IDENTIFICATION OF THE Ca^{2+}-DEPENDENT MODULATOR PROTEIN AS THE FOURTH SUBUNIT OF RABBIT SKELETAL MUSCLE PHOSPHORYLASE KINASE

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1. Introduction

Kakuichi et al. [1] and Cheung [2,3] were the first to demonstrate the presence of a factor in brain homogenates, which in the presence of Ca^{2+}, stimulated the activity of one of the cyclic nucleotide phosphodiesterases of this tissue. This factor was subsequently shown to be a small heat stable calcium binding protein, which was present in high concentrations in a wide variety of animal tissues [4–6]. Following its purification to apparent homogeneity from bovine brain [7] and bovine heart [8], it was noted that its physico-chemical properties were very similar to the calcium-binding subunit of rabbit skeletal muscle troponin, troponin-C, the protein which confers calcium sensitivity to actomyosin ATPase [9,10]. This idea was substantiated by the determination of the amino acid sequence of the 'calcium-dependent modulator' from bovine brain [11] and rat testis [12], which showed extensive homology with troponin-C, and by the finding that the 'modulator' could substitute for troponin-C in restoring calcium sensitivity to actomyosin ATPase in reconstituted systems [13]. Troponin-C can also substitute for the 'modulator' in the activation of cyclic nucleotide phosphodiesterase, although a 600-fold higher concentration is required [14].

Over the past two or three years it has become apparent that the 'modulator' is involved in the regulation of several other systems which are controlled by Ca^{2+}. It activates adenylate cyclase from bovine brain [15,16], activates the membrane-bound Ca^{2+}-dependent ATPase of human red blood cells [17,18] and stimulates erythrocyte membrane calcium transport [19], and it is a subunit of the Ca^{2+}-dependent myosin light chain kinase of smooth muscle [20] and skeletal muscle [21].

These discoveries suggest that the 'modulator' may be involved in further metabolic processes which are controlled by Ca^{2+}. Skeletal muscle phosphorylase kinase is activated by micromolar concentrations of Ca^{2+} and this activation reaction is believed to represent the mechanism by which glycogenolysis and muscle contraction are synchronized. Phosphorylase kinase was reported by both this laboratory [22] and by Hayakawa et al. [23,24] to be composed of three types of subunit, termed α, β and γ, which had molecular weights of 145 000, 128 000 and 45 000 respectively [22], the smallest active species being the dodecamer $(\alpha\beta\gamma)_4$ [22]. This structure would appear to exclude the involvement of the 'modulator' (mol. wt 16 700) in the regulation of phosphorylase kinase activity by Ca^{2+}. However since the modulator is very small and very acidic, it would only be expected

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to stain faintly compared to the other subunits, even if it were present at an equimolar concentration. In addition, it would have been expected to migrate with the bromophenol blue dye front on the 5% polyacrylamide gels that were used previously for the resolution of the α-, β- and γ-subunits [22].

In this paper we demonstrate that rabbit skeletal muscle phosphorylase kinase does indeed contain a fourth component, termed the δ-subunit, which appears to be identical with the 'modulator protein'.

2. Materials and methods

Phosphorylase kinase [22], troponin-C [25], the P-light chain of myosin [27] and 'modulator'-free myosin light chain kinase [28] were homogeneous preparations isolated from rabbit skeletal muscle. A pure preparation of the Ca^{2+}-dependent 'modulator' [10] and a partially purified preparation of 'modulator'-sensitive cyclic nucleotide phosphodiesterase [29] were isolated from bovine brain. Phosphorylase kinase was assayed at pH 8.6 [22] and cyclic nucleotide phosphodiesterase was measured by a standard procedure [10].

Polyacrylamide gel electrophoresis was carried out as in [22] on 10% gels, and the gels were scanned using a Joyce-Loebl Chromoscan 200. Amino acid analyses were performed on a Beckman Multichrom analyser using a single column separation system and 'Locarte' ion-exchange resin.

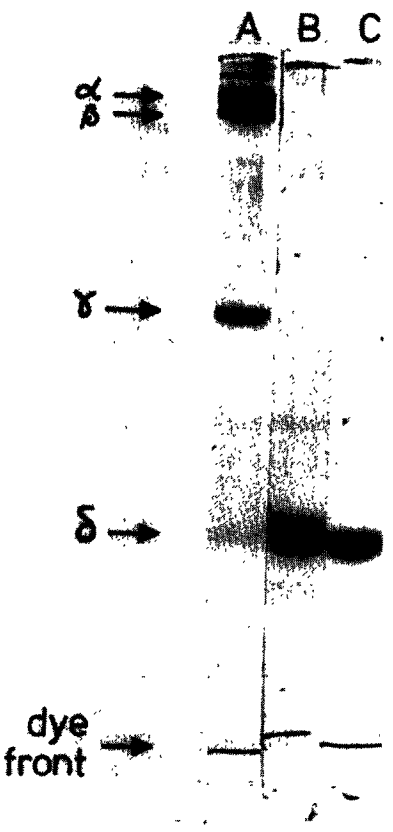

Fig.1. Electrophoresis on 10% polyacrylamide gels in the presence of sodium dodecyl sulphate. (A) phosphorylase kinase; (B) purified δ-subunit; (C), bovine brain modulator protein. Migration is from top to bottom and the gels were stained with Coomassie blue.

3. Results

3.1. *Detection of the δ-subunit of phosphorylase kinase*

Phosphorylase kinase was subjected to electrophoresis on 10% polyacrylamide gels in the presence of sodium dodecyl sulphate, instead of the 5% gels used in [22]. The α-, β- and γ-subunits were all present, as expected, but in addition, a much smaller component was also visible (fig.1A). This component, termed δ, migrated with the bromophenol blue dye front on 5% polyacrylamide gels (not illustrated).

When individual fractions from the final step of the purification of phosphorylase kinase, gel filtration on Sepharose 4B, were examined by polyacrylamide gel electrophoresis, the δ-subunit like the α-, β- and γ-subunits was found to follow the peak of phosphorylase kinase exactly. In order to obtain an initial estimate of the relative concentration of the δ-subunit, polyacrylamide gels from several different phosphorylase kinase preparations were scanned using a densitometer. Taking the molecular weight of the γ- and δ-subunits as 45 000 and 17 000, respectively, and assuming that Coomassie blue stains these two components uniformly, the molar ratio γ/δ was estimated to be 1:0.55.

The purified 'modulator' from bovine brain was found to have an electrophoretic mobility identical to that of the δ-subunit on 10% polyacrylamide gels

(fig.1C), and mixtures of the two components could not be resolved (not illustrated). In contrast, rabbit skeletal muscle troponin-C migrated slightly slower than either the δ-subunit or the 'modulator' and could just be resolved from these two components by electrophoresis (not illustrated).

Since the 'modulator' is known to be heat stable, phosphorylase kinase (10.0 mg/ml), which had been dialysed against 50 mM Tris–HCl/200 mM NaCl/ 1.0 mM EDTA/15 mM mercaptoethanol, pH 7.0, was heated in a boiling water bath for 2 min. The temperature of the solution rose to 85°C during this incubation and the thick white suspension that formed, was then removed by centrifugation at 20 000 × *g* for 15 min. The supernatant contained highly purified δ-subunits, the α-, β- and γ-subunits having been precipitated completely by the heat treatment.

The supernatant was applied to a 3 × 1 cm column of DEAE–Sephadex A-50 (Pharmacia) equilibrated in 50 mM Tris–HCl/200 mM NaCl/1.0 mM EDTA/15 mM mercaptoethanol, pH 7.0, at room temperature (20°C). The column was washed with the same buffer containing 300 mM NaCl and eluted with buffer containing 600 mM NaCl. The 600 mM NaCl eluate contained the δ-subunit, which was homogeneous by the criterion of polyacrylamide gel electrophoresis (fig.1B), faint minor impurities of higher molecular weights having been eliminated at 200 mM NaCl. The 'modulator' from bovine brain was also eluted from DEAE–Sephadex at the same salt concentration [10].

3.2. *Amino acid composition and spectral properties of the δ-subunit*

The amino acid compositions of the purified δ-subunit, the 'modulator' from bovine brain and troponin-C from rabbit muscle are given in table 1. The composition of the δ-subunit and 'modulator' were almost identical but were distinct from troponin-C. The high threonine/serine ratio, high

Table 1
Amino acid composition of the δ-subunit of phosphorylase kinase

Amino acid	δ-Subunit[a]		'Modulator' protein[b]	Troponin-C[c]
Aspartic acid	23.5	(24)	23	22
Threonine	11.8	(12)	12	6
Serine	5.1	(5)	4	7
Glutamic acid	27.0	(27)	27	31
Proline	2.1	(2)	2	1
Glycine	11.0	(11)	11	13
Alanine	11.1	(11)	11	13
Valine	7.3	(7)	7	7
Methionine	8.2	(8)	9	10
Isoleucine	7.6	(8)	8	9
Leucine	9.0	(9)	9	9
Tyrosine	1.8	(2)	2	2
Phenylalanine	7.3	(7)	8	10
Histidine	1.0	(1)	1	1
Trimethyllysine[d]	0.58	(1)	1	0
Lysine	7.1	(7)	7	9
Arginine	5.8	(6)	6	7
Cysteine	0	(0)	0	1
Tryptophan	0	(0)	0	0
		149	148	158

[a] Average of two different preparations
[b] From the amino acid sequence of the bovine brain protein [11]
[c] From the amino acid sequence of rabbit skeletal muscle protein [30]
[d] The colour value of trimethyllysine was assumed to be the same as histidine

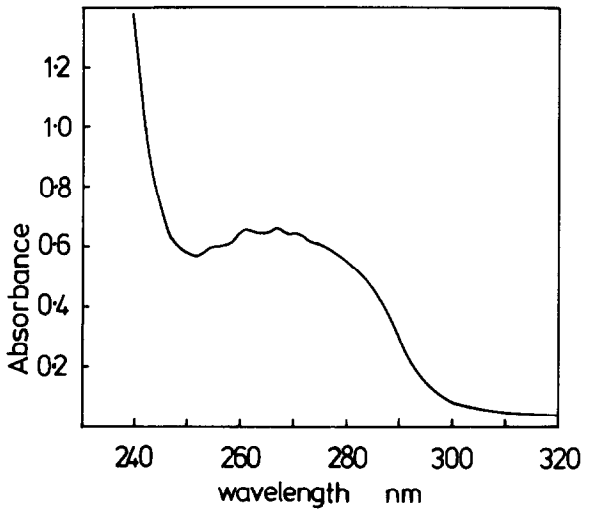

Fig.2. Ultraviolet absorption spectrum of the purified δ-subunit.

methionine, high phenylalanine and low tyrosine contents are very distinctive. A consequence of the proportion of aromatic amino acids is that the δ-subunit (like the 'modulator') shows the fine spectrum of phenylalanine with A_{max} at 255, 261, 267 and 271 nm (fig.2).

The amino acid analysis of the 'modulator' from bovine brain showed an unusual component that was shown to be trimethyllysine [31]. This component was eluted at 155 min, 2.5 min after histidine and 4 min before lysine. The δ-subunit contained the same unusual peak (table 1) which is presumed to be trimethyllysine.

3.3. *Molar proportion of the δ-subunit*

The δ-subunit should represent 5% of the phosphorylase kinase molecule by weight, if it is present at an equimolar concentration with the α-, β- and γ-subunits. 140 mg phosphorylase kinase should therefore contain 7.0 mg δ-subunit. When this quantity of enzyme was subjected to the heat treatment and chromatography on DEAE–Sephadex, 5.0 mg of purified δ-subunit was isolated, as measured by amino acid analysis. Since the δ-subunit would not be expected to be recovered quantitatively by the purification procedure, the results demonstrate that the δ-subunit is present at near stoichiometric concentrations with the other three subunits.

3.4. *Effect of the δ-subunit on cyclic nucleotide phosphodiesterase activity*

The purified δ-subunit was able to stimulate the activity of the 'modulator'-sensitive cyclic nucleotide phosphodiesterase, to almost the same extent as the 'modulator' from bovine brain. The activation required Ca^{2+}, and the basal activity was stimulated about 10-fold (table 2).

Purified phosphorylase kinase was also able to activate cyclic nucleotide phosphodiesterase in the presence of Ca^{2+} (not illustrated).

3.5. *Effect of the δ-subunit on the activity of myosin light chain kinase*

Skeletal muscle myosin light chain kinase is almost completely dependent on the Ca^{2+}-dependent 'modulator' for activity [21]. The purified δ-subunit activated myosin light chain kinase in an identical manner to the 'modulator' from bovine brain. A solution of myosin light chain kinase (3.7 nM) was half-maximally activated by δ-subunit (1.5 nM) indicating that the two proteins form stoichiometric complexes in the assay (fig.3).

Purified phosphorylase kinase also activated myosin light chain kinase to the same extent as either the purified δ-subunit or the 'modulator' (table 3). The slight phosphorylation of the P-light chain by phosphorylase kinase itself may either represent trace contamination with myosin light chain kinase, or slight phosphorylation of the light

Table 2
Effect of the δ-subunit of phosphorylase kinase (δ) and the Ca^{2+}-dependent modulator (MP) on the activity of 'modulator'-sensitive cyclic nucleotide phosphodiesterase

Additions	Relative activity
EGTA	1
Ca^{2+}	1
EGTA + MP	1
EGTA + δ	1
Ca^{2+} + MP	10
Ca^{2+} + δ	11

Activity measurements were carried out at 2.0 mM cyclic AMP in the presence of either EGTA (4.0 mM) or Ca^{2+} (2.0 mM). The concentration of modulator protein and δ-subunit in the assays were 20 μg/ml and 30 μg/ml, respectively, which are in excess of that required for maximal activation of cyclic nucleotide phosphodiesterase

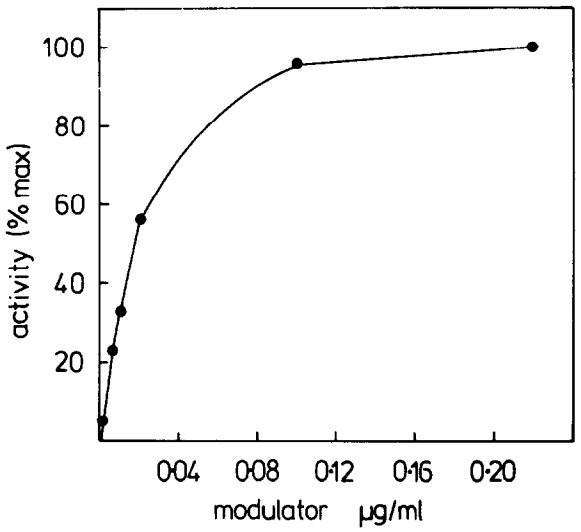

Fig.3. Effect of the purified δ-subunit on the activity of myosin light chain kinase. The assays contained 3.7 nM myosin light chain kinase (0.29 μg/ml) and varying quantities of the δ-subunit. 100% activity corresponds to a specific activity for myosin light chain kinase of 12 μmol phosphate incorporated/mg.

chain by the high concentrations of phosphorylase kinase in the assay (1000-fold higher than would be used to monitor the conversion of phosphorylase *b* to *a*).

3.6. *Interaction of the δ-subunit with troponin-I and troponin-T*

The δ-subunit formed complexes with troponin-I and troponin-T in the presence of Ca^{2+} on polyacrylamide gels that were indistinguishable from those formed with the 'modulator' from bovine brain (not illustrated) as reported by Amphlett et al. [13].

3.7. *Effect of the Ca^{2+}-dependent modulator on the activity of phosphorylase kinase*

Phosphorylase kinase 2 μM (0.66 mg/ml) was mixed with modulator protein 30 μM (0.5 mg/ml) and the solutions were then assayed for phosphorylase kinase activity at a further dilution of 1:3000. The activity of three different preparations of phosphorylase kinase was found to be increased by 61 ± 10% by the 'modulator' in the presence of Ca^{2+}. In the presence of EGTA, phosphorylase kinase was almost completely inactive in the presence and absence of the 'modulator'.

Table 3
Effect of phosphorylase kinase on the activity of myosin light chain kinase

Additions to assay	Activity (% max)
MLCK	0
PhK	8
Boiled PhK	0
PhK + MLCK	100
Boiled PhK + MLCK	100
MP + MLCK	100

The assays contained Ca^{2+} (0.1 mM) and the purified P-light chain (2 mg/ml) [28]. The concentrations of myosin light chain kinase (MLCK), phosphorylase kinase (PhK) and Ca^{2+}-dependent modulator protein (MP) were 0.29 μg/ml, 200 μg/ml and 0.17 μg/ml, respectively. These values correspond to molar concentrations of 3.8 nM, 600 nM and 10 nM, respectively. 100% activity corresponds to a specific activity for myosin light chain kinase of 12 μmol phosphate incorporated/min/mg. Control assays were carried out in all cases, in which the P-light chains were omitted. This corrected for slight incorporation of phosphate into the phosphorylase kinase and myosin light chain kinase

4. Discussion

The results presented here demonstrate that in addition to the α-, β- and γ-subunits, rabbit skeletal muscle phosphorylase kinase contains a fourth component termed the δ-subunit. This component has been shown to be very similar or identical to the Ca^{2+}-dependent 'modulator' by a variety of criteria. These include its heat stability, behaviour on ion-exchange chromatography, electrophoretic mobility on polyacrylamide gels, amino acid composition and ultraviolet absorption spectrum, ability to activate 'modulator'-sensitive cyclic nucleotide phosphodiesterase and myosin light chain kinase, and ability to form complexes with troponin-I and troponin-T.

Although the yield of the purified δ-subunit suggested that it was present in near stoichiometric amounts with the other three subunits of phosphorylase kinase, the addition of excess 'modulator' increased the phosphorylase kinase activity by a further 60%. One explanation for this result is that

purified phosphorylase kinase only contains 0.6–0.7 molecules of δ-subunit per αβγ-unit.

The concentration of the Ca^{2+}-dependent 'modulator' is very low in skeletal muscle compared to tissues such as heart and brain [4,5], and Yagi et al. [21] have reported that rabbit skeletal muscle contains only 30 mg 'modulator'/1000 g tissue. However, as phosphorylase kinase comprises nearly 1% of the soluble protein in dilute EDTA extracts of muscle [22] about 20 mg 'modulator'/1000 g muscle should be bound to phosphorylase kinase. Furthermore, the molar concentration of myosin light chain kinase in skeletal muscle is about half the concentration of phosphorylase kinase [28]. These two protein kinases may therefore be the major 'modulator'-binding proteins in skeletal muscle.

The interaction between phosphorylase kinase and the 'modulator' appears to be rather tight even in the absence of Ca^{2+}, since 2.0 mM EDTA was present in the buffers throughout the purification. Furthermore, if the EDTA was replaced by 20 mM EGTA at the final two steps (precipitation with 30% ammonium sulphate and gel filtration on Sepharose 4B) the proportion of the δ-subunit in the purified enzyme was not decreased. A preliminary attempt to resolve the δ-subunit from phosphorylase kinase by chromatography on troponin-I–Sepharose in the presence of Ca^{2+}, was also unsuccessful.

The strong interaction between the 'modulator' and phosphorylase kinase even in the absence of Ca^{2+} is surprising since both cyclic nucleotide phosphodiesterase [29] and myosin light chain kinase [21] are resolved from the 'modulator' during the purification of these enzymes. Furthermore the phosphodiesterase can be bound to columns of 'modulator'–Sepharose in the presence of Va^{2+} and eluted with buffers containing EGTA [31].

The possibility that phosphorylase kinase may bind the 'modulator' in a different manner to other proteins is also suggested by the observation that the 'modulator'–Sepharose in the presence of Ca^{2+} and chain kinase and cyclic nucleotide phosphodiesterase while it is attached to phosphorylase kinase (table 3). However since each molecule of 'modulator' binds four molecules of Ca^{2+} with equal affinity [32], an intriguing alternative idea that a single molecule of 'modulator' can activate two different 'modulator'-sensitive enzymes simultaneously. This idea also raises the possibility that several different proteins involved in biologically related events, could form a complex in vivo in the presence of Ca^{2+}.

The present work demonstrates that phosphorylase kinase is composed of four, and not three subunits, and that it possesses the structure $(\alpha\beta\gamma\delta)_4$, molecular weight 1 340 000. Convincing evidence has recently been presented that the γ-subunit is the catalytic subunit of phosphorylase kinase ([33], reviewed [34]), and it has been known for several years that the α- and β-subunits have a regulatory function in that they hold the enzyme in an inactive conformation [22]. Phosphorylation of the β-subunit by cyclic AMP-dependent protein kinase, or limited proteolysis of the α-subunit by proteinases such as trypsin, activates the enzyme 50–100-fold in the presence of Ca^{2+}, although in the absence of Ca^{2+} all forms of the enzyme are completely inactive [22,35]. The present work suggests that the regulation by Ca^{2+} is likely to be achieved by the binding of this divalent cation to the δ-subunit. In the case of the muscle contractile apparatus, the binding of Ca^{2+} to troponin-C causes it to interact more strongly with troponin-I, which relieves the inhibition of actomyosin ATPase by this component. It is therefore tempting to speculate that the binding of Ca^{2+} to the δ-subunit causes it to interact more strongly with one of the regulatory subunits (α or β) thereby relieving the inhibition on the catalytic (γ) subunit. The finding that the active γ-subunit is fully active in the absence of Ca^{2+} (D. J. Graves, personal communication) is of course entirely consistent with this concept.

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