 4), these conditions are not physiological, and it is not possible to estimate how active protein phosphatases-I and -II are against these substrates *in vivo*. However, two points can be made. Firstly, the activity of protein phosphatase-II against the α -subunit of phosphorylase kinase is very high compared with its activity against the other phosphorylation sites of the enzymes of glycogen

metabolism (Antoniw *et al.*, 1977). It therefore seems likely that the α -subunit of phosphorylase kinase represents a physiological substrate for this enzyme. The function of protein phosphatase-II seems to be to maintain phosphorylase kinase in a high-activity form (Antoniw & Cohen, 1976) and so promote glycogenolysis. Since this is in direct contrast with the functions of phosphorylase phosphatase and glycogen synthase phosphatase, the slight activity of protein phosphatase-II against phosphorylase *a* or glycogen synthase *b* may merely represent 'non-specific' dephosphorylation of no relevance *in vivo*. Secondly, the activity of protein phosphatase-I is so poor against all phosphoprotein substrates other than histones (Antoniw *et al.*, 1977) that this enzyme may not be relevant to glycogen metabolism.

It is, however, important to note that all three protein phosphatases show at least trace activity against almost any phosphoprotein that has so far been tested. Krebs (1973) originally proposed five criteria that should be met before an effect mediated by cyclic AMP could be said to take place through a protein-phosphorylation mechanism. The fifth of these was that a protein phosphatase should exist that reverses the phosphorylation catalysed by cyclic AMP-dependent protein kinase. The present analysis of the specificities of protein phosphatases in skeletal muscle suggests that this criterion is always likely to be met, and that it may be difficult to decide which of several such enzymes is the 'relevant' phosphatase *in vivo*.

The function of the protein phosphatase inhibitor is not yet known. However, its high degree of specificity for protein phosphatase-III suggests that it may be useful in distinguishing between different classes of protein phosphatases in mammalian tissue, and that it may become as useful in this respect as the protein inhibitor of cyclic AMP-dependent protein kinase.

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