Multisite phosphorylation of glycogen synthase from rabbit skeletal muscle

Phosphorylation of site 5 by glycogen synthase kinase-5 (casein kinase-II) is a prerequisite for phosphorylation of sites 3 by glycogen synthase kinase-3

Colin Picton*, James Woodgett, Brian Hemmings and Philip Cohen+

Department of Biochemistry, Medical Sciences Institute, University of Dundee, Dundee DD1 4HN, Scotland

Received 4 November 1982

Glycogen synthase kinase-5 (casein kinase-II) phosphorylates glycogen synthase on a serine termed site 5. This residue is just C-terminal to the 3 serines phosphorylated by glycogen synthase kinase-3, which are critical for the hormonal regulation of glycogen synthase in vivo. Although phosphorylation of site 5 does not affect the catalytic activity, it is demonstrated that this modification is a prerequisite for phosphorylation by glycogen synthase kinase-3. Since site 5 is almost fully phosphorylated in vivo under all conditions, the role of glycogen synthase kinase-5 would appear to be a novel one in forming the recognition site for another protein kinase

Glycogen synthase *Casein kinase* *Adrenalin* *Insulin* *Cyclic AMP* *Calmodulin*

1. INTRODUCTION

Glycogen synthase is the best documented example of 'multisite phosphorylation', a phenomenon that is being encountered with increasing frequency [1]. The enzyme is phosphorylated on 7 serine residues by 6 protein kinases [1–4]. Cyclic AMP-dependent protein kinase phosphorylates sites 1a, 1b and 2 (site 1a >> site 2 > site 1b), phosphorylase kinase, glycogen synthase kinase-4 and a specific calmodulin dependent glycogen synthase kinase site 2, glycogen synthase kinase-3 sites (3a + 3b + 3c), and glycogen synthase kinase-5 site 5. Site 2 is 7 residues from the N-terminus of the protein, while sites 3a, 3b, 3c, 5, 1a and 1b are 30, 34, 38, 46, 87 and 100 residues respectively from the *N*-terminus of a large cyanogen bromide peptide near the C-terminus of glycogen synthase [5].

The phosphorylation of glycogen synthase decreases its activity. Phosphorylation of sites (3a + 3b + 3c) or site 2 produces larger changes than site 1a, but the effects are cumulative, so that even greater decreases in activity are observed when all five sites are labelled. Phosphorylation of site 1b or 5 does not alter the kinetic properties [1,2].

All 7 serine residues are phosphorylated in vivo [6]. Glycogen synthase isolated from normally fed or 24 h starved animals contains slightly < 3 mol phosphate/mol subunit of which > 40% is present in sites (3a + 3b + 3c) [6,7]. Activation produced in vivo by acute administration of insulin is associated with the loss of 0.4–0.5 mol phosphate/mol subunit from sites (3a + 3b + 3c), without a significant change in the phosphate con-

+ To whom correspondence should be addressed

* Present address: Searle Research and Development, PO Box 53, Lane End Road, High Wycombe, Bucks, HP12 4HL, England

Published by Elsevier Biomedical Press
00145793/82/0000—0000/$2.75

tent of any other site [7]. Injection of adrenalin increases the phosphate content to slightly above 5 mol/mol subunit. Most of the increase occurs at sites (3a + 3b + 3c) (increased by ~1.2 mol phosphate/mol subunit) and site 2 (increased by ~0.6 mol phosphate/mol subunit), with only small increases at sites 1a and 1b (both increased by ~0.25 mol phosphate/mol subunit) and none at site 5 [6].

The increased phosphorylation at sites (3a + 3b + 3c) in response to adrenalin was unexpected. The effects of this hormone are mediated by cyclic AMP in skeletal muscle, yet the activity of glycogen synthase kinase-3 is unaffected by this second messenger [2]. One possibility is that increased phosphorylation of sites 1a, 1b and 2 alters the conformation of glycogen synthase in such a way as to facilitate the phosphorylation of sites (3a + 3b + 3c) by glycogen synthase kinase-3. This idea prompted a systematic study of the effects of phosphorylation at one site, on the rates of phosphorylation of other sites. The results of these studies are reported here and demonstrate that sites (3a + 3b + 3c) cannot be phosphorylated in vitro unless site 5 is phosphorylated.

2. MATERIALS AND METHODS

2.1. *Protein preparations*

Glycogen synthase *a* [8,9], phosphorylase kinase [10], glycogen synthase kinase-3 [11], glycogen synthase kinase-4 [2], glycogen synthase kinase-5 [2], the catalytic subunit of cyclic AMP-dependent protein kinase [12] and its specific inhibitor protein [13], and the calmodulin-dependent glycogen synthase [3] were purified from rabbit skeletal muscle by standard procedures. A further sample of glycogen synthase kinase-5 (casein kinase-II) isolated from rabbit reticulocytes, was a gift from Dr Jolinda Traugh (Department of Biochemistry, University of California, Riverside). Calmodulin was purified from sheep brain.

2.2. *Phosphorylation of glycogen synthase* a

Preparations of glycogen synthase were passed through phosphocellulose to remove trace endogenous glycogen synthase activities [14]. The assays (0.1 ml) were carried out at 0.4 mg glycogen synthase/ml in 50 mM Tris–HCl (pH 7.0)–0.1% (v/v) 2-mercaptoethanol, 3.0 mM $MgCl_2$ and 0.1 mM [γ-^{32}P]ATP unless stated otherwise. EGTA (0.2 mM) was present, except in reactions involving phosphorylase kinase and the calmodulin-dependent glycogen synthase kinase, which contained 0.1 mM $CaCl_2$. Calmodulin (10 μg/ml) was added to assays of the calmodulin-dependent glycogen synthase kinase. The inhibitor protein and heparin (2 μg/ml) were included in all incubations to inhibit any trace cyclic AMP-dependent protein kinase or glycogen synthase kinase-5 activity [2], respectively (except in reactions involving these two enzymes). The reactions were initiated with ATP and terminated and analysed as in [15]. Reaction blanks in which glycogen synthase kinase or glycogen synthase were omitted were included, but were negligible in virtually all the experiments. Rates of phosphorylation were generally linear up to 0.1–0.2 mol/mol subunit, and the extent of phosphorylation was kept within this limit. The phosphorylation stoichiometry was calculated as in [15]. One unit of glycogen synthase kinase activity was that amount which catalysed the incorporation of 1.0 nmol phosphate/min into glycogen synthase *a*.

2.3. *Dephosphorylation of glycogen synthase* a

Glycogen synthase *a* (2 mg/ml) was dialysed against 10 mM Tris–HCl (pH 6.5)–0.1% (v/v) 2-mercaptoethanol and incubated with potato acid phosphatase (4 μg/ml) for 18 h at 20°C to dephosphorylate site 5. Control incubations were carried out in which potato acid phosphatase was omitted. The alkali-labile phosphate contents of the preparations were determined. The dephosphorylated and control preparations of glycogen synthase *a* were then phosphorylated by the different glycogen synthase kinases as in section 2.2., except that 10 mM potassium phosphate (pH 7.5) was included in the assays to completely inhibit potato acid phosphatase. Potato acid phosphatase was also added to the control preparations (after the addition of potassium phosphate) to ensure that the compositions of all incubations were identical.

2.4. *Materials and other analytical procedures*

The sources of all materials are given in [2]. The assay of glycogen synthase [7,16], and measurements of alkali labile phosphate [2,17] were carried out by standard procedures.

3. RESULTS

3.1. *Effect of site-5 phosphorylation on the rate of phosphorylation of glycogen synthase by different glycogen synthase kinases*

Preparations of glycogen synthase *a* with activity ratios ± glucose-6P of ~0.8 still contain a considerable amount of alkali-labile phosphate (0.4–0.8 mol/mol subunit), located in the 2 sites that do not affect the kinetic properties (site 5 and site 1b) [6]. Most of the phosphate (usually ~75%) is in site 5 [6]. These sites are incompletely dephosphorylated during the isolation of glycogen synthase *a*, because they are very poor substrates for the protein phosphatases present in skeletal muscle [4,18].

Incubation with potato acid phosphatase for 18 h at 20°C (section 2.3.) released all the alkali-labile phosphate from glycogen synthase *a* (see fig. 1) without any detectable change in the activity of the enzyme, measured in the presence or absence of glucose-6P. The glycogen synthase protein (M_r 86000) showed no sign of proteolysis after the incubations, as judged by the absence of degradation products (M_r 77000 and 69000 [4,8], when examined by SDS–polyacrylamide gel electrophoresis (not shown). These experiments confirm that phosphorylation of site 5 does not alter the kinetic properties of glycogen synthase.

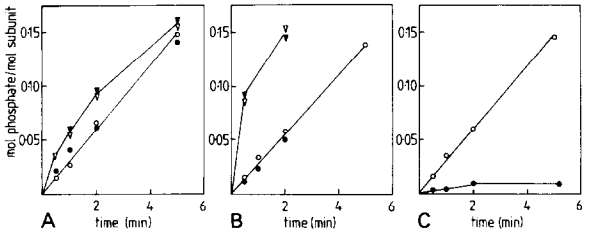

Fig. 1. Phosphorylation of glycogen synthase *a* before (open symbols) and after (closed symbols) incubation with potato acid phosphatase. The glycogen synthase *a* preparation contained 0.8 mol phosphate/mol subunit before, and <0.02 mol phosphate/mol subunit after incubation with potato acid phosphatase as in section 2: (A) phosphorylated with phosphorylase kinase (circles) and the calmodulin-dependent glycogen synthase kinase (triangles); (B) phosphorylated with glycogen synthase kinase-4 (circles) and cyclic AMP-dependent protein kinase (triangles); (C) phosphorylated with glycogen synthase kinase-3.

The dephosphorylated and control preparations of glycogen synthase *a* were tested for their ability to be phosphorylated by different glycogen synthase kinases. Dephosphorylation of glycogen synthase *a* did not affect the rate of phosphorylation of site 2 by phosphorylase kinase, glycogen syn-

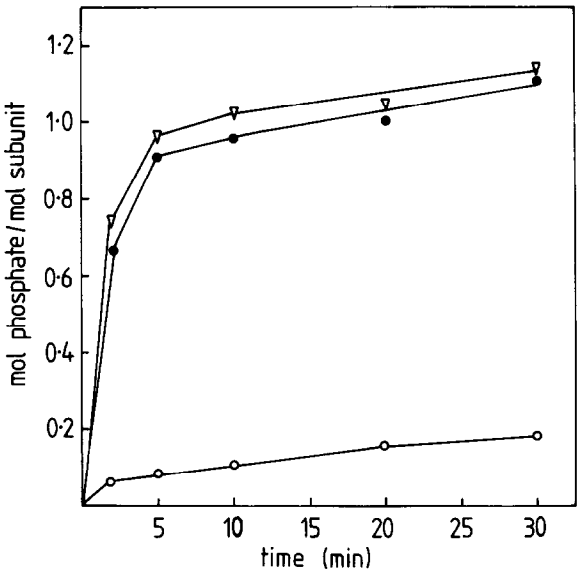

Fig. 2. Effect of dephosphorylation and rephosphorylation of site 5 on the rate of phosphorylation of glycogen synthase *a* by glycogen synthase kinase-3. Glycogen synthase *a* was treated with potato acid phosphatase (section 2.3.) and contained <0.04 mol phosphate/mol subunit. An aliquot of the preparation (0.4 mg/ml) was phosphorylated to 0.45 mol/mol subunit with glycogen synthase kinase-5 in 50 mM Tris–HCl (pH 7.5) containing 10 mM potassium phosphate (pH 7.5) 200 mM NaCl, inhibitor protein, 0.2 mM EGTA, 6.0 mM Mg^{2+} and 0.05 mM ATP (unlabelled). The phosphorylation stoichiometry was established by a separate parallel incubation with [γ-^{32}P]ATP. The reaction (0.07 ml) approached a plateau after 30 min and was terminated by the addition of a 0.1 mg/ml solution of heparin (0.01 ml). Glycogen synthase kinase-3 was added, followed by 1.0 mM [γ-^{32}P]ATP (0.01 ml). (▽—▽) Glycogen synthase *a* preparation not treated with potato acid phosphatase nor phosphorylated by glycogen synthase kinase-5; (●—●) glycogen synthase *a* preparation incubated with potato acid phosphatase and rephosphorylated with glycogen synthase kinase-5; (○—○) glycogen synthase *a* treated with potato acid phosphatase, but not rephosphorylated with glycogen synthase kinase-5.

thase kinase-4 and the calmodulin-dependent glycogen synthase kinase, or the phosphorylation of site 1a by cyclic AMP-dependent protein kinase (figs. 1A,B) but virtually abolished phosphorylation of the enzyme by glycogen synthase kinase-3 (fig. 1C). Rephosphorylation of site 5 with glycogen synthase kinase-5, restored the ability to be phosphorylated by glycogen synthase kinase-3 (fig. 2).

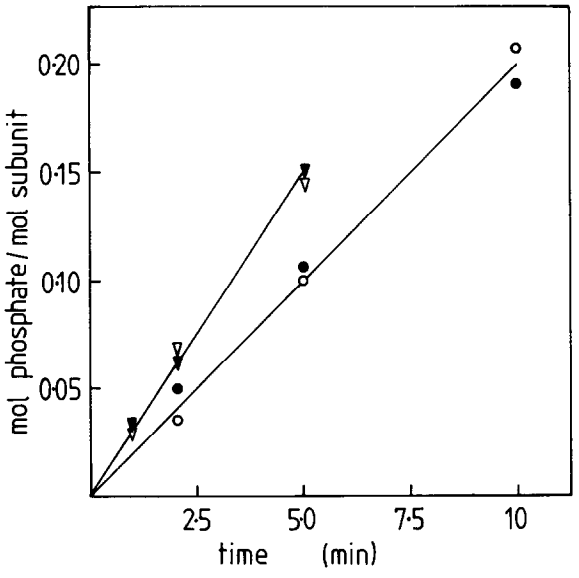

Fig. 3. Effect of phosphorylation by cyclic AMP-dependent protein kinase (triangles) or phosphorylase kinase (circles) on the rate of phosphorylation of glycogen synthase by glycogen synthase kinase-3. Glycogen synthase was phosphorylated with the catalytic subunit of cyclic AMP-dependent protein kinase (10 U/ml) to 2.15 mol/mol subunit [site 1a = 0.7, site 1b = 0.65, site 2 = 0.8 mol/mol subunit [23] or with phosphorylase kinase (10 μg/ml) to 0.45 mol/mol subunit, using the phosphorylation assay described in section 2.2. except that unlabelled ATP was used. The phosphorylation stoichiometries were established by separate parallel incubations with [γ-^{32}P]ATP. The reactions were terminated by the addition of either the inhibitor protein or 5.0 mM EGTA (0.01 ml). Glycogen synthase kinase-3 (5 μl) was then added, followed by 1.0 mM [γ-^{32}P]ATP (0.01 ml). Rates of phosphorylation (open symbols) were then compared to control incubations (closed symbols) in which either cyclic AMP-dependent protein kinase or phosphorylase kinase had been omitted.

3.2. *Effect of phosphorylation of sites 1a, 1b and 2 on the rate of phosphorylation of sites (3a + 3b + 3c)*

Phosphorylation of sites 1a, 1b and 2 by cyclic AMP-dependent protein kinase or site 2 with phosphorylase kinase had no effect on the rate of phosphorylation of sites (3a + 3b + 3c) by glycogen synthase kinase-3 (fig. 3). Conversely phosphorylation of sites (3a + 3b + 3c) by glycogen synthase kinase-3 did not affect the rate of phosphorylation of site 2 by phosphorylase kinase, glycogen synthase kinase-4 or the calmodulin-dependent glycogen synthase kinase or site 1a by cyclic AMP-dependent protein kinase (not shown).

The inclusion of glycogen (10 mg/ml) or glucose-6P (1.0 mM) in the assays did not significantly affect the activity of any glycogen synthase kinase (not shown).

4. DISCUSSION

Glycogen synthase kinase-5 [2] is identical to an enzyme that has a ubiquitous tissue distribution, and has been variously termed casein kinase TS [19], casein kinase-II [20], casein kinase-G [21], casein kinase N-II [22] and troponin-T kinase [23,24]. Several physiological substrates of this enzyme have been identified including glycogen synthase [6,7], the R_{II}-subunit of cyclic AMP-dependent protein kinase [25,26] and troponin-T [24,27] in muscle, and the β-subunit of protein synthesis initiation factor eIF-2 in reticulocytes [28,29]. However, the function of glycogen synthase kinase-5 has remained problematic, since none of the above phosphorylations appear to modify the activities of these proteins. Furthermore, all the physiological substrates as normally isolated contain substantial amounts of phosphate in the site(s) phosphorylated by glycogen synthase kinase-5, even though no precautions are taken to prevent the action of endogenous protein phosphatases. These sites therefore seem to be extremely poor substrates for protein phosphatases in mammalian tissues, suggesting that they may be rather stable modifications that do not turn over rapidly in vivo. This certainly appears to be the case for glycogen synthase, since phosphorylation of site 5 is not significantly affected by acute administration of adrenalin and insulin, or by starvation–refeeding [6,7].

This work suggests that one of the functions of glycogen synthase kinase-5 is to phosphorylate residues that can then act as recognition sites for glycogen synthase kinase-3 (and perhaps other protein kinases). This idea is supported by recent studies of the phosphorylation of the R_{II}-subunit of cyclic AMP-dependent protein kinase. Glycogen synthase kinase-5 phosphorylates serine-74 and serine-76, and glycogen synthase kinase-3 serine-44 and serine-47. However, the rate of phosphorylation of serine-44/serine-47 is decreased at least 10-fold, if serines-74 and 76 are first dephosphorylated [25].

The phosphorylation of glycogen synthase *a* by glycogen synthase kinase-3 in vitro generally reaches a plateau at 1.0–1.5 mol/mol subunit, despite the fact that 3 serine residues are phosphorylated [11,15]. Furthermore, at the end of the reaction the tryptic peptide containing sites (3a + 3b + 3c) is entirely in the triply phosphorylated state [11]. This puzzling result can now be explained, since the phosphorylation of glycogen synthase kinase-3 will plateau at a level determined by the extent of phosphorylation of site 5. The present work can also account for the failure of glycogen synthase kinase-3 to phosphorylate a synthetic hexadecapeptide containing sites (3a + 3b + 3c) but not site 5 [11].

Glycogen synthase kinase-3 phosphorylates the R_{II}-subunit of cyclic AMP-dependent protein kinase at 10–15% of the rate of glycogen synthase [25], but 9 further proteins that are excellent substrates for other protein kinases are not phosphorylated at all [11,15]. The necessity for a phosphoserine residue C-terminal to the sites phosphorylated by glycogen synthase kinase-3 may underlie its high specificity. It may also explain why 3 closely spaced serine residues in glycogen synthase are phosphorylated by this enzyme. On the other hand, the specificity of glycogen synthase kinase-3 may now need to be re-examined if potential substrates must first be phosphorylated by glycogen synthase kinase-5 (or other protein kinases).

The rate of phosphorylation of sites (3a + 3b + 3c) was unaffected by prior phosphorylation of sites 1a, 1b and 2 (fig. 3) or vice versa. The increased phosphorylation of sites (3a + 3b + 3c) that occurs in vivo in response to adrenalin cannot therefore be explained by this mechanism, and is most likely to involve an inhibition of protein phosphatase-1 [1,6].

ACKNOWLEDGEMENTS

This work was supported by a Programme Grant from the Medical Research Council, London, and by the British Diabetic Association. Glycogen synthase *a*, and the inhibitor protein were provided by Miss Caroline Mackie and Dr Peter Parker in this laboratory.

REFERENCES

[1] Cohen, P. (1982) Nature 296, 613–620.
[2] Cohen, P., Yellowlees, D., Aitken, A., Donella-Deana, A., Hemmings, B.A. and Parker, P.J. (1982) Eur. J. Biochem. 124, 21–35.
[3] Woodgett, J., Tonks, N.K. and Cohen, P. (1982) FEBS Lett. 148, 5–11.
[4] Embi, N., Parker, P.J. and Cohen, P. (1982) Eur. J. Biochem. 115, 405–413.
[5] Picton, C., Aitken, A., Bilham, T. and Cohen, P. (1982) Eur. J. Biochem. 124, 37–45.
[6] Parker, P.J., Embi, N., Caudwell, F.B. and Cohen, P. (1982) Eur. J. Biochem. 124, 47–55.
[7] Parker, P.J., Caudwell, F.B. and Cohen, P. (1983) Eur. J. Biochem. in press.
[8] Nimmo, H.G., Proud, C.G. and Cohen, P. (1976) Eur. J. Biochem. 68, 21–30.
[9] Caudwell, F.B., Antoniw, J.F. and Cohen, P. (1978) Eur. J. Biochem. 86, 511–518.
[10] Cohen, P. (1973) Eur. J. Biochem. 34, 1–14.
[11] Hemmings, B.A., Yellowlees, D., Kernohan, J.C. and Cohen, P. (1981) Eur. J. Biochem. 119, 443–451.
[12] Beavo, J.A., Bechtel, P.J. and Krebs, E.G. (1974) Methods Enzymol. 38, 299–308.
[13] Nimmo, G.A. and Cohen, P. (1978) Eur. J. Biochem. 87, 341–351.
[14] Soderling, T.R., Srivastava, A.K., Bass, M.A. and Khatra, B.S. (1979) Proc. Natl. Acad. Sci. USA 76, 2536–2540.
[15] Embi, N., Rylatt, D.B. and Cohen, P. (1980) Eur. J. Biochem. 107, 519–527.
[16] Nimmo, H.G., Proud, C.G. and Cohen, P. (1976) Eur. J. Biochem. 68, 31–44.
[17] Guy, P.S., Cohen, P. and Hardie, D.G. (1981) Eur. J. Biochem. 114, 399–405.
[18] Ingebritsen, T.S. and Cohen, P. (1982) Eur. J. Biochem. submitted.
[19] Meggio, F., Donella-Deana, A. and Pinna, L.A. (1977) FEBS Lett. 75, 192–196.

[20] Hathaway, G.M. and Traugh, J.A. (1979) J. Biol. Chem. 254, 762–768.
[21] Cachet, C., Job, D., Pirallet, F. and Chambaz, E.M. (1980) Endocrinology 106, 750–757.
[22] Thornburg, W. and Lindell, T.J. (1977) J. Biol. Chem. 252, 6660–6665.
[23] Risnik, V.V., Dobrovolskii, A.B., Gusev, N.B. and Severin, E.S. (1980) Biochem. J. 191, 851–854.
[24] Pinna, L.A., Meggio, F. and Deduikina, M.M. (1981) Biochem. Biophys. Res. Commun. 87, 114–120.
[25] Hemmings, B.A., Aitken, A., Cohen, P., Rymond, M. and Hofmann, F. (1982) Eur. J. Biochem. 127, 473–481.
[26] Carmichael, D.F., Geahlen, R.L., Allen, S.M. and Krebs, E.G. (1982) J. Biol. Chem. 257, 10440–10445.
[27] Pearlstone, J.R., Carpenter, M.R., Johnson, P. and Smillie, L.B. (1976) Proc. Natl. Acad. Sci. USA 73, 1902–1906.
[28] Tuazon, P.T., Merrick, W.C. and Traugh, J.A. (1980) J. Biol. Chem. 255, 10954–10958.
[29] DePaoli-Roach, A.A., Roach, P.J., Pham, K., Kramer, G. and Hardesty, B. (1981) J. Biol. Chem. 256, 8871–8874.