GLYCOGEN SYNTHASE KINASE-2 FROM RABBIT SKELETAL MUSCLE IS ACTIVATED BY THE CALCIUM-DEPENDENT REGULATOR PROTEIN

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1. Introduction

Glycogen synthase kinase-2 is a protein kinase which contaminates highly-purified preparations of glycogen synthase [1]. Unlike cyclic AMP-dependent protein kinase which also phosphorylates glycogen synthase [2,3], its activity is unaffected either by cyclic AMP or by the heat stable protein which inhibits cyclic AMP-dependent protein kinase specifically [1]. Moreover, it has a higher K_m for ATP and different nucleoside triphosphate specificity [1] and it phosphorylates preferentially a serine residue distinct from either of the two sites phosphorylated by cyclic AMP-dependent protein kinase [4,5].

The phosphorylation of glycogen synthase by cyclic AMP-dependent protein kinase is likely to represent the physiological mechanism by which glycogen synthase is inhibited by adrenaline [6–8]. However, the physiological role of glycogen synthase kinase-2 is unclear, since the mechanism by which this enzyme is regulated is unknown. In this paper we demonstrate that the phosphorylation of glycogen synthase by endogenous glycogen synthase kinase-2 is markedly stimulated by the calcium-dependent regulator protein (termed CDR) in the presence of Ca^{2+}, suggesting the possibility that this enzyme could represent a mechanism for the regulation of glycogen synthase activity in response to nervous stimulation.

2. Materials and methods

Glycogen synthase was purified to near homogeneity (method 2) [9] following the modifications described in [10]. The protein inhibitor of cyclic AMP-dependent protein kinase was obtained as a byproduct of the preparation of protein phosphatase inhibitor-1 [11]. CDR was a homogeneous preparation prepared from phosphorylase kinase [12,13] or bovine brain [14]. The bovine brain CDR was a generous gift from Professor T. C. Vanaman (Department of Microbiology and Immunology, Duke University).

The phosphorylation of glycogen synthase was carried out at pH 7.0 in the following incubation: glycogen synthase 1.0 mg/ml, sodium glycerophosphate 10.0 mM, EDTA 0.4 mM, magnesium acetate 3.0 mM, sufficient protein kinase inhibitor to inhibit endogenous cyclic AMP-dependent protein kinase activity completely, and either [^{32}P]ATP 1.0 mM to measure the phosphorylation, or cold ATP 1.0 mM to measure activity changes. The reactions also contained either 0.1 mM ethyleneglycol bis (2-aminoethylether) *N,N'* tetracetic acid (EGTA) or 0.1 mM $CaCl_2$. CDR was 1.0 μM (16.7 μg/ml). The stoichiometry of phosphate incorporation was calculated using an absorbance index, $A^{1\%}_{280\ nm}$, of 13.4, and a subunit mol. wt 88 000 [9]. For analysis of the phosphorylation sites, the reactions were terminated by the addition of 0.1 vol. 100% trichloroacetic acid. The trichloroacetic acid precipitated protein was washed, digested with either trypsin or chymotrypsin, and analysed by gel filtration on Sephadex G-50 [5]. Identification of the position of the phosphoserine in phosphopeptides was carried out as in [4,5].

Glycogen synthase activity was measured at pH 6.8 in potassium phosphate 10.0 mM, KCl 0.15 M, NaF 50 mM, EDTA 1.0 mM, [^{14}C]UDPG 5.0 mM

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and glycogen 10 mg/ml [15]. The activity ratio of glycogen synthase is defined as the activity in the absence of glucose 6-phosphate relative to the activity in the presence of 10 mM glucose 6-phosphate.

3. Results

3.1. *The phosphorylation of glycogen synthase by endogenous glycogen synthase kinase-2 is stimulated by the calcium-dependent regulator protein*

Incubation of purified glycogen synthase with Mg-ATP in the presence of an excess of the specific protein inhibitor of cyclic AMP-dependent protein kinase, resulted in a slow phosphorylation of the protein. This reaction is catalysed by the cyclic AMP-independent protein kinase which has been termed glycogen synthase kinase-2 (fig.1) [1]. Under these conditions, the phosphate becomes covalently attached to a single serine residue located seven amino acids from the N-terminus of the polypeptide chain [5]. As reported in [1], the rate of phosphorylation was completely unaffected by the inclusion of either 0.1 mM EGTA or 0.1 mM Ca^{2+} in the assays. However following the addition of both 0.1 mM Ca^{2+} and CDR the rate of phosphorylation was stimulated. The degree of stimulation, which varied considerably from preparation to preparation, was difficult to measure exactly, since the phosphorylation was not linear with time. However, based on the time required to incorporate 0.1 molecules of phosphate per subunit in the standard assay, it was estimated to range from 2–20-fold in the ten different glycogen synthase preparations that were tested. Two preparations representing the extremes of the stimulation observed, are illustrated in fig.1. Half maximal stimulation occurred at ~1.0 μg/ml CDR (0.06 μM). In all preparations, either Ca^{2+} alone, or CDR in the presence of EGTA, had no effect on the basal level of phosphorylation.

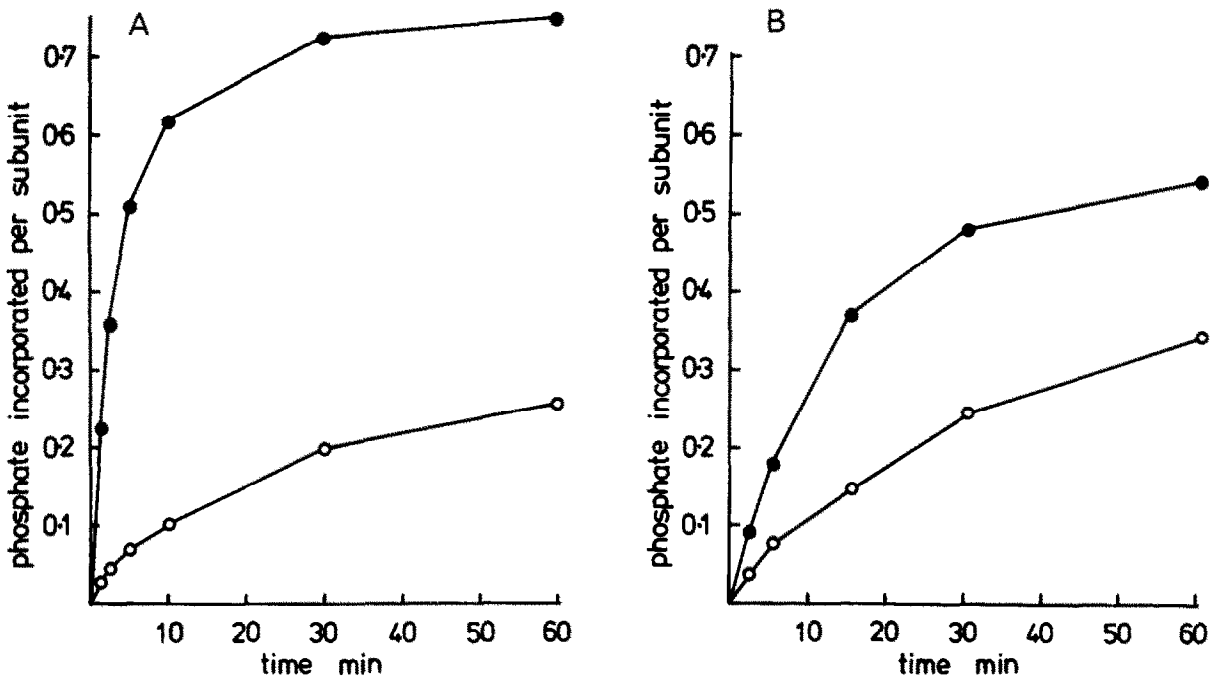

Fig.1. Effect of CDR and Ca^{2+} on the phosphorylation of glycogen synthase by endogenous glycogen synthase kinase-2. The open circles show the phosphorylation in the presence of (a) EGTA, (b) Ca^{2+}, or (c) CDR + EGTA, which were indistinguishable. The closed circles show the phosphorylation in the presence of CDR and Ca^{2+}. Figures 1A and 1B illustrate the variable stimulation by CDR and Ca^{2+} observed in different preparations.

3.2. *Glycogen synthase is phosphorylated at the same amino acid residue in the presence and absence of CDR*

Glycogen synthase was phosphorylated by incubating several different preparations with Mg-ATP in the presence and absence of CDR. The reactions were terminated by the addition of trichloroacetic acid, and the protein digested with either trypsin or chymotrypsin and subjected to gel filtration on Sephadex G-50 [5]. The profiles obtained after enzymic digestion of one preparation are illustrated in fig.2. After digestion with trypsin or chymotrypsin single phosphopeptides termed T1 and C1 were observed, which possessed v/v_0 values of 1.8 and 2.0, respectively, irrespective of whether the phosphorylation was carried out in the presence (fig.2A,B) or absence (fig.2C,D) or CDR and Ca^{2+}. We have shown [5] that in the absence of CDR a single serine is phosphorylated under these conditions and the amino acid sequences of peptide T1 and C1 are:

T1 Thr–Leu–Ser(P)–Val–Ser–Ser–Leu–Pro–Gly

C1 Ser(P)–Val–Ser–Ser–Leu–Pro–Gly

The site of phosphorylation was shown to be residue 1 in peptide C1 and residue 3 in peptide T1 following phosphorylation in the presence or absence of CDR, using the exact methodology described in [5]. This result was confirmed using several different prepara-

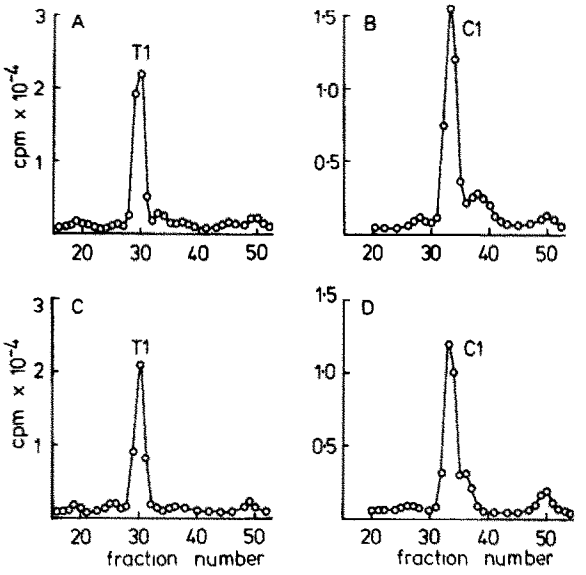

Fig.2. Gel filtration on Sephadex G-50 Superfine (65 × 2 cm) of tryptic (A,C) and chymotryptic (B,D) phosphopeptides obtained after phosphorylation of glycogen synthase by endogenous glycogen synthase kinase-2. Figures 2A and 2B were obtained after phosphorylation in the presence of CDR and Ca^{2+} to 0.32 and 0.20 molecules per subunit, respectively. Figures 2C and 2D were obtained after phosphorylation in the presence of EGTA to 0.23 and 0.16 molecules per subunit, respectively. The column was equilibrated with 25 mM ammonium bicarbonate. Fractions of 2.9 ml were collected and the flow rate was 15 ml/h. The synthase preparation used in this experiment was stimulated 7-fold by CDR and Ca^{2+} and the phosphorylations were therefore carried out for different periods of time.

Table 1
Effect of phosphorylation of serine-7 by endogenous glycogen synthase kinase-2, on the activity ratio of glycogen synthase

Preparation	Alkali labile phosphate per subunit[a]	Phosphate incorporated per subunit[b]	Initial activity ratio[c]	Final activity ratio[c]
1	0.42	0.65	0.64	0.26
2	0.43	0.65	0.64	0.54
3	0.40	0.74	0.73	0.47
4	0.38	0.69	0.63	0.48
5	0.40	0.58	0.69	0.39
6	0.36	0.67	0.60	0.40

[a] Determined prior to phosphorylation by the method in [16]

[b] By endogenous glycogen synthase kinase-2 activity in the presence of CDR and Ca^{2+}. The incubations were carried out for 60 min by which time the phosphorylation had reached a plateau

[c] Measured in the presence and absence of glucose 6-phosphate as in section 2. Control experiments in which Mg-ATP was omitted showed no decrease in the activity or activity ratio over the same time period

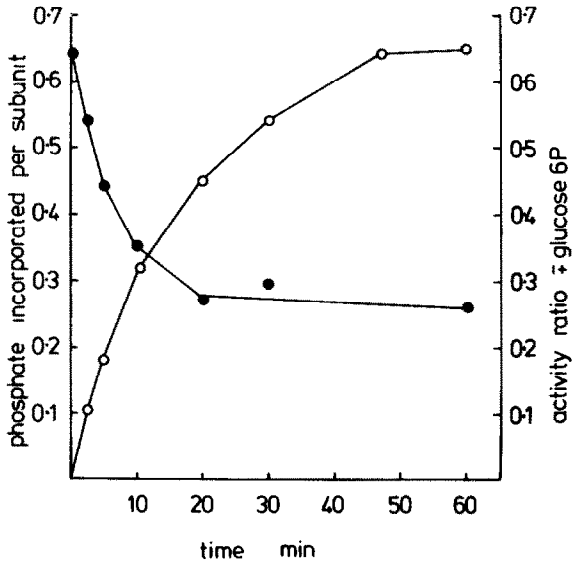

Fig.3. Phosphorylation (○) and inactivation (●) of glycogen synthase by endogenous glycogen synthase kinase-2 in the presence of CDR and Ca^{2+}. In the absence of Mg-ATP no change in the activity was observed.

tions of glycogen synthase which differed in the degree to which the phosphorylation was stimulated by CDR.

3.3. *Glycogen synthase kinase-2 decreases the activity ratio of glycogen synthase*

In our initial papers describing the identification of glycogen synthase kinase-2, we reported that the phosphorylation of glycogen synthase by this enzyme did not decrease the activity ratio −glucose 6-phosphate [1,15]. In the present work, however, phosphorylation by endogenous glycogen synthase kinase-2 did lead to inactivation, although the decrease in activity was somewhat variable (table 1). The incorporation of phosphate was inversely proportional to the decrease in activity (fig.3).

Each of the glycogen synthase preparations was phosphorylated on serine 7, as judged by gel filtration and peptide mapping of the tryptic phosphopeptides. Furthermore, each preparation of glycogen synthase *a* showed a single major band of mol. wt 88 000 when subjected to polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate (not illustrated), and a similar content of alkali-labile phosphate (table 1). This indicates that the variable inactivation by glycogen synthase kinase-2 is unlikely to be the result of either proteolysis or a variable content of phosphate in the starting material. As discussed in [16], it appears that the regulatory properties of glycogen synthase can become altered during the isolation of the enzyme, so that phosphorylation by glycogen synthase kinase-2 does not always lead to inactivation.

4. Discussion

The results described here show that the phosphorylation of glycogen synthase by endogenous cyclic AMP-independent protein kinase is stimulated by CDR in the presence of Ca^{2+}. The degree of stimulation was variable (fig.1), but whether it was 2-fold or 20-fold, there was no effect of Ca^{2+} alone, nor was there any demonstrable inhibition of the basal phosphorylating activity by EGTA. These experiments demonstrate that the variable stimulation is not due to the co-purification of trace amounts of CDR with the glycogen synthase, and that the phosphorylation must be catalysed by two types of activity. Furthermore, since the basal phosphorylation in the presence of EGTA and the CDR-stimulated activity in the presence of Ca^{2+} preferentially phosphorylate the same amino acid residue (serine-7) it seems most likely that the two types of activity are different forms of the same enzyme. One possibility, is that the two forms have the structures C (active) and C-I (inactive), respectively, where I is an inhibitory subunit which not only functions to hold C in an inactive form, but also interacts with CDR in the presence of Ca^{2+}. C-I would therefore require both CDR and Ca^{2+} for activity, while C would be insensitive to CDR and Ca^{2+} and be fully active in the presence of EGTA. It is however also possible that the CDR-insensitive component is simply a proteolytically-modified fragment which has lost the ability to be regulated by CDR.

The results illustrated in fig.3 show that the phosphorylation of serine-7 is associated with the conversion of glycogen synthase to a form which is more dependent on glucose 6-phosphate for activity. The finding that a Ca^{2+}-activated protein kinase inactivates glycogen synthase, suggests that this reaction could

determine the rate of glycogen synthesis during muscle contraction. It will therefore be important to measure the degree of phosphorylation of serine-7 in vivo in resting and contracting muscle.

A cyclic AMP-independent glycogen synthase kinase has been purified 4000-fold from rabbit skeletal muscle in this laboratory [16], which catalyses the inactivation of glycogen synthase. Although this enzyme was thought to be identical to the glycogen synthase kinase-2 which contaminates purified glycogen synthase [1,16], further experiments have demonstrated that it is a distinct enzyme, termed glycogen synthase kinase-3. The evidence in support of this statement will be presented in a subsequent publication (D.B. R., P. C., in preparation).

Acknowledgements

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References

[1] Nimmo, H. G. and Cohen, P. (1974) FEBS Lett. 47, 162–166.
[2] Soderling, T. R., Hickenbottom, J. P., Reimann, E. M., Hunkder, F. L., Walsh, D. A. and Krebs, E. G. (1970) J. Biol. Chem. 245, 6317–6328.
[3] Schlender, K. K., Wei, S. H. and Villar-Palasi, C. (1969) Biochim. Biophys. Acta 191, 272–278.
[4] Proud, C. G., Rylatt, D. B., Yeaman, S. J. and Cohen, P. (1977) FEBS Lett. 80, 435–442.
[5] Rylatt, D. B. and Cohen, P. (1979) FEBS Lett. 98, 71–75.
[6] Craig, J. W. and Larner, J. (1964) Nature 202, 971–973.
[7] Danforth, W. H. (1965) J. Biol. Chem. 240, 588–593.
[8] Cohen, P. (1979) Curr. Top. Cell. Reg. 14, 117–196.
[9] Nimmo, H. G., Proud, C. G. and Cohen, P. (1976) Eur. J. Biochem. 68, 21–30.
[10] Caudwell, B., Antoniw, J. F. and Cohen, P. (1978) Eur. J. Biochem. 86, 511–518.
[11] Nimmo, G. A. and Cohen, P. (1978) Eur. J. Biochem. 87, 341–351.
[12] Cohen, P. (1973) Eur. J. Biochem. 34, 1–14.
[13] Cohen, P., Burchell, A., Foulkes, J. G., Cohen, P. T. W., Vanaman, T. C. and Nairn, A. C. (1978) FEBS Lett. 92, 287–293.
[14] Watterson, D. M., Harreston, W. G., Keller, P. M., Sharief, F. and Vanaman, T. C. (1976) J. Biol. Chem. 251, 2501–2513.
[15] Cohen, P., Antoniw, J. F., Nimmo, H. G. and Yeaman, S. J. (1976) CIBA Found. Symp. 41, 281–295.
[16] Nimmo, H. G., Proud, C. G. and Cohen, P. (1976) Eur. J. Biochem. 68, 31–44.