CLASSIFICATION OF AN eIF-2 PHOSPHATASE AS A TYPE-2 PROTEIN PHOSPHATASE

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1. Introduction

As more and more proteins are reported to undergo regulation by phosphorylation–dephosphorylation, the number of protein phosphatases described in the literature has increased exponentially. However most of these enzymes have been reported to dephosphorylate several substrates, and this has raised the possibility that there are relatively few protein phosphatase catalytic subunits in mammalian tissues. This idea is supported by the observation that several high M_r protein phosphatases each contain a catalytic subunit of M_r 30 000–35 000 [1–5], which can be generated by the addition of ethanol at room temperature [1,2,5], freezing and thawing in 0.2 M 2-mercaptoethanol [3,4] or treatment with urea [6]. This subunit has been termed protein phosphatase C [1], and found to possess a broad substrate specificity [1,7]. However, this preparation is a mixture of two distinct enzymes with very similar physical properties [8]. Furthermore, these activities are closely related to two enzymes termed protein phosphatase-1 and protein phosphatase-2, which have been implicated in the regulation of glycogen metabolism in mammalian muscle [9–11].

The two enzymes termed type-1 and type-2 can be distinguished readily by the use of two criteria:

1. Type-1 protein phosphatase is potently inhibited by two proteins termed inhibitor-1 and inhibitor-2 which do not affect the activity of type-2 protein phosphatase [12–15].

2. Type-1 protein phosphatase dephosphorylates the β-subunit of phosphorylase kinase much faster than the α-subunit, whereas type-2 protein phosphatase dephosphorylates the α-subunit much faster than the β-subunit [9–11].

These two criteria appear to be essential for the initial classification of all protein phosphatases.

The α-subunit of protein synthesis initiation factor eIF-2 is phosphorylated in reticulocyte lysates by a protein kinase known as eIF-2 α-kinase, and the phosphorylation of this subunit appears to co-ordinate protein synthesis with the overall metabolic state of the cell [16]. We have purified an eIF-2 phosphatase to apparent homogeneity; it is composed of two different subunits of M_r 60 000 and M_r 38 000 [17]. Here, the above criteria are used to classify this enzyme as a type-2 protein phosphatase.

2. Materials and methods

2.1. *Protein preparations*

Initiation factor eIF-2 [18] and eIF-2 phosphatase [17] were purified to homogeneity and eIF-2 α-kinase [19] was partially purified from rabbit reticulocyte lysates. ^{32}P-Labelled eIF-2 was prepared by phosphorylation with eIF-2 α-kinase, dialysed against 50 mM Tris–HCl–50 mM mercaptoethanol (pH 7.0) (solution A) and stored at 0°C. eIF-2 phosphatase was stored at –180°C in 20 mM Tris–HCl (pH 7.5) containing 0.1 M KCl, 0.1 mM EDTA, 1.0 mM dithiothreitol and 10% glycerol. All other proteins and ^{32}P-labelled substrates were prepared as in [8].

2.2. *Assay of protein phosphatases and inhibitor proteins*

The phosphorylase phosphatase, phosphorylase kinase phosphatase and eIF-2 phosphatase activities of protein phosphatase-1 and eIF-2 phosphatase were measured as in [8] in the presence of either 1.0 mM EDTA or 1.0 mM free $MnCl_2$. The final concentrations of ^{32}P-labelled substrates were: phosphorylase *a*

(1.0 mg/ml); eIF-2 (0.002 mg/ml); and phosphorylase kinase (0.8 mg/ml). The protein phosphatase activities were also measured in the presence of inhibitor-1 and inhibitor-2 as in [8].

3. Results

3.1. *Substrate specificity of eIF-2 phosphatase*

Homogeneous preparations of eIF-2 phosphatase catalysed the dephosphorylation of both phosphorylase *a* and phosphorylase kinase. The initial rate of dephosphorylation of all 3 substrates (measured as % phosphate released) in the presence of 1.0 mM EDTA was similar (eIF-2 phosphatase/phosphorylase phosphatase/α-phosphorylase kinase phosphatase = 100:50:60). The inclusion of $MnCl_2$ (1.0 mM) instead of 1.0 mM EDTA, stimulated the phosphorylase kinase phosphatase activity 5-fold and the phosphorylase phosphatase activity 2-fold.

Protein phosphatase-1 also catalysed the dephosphorylation of eIF-2 at comparable rates to phosphorylase *a* and phosphorylase kinase. The initial rates of dephosphorylation (measured as % phosphate released) in the presence of 1.0 mM EDTA were similar (eIF-2 phosphatase/phosphorylase phosphatase/β-phosphorylase kinase phosphatase = 100:120:180). The inclusion of $MnCl_2$ (1.0 mM) instead of 1.0 mM EDTA stimulated the phosphorylase kinase phosphatase activity 2-fold but inhibited the phosphorylase phosphatase activity by 40%.

3.2. *The phosphorylase kinase phosphatase activity of eIF-2 phosphatase*

The rate of phosphate release from the α- and β-subunits of phosphorylase kinase by eIF-2 phosphatase is shown in fig.1. eIF-2 phosphatase was highly specific for the α-subunit. This site was 30% dephosphorylated in 10 min, whereas the β-subunit was hardly dephosphorylated after 90 min.

3.3. *Effect of inhibitor-1 and inhibitor-2 on the phosphorylase phosphatase activity of eIF-2 phosphatase and protein phosphatase-1*

Inhibitor-1 had no effect on the activity of eIF-2 phosphatase, even at concentrations 60-fold higher than those which inhibited protein phosphatase-1 by 50% (fig.2A). Inhibitor-2 only inhibited eIF-2 phosphatase by 5–10% at concentrations 50-fold higher than those which inhibited protein phosphatase-1 by 50% (fig.2B).

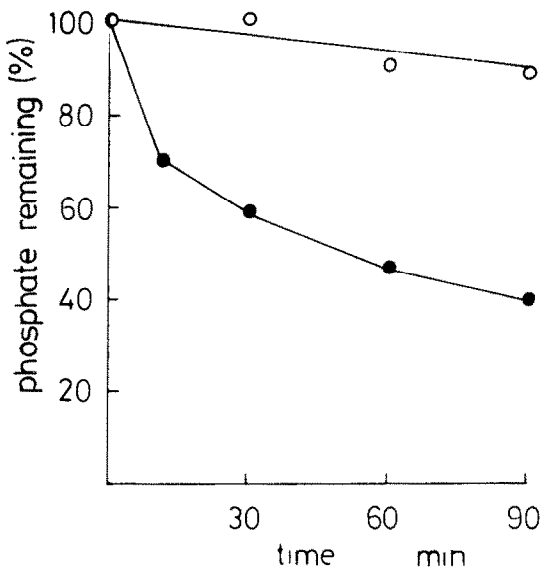

Fig.1. Dephosphorylation of phosphorylase kinase by eIF-2 phosphatase. ^{32}P-Labelled phosphorylase kinase containing 1.0 mol phosphate in the α-subunit and 0.8 mol phosphate in the β-subunit was incubated with eIF-2 phosphatase under the standard assay conditions. At the times indicated, aliquots were analysed for the ^{32}P-radioactivity remaining in each subunit, as described in [8]. (•) α-subunit; (○) β-subunit.

4. Discussion

In [8], a set of criteria were established for classifying protein phosphatases based on the likelihood that they contain one of two types of basic catalytic subunit, termed type-1 and type-2. This paper describes the first application of these criteria to a homogeneous protein phosphatase of unknown classification. The results clearly demonstrate the enzyme is a type-2 and not a type-1 protein phosphatase.

eIF-2 phosphatase is composed of two subunits M_r 60 000 and M_r 38 000 [17] and although the catalytic subunit has not yet been identified, it is likely to be the component of M_r 38 000 [8]. Thus in this enzyme the type-2 catalytic subunit is complexed with a larger subunit, which may have a regulatory function. The M_r of eIF-2 phosphatase (=95 000) remains constant at each step of the purification and is about 50% the size of protein phosphatase-2 from rabbit skeletal muscle [10].

eIF-2 phosphatase is not specific for eIF-2 and not only dephosphorylates glycogen phosphorylase and phosphorylase kinase, but a variety of other phospho-

proteins (unpublished). Nevertheless, the enzyme may be much more specific in vivo. It has been demonstrated that eIF-2 phosphatase can only dephosphorylate the α-subunit of eIF-2 in reticulocyte lysates, whereas it dephosphorylates both the α- and β-subunits of purified eIF-2 [17]. The binding of eIF-2 to another component of the protein synthetic machinery may prevent the dephosphorylation of the β-subunit of eIF-2 by eIF-2 phosphatase in vivo.

Protein phosphatase-1 from skeletal muscle dephosphorylates eIF-2 at a comparable rate to eIF-2 phosphatase, when normalized to the rate of dephosphorylation of phosphorylase (see section 3). Since protein phosphatase-1 is present in reticulocytes (J. G. F., unpublished) this raises the question of which enzyme dephosphorylates the α-subunit of eIF-2 in vivo. Since large losses of activity were encountered during the purification of eIF-2 phosphatase [17], the possibility that a second eIF-2 phosphatase (protein phosphatase-1) was lost, cannot yet be excluded.

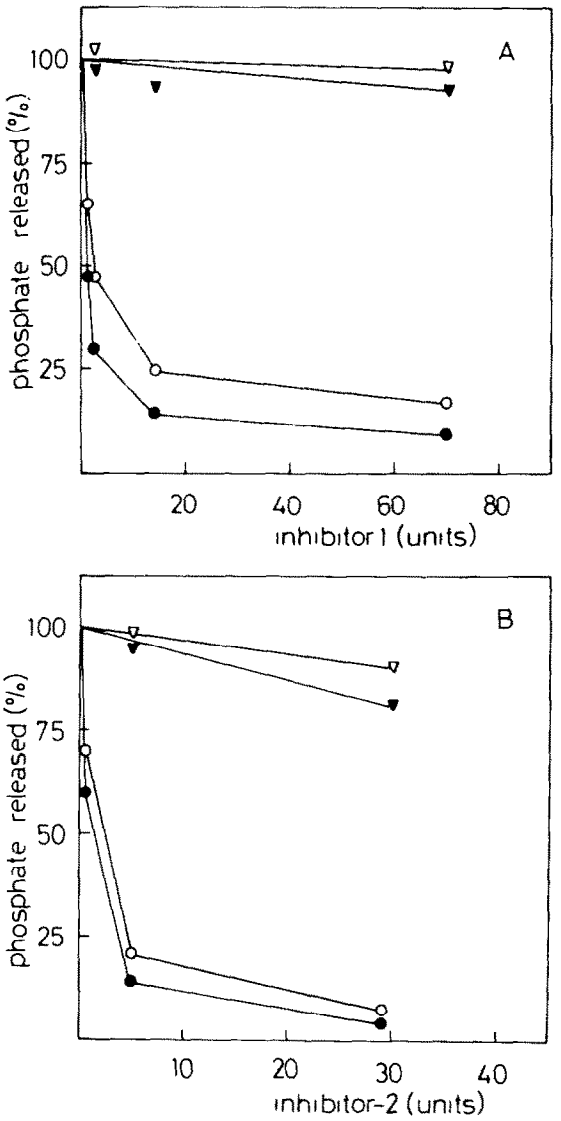

Fig.2. Effect of inhibitor-1 (A) and inhibitor-2 (B) on the phosphorylase phosphatase activity of protein phosphatase-1 (○,●) and eIF-2 (▽,▼). The assays were done as in section 2. Since the inhibition of protein phosphatase-1 depends on the concentration of the enzyme in the assay, experiments were carried out at two different enzyme concentrations with both phosphatases. (○,▽) Assays containing 0.02 units of protein phosphatase; (●,▼) assays containing 0.004 units.

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