PHOSPHORYLATION OF GLYCOGEN SYNTHASE BY PHOSPHORYLASE KINASE

Stoichiometry, specificity and site of phosphorylation

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1. Introduction

The enzyme glycogen synthase contains multiple phosphorylation sites per tetrameric subunit which can be phosphorylated by cAMP-dependent and cAMP-independent protein kinases (reviewed [1]). On the basis of analysis of tryptic and CNBr [^{32}P]-peptides we have defined two phosphorylation domains per 90 000 dalton subunit [2]. The trypsin-sensitive domain of M_r 17 000 near the subunit C-terminus contains two or more phosphorylation sites [3,4] which are preferentially phosphorylated by the cAMP-dependent protein kinase. The trypsin-insensitive domain of 10 000 daltons is preferentially phosphorylated by a cAMP-independent synthase kinase [2].

Recently, a CDR-dependent synthase kinase has been reported [5,6] which now appears to be identical with phosphorylase kinase which can also catalyze phosphorylation and inactivation of glycogen synthase [7–9]. In this paper, antiserum to phosphorylase kinase was used to confirm the conclusion that phosphorylase kinase itself catalyzes phosphorylation of glycogen synthase. It is also shown that the presence of phosphorylase inhibits the inactivation of glycogen synthase by phosphorylase kinase and that the phosphorylation site in the trypsin–insensitive domain of glycogen synthase is serine residue no. 7 from the N-terminus. It is concluded that probably the same catalytic site in phosphorylase kinase catalyzes phosphorylation of phosphorylase and glycogen synthase.

Abbreviations: cAMP, adenosine 3′,5′-monophosphate; CDR, calcium-dependent regulatory protein; PTH, phenylthiohydantoin

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2. Methods

Glycogen synthase was purified from rabbit skeletal muscle through the affinity chromatography [2] and phosphocellulose chromatography [8] steps. This procedure yields glycogen synthase which is free of contaminating synthase kinase and phosphatase activities. Phosphorylase kinase from rabbit skeletal muscle was purified according to [10] and phosphorylase *b* as in [11]. Liver phosphorylase kinase was obtained from Drs T. D. Chrisman and E. G. Krebs [12] and antiserum to rabbit muscle phosphorylase kinase from Dr L. M. G. Heilmeyer [13]. Regulatory subunit of cAMP-dependent protein kinase was prepared by Dr J. D. Corbin. Phosphorylation of glycogen synthase by phosphorylase kinase was performed as in [8]. The stoichiometry of synthase phosphorylation, trypsin-insensitive [^{32}P]synthase, and the synthase activity ratio were determined as in [2]. Phosphorylase was assayed in the absence and presence of 5′-AMP as in [14]. [γ-^{32}P]ATP was prepared according to [15]. The [^{32}P]synthase was sequenced from the amino terminus in a Beckman Model 890 B Sequencer using the method in [16].

3. Results and discussion

We have reported that phosphorylation of glycogen synthase by skeletal muscle phosphorylase kinase (0.003–0.03 μM) resulted in incorporation of ~0.5–0.6 mol ^{32}P/synthase subunit associated with partial inactivation of synthase [8]. This phosphorylation was almost exclusively into the trypsin-insensitive domain. With 3500-fold purified liver phosphorylase kinase [12] as catalyst, we have also obtained incorporation of 0.5–0.6 mol ^{32}P/mol synthase subunit resulting in a decrease of the synthase activity ratio from 0.8–0.4 (results not shown). Using 0.28 μM activated muscle phosphorylase kinase (pH 6.8/8.6 activity ratio = 0.6) as catalyst, synthase phosphorylation was biphasic (fig.1) with rapid incorporation of 0.5–0.6 mol ^{32}P/90 000 g into the trypsin-insensitive domain followed by a much slower phosphorylation of the trypsin-sensitive domain. Synthase inactivation as measured by the synthase activity ratio correlated exactly with phosphorylation of the trypsin-insensitive domain. The slow phosphorylation of the trypsin-sensitive domain was not due to the small amount of cAMP-dependent protein kinase which normally contaminates phosphorylase kinase since this phosphorylation was not blocked by the heat-stable inhibitor [17] of cAMP-dependent protein kinase or by added regulator subunit of cAMP-dependent protein kinase (results not shown).

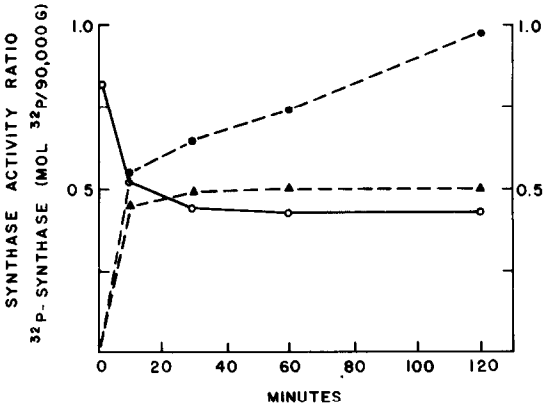

Fig.1. Glycogen a_4-synthase (0.5 mg/ml) was phosphorylated at pH 8.6 using activated rabbit skeletal muscle phosphorylase kinase (90 μg/ml, pH 6.8/8.6 activity ratio = 0.6). At the indicated times, aliquots were removed and analyzed for 8 total [^{32}P]synthase (●—●); trypsin-insensitive [^{32}P]synthase (▲– – – –▲); synthase activity ratio (○———○).

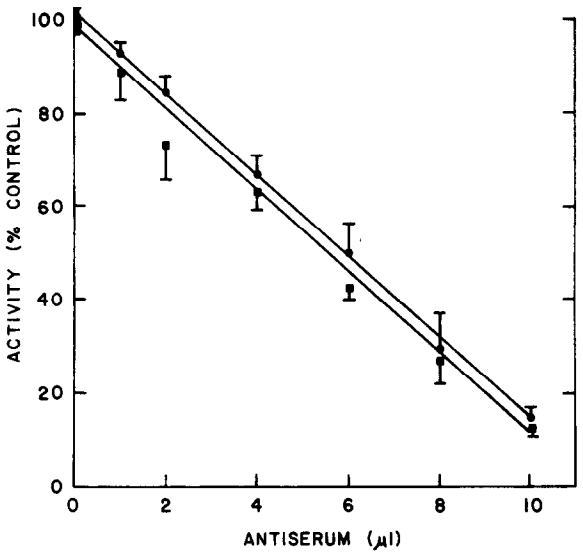

Fig.2. Phosphorylase kinase (14 μg) was incubated with the indicated amount of antiserum (6.5 μg/μl) plus control serum (total serum protein of 6.5 μg/μl) at 5°C for 2 h in 0.1 ml. The mixture was centrifuged and the supernatant was diluted 20-fold and assayed at pH 8.6 for phosphorylation of glycogen synthase (circles) and phosphorylase (squares) at 0.6 mg substrate/ml.

If the synthase phosphorylation is being catalyzed by phosphorylase kinase, antiserum to purified phosphorylase kinase should give identical inhibition of phosphorylation of phosphorylase and glycogen synthase as observed in fig.2. Antiserum had no effect on synthase phosphorylation catalyzed by cAMP-dependent protein kinase catalytic subunit.

Using 50 μM synthase and 160 μM phosphorylase, there was significant inhibition of each substrate towards conversion of the other (fig.3). These results as well as the antibody studies suggest that the same catalytic site in phosphorylase kinase catalyzes phosphorylation of glycogen synthase and phosphorylase. It was concluded [13] that different catalytic sites in phosphorylase kinase catalyze phosphorylation of phosphorylase and troponin I. Also, phosphorylase has been shown [18] to stimulate rather than inhibit the rate of the autocatalytic activation of phosphorylase kinase.

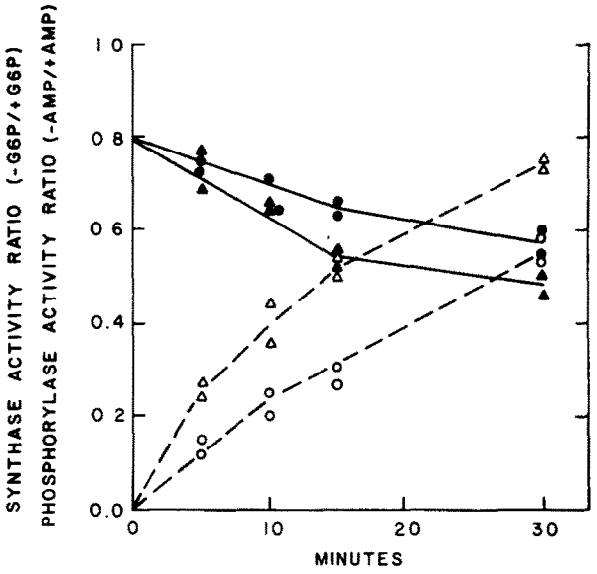

Fig.3. Interconversions of glycogen synthase (solid symbols) and phosphorylase (open symbols) in the absence (triangles) and presence (circles) of each other.

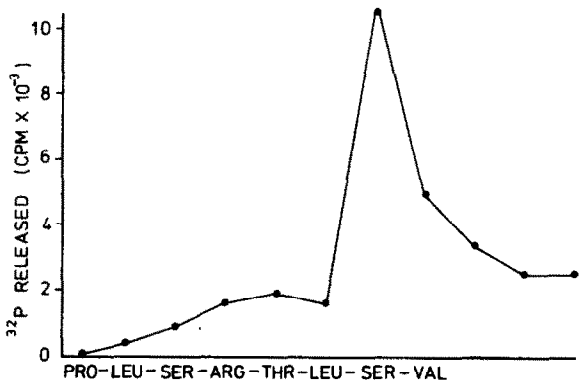

Fig.4. Sequence analysis of phosphorylated glycogen synthase. Glycogen synthase was phosphorylated using phosphorylase kinase and [γ-^{32}P]ATP. The [^{32}P]synthase was sequenced from the N-terminus (see section 2). An aliquot of the chlorobutane wash at each cycle was counted for ^{32}P and the remainder was used for identification of the PTH amino acid.

Glycogen synthase was phosphorylated to 0.5 mol ^{32}P/90 000 g using [γ-^{32}P]ATP and phosphorylase kinase. The intact synthase subunit was subjected to automated amino acid sequence analysis (see section 2). An aliquot of the chlorobutane wash from the sequenator cup was counted for ^{32}P and the remainder used for identification of the PTH-amino acid. A peak of ^{32}P was found at cycle no. 7 which corresponded to a serine residue (fig.4). The sequence that was obtained through valine no. 8 agreed with the reported N-terminal sequence [19].

lack of quantitative recovery is due to hydrolysis of PTH-phosphoserine to $^{32}P_i$ which extracts poorly in the chlorobutane wash [4]. While this work was in progress Rylatt and Cohen [20] reported that a CDR-dependent kinase contaminating their glycogen synthase preparation phosphorylates serine residue 7. The homology surrounding these phosphorylation sites is consistent with similar K_m values of phosphorylase kinase for glycogen synthase and phosphorylase with ~2.3-fold higher V_{max} for phosphorylase than for synthase [8].

Synthase [19]: Arg–Thr–Leu–$\overset{7}{\text{Ser(P)}}$–Val–Ser–Ser–Leu

Phosphorylase [20]: Lys–Gln–Ile–$\overset{14}{\text{Ser(P)}}$–Val–Arg–Gly–Leu

We concluded that serine no. 7 in glycogen synthase was phosphorylated by phosphorylase kinase. We cannot exclude the possibility of other phosphorylated sites since our recovery of ^{32}P at turn 7 was not quantitative. We believe, however, that the

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