

PHOSPHORYLATION OF GLYCOGEN SYNTHASE IN RABBIT MUSCLE

Effects of epinephrine and diabetes

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1. Introduction

Glycogen synthase, the rate-limiting enzyme of glycogenesis, has been purified from skeletal muscle and studied extensively over the past decade. Investigations on the *in vitro* phosphorylation have shown that glycogen synthase contains multiple phosphorylation sites per subunit which can be phosphorylated by three classes of protein kinases: cAMP-dependent protein kinase; Ca^{2+} -dependent protein kinases; and cAMP-independent protein kinase (reviews [1–3]). Analysis of tryptic and CNBr [^{32}P]peptides indicate the presence of a trypsin-sensitive phosphorylation region near the C-terminus and a trypsin-insensitive phosphorylation region near the N-terminus of the synthase subunit [1,4,5].

The phosphorylation state of glycogen synthase *in vivo* has not been thoroughly investigated. Insulin treatment of rabbits decreased the phosphate content of the muscle synthase from 1.33–0.87 mol P_i /mol subunit [6]. Epinephrine injection into rabbit increased the skeletal muscle synthase phosphate content from 2.2–3.1 mol P_i /mol subunit [3]. Here we show that the phosphorylation state of glycogen synthase in rabbit muscle is increased by epinephrine and by the diabetic state. However, the distribution of the phosphate between the trypsin-sensitive and trypsin-insensitive regions differ in these two conditions. The data indicate that the cAMP-dependent protein kinase

is involved in epinephrine action but probably not in insulin action.

2. Methods

New Zealand female rabbits (3–4 kg body wt) were used. Intravenous injection of epinephrine (66 $\mu\text{g/kg}$) was at 30 s prior to injection of a lethal dose (70 mg/kg) of nembutal. Control and diabetic rabbits were sacrificed by injection of propranolol (10 mg/kg). Diabetes was induced by intravenous injection of 2 doses of alloxan (80 mg/kg each) 2 h apart. Blood glucose values of diabetic rabbits were ≥ 350 mg% compared to 80–120 mg% of control rabbits. The diabetic condition was maintained 5–7 days before use of the animals.

Immediately following injection of nembutal the rabbits were bled from the jugular vein, skinned, and the hindleg muscle excised, ground and homogenized in cold 4 mM EDTA, 1 mM EGTA, 50 mM NaF, 10 mM β -glycerol phosphate, 30 mM 2-mercaptoethanol (pH 7.0). The time elapse between nembutal injection and completion of homogenization was < 5 min. Synthase was then purified in the cold as in [7] except that 50 mM NaF was included in all buffers, and the acid precipitation was at pH 5.8 rather than 6.1. The amylase digestion of the glycogen pellet was modified in that amylase was 0.5 mg/ml, no Mg^{2+} was present, and the digestion time was 30 min. The purified synthase showed a single major protein band at $\sim 90\,000 M_r$ and a minor band at $\sim 45\,000 M_r$ when subjected to SDS disc-gel electrophoresis [7].

Glycogen synthase was assayed as in [7]. Alkali-labile phosphate in purified glycogen synthase was

Abbreviations: cAMP, adenosine 3',5'-monophosphate; TS, trypsin-sensitive; TI, trypsin-insensitive; G-6-P, glucose-6-phosphate, SDS, sodium dodecyl sulfate; M_r , relative molecular mass

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determined as in [9]. Phosphate analyses were done on synthase before and after trypsinization (0.125 mg trypsin/ml for 15 min, see [10]) to determine trypsin-sensitive and trypsin-insensitive phosphate. To verify that the limited trypsinization was working as in [4], aliquots of synthase from control, epinephrine-treated, and diabetic rabbits were phosphorylated using $[\gamma\text{-}^{32}\text{P}]\text{ATP}$. Each sample was analyzed for trypsin-sensitive and trypsin-insensitive ^{32}P as above. A second aliquot of each was hydrolyzed with CNBr and the ^{32}P peptides separated by SDS disc-gel electrophoresis in [4]. In all 3 samples the distribution of ^{32}P as trypsin-sensitive or trypsin-insensitive corresponded with the distribution of ^{32}P between the high and low M_r CNBr ^{32}P peptides.

3. Results and discussion

Many types of control experiments were performed to ensure that the phosphorylation state of glycogen synthase was not altered by killing of the rabbits, removal and/or homogenization of the muscle, and purification of the enzyme. Details of these controls will be published elsewhere and only summarized here. Most of these controls relied on the fact that measurement of the synthase activity ratio plus the K_a for G-6-P would indicate any alteration of the phosphorylation state of the enzyme [11–14]. These two kinetic properties of synthase were measured in muscle extracts from the standard homogenization; muscle frozen in situ at the temperature of liquid nitrogen and homogenized at -20°C in 60% glycerol [15]; and extracts from rabbits sacrificed using propranolol or nembutal. These kinetic parameters were

Table 1
Kinetic parameters of glycogen synthase during purification

Fraction	Activity ratio (-G-6-P/+G-6-P)	G-6-P K_a (μM)
Frozen muscle extract	0.23	182
Fresh muscle extract	0.24	170
pH 5.8 precipitate	0.24	174
Glycogen pellet	0.21	170
DEAE-cellulose (peak tube)	0.22	183
Purified enzyme	0.24	175

Glycogen synthase was purified from a fresh muscle extract, and the synthase activity ratio and G-6-P K_a were determined at each stage. In addition a sample of muscle was frozen in situ, powdered, and homogenized at -20°C [15] before determination of the synthase kinetic parameters

also determined at each stage of the purification procedure (table 1). All these control experiments indicated that the phosphorylation state measured in the purified enzyme was a true reflection of the in vivo phosphorylation state.

The synthase activity ratios and K_a for G-6-P (table 2) suggested that synthase from control animals was already partially phosphorylated since non-phosphorylated synthase has a ratio of ~ 0.8 and a K_a of 5–10 μM [1]. Phosphate analyses showed that synthase from controls contained ~ 2.4 mol P_i /subunit. As expected, epinephrine treatment produced a decrease in the synthase activity ratio, increase in G-6-P K_a value, and the phosphate content was elevated to ~ 3.9 mol/mol subunit. These results are in general agreement with [3,16]. The somewhat higher phosphate contents and lower activity ratios of the latter study may reflect the fact that they used perfused rat

Table 2
Glycogen synthase was purified from control, epinephrine-treated, and diabetic rabbits

Treatment	Activity ratio (-G-6-P/+G-6-P)	G-6-P K_a (μM)	Phosphate content (mol/mol subunit)		
			Total	TS	TI
Control (6)	0.24 ± 0.03	166 ± 39	2.35 ± 0.29	0.95 ± 0.10	1.40 ± 0.12
Epinephrine (5)	0.07 ± 0.02^a	768 ± 117^a	3.85 ± 0.26^a	2.09 ± 0.14^a	1.75 ± 0.09^c
Diabetic (6)	0.11 ± 0.02^a	443 ± 74^b	3.91 ± 0.25^a	1.08 ± 0.12^c	2.84 ± 0.13^a

^a $p < 0.005$ compared to control; ^b $p < 0.025$ compared to control; ^c not statistically different from control ($p > 0.05$)

The synthase was characterized in terms of its activity ratio in the absence and presence of G-6-P; apparent K_a for G-6-P; alkali-labile phosphate content; and distribution of the phosphate between the trypsin-sensitive (TS) and trypsin-insensitive (TI) regions. Numbers in parenthesis indicate the number of synthase preparations in each group. Each synthase preparation contained hindleg muscle from 2 rabbits

hindlimb. Synthase from diabetic rabbits also has a depressed activity ratio, elevated K_a for G-6-P, and an increased phosphate content to 3.9 mol/mol subunit (table 2).

The distribution of the phosphate between the trypsin-sensitive (TS) and trypsin-insensitive (TI) regions is very interesting. The cAMP-dependent protein kinase has a relative preference for phosphorylation of the TS region whereas the Ca^{2+} -dependent and cAMP-independent kinases phosphorylate predominantly the TI region [1,4,17]. The distribution of phosphate in synthase from control rabbits was 1.4 mol TI to 1.0 mol TS suggesting that synthase kinases other than the cAMP-dependent kinase are most active in the basal state. We have estimated in muscle extracts that under basal conditions of low cAMP and Ca^{2+} concentrations the cAMP-independent kinase(s) constitutes the major synthase kinase activity (see fig.3 of [1]). With epinephrine treatment the TI phosphate did not increase significantly (1.75 mol) but TS phosphate doubled to 2.1 mol. This is entirely consistent with the known specificity of the cAMP-dependent kinase for the TS region [4], and the known activation of this kinase by epinephrine in perfused rat muscle [18]. In response to diabetes the phosphate content of the TS region remained about the same as control (1.1 vs 1.0) but TI phosphate doubled to 2.8. Thus, in the control rabbit insulin acts to decrease phosphorylation of the TI region. This conclusion is supported by preliminary results which show that insulin treatment of normal rabbits does specifically decrease the TI phosphate but not the TS phosphate. This effect of insulin could be through stimulation of a phosphatase which is specific for the TI region. We have reported that a high M_r phosphatase from skeletal muscle preferentially dephosphorylates the TI region [18,19]. Alternatively, insulin could be inhibiting a protein kinase which preferentially phosphorylates the TI region. Our data are not consistent, however, with a mechanism in which insulin generates a factor which is inhibitory to the cAMP-dependent kinase [21,22]. Further studies on the effects of insulin on this system are in progress.

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