GLYCOGEN SYNTHETASE KINASE 2 (GSK 2); THE IDENTIFICATION OF A NEW PROTEIN KINASE IN SKELETAL MUSCLE

H. G. NIMMO and Philip COHEN
Department of Biochemistry, Medical Sciences Institute, The University, Dundee DD1 4HN, Scotland

Received 20 August 1974

1. Introduction

Glycogen synthetase (EC 2.4.1.11) is regulated through a phosphorylation-dephosphorylation mechanism [1]. The dephosphorylated *I*-form is fully active in both the presence and absence of glucose-6-phosphate, and can be converted to the phosphorylated *D*-form, which has an absolute requirement for glucose-6-phosphate, by incubation with magnesium ions, ATP and the cyclic AMP dependent protein kinase which also activates phosphorylase kinase [2,3]. The proportion of glycogen synthetase *D* in skeletal muscle is increased by the administration of adrenalin [4]. This presumably acts through the elevation in cyclic AMP levels [5,6] and activation of cyclic AMP dependent protein kinase [7] which have been shown to occur in vivo in response to the hormone.

The reconversion of glycogen synthetase *D* to *I* is under the control of insulin [8], but it appears to be independent of the effect of this hormone on glucose transport into muscle [4]. It is also unlikely that the effect is explainable in terms of an inactivation of cyclic AMP dependent protein kinase resulting from a decreased availability of cyclic AMP, because cyclic AMP levels in muscle not only do not decrease in the presence of insulin, but even show a slight rise [9].

Theoretically, the elevation of synthetase *I* levels by insulin could be achieved in one of three different ways. Insulin could increase the activity of glycogen synthetase phosphatase, decrease the activity of cyclic AMP dependent protein kinase, or change the conformation of glycogen synthetase itself, rendering it a better substrate for the phosphatase or a less effective substrate for the kinase. Since an investigation of these possibilities required the use of both the *D* and *I* forms as substrates for the interconverting enzymes, a detailed study was undertaken of the phosphorylation of glycogen synthetase *I* in vitro. This has led to the discovery reported here, that glycogen synthetase preparations contain a new protein kinase, distinct from cyclic AMP dependent protein kinase and phosphorylase kinase, which is able to phosphorylate glycogen synthetase. The possible relationship between this new enzyme, termed glycogen synthetase kinase 2 (GSK 2) and the mechanism of action of insulin is discussed.

2. Materials and methods

2.1. *Enzymes*

Glycogen synthetase was isolated from rabbit skeletal muscle [10]. The preparation was essentially homogeneous by the criterion of dodecyl sulphate gel electrophoresis, and was greater than 95% in the *I*-form as judged by assays in the presence and absence of glucose-6-phosphate. Phosphorylase kinase and cyclic AMP dependent protein kinase (peak II from DEAE-cellulose) were purified from skeletal muscle as described previously [11]. The protein inhibitor of cyclic AMP dependent protein kinase was partially purified up to the 15% trichloroacetic acid precipitation step [12]. Histone F_1 from rabbit liver was a generous gift from Professor Thomas Langan, University of Denver.

2.2. *Protein phosphorylation*

Reactions were carried out at pH 7.0, 20°C in the following incubation mixture: sodium glycerophosphate 10 mM, EDTA 0.4 mM, [γ-^{32}P] ATP or GTP as indicated, and protein substrate 0.006–0.012 mM (glycogen synthetase, phosphorylase kinase or histone

F_1). Magnesium acetate was normally present in a ten fold excess over the nucleoside triphosphate, but for experiments in which initial rates of phosphorylation were measured, the free magnesium ion concentration was maintained at 10 mM. Where indicated, cyclic AMP (0.001 mM), cyclic AMP dependent protein kinase, and the inhibitor protein were also included. The reactions were initiated with the nucleoside triphosphate, and aliquots were removed at various times, and analysed for the total covalently bound phosphate incorporated [13]. In calculating the stoichiometry of phosphate incorporation, one mole of glycogen synthetase was taken as 88 000 g (the subunit molecular weight determined by gel electrophoresis [10], one mole of histone F_1 as 21 000 g, and one mole of phosphorylase kinase as the $\alpha\beta\gamma$ unit or 318 000 g of protein [11]. The absorbance index, $A^{1\%}_{280}$, of glycogen synthetase was taken as 13.5 [10] and that of phosphorylase kinase as 12.4 [11]. The initial rate of phosphorylation of each protein was found to be linear up to an incorporation of 0.1 moles of phosphate per mole protein.

In order to use ^{32}P-labelled glycogen synthetase as a substrate for trypsin, reactions were terminated by the addition of EDTA to 15 mM and ammonium sulphate to 45% saturation. The solutions were stored for 30 min at 0°C, and then centrifuged at 18 000 g for ten min. The precipitates were washed once with 45% ammonium sulphate, EDTA 1.0 mM pH 7.0, redissolved in Tris–HCl buffer 50 mM pH 7.5 containing EDTA 1.0 mM and dithiothreitol 1.0 mM, and dialysed against this buffer overnight at 4°C.

3. Results

3.1. *Phosphorylation of glycogen synthetase by endogenous protein kinases*

Incubation of purified glycogen synthetase or phosphorylase kinase with magnesium ions and [γ-^{32}P] ATP led to a slow incorporation of radioactivity into each protein, which was markedly stimulated by the addition of cyclic AMP (fig. 1). This showed that each preparation was contaminated with trace amounts of cyclic AMP dependent protein kinase. The endogenous activity was however *not* completely supressed by the addition of the specific protein inhibitor of cyclic AMP dependent protein kinase. In the case of phosphorylase kinase, the residual activity is thought to arise from the self phosphorylation of phosphorylase kinase catalysed by the enzyme itself [13]. In the case of glycogen synthetase however, a similar interpretation did not seem likely, and the results suggested that the glycogen synthetase preparations might be contaminated with a protein kinase distinct from the cyclic AMP dependent enzyme. This idea was tested by using the inhibitor protein to titrate the phosphorylation of histone catalysed by cyclic AMP dependent protein kinase and the endogenous phosphorylation of glycogen synthetase (fig. 2). It can be seen, that, whereas the phosphorylation of histone could be almost totally suppressed by the inhibitor protein, the endogenous phosphorylation of glycogen synthetase showed a component, accounting for about 50% of the total phosphorylation rate at 0.2 mM ATP,

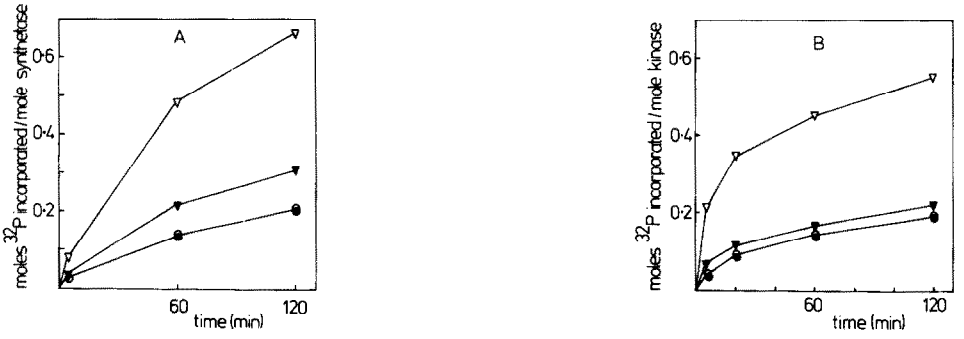

Fig. 1. Phosphorylation of (A) glycogen synthetase and (B) phosphorylase kinase by endogenous protein kinases upon addition of ATP to a final concentration of 0.2 mM. The reactions were carried out with cyclic AMP (0.001 mM) in the presence (○) or absence (▽) on inhibitor protein 60 μg/ml, or without cyclic AMP in the presence (●) or absence (▼) of inhibitor protein. The other reaction conditions are as given under Materials and methods.

Fig. 2. The effect of inhibitor protein on the phosphorylation of (A) histone F_1 and (B) glycogen synthetase. The reaction conditions were: (A) histone 0.25 mg/ml, cyclic AMP (0.001 mM), purified cyclic AMP dependent protein kinase: (B) glycogen synthetase 0.01 mg/ml, cyclic AMP 0.001 mM). Each reaction was initiated with ATP to a final concentration of 0.2 mM, and graphs show the initial rates of ^{32}P incorporation (moles/mole protein) plotted as a function of inhibitor protein concentration.

which was completely unaffected by the inhibitor protein.

Analysis of glycogen synthetase preparations showed a contamination with phosphorylase kinase of about 0.5% by weight. However, addition of purified phosphorylase kinase to increase this activity ten fold had no influence on the rate of phosphorylation of glycogen synthetase. Similarly, ethyleneglycol bis(2-aminoethyl ether)-*N*, *N'* tetracetic acid (EGTA) 0.1–1.0 mM which inhibits phosphorylase kinase, and calcium ions (0.1–1.0 mM) and glycogen (6 mg/ml) which activate phosphorylase kinase [14–16] did not affect the rate of phosphorylation of glycogen synthetase in the presence or absence of inhibitor protein. This indicated that a new protein kinase distinct from cyclic AMP dependent protein kinase and phosphorylase kinase was present as a trace contaminant in glycogen synthetase preparations. This enzyme is termed glycogen synthetase 2 (GSK 2) to distinguish it from cyclic AMP dependent protein kinase, which can be regarded as GSK 1.

3.2. *Properties of glycogen synthetase kinase 2*

The nucleoside triphosphate specificity of GSK 2 was examined by studying the endogenous phosphorylation of glycogensynthetase in the presence of the inhibitor protein at 60μg/ml. The K_m values for ATP and GTP were found to be 0.4 mM and 5.0 mM respectively, and approximately the same maximal reaction velocity was found in each case. Under equivalent conditions, the K_m of cyclic AMP dependent protein kinase for ATP was 0.024 mM, but this enzyme did not give a measurable rate of phosphorylation of glycogen synthetase when GTP was used as the phosphoryl donor.

In the presence of the inhibitor protein, and either ATP 2 mM or GTP 10 mM, the incorporation of ^{32}P by endogenous GSK 2 reached a plateau at 0.4–0.5 moles of phosphate per mole protein. The incorporation did not affect the activity of the enzyme which remained in the *I*-form throughout. This contrasted with the phosphorylation of glycogen synthetase by exogenous cyclic AMP dependent protein kinase, which rapidly reached a plateau after the incorporation of 1.0 ± 0.1 moles per mole protein, and converted glycogen synthetase *I* to *D* [10,2].

3.3. *Tryptic digestion of ^{32}P-labelled glycogen synthetase*

Glycogen synthetase was maximally phosphorylated by the action of either added cyclic AMP dependent protein kinase at 0.1 mM ATP, or by endogenous GSK 2 at 2 mM ATP in the presence of the inhibitor protein. Each preparation was then incubated with trypsin at a 1:1 molar ratio at pH 7.5, and the liberation of trichloroacetic acid soluble ^{32}P-labelled peptides was analysed as a function of time. When glycogen synthetase labelled with cyclic AMP dependent protein kinase was used, there was an extremely rapid release of ^{32}P-labelled peptides, which reached a plateau within a few minutes. However, with glycogen synthetase labelled using GSK 2 only 1–2% of the radioactivity was released in 5 min under equivalent conditions (fig. 3). Only

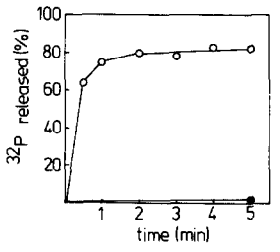

Fig. 3. Digestion of native ^{32}P-labelled glycogen synthetase (2.6 mg/ml) with trypsin (0.7 mg/ml). The graph shows the release of trochloroacetic acid soluble tryptic peptides at 20°C from glycogen synthetase phosphorylated with cyclic AMP dependent protein kinase (○) or GSK 2 (●), as a function of time. Glycogen synthetase was phosphorylated either in the presence of 0.1 mM ATP with 0.001 mM cyclic AMP and added cyclic AMP dependent protein kinase (○), or by endogenous GSK 2 in the presence of 2 mM ATP and inhibitor protein 60 g/ml (●). The preparations contained 0.95 moles ^{32}P/mole protein at zero time (○) or 0.43 moles ^{32}P/mole (●).

10% of the radioactivity became acid soluble after incubation with trypsin for 60 min, although 80% solubilisation could be achieved in 60 min, by digestion with chymotrypsin at a 1:1 molar ratio (not illustrated). These results clearly showed that the two protein kinases had labelled different sites on glycogen synthetase.

4. Discussion

The results establish the existence in skeletal muscle of a new protein kinase activity, termed GSK 2, which can phosphorylate glycogen synthetase. GSK 2 is not phosphorylase kinase, and can be distinguished from cyclic AMP dependent protein kinase on five counts: (1) it is not activated by cyclic AMP; (2) it is not inhibited by the inhibitor protein; (3) it does not appear to promote the conversion of glycogen synthetase *I* to *D*; (4) it has a different nucleoside triphosphate specificity; (5) it labels different site(s) on glycogen synthetase. GSK 2 phosphorylation 'plateaus' at lower than stoichiometric amounts: this may arise as a consequence of the low amount of endogenous GSK 2 activity present in the synthetase preparations or possibly because the site(s) labelled by GSK 2 are already partially phosphorylated.

The identification of GSK 2 at least partially explains the wide discrepancy in the content of phosphate bound covalently to glycogen synthetase *D* which has been reported previously [2,17]. Incubation of purified synthetase *I* with purified cyclic AMP dependent protein kinase at 0.6 mM ATP was reported to be accompanied by the rapid conversion to glycogen synthetase *D* and incorporation of close to one mole of phosphate per mole enzyme [2], in agreement with the present data. However, preparations of synthetase *D* which had been phosphorylated at an early stage in the purification by a prolonged three day incubation at 3°C with 10 mM ATP were found to contain six moles of phosphate per mole protein [17]. In this case at least some of the extra phosphate probably resulted from the combined action of the two endogenous protein kinases which label different sites on the enzyme.

When assayed under optimal conditions (i.e. high ATP), endogenous GSK 2 activity in purified glycogen synthetase preparations is about three times greater than endogenous cyclic AMP dependent protein kinase activity. Cyclic AMP dependent protein kinase is an extremely active enzyme, and is potentially capable of converting glycogen synthetase *I* to *D* within a few seconds in vivo. Since the purification of glycogen synthetase enriches the enzyme relative to cyclic AMP dependent protein kinase by a factor of more than 200, these results suggest that the activity of GSK 2 in muscle extracts is also likely to be appreciable. Furthermore, skeletal muscle extracts contain a protein phosphatase activity which catalyses the dephosphorylation of the site(s) labelled by GSK 2 at a rate as fast as or faster than that of the site(s) labelled by cyclic AMP dependent protein kinase (J. F. Antoniw, H. G. Nimmo and P. Cohen, unpublished experiments). It is therefore of interest to speculate about the possible role of GSK 2 phosphorylation.

Two cases are now known to exist in which protein function is regulated by multiple phosphorylations. The control of phosphorylase kinase activity by cyclic AMP dependent protein kinase involves the phosphorylation of the enzyme at two sites. The phosphorylation of the second site does not affect the activity of the enzyme directly, but greatly enhances the rate of dephosphorylation of the primary site and hence inactivation of the enzyme [18]; moreover, the phosphorylation of both sites occurs in vivo in response to adrenalin [7]. Secondly, it has been demonstrated that histone F_1 is phospho-

rylated in vivo by two distinct protein kinases. One of these is the cyclic AMP dependent protein kinase [19], and the other is a kinase, present only in growing cells, which phosphorylates the protein at sites distinct from that labelled by cyclic AMP dependent protein kinase [20]. Phosphorylation of histone F_1 by either kinase influences its interaction with DNA (T. A. Langan, personal communication). The recent discovery that insulin stimulates the phosphorylation of a 140 000 mol. wt. component in adipose tissue [21] suggests that the activation of a protein kinase may underlie the actions of insulin, as appears to be the case for adrenalin. It seems possible that GSK 2 is under the control of insulin and induces a functional alteration in glycogen synthetase which facilitates the conversion of glycogen synthetase *D* to *I*. The isolation of GSK 2 and an assessment of this possibility are in progress in this laboratory.

Acknowledgements

We wish to thank Mrs Carol Taylor for her excellent technical assistance. This work was supported by grants from the Science Research Council, London, and the British Diabetic Association.

References

[1] Friedman, D. L. and Larner, J. (1963) Biochemistry 2, 669–675.
[2] Soderling, T. R., Hickenbottom, J. P., Reimann, E. M., Hunkeler, 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] Danforth, W. H. (1965) J. Biol. Chem. 240, 588–593.
[5] Posner, J. B., Stern, R., and Krebs, E. G. (1965) J. Biol. Chem. 240, 1982–1985.
[6] Drummond, G. I., Harwood, J. P. and Powell, C. A. (1969) J. Biol. Chem. 244, 4235–4240.
[7] Yeaman, S. J. and Cohen, P. (1974) Biochemical Society Transactions, in press.
[8] Villar-Palasi, C. and Larner, J. (1960) Biochim. Biophys. Acta 39, 171.
[9] Larner, J., Villar-Palasi, C., Goldberg, N. D., Bishop, J. S., Huijing, F., Winger, J. I., Sasko, H. and Brown, N. D. (1968) Adv. Enzyme Regulation 6, 409–423.
[10] Nimmo, H. G. and Cohen, P. (1974), manuscript in preparation.
[11] Cohen, P. (1973) European J. Biochem. 34, 1–14.
[12] Walsh, D. A., Ashby, C. D., Gonzales, C., Calkins, C., Fischer, E. H. and Krebs, E. G. (1971) J. Biol. Chem. 246, 1977–1985.
[13] Walsh, D. A., Perkins, J. P., Brostrom, C. O., Ho, E. S. and Krebs, E. G. (1971) J. Biol. Chem. 246, 1968–1976.
[14] Brostrom, C. O., Hunkeler, F. L. and Krebs, E. G. (1971) J. Biol. Chem. 246, 1961–1967.
[15] Heilmeyer, L. M. G., Meyer, F., Haschke, R. H. and Fischer, E. H. (1970) J. Biol. Chem. 245, 6649–6656.
[16] Delange, R. J., Kemp, R. G., Riley, W. D., Cooper, R. A. and Krebs, E. G. (1968) J. Biol. Chem. 243, 2200–2208.
[17] Smith, C. H., Brown, N. D. and Larner, J. (1971) Biochem. Biophys. Acta 242, 81–88.
[18] Cohen, P. and Antoniw, J. F. (1973) FEBS Letters 34, 43–47.
[19] Langan, T. A. (1969) Proc. Natl. Acad. Sci. U.S. 64, 1276–1283.
[20] Langan, T. A. and Hohmann, P. (1974) Fed. Amer. Soc. Exp. Biol. 33, 1597–1597.
[21] Benjamin, W. B. and Singer, I. (1974) Biochim. Biophys. Acta 351, 28–41.