AMINO ACID SEQUENCES AT THE TWO SITES ON GLYCOGEN SYNTHETASE PHOSPHORYLATED BY CYCLIC AMP-DEPENDENT PROTEIN KINASE AND THEIR DEPHOSPHORYLATION BY PROTEIN PHOSPHATASE-III

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1. Introduction

It has been established that glycogen synthetase activity in skeletal muscle can be regulated by two distinct protein kinases. One of these is cyclic AMP-dependent protein kinase, while the other is an enzyme termed glycogen synthetase kinase-2 [1,2]. The phosphorylation of glycogen synthetase *a* in vitro by either kinase in the presence of ATP–Mg was found to reach a plateau when about one molecule of phosphate had been incorporated per subunit, and the resulting enzyme species were more dependent on the allosteric activator glucose-6-phosphate for activity. However cyclic AMP dependent protein kinase and glycogen synthetase kinase-2 produced different forms of glycogen synthetase, termed b_1 and b_2 respectively. These forms had different activity ratios (defined as the activity in the absence of glucose-6-phosphate relative to the activity in the presence of this effector) and different activation constants (K_a) for glucose-6-phosphate [2]. Moreover, the addition of both protein kinases resulted in the incorporation of almost two molecules of phosphate per subunit and the conversion of glycogen synthetase to a form, termed $b_{1,2}$, which was almost completely inactive in the absence of glucose-6-phosphate [2]. These results indicated that the two protein kinases phosphorylated different sites on the enzyme, although the reconversion of both glycogen synthetases b_1 and b_2 to glycogen synthetase *a* was catalysed by a single major activity in skeletal muscle, termed protein phosphatase-III [3,4].

The finding that two phosphorylation–dephosphorylation cycles regulate the activity of glycogen synthetase in vitro raises the question of which of the two cycles is operative under different metabolic conditions. The activity ratio of glycogen synthetase in vivo increases as the concentration of glycogen decreases, and vice versa [5]. It also increases in response to insulin and decreases in response to adrenalin [5–7]. However whether these changes involve the interconversion of glycogen synthetase *a* and b_1 or *a* and b_2 is not yet known. In order to solve this problem it is first necessary to characterize the sites that are phosphorylated by each protein kinase in vitro. In this paper we present such an analysis for the sites phosphorylated by cyclic AMP-dependent protein kinase. The results unexpectedly show that two sites on the enzyme are phosphorylated, and that only one of these is responsible for the decrease in the activity ratio.

2. Materials and methods

Glycogen synthetase *a* was purified by Method 2 of the procedure described by Nimmo et al. [8] and its purity was ca. 90% as judged by dodecyl sulphate–gel electrophoresis. Phosphorylation of the enzyme was carried out using the partially purified peak-1

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isoenzyme of cyclic AMP-dependent protein kinase [2] in the following incubation: Glycogen synthetase *a* (0.9 mg/ml), cyclic AMP-dependent protein kinase, sodium glycerophosphate 10 mM, EDTA 0.4 mM, EGTA 0.1 mM, cyclic AMP 0.02 mM, $MgCl_2$ 0.8 mM and [γ-^{32}P]ATP 0.1 mM. The quantity of cyclic AMP-dependent protein kinase was sufficient to achieve half-maximal phosphorylation in 2 min and maximal phosphorylation within 15 min. Phosphorylation by glycogen synthetase kinase-2 [traces of which contaminate purified glycogen synthetase *a* [1,2]] was estimated in a separate incubation in which cyclic AMP and cyclic AMP-dependent protein kinase were omitted, and an excess of the specific protein inhibitor of cyclic AMP-dependent protein kinase was added [2,9]. The incorporation of phosphate into glycogen synthetase was calculated using an absorbance index, $A^{1\%}_{280\,nm}$, of 13.4 to measure protein concentration and a subunit mol. wt 88 000 [8]. Protein phosphatase-III was a nearly homogeneous preparation (A. Burchell and P. Cohen, unpublished results).

High-voltage electrophoresis was carried out on Whatman 3 MM chromatography paper or on Eastman thin-layer cellulose sheets (No. 13255) at either pH 3.6 (pyridine/acetic acid/water, 1:10:190) or pH 6.5 (pyridine/acetic acid/water, 50:2:450). Descending paper chromatography employed butanol/pyridine/acetic acid/water, 90:60:18:72. [^{32}P]Phosphopeptides were detected by staining with fluorescamine or by autoradiography. Their electrophoretic mobilities (EM) were expressed relative to aspartic acid, and their net charge on thin-layer cellulose was calculated by standard procedures [10,11]. Chromatographic mobilities (CM) were expressed relative to phenol red. Elution of peptides was carried out with 6% acetic acid and amino acid sequences were determined by a micro-dansyl-Edman procedure [12]. The assignment of amides or location of phosphoserines was determined by measuring the net charge of peptides, at pH 6.5, after each cycle of the Edman degradation.

3. Results

3.1. *Amino acid sequences of the tryptic phosphopeptides*

Pilot experiments showed that the phosphorylation of different glycogen synthetase preparations by cyclic AMP-dependent protein kinase reached a plateau when 0.8–1.2 molecules of phosphate had been incorporated per subunit, of which no more than 5–10% was due to phosphorylation by glycogen synthetase kinase-2 (see Materials and methods). The formation of glycogen synthetase b_1 therefore resulted in the incorporation of about one molecule of phosphate per subunit, a very similar value to that reported previously from this laboratory [2]. Further pilot experiments showed that when ^{32}P-labelled glycogen synthetase b_1 was incubated with trypsin (Worthington, TPCK-treated), 85% of the radioactivity became soluble in 5% trichloroacetic acid within five min (fig.1). In contrast when ^{32}P-labelled glycogen synthetase produced by the action of endogenous glycogen synthetase kinase-2 was incubated with trypsin under identical conditions, only 20% of the counts were solubilized (fig.1). Therefore following phosphorylation and tryptic digestion > 90% of the phosphopeptides resulting from phosphorylation by cyclic AMP-dependent protein kinase were solubilized,

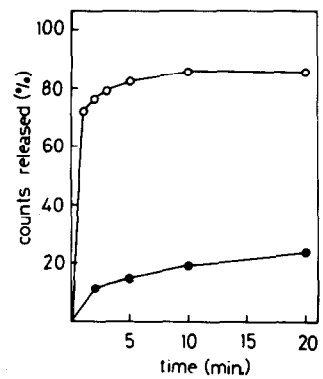

Fig.1. Digestion of native ^{32}P-labelled glycogen synthetase (1.0 mg/ml) with trypsin (0.03 mg/ml) in 50 mM glycerophosphate/2 mM EDTA/50 mM NaF/15 mM mercaptoethanol, pH 7.0, 25°C. The graph shows the release of phosphopeptides soluble in 5% trichloroacetic acid from glycogen synthetase phosphorylated with cyclic AMP-dependent protein kinase (○–○) or glycogen synthetase kinase-2 (●–●). The phosphorylations were carried out as described under Materials and methods except that for the phosphorylation by glycogen synthetase kinase-2, the ATP concentration was 1.0 mM and the free Mg^{2+} concentration was 2.0 mM. The preparations contained 1.1 molecules of phosphate per subunit (○–○) or 0.5 molecules of phosphate per subunit (●–●).

while contamination with phosphopeptides produced by glycogen synthetase kinase-2 was negligible. Only 5–10% of the 280 nm absorbance of the enzyme was released by trypsin under these conditions.

A phosphorylation reaction (170 ml) containing 150 mg glycogen synthetase was terminated by the addition of 0.1 vol. 0.1 M EDTA, and a further 0.1 vol. 0.5 M NaF was added to inactivate any endogenous protein phosphatase-III. The solution was concentrated to 20 ml by vacuum dialysis and passed through a column of Sephadex G-25 equilibrated in 50 mM sodium glycerophosphate-2 mM EDTA/50 mM NaF/15 mM mercaptoethanol, pH 7.0, to remove excess [γ-^{32}P]ATP. The native enzyme was then incubated with trypsin as described in fig.1, and the reaction was terminated after 5 min by addition of 0.1 vol. 50% trichloroacetic acid. The suspension was kept on ice for 15 min then centrifuged at 10 000 × g for 10 min. The supernatant was decanted, and the pellet extracted twice more with 10 ml portions of 5% trichloroacetic acid. The combined supernatants were extracted five times with an equal volume of ether, to remove trichloroacetic acid, and lyophilised. The residue was redissolved in 3.0 ml 1.0 M acetic acid, clarified by centrifugation at 15 000 × g for 2 min and passed through a column of Sephadex G-50. The elution profile (fig.2) showed the presence of

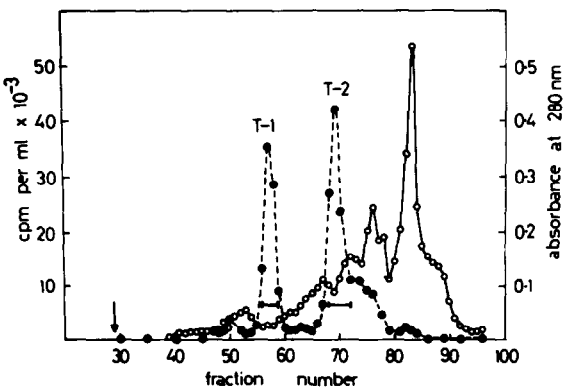

Fig.2. Gel filtration on Sephadex G-50 superfine (150 × 1.5 cm) of the phosphopeptides produced by digestion of glycogen synthetase b_1 (150 mg) with trypsin. (●- - -●) ^{32}P-Radioactivity; (○—○) $A_{280\,nm}$. The arrow denotes the position of the void volume, V_0, and the horizontal bars the fractions pooled after elution. Each fraction was 3.0 ml and the column was equilibrated and run in 1.0 M acetic acid.

two radioactive peaks, termed T-1 and T-2, which were present in similar amounts. The ratio of counts T-1/T-2 varied from 1:1.2 to 1:1.6 in five different glycogen synthetase preparations.

Peak T-1 (V/V_0 2.0) was further purified by electrophoresis on thin layer cellulose at pH 3.6. One major radioactive spot (EM –0.42) was obtained, which contained 80–90% of the total radioactivity, together with a second minor radioactive component (EM –0.24) which varied in amount from 10% to 20%. Both components had the same amino acid composition (not illustrated) and the same amino acid sequence through the first three residues (see below). The minor component is therefore presumed to result from the partial deamidation of an asparaginyl or glutaminyl side chain of the major component. The amino acid composition of the peptide showed that it contained 25 amino acids (table 1). Amino-terminal and sequence analysis of two different preparations showed the peptide, termed site-1, to be of high purity and yielded an unambiguous sequence through the first 10 residues:

Ser–Asn–Ser(P)–Val–Asp–Thr–Ser–Ser–Leu–Ser

Table 1
Amino acid compositions of tryptic phosphopeptides T-1 and T-2A$_1$

Amino Acid	T-1	T-2A$_1$
Aspartic Acid	2.80 (3)	0.20
Threonine	1.97 (2)	0.81 (1)
Serine	7.95 (8)	3.96 (4)
Glutamic Acid	3.22 (3)	0.30
Proline	2.41 (2)	0.15
Glycine	1.28 (1)	2.00 (2)
Alanine	1.25 (1)	0.97 (1)
Valine	1.00 (1)	
Leucine	2.58 (3)	
Histidine	0.2	
Lysine		0.87 (1)
Arginine	1.20 (1)	1.33 (1)
Cysteine		1.04 (1)
Total	25	11

Hydrolyses were carried out in 6 N HCl + 0.01 M phenol for 24 h. Compositions were determined on a Beckman Multichrom Analyser using a single column separation system. Serine and threonine were corrected for 10% and 5% destruction, respectively. Cysteine was determined as cysteic acid. Impurities below 0.1 mol. are omitted.

After the first and second cycles of the Edman degradation, electrophoresis at pH 6.5 showed the presence of single fluorescamine positive spots which coincided in position with the ^{32}P-radioactivity and which had a net charge of −3 identical to the original peptide. However, after the third cycle, the fluorescamine spot entirely lost its radioactivity, and migrated with a net charge of −2. The ^{32}P-radioactivity migrated in the same position as ^{32}P-labelled inorganic phosphate. These results coupled with the identification of dansyl-serine after the second cycle, showed that the phosphorylation had occurred on the serine three amino acids from the N-terminus and at no other site in the peptide. The results also showed that the phenylthiohydantoin derivative of phosphoserine is unstable and rapidly undergoes hydrolysis to inorganic phosphate.

The radioactive peak T-2 (V/V_0 2.4) was further purified by peptide mapping on paper using electrophoresis, at pH 3.6, followed by descending chromatography. Three radioactive peptides were detected by autoradiography, termed T-2A, T-2B and T-2C. The major component T-2A (EM −3.4, CM 0.05) contained about 70% of the radioactivity, while T-2B (EM −2.4, CM 0.05) and T-2C (EM −2.2, CM 0.34) each contained about 15%.

Peptide T-2A was further purified on thin layer cellulose, at pH 6.5. Autoradiography showed one major radioactive spot T-2A_1 containing 85% of the radioactivity (EM −0.31), and two minor radioactive components, T-2A_2 (EM −0.15) and T-2A_3 (EM −0.04). The amino acid composition of T-2A (table 1) showed that it contained 11 amino acids and that it could not be derived from T-1, since although smaller, it possessed one residue of lysine and one of cysteine, which were both absent from T-1. Amino terminal and sequence analysis showed this peptide, termed site-2, was of high purity and yielded the following partial sequence through the first three residues:

Arg–Ala–Ser(P)–

The identification of the serine three amino acids from the N-terminus of the peptide as the site of phosphorylation was based on the following evidence. Firstly, when the peptide was subjected to successive Edman Degradations on a Beckman 890C sequencer, the ^{32}P-radioactivity emerged at the third cycle (not illustrated). Secondly, the amino acid composition of the peptide (table 1) and its net charge of −1 at pH 6.5 indicated that not more than one site could be phosphorylated. Thirdly, the presence of arginine as the N-terminal amino acid of the tryptic peptide strongly indicated that the third amino acid must be phosphoserine. This same phenomenon has been found in three other substrates for cyclic AMP-dependent protein kinase [15,18] and it is now clear that the presence of phosphoserine two amino acids to the C-terminus of lysine or arginine completely prevents trypsin from cleaving at these basic residues [15].

Peptide T-2B was also further purified by electrophoresis, at pH 6.5 and showed two radioactive spots, T-2B_1 and T-2B_2, which had identical electrophoretic mobilities to peptides T-2A_2 and T-2A_3, respectively. Although sufficient quantities of these minor components could not be obtained for detailed analysis, the following evidence suggested that T-2A_2, T-2A_3, T-2B_1 and T-2B_2 were all related to T-2A_1 and were phosphorylated at the same residue. Firstly, peptides T-2A_2 and T-2A_3 both started with the sequence Arg–Ala–Ser(P). Secondly, peptides T-2B_1 and T-2B_2 had the same electrophoretic mobilities as T-2A_2 and T-2A_3 and they both contained arginine as the N-terminal amino acid. Thirdly, the qualitative amino acid compositions of all these peptides determined by the dansyl technique were similar to T-2A_1 (table 1). It is possible that these minor components result from modification or oxidation of the cysteine residue present in peptide T-2A_1 (table 1).

Peptide T-2C which comprised about 15% of the radioactivity in peak T-2 (fig.2) but no more than 10% of the total phosphate incorporated, could not be obtained in pure form and was not analysed further.

3.2. *Phosphorylation and dephosphorylation of glycogen synthetase by cyclic AMP-dependent protein kinase and protein phosphatase-III*

In order to investigate the relative rate of phosphorylation of site-1 and site-2, glycogen synthetase preparations which had been phosphorylated to different extents were incubated with trypsin and passed through Sephadex G-50. The results of these experiments are summarized in table 2. It can be seen that the rate of phosphorylation of site-2 was initially ten-times faster than site-1, that the phos-

Table 2
Rates of phosphorylation of site-1 and site-2

Molecules of phosphate per subunit Total	Site-1	Site-2	% Phosphorylation Site-2	% Conversion to glycogen synthetase b_1 [a]	Activity ratio ∓ glucose-6-P
0	0	0	0	0	0.75
0.21	0.015	0.195	39	42	0.49
0.40	0.09	0.31	62	57	0.40
0.86	0.36	0.50	100	100	0.13

[a] This calculation assumes that the decrease in the activity ratio is a linear function of the degree of phosphorylation

Glycogen synthetase was phosphorylated with cyclic AMP-dependent protein kinase and the reactions were stopped at various times by the addition of EDTA (10 mM) and NaF (50 mM). The proportion of phosphate in site-1 and site-2 was then analysed by tryptic digestion and gel-filtration on Sephadex G-50, as described in the text. Activities were measured in 10 mM phosphate, 0.15 M KCl, 50 mM NaF, 1.0 mM EDTA, 5 mM UDPG and 10 mg/ml glycogen [2] in the presence and absence of 10 mM glucose-6-phosphate. The activity ratio is defined as the activity in the absence of glucose-6-phosphate relative to the activity in the presence of glucose-6-phosphate

phorylation of site-1 only became rapid when about half-maximal phosphorylation of site-2 had taken place, and that the decrease in the activity ratio correlated with the degree of phosphorylation of site-2.

Glycogen synthetase b_1 preparations were then incubated with protein phosphatase-III, and the results are shown in fig.3 and 4.

Phosphorylation of glycogen synthetase *a* (activity ratio 0.75) yielded a preparation of glycogen synthe-

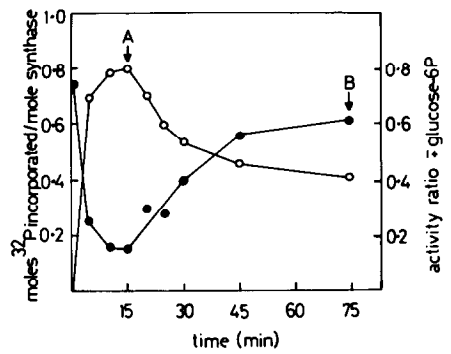

Fig.3. Phosphorylation and dephosphorylation of glycogen synthetase by cyclic AMP-dependent protein kinase and protein phosphatase-III. Glycogen synthetase was phosphorylated with cyclic AMP-dependent protein kinase and aliquots were analysed for the incorporation of phosphate (○–○) and the decrease in the activity ratio (●–●). The reaction which reached a plateau after 15 min was stopped by the addition of 0.05 vol. 0.1 M EDTA, pH 7.0. Protein phosphatase-III (0.02 vol.) in 50 mM Tris–HCl/1.0 mM EDTA/15 mM mercaptoethanol, pH 7.0, was added and further aliquots were analysed for the release of phosphate and increase in the activity ratio over the next 60 min. At points A (15 min) and B (75 min) further samples were removed and analysed for the presence of phosphate in site-1 and site-2 (fig.4).

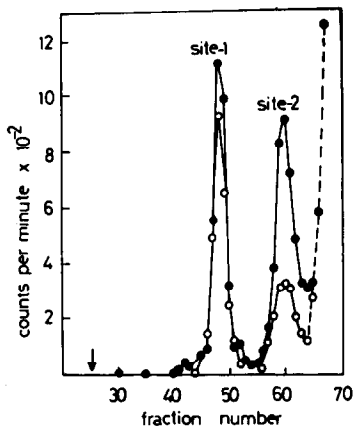

Fig.4. Relative rate of dephosphorylation of site-1 and site-2 by protein phosphatase-III. Aliquots obtained from point A and point B (fig.3) were digested with trypsin and subjected to gel filtration on Sephadex G-50 superfine (50 × 2 cm) equilibrated in 1.0 M acetic acid. The material at Point A (●–●), obtained before the addition of protein phosphatase-III, contained 0.8 molecules of phosphate per subunit. The material at point B (○–○) which had been incubated with protein phosphatase-III for 60 min, contained 0.42 molecules of phosphate per subunit. Each fraction was 2.8 ml. The large peak of radioactivity (- - -) emerging after site-2 is [γ-^{32}P]ATP. The arrow denotes the void volume (V_0).

Table 3
Amino acid sequences at the phosphorylation sites of substrates for cyclic AMP-dependent protein kinase

Substrate	Sequence	Reference
Phosphorylase kinase (β-subunit)	Ala–Arg–Thr $\overset{100}{\underset{8}{\text{Lys}}}$ –Arg–Ser–Gly–**Ser(P)**–$\overset{\text{Val}}{\underset{50}{\text{Ile}}}$–Tyr–Glu–Pro–Leu–Lys	[15]
Histone H2B	Lys–Lys–Arg–Lys–Arg–Ser(P)–Arg–Lys–Glu–**Ser(P)**–Tyr–Ser–Val–Tyr Val–Tyr–Lys	[15]
Pyruvate kinase (rat liver)	$\overset{35}{\text{}}$ Gly–Val– Leu –Arg–Arg–Ala–**Ser(P)**–Val–Ala–Glx–Leu	[15,16]
Inhibitor-1	$\overset{30}{\text{}}$ Ile–Arg– Arg –Arg–Arg–Pro–**Thr(P)**–Pro–Ala Thr	[17]
Glycogen synthetase (site-1)	$\overset{10}{\underset{\text{Arg}}{\text{Lys}}}$–Ser–Asn–**Ser(P)**–Val–Asp–Thr–Ser–Ser–Leu–Ser	This paper
Glycogen synthetase (site-2)	$\overset{80}{\underset{\text{Arg}}{\text{Lys}}}$–Arg–Ala–**Ser(P)**–	This paper
Phosphorylase kinase (α-subunit)	$\overset{20}{\text{}}$ Phe–Arg–Arg–Leu–**Ser(P)**–Ile–Ser–Thr–Glu–Ser–Glx–Pro	[15]
Histone H-1 (rat liver)	$\overset{8}{\text{}}$ Ala –Lys –Arg–Lys–Ala–**Ser(P)**–Gly–Pro–Pro–Val–Ser	[18]

The numbers above the phosphorylated residues refer to the rates at which the sites are phosphorylated relative to the β-subunit of phosphorylase kinase (100%) under a standard set of conditions [17]. Basic amino acids are underlined and the phosphorylated residue is shown in bold type

tase b_1 with an activity ratio of 0.15. Incubation of glycogen synthetase b_1 with protein phosphate-III for 60 min restored the activity ratio to 0.60 when 48% of the phosphate had been released and changed the ratio site-2/site-1 from 1.2:1 to 0.5:1. In a separate experiment, in which a different preparation of glycogen synthetase *a* (activity ratio 0.65) was used, incubation of glycogen synthetase b_1 (activity ratio 0.20) with protein phosphatase-III for 60 min completely restored the activity ratio to 0.65 when 65% of the phosphate had been released, and the ratio site-2/site-1 changed from 1.6:1 to 0.3:1 during this period (not illustrated). Very similar results were obtained in four different experiments. The results demonstrate that the activity of the enzyme is determined by the state of phosphorylation of site-2, and that this site is dephosphorylated about 20-fold more rapidly than site-1 by protein phosphatase-III. Complete dephosphorylation of site-1 does however take place when high concentrations of protein phosphatase-III are used (not illustrated).

4. Discussion

The results presented in this paper show that the phosphorylation of glycogen synthetase *a* by cyclic AMP-dependent protein kinase results in the phosphorylation of two distinct serines termed site-1 and site-2, which account for 90% of the total phosphorylation. The partial amino acid sequence of site-1 is consistent with the results of Huang and Krebs [13] who recently reported the complete amino acid sequence of this 26 residue tryptic peptide. However neither site-1 nor site-2 correspond to the sequence previously reported by Larner and Sanger [14] to represent the phosphorylation site of glycogen synthetase.

The structures of the phosphorylation sites for a number of the substrates of cyclic AMP-dependent protein kinase have been determined recently and a striking feature is the presence of at least two adjacent basic amino acids just N-terminal to the residue that is phosphorylated in each of the sequences (table 3). It is therefore of interest that site-2 must also show this structure, since it is a tryptic peptide starting with arginine. Site-1 must also contain at least one basic amino acid immediately N-terminal to the phosphoserine, but further extension of the sequence in the amino-terminal direction will clearly be necessary to establish whether or not two basic amino acids are present.

The finding that glycogen synthetase activity is determined by the state of phosphorylation of site-2, and that the phosphorylation of site-1 occurs more slowly after a lag period without any direct effect on the activity, bears a striking resemblance to the phosphorylation of phosphorylase kinase by cyclic AMP-dependent protein kinase. The activity of phosphorylase kinase correlates with the state of phosphorylation of the β-subunit (table 3), while the phosphorylation of the α-subunit (table 3) which also takes place more slowly after a lag period, regulates the rate of dephosphorylation of the β-subunit [19]. Since the dephosphorylation of the α- and β-subunits are catalysed by different enzymes [3,20], it will be important to investigate whether the phosphorylation of site-1 has any function, and whether it is dephosphorylated by another enzyme in vivo.

The phosphorylation of glycogen synthetase reaches a plateau when one molecule of phosphate has been incorporated per subunit (fig.3) and yet two sites are phosphorylated to a similar extent. Since glycogen synthetase is a tetramer formed from subunits of uniform size (mol. wt 88 000) [8], there are at least two explanations for this anomalous behaviour, assuming that the enzyme is indeed 90% pure, as indicated by a variety of electrophoretic and ultracentrifugal criteria [8]. Firstly, the phosphorylation of two sites per tetramer may alter the conformation such that the remaining two sites become unavailable for phosphorylation. Secondly, glycogen synthetase may be composed of two types of subunit, one of which contains site-1 and the other site-2. More detailed structural analyses should distinguish between these possibilities.

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