HORMONAL REGULATION OF GLYCOGEN SYNTHASE PHOSPHORYLATION IN PERFUSED RAT SKELETAL MUSCLE

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1. Introduction

There is much in vitro evidence that skeletal muscle glycogen synthase is regulated through phosphorylation–dephosphorylation mechanisms [1–3]. The number of phosphorylation sites as indicated by the amount of phosphate per mole of subunit has been reported to be between 1–6 [4–13]. However, all these studies have correlated the phosphate content and the activity of the purified enzyme after in vitro modification by protein kinases, and there have been only two brief reports on the phosphorylation state of the enzyme in vivo [3,14].

Using the rat hindlimb perfusion technique we have presented kinetic evidence that glycogen synthase is substantially phosphorylated in control skeletal muscle and that epinephrine causes further phosphorylation [15]. Here, we have measured the chemical phosphate of the enzyme purified from control and hormone-treated muscle and have found a good correlation between the phosphorylation state of the enzyme and its activity.

2. Methods

The method used for hindlimb perfusion has been described [10]. Hindlimbs from fed rats (180–220 g body wt) were perfused with Krebs–Henseleit bicarbonate buffer containing 33% aged human erythrocytes, 4% bovine serum albumin and 10 mM D-glucose. The muscles were perfused for 20 min without hormones and then infused with either saline or epinephrine (10^{-7} M) for 20 min. At the end of the treatment period, the muscles were quickly extracted and homogenized in ice-cold buffer comprising 5 mM EDTA, 100 mM NaF, 1 mM DTT and trasylol (42.5 units/l) at pH 7.0. Homogenates of muscles (~80 g) from 8 perfusions were used for each enzyme preparation.

Purification of glycogen synthase was similar to that in [17] up to the second glycogen pellet. The resuspended pellet was then put onto a con A–Sepharose column as in [18]. After amylase digestion the enzyme was finally applied to a DEAE–Sepharose column and eluted with a linear NaCl gradient.

Throughout the purification, glycogen synthase was assayed according to [19] and the K_a for G-6-P was obtained from plots of the data [15]. Protein was determined by the method in [20] and alkali-labile phosphate by the malachite-green technique [21]. SDS–polyacrylamide gel electrophoresis (7.5%) was done as in [22] in amediol–sulfate buffer. Regular polyacrylamide disc-gel electrophoresis was done as in [23] with the addition of 1 mM G-6-P in the upper reservoir buffer [17]. Protein bands in gels were detected by staining with Coomassie blue [24], and the presence of glycogen synthase was confirmed by the iodine activity stain [25] and also by measuring the enzyme activity eluted from sliced gel.

3. Results

Table 1 illustrates the purification scheme for gly-

Abbreviations: con A, concanavalin A; G-6-P, glucose-6-phosphate; SDS, sodium dodecyl sulfate; M_r relative molecular mass

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Table 1
Muscle glycogen synthase purification

Procedure	Total units	Units/mg protein	Yield (%)	Purification
1. Extract	121	0.03	100%	1
2. Acidification	58	0.22	48%	7
3. 1st Glycogen pellet	46	0.27	38%	9
4. 2nd Glycogen pellet	46	0.35	36%	12
5. Con A–Sepharose	22	7.20	18%	240
6. DEAE–Sepharose	5	40.0	4%	1330

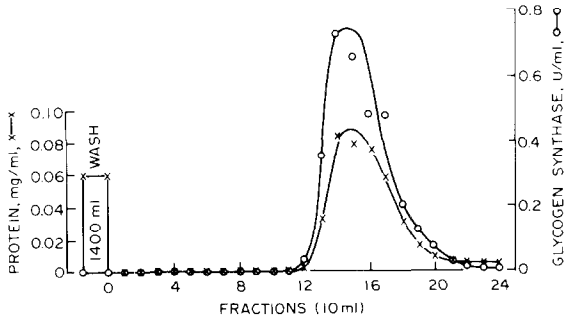

Fig.1. Con A–Sepharose profile: The column was washed overnight with buffer containing 50 mM Tris, 50 mM NaF, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM $MnCl_2$, 0.5 M NaCl, 1 mM DTT at pH 7.5. Glycogen synthase was then eluted with 50 mM Tris buffer containing 50 mM NaF, 1 mM DTT and 200 mM glucose at pH 7.5. The column dimensions were 5 × 10 cm and the flow rate was 70 ml/h.

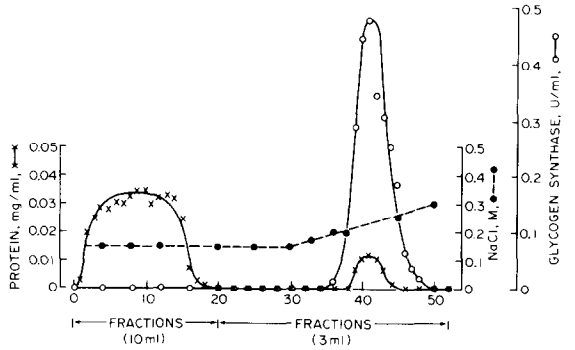

Fig.2. DEAE–Sepharose profile: The amylase-treated enzyme was applied to a DEAE–Sepharose column and eluted by a salt gradient using a 50 mM Tris buffer containing 5 mM EDTA, 50 mM NaF, 1 mM DTT at pH 7.5. The column dimensions were 2.5 × 5 cm and the flow rate was 30 ml/h.

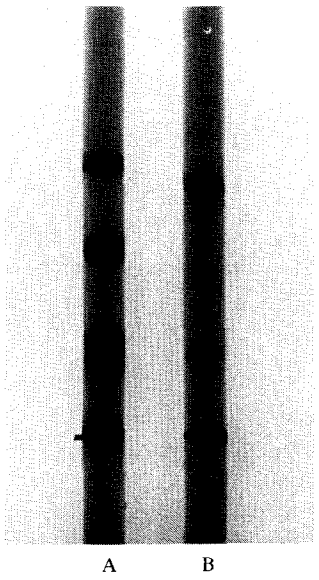

Fig.3. SDS–polyacrylamide gel electrophoresis (7.5%) of purified glycogen synthase: Electrophoresis was performed at 2 mA/tube using amediol–sulfate as the reservoir buffer. (A) Positions of subunits of marker proteins, i.e., phosphorylase (94 000 M_r), bovine serum albumin (67 000 M_r), ovalbumin (43 000 M_r) and carbonic anhydrase (30 000 M_r); (B) enzyme preparation.

cogen synthase. The enzyme was purified 1300-fold giving a final spec. act. of 40 U/mg protein with a total recovery of 4% of the initial activity. Con A–Sepharose chromatography was a major step in the purification procedure and resulted in a 20-fold purification. While the bulk of the protein appeared in the void volume (fig.1), glycogen synthase remained on the column and was eluted with 200 mM glucose.

Table 2
Glycogen synthase activity ratio and K_a for G-6-P during purification from control and epinephrine-perfused muscle

Procedure	Control		Epinephrine	
	Activity ratio	G-6-P K_a (mM)	Activity ratio	G-6-P K_a (mM)
1. Extract	0.14	0.15	0.04	1.20
2. Acidification	0.16	–	0.04	–
3. 1st Glycogen pellet	0.16	–	0.04	
4. 2nd Glycogen pellet	0.18	–	0.05	–
5. Con A–Sepharose	–	–	–	–
6. DEAE–Sepharose	0.16	0.13	0.04	0.95

Table 3
Phosphorylation state of glycogen synthase

Treatment	mol Phosphate/ 85 000 g protein
Control	3.1
Epinephrine	4.9

Another 5-fold purification was achieved by DEAE–Sepharose chromatography (fig.2), where the enzyme was eluted by a salt gradient (0.15–0.3 M NaCl) as a single symmetrical protein peak corresponding to the activity peak. SDS–polyacrylamide gel electrophoresis of the material in this peak showed a major 85 000 M_r band with an accompanying 40 000 M_r band (fig.3). Scans of gels showed that the minor band corresponded to 25% of total protein. This pattern is similar to that reported for the rabbit muscle enzyme [17]. Gel electrophoresis without detergent showed a single protein band corresponding to the glycogen synthase activity determined by iodine stain and activity assayed from sliced gel (not shown).

The kinetics of glycogen synthase isolated from control or epinephrine-treated muscle, as assessed by the activity ratio (–G-6-P/+G-6-P) and K_a for G-6-P, changed minimally or not at all throughout the purification procedure (table 2). The activity ratio was 0.14 in the control group and decreased to 0.04 after epinphrine treatment. The basal K_a for G-6-P was 0.15 mM and was increased by the hormone to 1.2 mM.

The measured chemical phosphate of the purified glycogen synthase is shown in table 3. The basal phosphate content in the control group was 3.1 mol/ 85 000 g protein. Epinephrine treatment resulted in the incorporation of almost 2 mol phosphate in addition.

4. Discussion

The usual procedure for purifying skeletal muscle glycogen synthase was modified by including con A–Sepharose chromatography as a major step. This approach gave us a 1300-fold purification of an enzyme approaching homogeneity (fig.3) with spec. act. 40 U/mg. More importantly, there was little or no change in the kinetics of the enzyme during the purification (table 2), suggesting that its phosphorylation state was not altered.

Phosphorylation of skeletal muscle glycogen synthase by cAMP-dependent protein kinase in vitro results in decreased enzyme activity [5–8,12,13]. Epinephrine treatment of intact skeletal muscle resulted in similar kinetic changes [15]; the kinetic properties of the enzyme in muscle perfused without hormones were consistent with a high degree of phosphorylation [15]. These data support that conclusion about the phosphorylation state of the control enzyme. Consistent with the current concept that epinephrine causes its metabolic effects in skeletal muscle via cAMP and cAMP-dependent protein kinase, the hormone caused a further increase in phosphate content. These findings in an isolated, perfused rat muscle preparation support the conclusions in [26]; i.e., that the enzyme isolated from skeletal muscle of control rabbits in vivo is phosphorylated to >2 mol phosphate/mol subunit and that epinephrine administration in vivo causes the incorporation of >1 mol phosphate in addition.

Glycogen synthase purified here from perfused rat skeletal muscle showed the presence of a 40 000 M_r protein on SDS–polyacrylamide gel electrophoresis. This protein cannot be phosphorylated by cAMP-dependent protein kinase, cAMP-independent protein kinase or phosphorylase kinase (T. R. Soderling, personal communication) and thus may not contain phosphate. Since this 40 000 M_r band was included in the measured protein, this means that the phosphate content of the 85 000 M_r subunit was probably underestimated. If this 40 000 M_r band is corrected for, the basal phosphate content in the control would increase to 4 mol/85 000 g protein, and epinephrine would cause the incorporation of 2.5 mol in addition. The enzyme purification technique is being modified in an attempt to produce an enzyme which shows only one band on SDS gel electrophoresis.

This study does not indicate the site(s) of phosphorylation. However, in [26] the control enzyme was phosphorylated mainly in the trypsin-insensitive region, consistent with our conclusion [15] that phosphorylation was mainly due to the activity of a cAMP-independent synthase kinase(s). As would be predicted from an increase in the activity of cAMP-dependent protein kinase, epinephrine increased the phosphate content of the trypsin-sensitive region of the enzyme [26,27]. Studies are in progress to determine the effects of insulin on the phosphorylation state of the enzyme in rat hindlimb muscle perfused without and with epinephrine.

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