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Comparison of calmodulin-dependent glycogen synthase kinase from skeletal muscle and calmodulin-dependent protein kinase-II from brain

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Calmodulin-dependent glycogen synthase kinase from rabbit skeletal muscle and calmodulin-dependent protein kinase-II from rat brain were found to have remarkably similar substrate specificities. Both protein kinases phosphorylated synapsin-I, glycogen synthase, smooth muscle myosin light chains, histone H1 and acetyl-CoA carboxylase at the same relative rates. Site-2 of glycogen synthase was preferentially phosphorylated by both enzymes, followed by a slower phosphorylation of site-1b. Each protein kinase catalysed a 2-fold activation of tryptophan 5-monooxygenase. Calmodulin-dependent protein kinase-II and glycogen synthase kinase exhibited similar immunological cross-reactivity in the presence of Ca^{2+} and calmodulin, using monoclonal antibody raised against the rat brain enzyme. In the absence of Ca^{2+} and calmodulin, cross-reactivity of glycogen synthase kinase was decreased, whereas that of calmodulin-dependent protein kinase-II was not. The two enzymes appear to represent different isoenzymes of a multifunctional calmodulin-dependent protein kinase that may mediate many of the actions of Ca^{2+} in mammalian tissues. The results demonstrate that calmodulin-dependent protein kinase-II is identical to calmodulin-dependent synapsin-I kinase-II, previously shown to be very similar to calmodulin-dependent glycogen synthase kinase [(1983) FEBS Lett. 163, 329–334].

Ca^{2+} *Calmodulin* *Protein phosphorylation* *Glycogen synthesis* *Neurotransmitter* *Catecholamine*

1. INTRODUCTION

A calmodulin-dependent protein kinase has been described that is involved in the activation of tryptophan 5-monooxygenase, the rate-limiting enzyme in the biosynthesis of serotonin in the brain [1]. This protein kinase was designated calmodulin-dependent protein kinase-II [2], since it eluted from gel filtration columns after phosphorylase kinase (kinase-I) but before myosin light chain kinase (kinase-III). However, in contrast to the narrow substrate specificities of phosphorylase kinase and myosin light chain kinase, kinase-II demonstrated a broad specificity, suggesting that it may mediate many of the actions of Ca^{2+} in the central nervous system [2].

Recently, a calmodulin-dependent protein kinase from skeletal muscle involved in the regulation of glycogen synthase [3] was compared to a calmodulin-dependent protein kinase from brain that phosphorylates a major postsynaptic membrane protein, termed synapsin-I [4,5]. Both enzymes possessed broad and remarkably similar substrate specificities and cross-reacted immunologically, while the 58-/60-kDa protein components in these preparations exhibited virtually identical 'phosphopeptide maps' [5].

These findings raised the question of whether glycogen synthase kinase and synapsin-I kinase are related to kinase-II [6]. We have therefore com-

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pared the properties of these enzymes in order to decide whether a single calmodulin dependent multi-protein kinase mediates many of the actions of Ca^{2+} in mammalian tissues.

2. MATERIALS AND METHODS

2.1. *Protein preparations*

Calmodulin-dependent glycogen synthase kinase was purified from rabbit skeletal muscle [3] and calmodulin-dependent protein kinase-II (kinase-II) from rat cerebral cortex [7]. Calmodulin was purified from sheep [8], bovine and rat brain [9] and glycogen synthase from rabbit skeletal muscle [10,11]. Tryptophan 5-monooxygenase was partially purified from rat brain stem [2]. This preparation was free of kinase-II, but contained sufficient amounts of the activator protein required to observe activation of tryptophan 5-monooxygenase after phosphorylation by kinase-II (revew [6,12]). Rat brain tubulin was purified by chromatography on phosphocellulose as in [13]. Microtubule-associated protein-2 (MAP2) was purified by chromatography on Bio-Gel A-1.5 m as in [14]. Myosin light chains were purified from chicken gizzard as in [15] or provided by Dr J. Kendrick-Jones (MRC Laboratory of Molecular Biology, Cambridge, England). Synapsin-I from bovine brain [16] was a gift from Dr P. Greengard (Rockefeller University, New York). Acetyl-CoA carboxylase from lactating rat mammary glands and histone H1 from calf thymus were provided by Dr D.G. Hardie (Dept. of Biochemistry, University of Dundee, Scotland). The sources of other substrate proteins and the procedures for their phosphorylation with [γ-^{32}P]ATP are given in [3].

2.2. *Activation of tryptophan 5-monooxygenase (2)*

The standard incubation mixture contained 50 mM Hepes buffer (pH 7.6), 0.5 mM ATP, 5 mM $Mg(CH_3COO)_2$, 0.12 mM $CaCl_2$, 0.1 mM EGTA, 500 nM calmodulin, 0.2 mM tryptophan, 0.3 mM 4-hydroxy-6-methyl-5,6,7,8-tetrahydropteridine, 0.05 mM $Fe(NH_4)_2(SO_4)_2$, 2 mM dithiothreitol, 50 g catalase, about 0.01 unit of tryptophan 5-monooxygenase and a suitable amount of kinase-II, in a final volume of 0.2 ml. The incubation was carried out at 30°C for 20 min. Units of activity are defined in [2].

2.3. *Preparation of monoclonal antibody to kinase-II and immunoprecipitation of protein kinases*

Staphylococcus aureus (Cowan I strain) was cultured as in [17] and the cells collected and fixed with formaldehyde essentially as in [18]. Hybridoma that secrete monoclonal antibody (IgM) directed against kinase-II were produced by fusion of NS-1 myeloma cells with spleen cells from mice that had been immunized with purified kinase II essentially as in [19].

Immunoprecipitations were carried out by incubating each protein kinase for 20 min at 4°C with varying amounts of monoclonal antibody in a solution containing 50 mM Hepes buffer (pH 7.6), 1 mM dithiothreitol, 1 mg/ml transferrin in the presence or absence of 0.2 mM $CaCl_2$ and 3 μM calmodulin. The final volume was 25 μl. Anti-mouse IgM (1 μl) and a 10% suspension of staphylococci (20 μl) were added, and the mixture incubated for 30 min at 4°C with shaking. After centrifugation at 20000 × g for 7 min, 30-μl aliquots of the supernatant were withdrawn and assayed for enzyme activity, using myosin light chains as substrate.

3. RESULTS

The substrate specificities of calmodulin-dependent glycogen synthase kinase and kinase-II are compared in table 1. The specificities of the two enzymes were virtually identical. Both protein kinases also phosphorylated tubulin and MAP-2, the K_m for the latter substrate being 0.3 μM for each enzyme.

Glycogen synthase is phosphorylated on 7 serine residues by at least 6 protein kinases [20–22]. The calmodulin-dependent glycogen synthase kinase phosphorylates two serines, the rapid phosphorylation of site-2 being followed by a slower phosphorylation of site-1b [3]. Glycogen synthase was phosphorylated to 0.2 mol/mol subunit with kinase-II, digested with cyanogen bromide and examined by SDS-polyacrylamide gel electrophoresis as in [21]. This experiment showed that ≃80% of the ^{32}P-radioactivity was contained in peptide CB-1 (containing site-2) and the remainder

Table 1

Comparison of the substrate specificities of calmodulin-dependent glycogen synthase kinase from skeletal muscle (GSK) and kinase-II (KII) from brain

Substrate	Concentration		Relative rates of phosphorylation (%)	
	(mg/ml)	(μM)	GSK	KII
Synapsin-I	0.1	1.2	590	620
Glycogen synthase	0.4	4.6	100	100
Myosin P-light chain (smooth muscle)	0.8	40.0	76	81
Histone H1	0.2	10.0	21	18
Acetyl-CoA carboxylase	0.11	0.4	8	10
Phosphorylase *b*	1.5	15.5	<0.1	<0.1
Phosvitin	2.0	50.0	<0.1	<0.1

Phosphorylation of each protein was carried out as in [3,28] and initial rate conditions were met for each substrate

in CB-2 (containing site-1b). These results were identical to those obtained with the calmodulin-dependent glycogen synthase kinase [3]. Glycogen synthase was then phosphorylated to 0.3 mol/mol subunit with kinase-II, digested with trypsin and analysed by high performance liquid chromatography as in [23]. As shown in fig.1B, the major peak of ^{32}P-radioactivity, eluting at 46% acetonitrile, corresponded to the tryptic peptide containing site-2, while the minor peak eluting at 23% acetonitrile corresponded to site-1b [23]. Identical results were obtained with the calmodulin-dependent glycogen synthase kinase (fig.1A). No peaks corresponding to the positions of the other 5 known phosphorylation sites [23] were observed.

Incubation of tryptophan 5-monooxygenase with kinase-II and Mg-ATP produced the expected 2-fold activation (fig.2). A similar activation was observed when kinase-II was substituted by glycogen synthase kinase (fig.2).

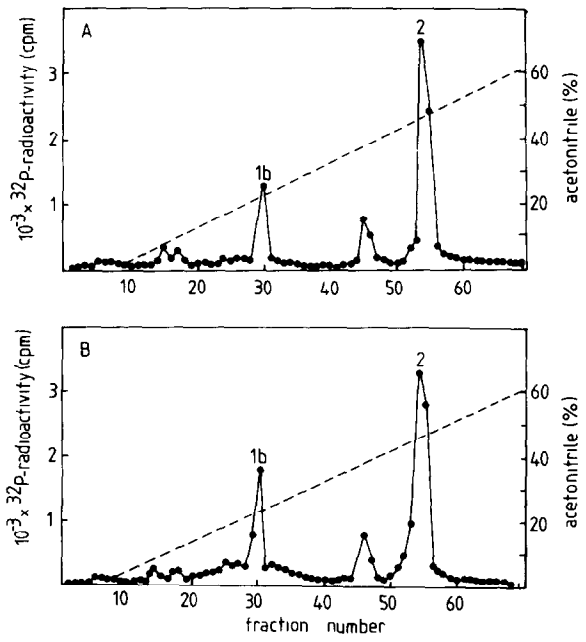

Fig.1. Separation of tryptic-phosphopeptides from glycogen synthase by HPLC. Glycogen synthase was phosphorylated to 0.16 mol/mol subunit with skeletal muscle calmodulin-dependent glycogen synthase kinase (A) or to 0.30 mol/mol subunit with kinase-II (B). The enzyme was digested with trypsin (Worthington) and analysed on a Waters bondapak C18 HPLC column (30 cm × 3.9 mm) as in [23]. The column was developed with a water/acetonitrile gradient (– – –) in the presence of 0.1% (v/v) trifluoroacetic acid. The flow rate was 1.0 ml/min, and fractions of 1.0 ml were collected and analysed by Cerenkov counting (●—●). The peptides eluting at 23 and 46% acetonitrile correspond to sites-1b and 2, respectively [23]. The minor peak at 38% acetonitrile is related to site-2, since it is also observed after phosphorylating glycogen synthase with either phosphorylase kinase or glycogen synthase kinase-4 (unpublished).

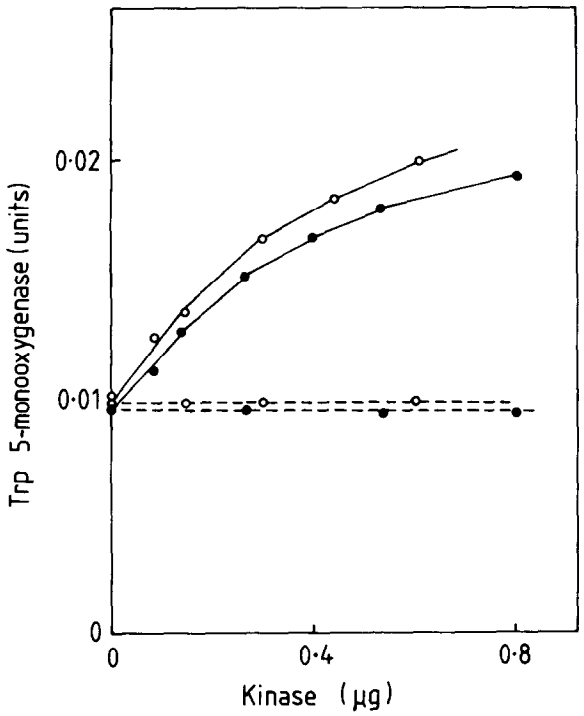

Fig.2. Activation of tryptophan 5-monooxygenase by calmodulin-dependent protein kinase-II and calmodulin-dependent glycogen synthase kinase. Tryptophan (Trp) 5-monooxygenase (70 g protein) was incubated with different amounts of kinase-II (○) and glycogen synthase kinase (●) under the standard conditions (solid lines). Control experiments were carried out without ATP (broken lines).

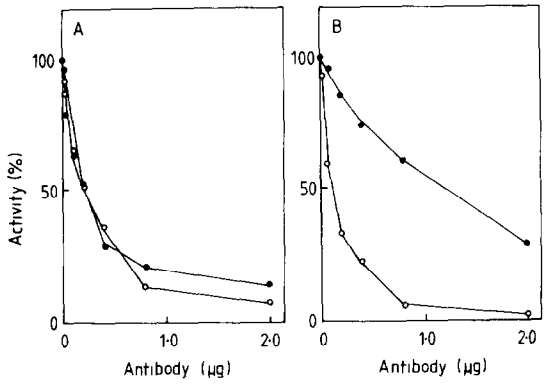

Fig.3. Immunotitration of kinase-II and glycogen synthase kinase. Kinase-II (18 ng) (○) and glycogen synthase kinase (22 ng) (●) were titrated with the indicated amounts of monoclonal antibody prepared against kinase-II in the presence (A) or absence (B) of Ca^{2+} and calmodulin as described in section 2. The enzyme activity was determined by measuring the phosphorylation of smooth muscle myosin light chain (1.0 mg/ml).

Kinase-II and glycogen synthase kinase were immunotitrated with monoclonal antibody prepared against kinase-II. Both protein kinases exhibited the same cross-reactivity in the presence of Ca^{2+} and calmodulin (fig.3A). However, in the absence of Ca^{2+} and calmodulin, cross-reactivity of glycogen synthase kinase was decreased, whereas that of kinase-II remained unchanged (fig.3B).

4. DISCUSSION

The present study was prompted by several observations that suggested similarities between calmodulin-dependent glycogen synthase kinase from skeletal muscle and calmodulin-dependent protein kinase-II from rat brain. For example, although the procedures for isolating these two enzymes [3,7] were developed independently, identical steps are employed in their purification (although not in the same order). Both enzymes are precipitated at <40% ammonium sulphate, eluted from phosphocellulose at 0.28–0.3 M NaCl, and have relative molecular masses in excess of 500 kDa. A close relationship between these two protein kinases has now been established by the present study. The enzymes have identical substrate (table 1) and site (fig.1) specificities and cross-react immunologically (fig.3). Differences in cross-reactivity were observed in the absence of Ca^{2+} and calmodulin (fig.3B). However, whether this reflects a tissue or a species difference remains to be elucidated.

We have demonstrated that calmodulin-dependent glycogen synthase kinase from rabbit skeletal muscle is closely related to synapsin-I kinase-II from rat brain [5]. The present data (table 1) therefore suggest that synapsin-I kinase-II and calmodulin-dependent protein kinase-II are the same enzyme. This view is reinforced by the comigration of these two protein kinases on SDS polyacrylamide gels (not shown). Both preparations show a minor 60-kDa and a major 50-kDa component when electrophoresis is carried out as in [24]. However, the apparent molecular mass of the major component is 55 kDa when the electrophoretic system in [25] is employed, as reported in [7].

Authors in [26] recently examined the substrate specificity of synapsin-I kinase-II from rat brain and reported that it was unable to use rabbit mus-

cle glycogen synthase as substrate. The reason for this discrepancy with our own findings ([5] table 1) is unclear. However, it could be related to their use of commercial glycogen synthase, since we have found Sigma glycogen synthase (lot number 111F-9710) to be a much poorer substrate for calmodulin-dependent glycogen synthase kinase (10% of the rate) than the glycogen synthase purified in our laboratory (unpublished).

Based on the ability of tissue extracts to activate tryptophan 5-monooxygenase in the presence of Ca^{2+}-calmodulin and Mg-ATP, it was previously concluded that calmodulin-dependent protein kinase-II kinase was absent from skeletal muscle [12,27]. The failure to detect activity appears to be due to the buffer used to extract the tissue. After inclusion of 50 mM NaCl [28] or 0.1% Triton in the homogenisation medium, significant amounts of activity are detectable (unpublished).

A calmodulin-dependent protein kinase active towards smooth muscle myosin light chains, glycogen synthase and MAP-2 has been purified from rat brain by authors in [29–31]. This enzyme, which has a subunit molecular mass of 49 kDa [29], is almost certainly identical to synapsin-I kinase-II/calmodulin-dependent protein kinase-II. A calmodulin-dependent glycogen synthase kinase has been purified from rat liver [32] that is closely related to the skeletal muscle enzyme (discussed in [3,28]). Recently, the liver preparation has also been shown to exhibit a broad specificity and to phosphorylate proteins such as synapsin-1 and MAP-2 [33].

In summary, the present results and those presented previously [5,6,33], strengthen the view that many of the actions of Ca^{2+} are mediated by a widely distributed multifunctional calmodulin-dependent protein kinase.

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