THE BROAD SPECIFICITY PROTEIN PHOSPHATASE FROM MAMMALIAN LIVER

Separation of the M_r 35 000 catalytic subunit into two distinct enzymes

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1. Introduction

The reversible phosphorylation of enzymes is recognized to be one of the major general mechanisms by which cellular metabolism is regulated by neural and hormonal stimuli. An important generality that seems to be emerging is that enzymes in biodegradative pathways are activated and enzymes in biosynthetic pathways inactivated by phosphorylation [1,2]. This suggests that different metabolic pathways may be regulated in a co-ordinated manner by common protein kinases and protein phosphatases. This concept is already established in the case of cyclic AMP-dependent protein kinase, which mediates the actions of hormones which use cyclic AMP as a second messenger [1,3].

There is increasing evidence that protein phosphatases are also important targets for hormone action [1]. The current dogma is that a single broad specificity catalytic subunit (M_r 35 000) exists in liver, skeletal muscle and heart muscle, which is capable of reversing phosphorylation reactions catalysed by both cyclic AMP-dependent and -independent protein kinases [4–9]. This enzyme, which has been termed protein phosphatase C [10], has been obtained in highly purified form from each of these tissues using an 80% ethanol precipitation at room temperature at an early stage of the preparation. This treatment not only denatures many contaminating proteins, but is also believed to dissociate the catalytic subunit from higher M_r complexes which may contain regulatory subunits [10].

In this laboratory two protein phosphatases have been implicated in the regulation of glycogen metabolism in rabbit skeletal muscle. Protein phosphatase-1 accounts for 80–90% of the phosphorylase phosphatase activity, 70–80% of the glycogen synthase phosphatase activity, and >90% of the activity against the β-subunit of phosphorylase kinase in this tissue. Protein phosphatase-2, on the other hand, possesses >90% of the activity against the α-subunit of phosphorylase kinase, but also has some phosphorylase phosphatase and glycogen synthase phosphatase activity [11–13]. The enzymes can also be distinguished by the use of two heat-stable proteins termed inhibitor-1 and inhibitor-2, which only inhibit protein phosphatase-1 [13–15].

Here, we demonstrate that protein phosphatase C isolated from either rat liver or rabbit liver is not a single enzyme. It consists of at least two different low M_r protein phosphatases whose properties suggest that they are either the catalytic subunits or fragments of protein phosphatase-1 and protein phosphatase-2.

The criteria used to distinguish these enzymes should be of general significance for the classification of all protein phosphatases.

2. Methods

2.1. *Protein preparation*

Phosphorylase *b* [16], phosphorylase kinase [17], inhibitor-2 [18] and the active phosphorylated form of inhibitor-1 [19] were homogeneous preparations from rabbit skeletal muscle. The catalytic subunit of cyclic AMP-dependent protein kinase was purified by chromatography on CM-Sephadex [20]. ^{32}P-Labelled phosphorylase *a* was prepared from phosphorylase *b* using phosphorylase kinase [12]. ^{32}P-Labelled

phosphorylase kinase was prepared using the catalytic subunit of cyclic AMP-dependent protein kinase and contained about 1 molecule of phosphate in each α-subunit and 1 molecule in each β-subunit [15,18]. Bovine erythrocyte carbonic anhydrase, hen egg ovalbumin and bovine serum albumin were purchased from Sigma.

2.2. *Purification of protein phosphatases*

Protein phosphatase C was prepared from rabbit and rat livers as in [4] up to and including the second DEAE-Sephadex step. The rabbit liver protein phosphatase C used for most of the studies had spec. act. 3500 U/mg. It contained a major protein-staining band and a number of minor bands when examined by electrophoresis on 10% polyacrylamide gels in the presence of sodium dodecyl sulphate (SDS). The major band (M_r 37 000) migrated between ovalbumin (M_r 43 000) and carbonic anhydrase (M_r 29 500). Preparations of protein phosphatase C from rat liver had lower specific activities (about 1000–1500 U/mg) and that used for most of the studies was prepared by T. S. I. in the laboratory of Professor David Gibson, Department of Biochemistry, Indiana University School of Medicine, USA. As expected, rat liver (fig.1) and rabbit liver protein phosphatase C were eluted as single peaks of activity (M_r 34 000) when subjected to gel filtration on Sephadex G-100 Superfine.

Protein phosphatase-1 was isolated from rabbit skeletal muscle by an improved procedure [21] and had spec. act. 3500 U/mg. It was stored at −20°C in solution A (50 mM Tris–HCl (pH 7.0)–1.0 mM EDTA–0.3% (v/v) 2-mercaptoethanol) containing 6.0 mM $MnCl_2$ and 50% ethanediol. Protein phosphatase-1 was eluted from the final gel filtration on Sephadex G-200 as two different M_r forms (M_r 70 000–75 000 and M_r 35 000–45 000). The smaller form was used here, although both had identical enzymatic properties (see section 4).

All protein phosphatase preparations were completely free of inhibitor-1 and inhibitor-2.

2.3. *Assay of protein phosphatases*

Immediately prior to assay, enzymes were diluted appropriately in solution A containing 2 mg bovine serum albumin/ml) The diluted protein phosphatase (0.02 ml) was mixed with 0.02 ml solution A containing 0.01% Brij 35 [18] and incubated for 10 min at 30°C. The reactions were initiated with 0.02 ml ^{32}P-labelled phosphorylase *a* (3.0 mg/ml) in solution A and then carried out as in [18,19]. One unit of activity is that amount which releases 1.0 nmol phosphate/min.

In experiments where ATP, inhibitor-1 or inhibitor-2 were added, these were included in the 0.02 ml solution A containing 0.01% Brij 35, and added at the start of the 10 min pre-incubation. When inhibitor-1 was added, the pre-incubation contained ^{32}P-labelled phosphorylase *a* and the reaction was initiated with protein phosphatase which had been pre-incubated with solution A for 90 min at 30°C. This was done to minimize the dephosphorylation and inactivation of inhibitor-1 [15].

One unit of inhibitor-1 or inhibitor-2 is that amount which inhibits 0.02 units of phosphorylase phosphatase activity by 50% in the standard assay.

Assays of phosphorylase kinase phosphatase were identical to phosphorylase phosphatase except that 2.0 mM $MnCl_2$ was included and the final concentration of ^{32}P-labelled phosphorylase kinase was 0.8 mg/ml.

3. Results

Protein phosphatase C from rat and rabbit liver behaved as a single enzyme throughout the 6 purification steps [4], and also on a subsequent gel filtration (fig.1). Nevertheless, the evidence presented below indicates that each preparation is a mixture of two distinct low M_r phosphatases.

3.1. *Effects of inhibitor-1 and inhibitor-2 on the activities of protein phosphatase C and protein phosphatase-1*

The effect of inhibitor-2 on the phosphorylase phosphatase activities of 3 protein phosphatase preparations is illustrated in fig.2. As expected, protein phosphatase-1 was almost completely inhibited by 100 units of inhibitor-2. In contrast the activities of rat liver and rabbit liver protein phosphatase C were only inhibited 50% and 25%, respectively. Nevertheless, half-maximal effects were observed in each case with only 1.0 units of inhibitor-2. A similar result was obtained with inhibitor-1 (not shown). Since inhibitor-1 and inhibitor-2 appear to be highly specific for protein phosphatase-1 [13], the results suggested that only 50% and 25% of the phosphorylase phosphatase activity in the rat and rabbit liver protein

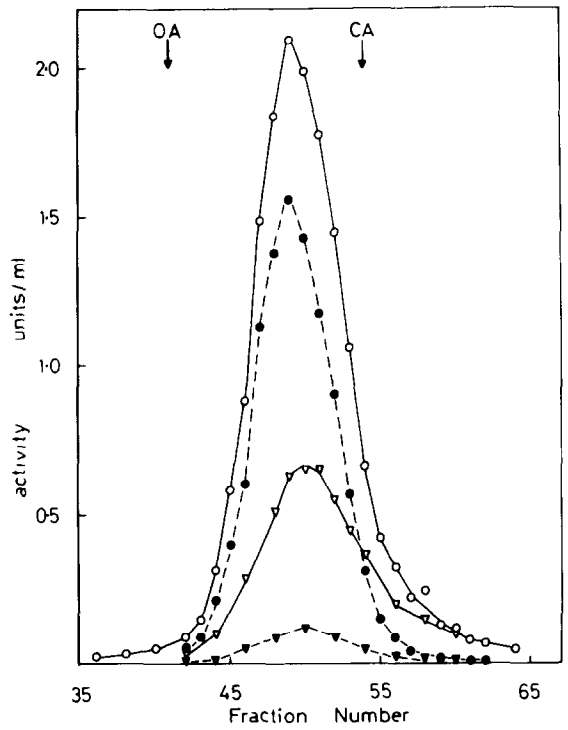

Fig.1. Gel filtration of rat liver protein phosphatase C on Sephadex G-100 Superfine (41.5 × 1.9 cm). The column was equilibrated in solution A containing 6 mM $MnCl_2$ and 0.01% Brij 35 as stabilising agents. After 31 ml buffer had passed through the column, 0.9 ml fractions were collected at 3.0 ml/h flow rate. The column was assayed for phosphorylase activity in the presence of 10 units of inhibitor-2 (●---●) after pre-incubation with 0.1 mM ATP (▼——▼), in the presence of inhibitor-2 after pre-incubation with ATP (▼---▼) or with no additions (○——○). The position of the marker proteins ovalbumin (OA, M_r 43 000) and carbonic anhydrase (CA, M_r 29 500) are denoted by arrows.

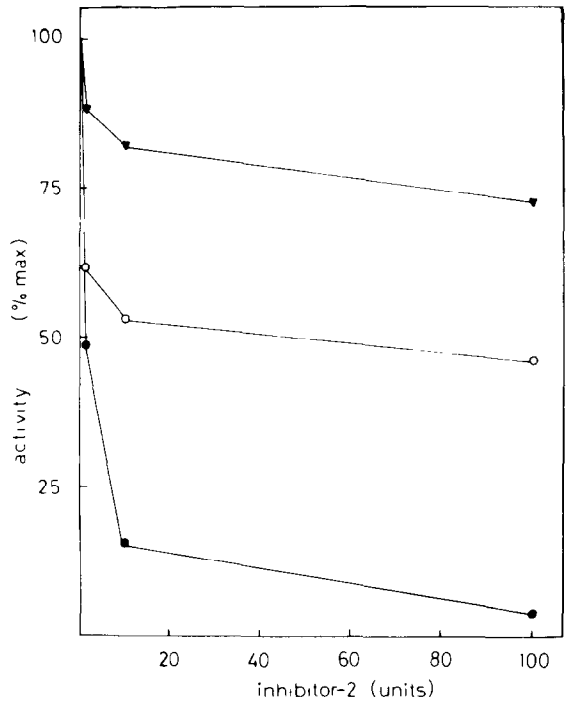

Fig.2. Influence of inhibitor-2 on the activity of protein phosphatases. Skeletal muscle protein phosphatase-1 (●——●), rat liver protein phosphatase C (○——○), rabbit liver protein phosphatase C (▼——▼). All the assays contained 0.01 units of phosphorylase phosphatase activity.

phosphatase C preparations was catalysed by protein phosphatase-1, and that the remainder of the activity was due to another enzyme.

3.2. *Dephosphorylation of the α- and β-subunits of phosphorylase kinase by protein phosphatase C and protein phosphatase-1*

The results of these experiments are illustrated in fig.3. As reported [11–13] protein phosphatase-1 was highly specific for the β-subunit of phosphorylase kinase, the dephosphorylation of the α-subunit being an order of magnitude slower. The dephosphorylation of both subunits was blocked by inhibitor-2 (fig.3C).

Rabbit liver protein phosphatase C dephosphorylated the α-subunit 9-fold faster than the β-subunit and this reaction was only inhibited slightly by inhibitor-2. The slow dephosphorylation of the β-subunit was inhibited to a significantly greater extent (fig.3A).

Rat liver protein phosphatase C dephosphorylated the α- and β-subunits at similar rates. The dephosphorylation of the β-subunit but not the α-subunit was largely blocked by inhibitor-2 (fig.3B).

The phosphorylase kinase phosphatase assays were done in the presence of Mn^{2+}. If the divalent cation was excluded, the dephosphorylation of the α-subunit by rabbit liver protein phosphatase C was decreased by at least 1 order of magnitude. In contrast the phosphorylase phosphatase activity was only slightly decreased in the absence of Mn^{2+}.

These results strongly supported the conclusions drawn from the effects of inhibitors on the phosphorylase phosphatase activity (fig.2). The finding that the dephosphorylation of the β-subunit was

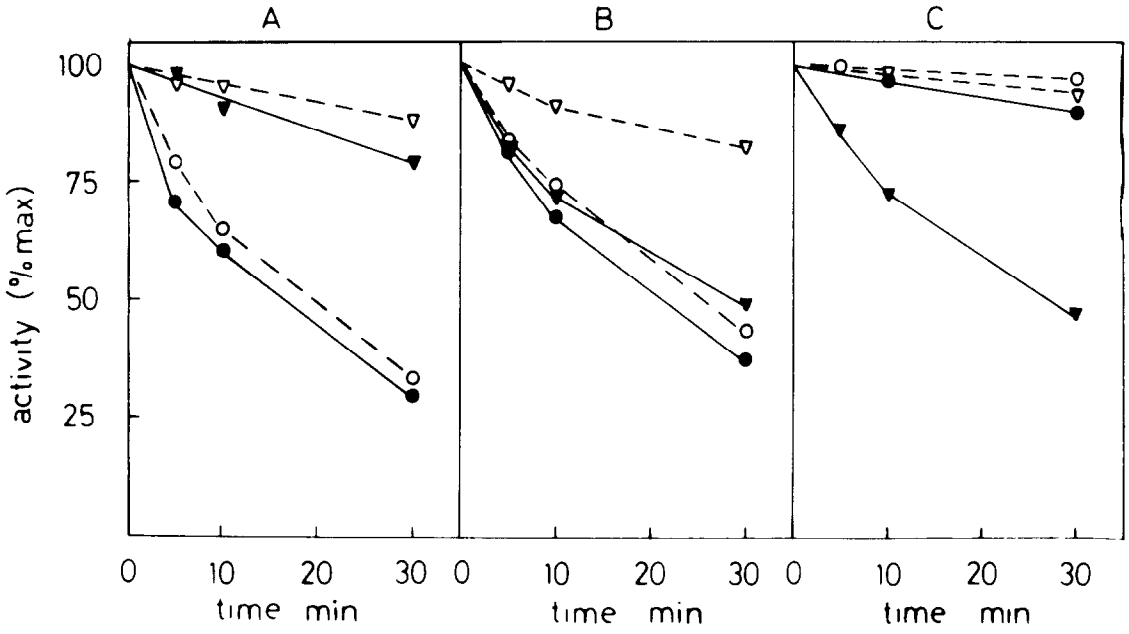

Fig.3. Dephosphorylation of the α- and β-subunits of phosphorylase kinase by protein phosphatases: (A) rabbit liver protein phosphatase C; (B) rat liver protein phosphatase C; (C) rabbit muscle protein phosphatase-1. The assays were done in the presence of $MnCl_2$ in either the absence (●——●, ▼——▼) or the presence (○ – –○, ▽ – – ▽) of 100 units of inhibitor-2. The dephosphorylation of the α-subunit is shown by circles and the dephosphorylation of the β-subunit by triangles. At various times aliquots were analysed for ^{32}P-radioactivity released. Further aliquots were denatured in SDS and subjected to polyacrylamide gel electrophoresis. The gels were stained with Coomassie blue, destained, and the bands corresponding to the α- and β-subunits were sliced out and analysed by Cerenkov counting. The ^{32}P-radioactivity in each subunit was computed from the total ^{32}P-radioactivity released and the α/β ratio determined by gel electrophoresis. The ordinate shows the phosphate remaining in either subunit as a percentage of the maximum (% max).

blocked by inhibitor-2 demonstrated that the rat liver and to a lesser extent the rabbit liver protein phosphatase C, contained protein phosphatase-1. However, the presence of a powerful inhibitor-insensitive α-phosphorylase kinase phosphatase activity in both preparations confirmed the presence of a second enzyme. The properties of this enzyme suggested that it was related to protein phosphatase-2 from rabbit muscle [13].

3.3. *Effect of ATP on the activities of protein phosphatase C and protein phosphatase-1*

Protein phosphatases from a variety of tissues have been shown to undergo a time-dependent inactivation when pre-incubated with ATP, which can be reversed by incubation with either Mn^{2+} or Co^{2+} [22–25]. Here, we found that half maximal inhibition of muscle protein phosphatase-1 required a 20-fold higher concentration of ATP (0.35 mM) than did rabbit liver protein phosphatase C (predominantly protein phosphatase-2). At 0.1 mM ATP, inhibition of protein phosphatase-2 was >90% while that of protein phosphatase-1 was only 25–30%.

3.4. *Gel filtration of rat liver protein phosphatase C and protein phosphatase-1 on Sephadex G-100 Superfine*

Rat liver protein phosphatase C was subjected to gel filtration and the fractions were assayed for phosphorylase phosphatase activity either in the presence of inhibitor-2 (10 units) to measure protein phosphatase-2 or after pre-incubation with 0.1 mM ATP to measure protein phosphatase-1. The results demonstrated that the two enzymes have very similar M_r values although protein phosphatase-1 (M_r 33 000) reproducibly appeared one or two tubes later than protein phosphatase-2 (M_r 34 000). The use of ATP to inhibit protein phosphatase-2 specifically was validated by the observation that in the presence of 0.1 mM ATP the phosphorylase phosphatase activities

of the peak fractions were inhibited >90% by inhibitor-2, while in the absence of ATP, the inhibition was only 40% (fig.1).

The protein phosphatase-1 from rabbit skeletal muscle used in these studies was also passed through Sephadex G-100 Superfine. Its elution position was identical to protein phosphatase C yielding M_r 33 000–34 000.

3.5. *Chromatography of rat liver protein phosphatase C on DEAE-cellulose*

Although the purification of protein phosphatase C involves two chromatographies on DEAE–Sephadex, stepwise elutions from 0.18–0.24 M NaCl are used in each case. Rat liver protein phosphatase C was therefore subjected to gradient elution from DEAE-cellulose in a further attempt to separate the two protein phosphatases contained in this preparation. The results (fig.4) demonstrated a partial resolution, protein phosphatase-1 eluting at 0.15 M NaCl and protein phosphatase-2 at 0.17 M NaCl. These two enzymes were identified by assaying for phosphorylase phosphatase in the presence of either ATP or inhibitor-2 (fig.4A) as well as by measuring the α- and β-phosphorylase kinase phosphatase activities (fig.4B).

4. Discussion

These results demonstrate that protein phosphatase C isolated from mammalian liver contains at least two protein phosphatases (M_r 33 000–34 000). They have quite distinct enzymatic properties, but very similar physical properties, which is the reason they are not resolved by the standard purification procedure [4]. One enzyme appears to be identical to protein phosphatase-1 from skeletal muscle based on its sensitivity to inhibitor-1 and inhibitor-2 and specificity for the β-subunit of phosphorylase kinase. The second enzyme appears to be related to protein phosphatase-2 from skeletal muscle based on its lack of sensitivity to the inhibitor proteins and specificity for the α-subunit of phosphorylase kinase [13].

These findings raise several important questions concerning the nature of low M_r broad specificity protein phosphatases that have been isolated by other laboratories. Several groups have purified 'protein phosphatase C' to apparent homogeneity from mammalian liver and heart muscle [4–8]. These preparations all showed a single protein staining band when examined by SDS–polyacrylamide gel electrophoresis (M_r 30 000–35 000). However none of the criteria used in this present work, that would have distin-

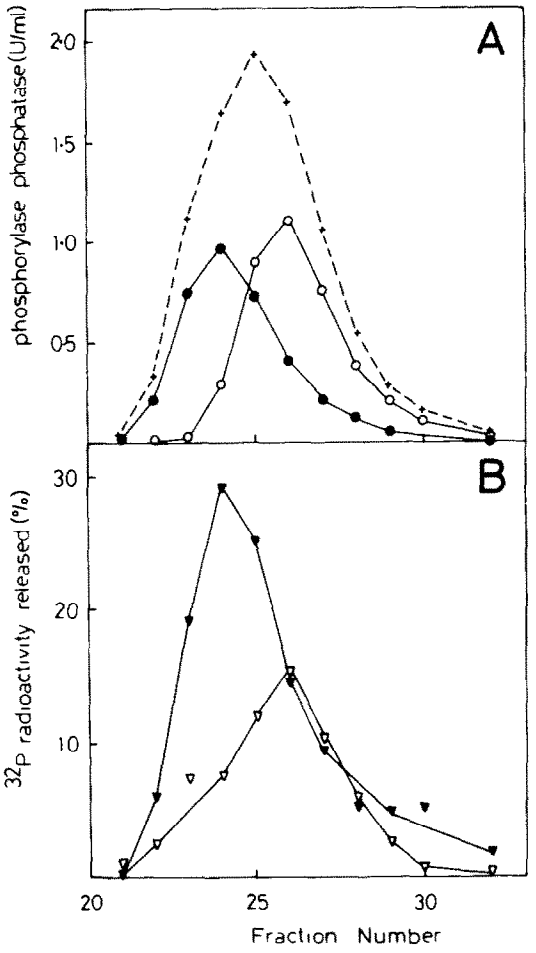

Fig.4. Chromatography of rat liver protein phosphatase C on DEAE-cellulose (4.5 × 1.2 cm). The column was equilibrated in 10 mM Tris–HCl (pH 7.0) containing 0.1 mM EDTA, 3.0 mM $MnCl_2$, 0.01% Brij 35 and 0.1% (v/v) 2-mercaptoethanol and the samples applied in the same buffer. The column was developed with a linear NaCl gradient from 0–0.4 M NaCl in 100 ml total vol. The flow rate was 30 ml/h and 2.0 ml fractions were collected. The fractions were assayed for phosphorylase phosphatase activity (A) with no additions (+––+), in the presence of 50 units of inhibitor-2 (○——○), or after preincubation with 0.1 mM ATP (●——●). They were also assayed for phosphorylase kinase phosphatase activity (B). The dephosphorylation of the α-and β-subunits is shown by ▽ and ▼, respectively. The ordinate shows the % release from each subunit in a 20 min assay, and the experiments were performed as described in the legend to fig.3.

guished protein phosphatase-1 from protein phosphatase-2, were employed. The composition of these preparations is therefore unclear. On the other hand, a broad specificity protein phosphatase was isolated from rat liver (M_r 35 000) [9] whose activity was unaffected by inhibitor-1 and inhibitor-2 and this enzyme is likely to be protein phosphatase-2.

Measurements of phosphorylase phosphatase done in gel filtered liver and muscle extracts at high dilution, and in the presence and absence of inhibitor proteins, has demonstrated that protein phosphatase-1 accounts for 50% and 90%, respectively, of the activities when assays contain 1.0 mM EDTA [26]. When extracts contain 1.0 mM $MnCl_2$, protein phosphatase-1 accounts for 25% and 80% of the activity, respectively, since protein phosphatase-2 is stimulated about 2-fold by $MnCl_2$ whereas protein phosphatase-1 is slightly inhibited. If skeletal muscle or liver extracts are treated with 80% ethanol at room temperature, protein phosphatase-1 is lost to a variable extent whereas the phosphorylase phosphatase activity of protein phosphatase-2 is increased about 2-fold. The recovery of protein phosphatase-1 from ion-exchange and gel-filtration columns can also be much lower than protein phosphatase-2, particularly if EDTA is present in the buffers. The classical procedure [4], on which many subsequent purifications have been based, therefore tends to select for protein phosphatase-2 over protein phosphatase-1, and indeed some preparations of rat liver protein phosphatase C contained much less protein phosphatase-1 than that used here. It is not clear whether chromatography on hexane–diamine Sepharose [4] or histone–Sepharose [8] which have been used as a final purification step by several workers, would remove one of the two enzymes.

Protein phosphatase C preparations have been shown to dephosphorylate many proteins, including the enzymes glycogen phosphorylase, phosphorylase kinase, glycogen synthase [27], the regulatory subunit of cyclic AMP-dependent protein kinase [5], pyruvate kinase [9], acetyl-CoA carboxylase [28], hydroxymethylglutaryl (HMG) CoA reductase [29], HMG–CoA reductase kinase [29] and hormone-sensitive triglyceride lipase [30]. For this reason 'protein phosphatase C' has been implicated in the control of a variety of metabolic pathways. Since protein phosphatase-1 and protein phosphatase-2 have broad, but quite distinct substrate specificities, it is essential to define whether protein phosphatase-1 or protein phosphatase-2 was the major dephosphorylating activity in each of these systems. In particular, the rat liver protein phosphatase C preparation used here, was also used to dephosphorylate HMG-CoA reductase [29], HMG-CoA reductase kinase [29] and acetyl-CoA carboxylase [28].

The protein phosphatase-1 present in liver 'protein phosphatase C' has apparent M_r 33 000 (fig.1), as did the skeletal muscle protein phosphatase-1 used here. However protein phosphatase-1 has also been isolated in higher M_r forms (45 000, 75 000 and 125 000) [12,13]. It is not yet clear whether the smaller forms are fragments [31] or subunits of the larger species. However all forms have the same substrate specificities [12,32,33] and sensitivity to the inhibitor proteins [12,13].

Several high M_r protein phosphatases can be separated by chromatography of tissue extracts on DEAE-cellulose, and dissociated to more active M_r 35 000 catalytic subunits [10,34–38]. Our experiments suggest that most, if not all, of these enzymes contain a type-2 catalytic subunit. The high M_r forms of protein phosphatase-2 differ considerably in their relative activities towards phosphorylase *a* and phosphorylase kinase, and one of these corresponds to the protein phosphatase-2 isolated from rabbit skeletal muscle [11–13]. The possibility that the different forms of protein phosphatase-2 contain distinct regulatory subunits which determine the molecular size and substrate specificity is under investigation.

In [33] the criteria used to distinguish protein phosphatase-1 and -2 have been applied to a homogeneous protein phosphatase from reticulocytes. On the basis of the results, this enzyme is classified as a type-2 protein phosphatase.

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