Ca^{2+}, calmodulin-dependent phosphorylation of glycogen synthase by a brain protein kinase

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A Ca^{2+}, calmodulin-dependent protein kinase from brain with a M_r of 640000 is capable of phosphorylating glycogen synthase from skeletal muscle. The reaction was inhibited by the addition of 1 mM EGTA and 50 μM trifluoperazine, but not by protein kinase inhibitor and heparin. The amount of phosphate incorporated into glycogen synthase was 1.4 mol/mol subunit. The phosphorylation sites of glycogen synthase were cyanogen bromide-treated peptides CB-1 and CB-2 and only the seryl residue was phosphorylated.

Ca^{2+} Calmodulin-dependent protein kinase Glycogen synthase Protein phosphorylation Brain

1. INTRODUCTION

Myosin light chain kinase was first discovered in skeletal muscle [1] and subsequently in smooth and cardiac muscles and non-muscle tissues. We isolated two Ca^{2+}, calmodulin-dependent protein kinases from rat brain, one of which had a M_r of 640000 (640 kDa enzyme) by gel filtration analysis and was purified to apparent homogeneity [2,3]. A similar enzyme was found in rat anterior pituitary gland [4]. The enzyme was capable of phosphorylating several exogenous and endogenous substrates [3]. The phosphorylation of microtubule-associated proteins by the enzyme induced an inhibition of microtubule assembly [5].

Enzymes have been isolated from rabbit liver [6,7] and rabbit skeletal muscle [8] which phosphorylated glycogen synthase from rabbit skeletal muscle in a Ca^{2+}, calmodulin-dependent manner. In view of their physicochemical properties and substrate specificities, these protein kinases from the liver, skeletal muscle and brain are apparently distinct from 'myosin light chain kinase' in muscle tissues. The present communication describes the phosphorylation of glycogen synthase by the 640 kDa enzyme from brain.

2. MATERIALS AND METHODS

2.1. *Materials*

Calmodulin was prepared from bovine brain [9]. A Ca^{2+}, calmodulin-dependent protein kinase (640 kDa enzyme) was purified from rat brain as in [3]. The purified 640 kDa enzyme was completely free of cyclic AMP-dependent protein kinase, phospholipid-dependent protein kinase and phosphorylase kinase activities. Its specific activity was 293 $nmol.mg^{-1}.min^{-1}$ with chicken gizzard myosin light chain as substrate. Dephosphorylated glycogen synthase was prepared from rabbit skeletal muscle [10]. The specific activity of the purified glycogen synthase was 18.5 $\mu mol.mg^{-1}.min^{-1}$, and the value was in good agreement with that in [10]. Phosphorylase *b*, protein kinase inhibitor and heparin were purchased from Sigma. Trifluoperazine was a gift of Yoshitomi Pharmaceuticals. [γ-^{32}P]ATP was prepared as in [11]. [UDP-^{14}C(U)]Glucose was obtained from New England Nuclear.

2.2. *Assay for protein phosphorylation and glycogen synthase*

The incubation for protein phosphorylation was

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carried out as in [3,12]. When indicated, the reaction was terminated by the addition of the SDS sample buffer [13]. Samples were subjected to SDS–polyacrylamide gel electrophoresis (SDS–PAGE) in 9% acrylamide [13], followed by autoradiography [14]. The activity of glycogen synthase was determined in the presence of 10 mM glucose-6-phosphate as in [15].

2.3. *Determination of phosphorylation site and amino acid residue*

Phosphorylation sites of glycogen synthase were determined by treating with cyanogen bromide [16]. Phosphorylated glycogen synthase was extracted from a stained protein band on SDS–PAGE [17], and subjected to partial acid hydrolysis and high voltage paper electrophoresis [18].

2.4. *Other methods*

Polyacrylamide disc gel electrophoresis for non-denaturing protein was carried out in 5% acrylamide [19]. Protein was determined as in [20].

3. RESULTS AND DISCUSSION

3.1. *Phosphorylation of glycogen synthase by the 640 kDa enzyme and its coincidence with enzyme activity*

Fig.1 shows the staining pattern of glycogen synthase by SDS–PAGE and the autoradiograph. Glycogen synthase migrated as one major and several minor bands [21]. Endogenous phosphorylation of glycogen synthase was hardly observed. The addition of the 640 kDa enzyme increased the Ca^{2+}, calmodulin-dependent phosphorylation of glycogen synthase. The stained band of glycogen synthase coincided well with phosphate incorporation (fig.1). The 640 kDa enzyme did not phosphorylate phosphorylase *b*.

To confirm that the phosphorylated protein was glycogen synthase, the following experiments were performed. Glycogen synthase was subjected to polyacrylamide disc gel electrophoresis under non-denaturing conditions. The main protein band migrated at about 14–18 mm from the origin, which coincided well with glycogen synthase activity and Ca^{2+}, calmodulin-dependent protein phosphorylation (fig.2).

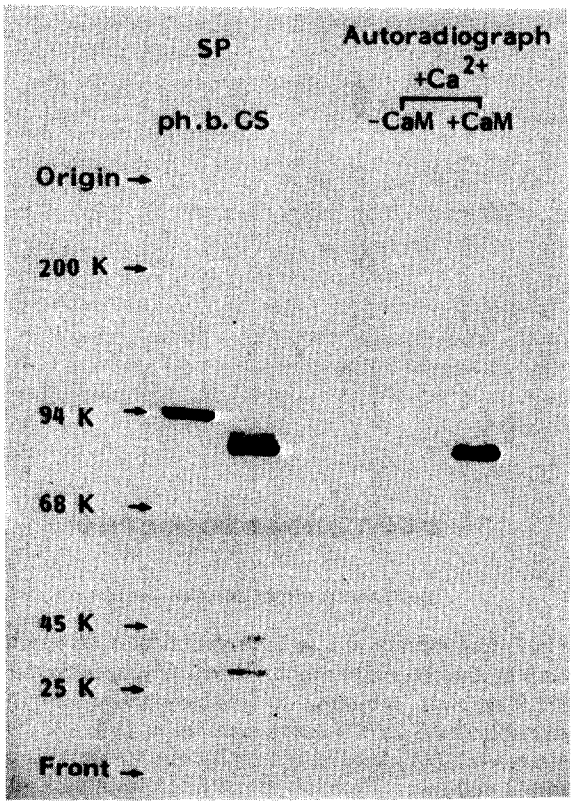

Fig.1. SDS–PAGE of the phosphorylated glycogen synthase and autoradiography of the gel. Glycogen synthase (27 μg) was incubated with 0.25 μg of the 640 kDa enzyme and 0.02 mM [γ-^{32}P]ATP in the presence or absence of calmodulin (CaM), under standard conditions. After termination of the reaction and heat treatment, aliquots (0.04 ml) were subjected to SDS–PAGE, followed by autoradiography. Standard proteins used were myosin (200 kDa), phosphorylase *b* (94 kDa), bovine serum albumin (68 kDa), ovalbumin (45 kDa), and chymotrypsinogen A (25 kDa). SP: a staining pattern of phosphorylase *b* (ph.b) and glycogen synthase (GS).

3.2. *Effects of several agents on glycogen synthase phosphorylation by the 640 kDa enzyme*

The effects of several agents on phosphorylation of glycogen synthase by the 640 kDa enzyme were examined (table 1). EGTA (Ca^{2+} chelator) and trifluoperazine (calmodulin inhibitor) inhibited the phosphorylation, whereas protein kinase inhibitor (cyclic AMP-dependent protein kinase inhibitor) and heparin (casein kinase inhibitor) had little effect on the phosphorylation.

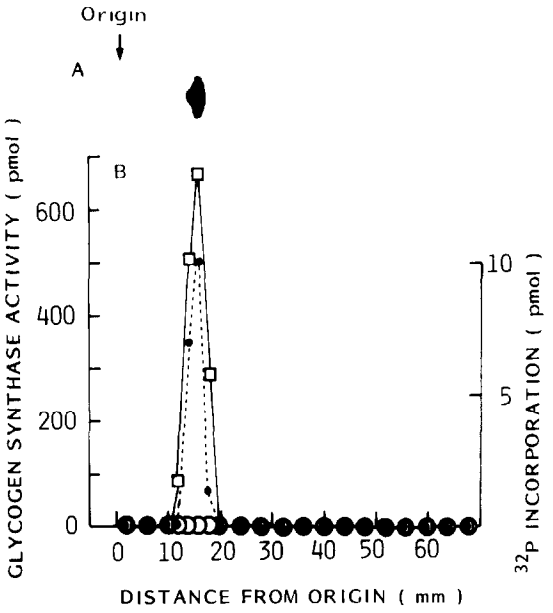

Fig.2. Coincidence of a main protein band with enzyme activity and protein phosphorylation. Glycogen synthase (21.5 μg) was subjected to polyacrylamide disc gel electrophoresis in 5% acrylamide as in [19]. (A) The stained gel; (B) after the electrophoresis, the gel was cut into 2-mm slices. Protein was extracted from each slice with 0.2 ml of 50 mM Tris–HCl buffer (pH 7.8) and assayed for glycogen synthase activity (□—□) and for protein phosphorylation by the 640 kDa enzyme (0.5 μg) in the presence (●---●) or absence (○) of calmodulin, under standard conditions. The value was corrected for that of the 640 kDa enzyme alone.

3.3. *Phosphate amount incorporated into glycogen synthase by the 640 kDa enzyme*

Fig.3 shows the time course of glycogen synthase phosphorylation by the 640 kDa enzyme. A small amount of endogenous phosphorylation occurred in the absence of 640 kDa enzyme. At 30-min incubation, the reaction reached a plateau. The amount of phosphate incorporated into glycogen synthase was about 1.4 mol/mol subunit, indicating that at least 2 sites/subunit are phosphorylated.

3.4. *Phosphorylation site of glycogen synthase by the 640 kDa enzyme*

The sites on glycogen synthase phosphorylated by the 640 kDa enzyme were examined. Fig.4 shows the autoradiograph of the sample treated by

Table 1

Effects of various agents on the phosphorylation of glycogen synthase by the 640 kDa enzyme

Additions	Concentration	$+Ca^{2+}$	
		−CaM	+CaM
		(pmol/min)	
None		0	15.3
EGTA	1 mM	0	1.3
Trifluoperazine	50 μM	2.3	0
Protein kinase inhibitor	75 μg/ml	0	16.1
Heparin	10 μg/ml	2.0	12.3

Glycogen synthase (23.2 μg protein) was phosphorylated with 0.5 μg of the 640 kDa enzyme and the indicated additions in the presence or absence of 1 μg of calmodulin (CaM) with 0.1 mM Ca^{2+}, under standard conditions. Values were corrected for those determined in the presence of glycogen synthase or the 640 kDa enzyme alone, and represent means of triplicate determinations

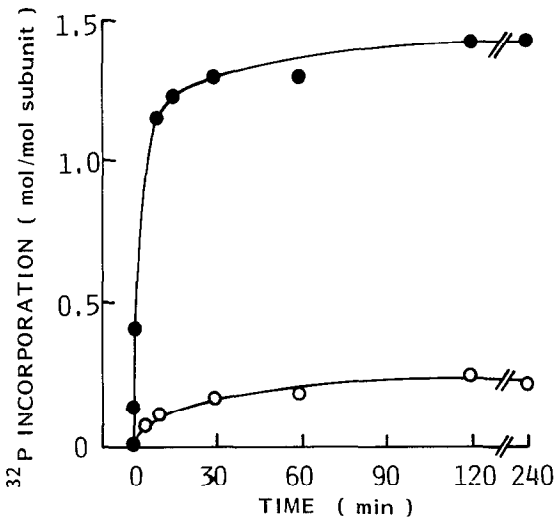

Fig.3. Time course of glycogen synthase phosphorylation by the 640 kDa enzyme. Glycogen synthase (17.0 μg) was phosphorylated with 2 μg of the 640 kDa enzyme, 0.5 mM [γ-^{32}P]ATP, 0.1 mM Ca^{2+} and 1 μg of calmodulin, under standard conditions (●—●). The value was calculated using a M_r of the subunit as 85000, and was corrected for that of the 640 kDa enzyme alone. The phosphorylation of glycogen synthase without the addition of the 640 kDa enzyme was represented (○—○).

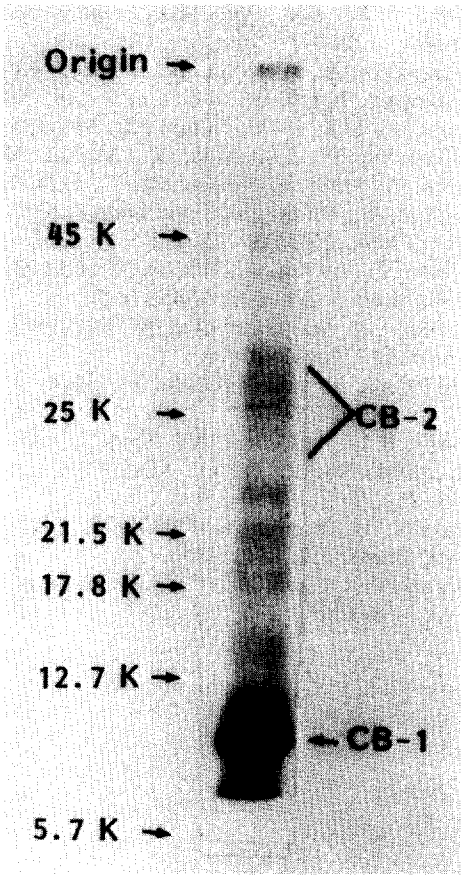

Fig.4. Phosphorylation site of glycogen synthase. Glycogen synthase (135 μg/ml) was phosphorylated with 10 μg/ml of the 640 kDa enzyme, 0.02 mM [γ-^{32}P]ATP, 0.1 mM Ca^{2+}, 5 μg/ml of calmodulin and 0.1 mM heparin, under standard conditions. After cyanogen bromide treatment, an aliquot corresponding to 21.6 μg of glycogen synthase was subjected to SDS–PAGE in 15% acrylamide. Standard proteins used were ovalbumin (45 kDa), chymotrypsinogen A (25 kDa), soybean trypsin inhibitor (21.5 kDa), myoglobin (17.8 kDa), cytochrome *c* (12.7 kDa) and insulin (5.7 kDa).

cyanogen bromide after glycogen synthase phosphorylation. Phosphate was incorporated into both CB-1 (M_r 9000) and CB-2 (M_r 24000–29000) and CB-1 was more extensively phosphorylated. These results are consistent with those described above, in that at least two sites per subunit are phosphorylated. Phosphorylation of site 2 (CB-1) or sites 1a and (3a + 3b + 3c) (CB-2) are known to influence the enzyme activity and the effects are additive [22]. This suggests that the phosphorylation of glycogen synthase by the brain Ca^{2+}, calmodulin-dependent protein kinase affects the enzyme activity.

3.5. *Phosphoamino acid residue in glycogen synthase*

The 640 kDa enzyme phosphorylated both seryl and threonyl residues in tubulin and microtubule-associated proteins, while cyclic AMP-dependent protein kinase phosphorylated only seryl residues (H. Yamamoto and E. Miyamoto, unpublished). We therefore examined whether seryl and threonyl residues in glycogen synthase are phosphorylated by the 640 kDa enzyme. Phosphate was only incorporated into seryl residues in glycogen synthase.

We here indicate that glycogen synthase from rabbit skeletal muscle can be phosphorylated in a Ca^{2+}, calmodulin-dependent manner by the 640 kDa enzyme from rat brain. Ca^{2+}, calmodulin-dependent phosphorylation of glycogen synthase was reported earlier [6–8]. The 640 kDa enzyme from brain [3] and glycogen synthase kinases from liver [6,7] and skeletal muscle [8] both have large M_r-values on gel filtration. All 3 enzymes incorporate phosphate into both CB-1 and CB-2, have relatively broad substrate specificities, and do not utilize phosphorylase *b* as substrate. It appears that these 3 enzymes belong to the same class of Ca^{2+}, calmodulin-dependent protein kinase and differ from myosin light chain kinase. However, the brain and liver enzymes differ with respect to molecular masses by sucrose density gradient centrifugation, sedimentation coefficients and subunit structure. The substrate specificity for each enzyme may also not be completely identical. Further study will be needed to compare the enzymes.

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