EVIDENCE FOR THE INVOLVEMENT OF PROTEIN PHOSPHATASE-1 IN THE REGULATION OF METABOLIC PROCESSES OTHER THAN GLYCOGEN METABOLISM

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1. Introduction

Cyclic AMP dependent protein kinase was originally discovered as the trace endogenous contaminant in phosphorylase kinase, that was responsible for activation of the latter enzyme following incubation with cyclic AMP, ATP and magnesium ions [1]. Subsequently, cyclic AMP dependent protein kinase was found to regulate the activity of other proteins, and it is now firmly established that this enzyme mediates most, if not all, of the intracellular actions of hormones which work through cyclic AMP, according to the following series of reactions [2]:

$$\text{hormone} \rightarrow \begin{matrix}\text{raised}\\ \text{cyclic}\\ \text{AMP}\end{matrix} \rightarrow \begin{matrix}\text{activated}\\ \text{protein}\\ \text{kinase}\end{matrix} \rightarrow$$

$$\begin{matrix}\text{phosphorylated}\\ \text{protein}\end{matrix} \rightarrow \begin{matrix}\text{physiological}\\ \text{response}\end{matrix}$$

One of the first pieces of evidence which implicated cyclic AMP dependent protein kinase in the regulation of cellular processes other than glycogen metabolism, was the finding that its activity was relatively constant over a wide range of mammalian tissues, and was high even in those tissues where glycogen metabolism was of very minor importance [3]. This initiated the search for other physiological substrates for the enzyme, and well established examples now include histone H1 and L-type pyruvate kinase (liver), cholesterol esterase (adrenal cortex), triglyceride lipase (adipose tissue), troponin-I (cardiac muscle) and acetyl CoA carboxylase (mammary gland) [4,5].

These findings have raised the question of whether the dephosphorylation of each of these proteins is catalysed by a single protein phosphatase activity. The possibility that this could be so, again relies heavily on information available from studies of the regulation of glycogen metabolism in skeletal muscle. This laboratory has demonstrated that a single major enzyme in skeletal muscle, termed protein phosphatase-1*, catalyses each of the dephosphorylations which inhibit glycogenolysis or activate glycogen synthesis [4,6–8]. It therefore not only reverses the phosphorylation of phosphorylase kinase and glycogen synthetase catalysed by cyclic AMP dependent protein kinase but it also reverses the phosphorylation of phosphorylase catalysed by phosphorylase kinase and the phosphorylation of glycogen synthetase catalysed by glycogen synthetase kinase-2 [4,6] i.e. protein kinases whose activities are not regulated by cyclic nucleotides.

The ability of protein phosphatase-1 to catalyse a number of functionally related dephosphorylations in glycogen metabolism demonstrates that this enzyme has a rather broad specificity, and the idea that it may dephosphorylate other enzymes in vivo is particularly attractive since all interconvertible enzymes involved in biodegradative pathways are activated by phosphorylation, while all interconvertible enzymes which catalyse reactions in biosynthetic pathways are inactivated by phosphorylation [4].

The investigation of this possibility has been

* This enzyme was previously termed protein phosphatase-III [4,6]. The reasons for the change in nomenclature are discussed in [7]

facilitated by the recent discovery that skeletal muscle contains two heat stable proteins, termed inhibitor-1 and inhibitor-2, which appear to specifically inhibit protein phosphatase-1. The inhibitions are reversible and occur with K_i values in the nM range. Inhibitor-1 is only effective after it has been phosphorylated by cyclic AMP dependent protein kinase [8–10].

In this paper, we have measured the ability of mammalian tissue extracts to dephosphorylate muscle phosphorylase *a*, and have tested the susceptibility of this activity to inhibition by highly purified preparation of inhibitor-1 and inhibitor-2. The results strongly support the idea of a wider role for this enzyme in the regulation of metabolism.

2. Materials and methods

2.1. *Protein and enzyme preparations*

Phosphorylase *b* [11], phosphorylase kinase [12] and inhibitor-1 [10] were purified to homogeneity from rabbit skeletal muscle, and ^{32}P-labelled phosphorylase *a* (5×10^7 cpm per μmol) was prepared from phosphorylase *b* using purified phosphorylase kinase and [γ-^{32}P]ATP [6]. The protein inhibitor of cyclic AMP dependent protein kinase was partially purified as a byproduct of the purification of inhibitor-1 [10]. Inhibitor-2 also copurified through the first three steps of this procedure and was then further purified by gel filtration on Sephadex G-100 at pH 8.5 and chromatography on DEAE-cellulose at pH 5.0. It was enriched 30 000-fold over the muscle extracts and had a purity of 70–80% (J. G. Foulkes and P. Cohen, unpublished work). Protein phosphatase-1 was purified 300-400 fold from rabbit skeletal muscle [6,13].

2.2. *Preparation of tissue extracts*

New Zealand White rabbits were killed by an intravenous injection of nembutal, exsanguinated, and the skeletal muscle and other tissues were excised and chilled in ice. The skeletal muscle was minced and homogenised in a Waring Blender with 2.0 vol. of 4.0 mM EDTA/15 mM mercaptoethanol pH 7.0. The other tissues were chopped finely with scissors and homogenized in an 'Ultra-Turrex' homogeniser (Janke and Kunkel) using 2.5 vol. of the same buffer. The homogenates were centrifuged at 10 000 × *g* for 30 min and passed through glass wool to remove fat.

2.3. *Measurement of phosphorylase, phosphorylase kinase and cyclic AMP dependent protein kinase activities in tissue extracts*

Phosphorylase kinase was assayed at pH 8.6 [12] and phosphorylase in the direction of glycogen synthesis with 5′-AMP [14]. Cyclic AMP dependent protein kinase was assayed using a mixed histone substrate (Sigma type IIA) [8]. The control incubations were carried out in the presence of a large excess of the protein kinase inhibitor.

2.4. *Measurement of phosphorylase phosphatase activity in tissue extracts*

Tissue extracts contain low molecular weight compounds which are powerful inhibitors of protein phosphatase-1 [10]. In order to obtain reliable estimates of the activity, extracts (2.0 ml) were therefore passed through a column of Sephadex G-25 superfine (15 × 1 cm) equilibrated in 50 mM Tris–HCl/1.0 mM EDTA/15 mM mercaptoethanol, pH 7.0 prior to assay. The assays were also carried out at a final dilution over the tissue extracts of at least 1:60, this dilution being sufficient to eliminate inhibition of protein phosphatase-1 by high molecular weight compounds present in each extract.

Phosphorylase phosphatase activity was assayed in the presence and absence of inhibitor-1 and inhibitor-2 as described previously [6,8,10]. Manganese chloride was omitted from all assays.

3. Results

3.1. *Enzyme activity measurements in tissue extracts*

The phosphorylase, phosphorylase kinase, phosphorylase phosphatase and cyclic AMP dependent protein kinase activities are given in table 1. Phosphorylase and phosphorylase kinase were only measured in tissue extracts where the phosphorylase was of the 'muscle' or 'brain' type, and not the 'liver' type. The activities of the *b*-form and *a*-form of these two isoenzymes are very similar when assays are carried out in the presence of 5′-AMP [16]. The activities should therefore measure the relative concentration of phosphorylase in these tissues. The

Table 1
Relative specific activities of phosphorylase, phosphorylase kinase, phosphorylase phosphatase and cyclic AMP dependent protein kinase in rabbit tissue extracts

Tissue	Phosphorylase	Phosphorylase kinase	Phosphorylase phosphatase	Cyclic AMP dependent protein kinase
Skeletal muscle	100	100	100	100
Cardiac muscle	10	5.3	47	120
Brain	5.6	2.3	55	250
Mammary gland[a]	2.3	2.2	45	170
Adipose tissue[b]	0.5	0.3	52	430
Liver			37	
Kidney			47	
Lung			45	

[a] During peak lactation
[b] Supra-renal adipose tissue

The results are the average of values from at least three different extracts and are expressed relative to skeletal muscle = 100. Protein concentrations were determined by the method of Bradford [15]. The specific activities in skeletal muscle were: phosphorylase (5.6 U/mg), phosphorylase kinase (84 U/mg), phosphorylase phosphatase (0.96 U/mg), cyclic AMP dependent protein kinase (0.08 U/mg). One unit of activity is the number of nmol (phosphorylase kinase, phosphorylase phosphatase, cyclic AMP dependent protein kinase) or μmol (phosphorylase) of product produced per min

specific activities of phosphorylase and phosphorylase kinase were found to vary 200–300 fold in the five tissues studied (table 2), but the activity ratio phosphorylase/phosphorylase kinase was rather constant. In contrast, the specific activity of phosphorylase phosphatase only varied 2–3 fold, while the activity ratio phosphorylase/phosphorylase phosphatase varied by 100–fold. The distribution of phosphorylase phosphatase activity did not therefore parallel that of phosphorylase and phosphorylase kinase. In this respect it resembled cyclic AMP dependent protein kinase, which was high in tissues where the glycogenolytic enzymes were very low (table 1).

3.2. *Influence of inhibitor-1 and inhibitor-2 on the phosphorylase phosphatase activity of tissue extracts*

These two proteins inhibited the phosphorylase phosphatase activity of all tissue extracts examined (fig.1). One unit of inhibitor-1, which corresponds to a concentration of only 5 nM or 0.1 μg/ml in the assay, inhibited phosphorylase phosphatase activity in the tissue extracts by 30–46%. One unit of inhibitor-2, corresponding to a concentration of about 12 nM or

Table 2
Effect of inhibitor-1 and inhibitor-2 (10 units) on the activity of phosphorylase phosphatase in rabbit tissue extracts

Tissue extract	Inhibition (%)	
	Inhibitor-1	Inhibitor-2
Skeletal muscle	68	78
Mammary gland[a]	62	69
Cardiac muscle	63	73
Liver	57	66
Brain	53	64
Kidney	66	62
Lung	62	72
Adipose tissue[b]	71	85
Protein Phosphatase-1[c]	80	90

[a] During peak lactation
[b] Supra-renal adipose tissue
[c] Purified 300-fold from skeletal muscle [6,13]

The assays contained 0.01 ± 0.004 U of phosphorylase phosphatase One unit of inhibitor is that amount which inhibits 0.02 U of partially purified protein phosphatase-1 by 50% in the standard assay. The results with each tissue are the average of experiments carried out with three different extracts

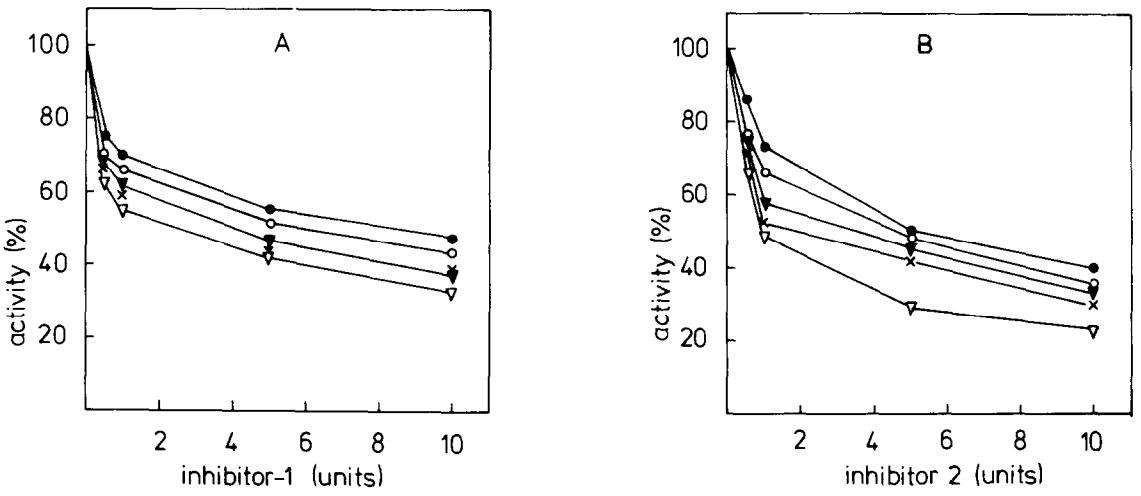

Fig.1. Influence of inhibitor-1 (A) and inhibitor-2 (B) on the phosphorylase phosphatase activity of rabbit tissue extracts. Each extract was suitably diluted so that the assays contained 0.01 ± 0.004 units of phosphorylase phosphatase. One unit of inhibitor is that amount which inhibits 0.02 units of partially purified protein phosphatase-1 by 50% in the standard assay. Symbols: •, brain; ○, liver; ▼, heart; × mammary gland; ▽ skeletal muscle.

0.4 μg/ml in the assay, inhibited the activities by 27–52%.

The inhibition of phosphorylase phosphatase in the tissue extracts was a little less than with partially purified protein phosphatase-1 (table 2). This discrepancy and the slight variation from tissue to tissue, could be explained in a number of ways. For example, inhibitor-1 and inhibitor-2 may interact with proteins other than protein phosphatase-1. Alternatively, tissue extracts may contain protein phosphatases which have phosphorylase phosphatase activity, but are unaffected by inhibitor-1 or inhibitor-2. This is certainly the case in skeletal muscle, which contains two additional protein phosphatases which have slight phosphorylase phosphatase activity and are insensitive to the inhibitor proteins [6,8].

4. Discussion

The results presented in this paper have shown that the phosphorylase phosphatase activity of rabbit tissues is remarkably constant even in tissues where the phosphorylase and phosphorylase kinase activities are very low (table 1). Furthermore these activities are extremely sensitive to inhibitor-1 and inhibitor-2, which inhibit protein phosphatase-1 specifically. This information allows the following inferences to be made. Firstly, protein phosphatase-1 or a closely analogous protein is present at a very similar concentration in all of these tissues. Secondly, this enzyme is likely to dephosphorylate protein substrates other than those involved in glycogen metabolism. Severson et al. [17] have stated that a highly purified preparation of phosphorylase phosphatase from rabbit liver was able to inactivate hormone sensitive triglyceride lipase from chicken adipose tissue, which had been activated by cyclic AMP dependent protein kinase. Ingebritsen et al. [18] have also reported that liver microsomal hydroxymethylglutaryl CoA reductase which had been inactivated by incubation with ATP and magnesium ions could be reactivated by a protein phosphatase preparation, which appeared to copurify with liver phosphorylase phosphatase. Similarly, protein phosphatase-1 from skeletal muscle has been found to catalyse the dephosphorylation of acetyl CoA carboxylase from mammary gland, which has been phosphorylated by either cyclic AMP dependent protein kinase or by acetyl CoA carboxylase kinase-2([5], D.G. Hardie and P. Cohen, unpublished experiments from this laboratory). These observations strengthen the view that protein phosphatase-1 may

have a number of physiological substrates, and the identification of more of these proteins should help in the development of an integrated view of the regulation of intermediary metabolism in mammalian tissues.

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