AMINO ACID SEQUENCE AT THE SITE ON RABBIT SKELETAL MUSCLE GLYCOGEN SYNTHASE PHOSPHORYLATED BY THE ENDOGENOUS GLYCOGEN SYNTHASE KINASE-2 ACTIVITY

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1. Introduction

Purified preparations of glycogen synthase (EC 2.4.1.11) have been shown to be contaminated with two types of protein kinase that phosphorylate the enzyme. One of these kinases was cyclic AMP-dependent protein kinase (EC 2.7.1.37), while the other was an activity termed glycogen synthase kinase-2. The latter enzyme could be distinguished from cyclic AMP-dependent protein kinase in a number of ways. Its activity was unaffected by cyclic AMP or by the specific protein inhibitor of cyclic AMP-dependent protein kinase, it had a higher K_m for ATP, and a different nucleoside triphosphate specificity [1].

Following digestion of the native enzyme with trypsin, the sites phosphorylated by cyclic AMP-dependent protein kinase were found to be released much more rapidly than those phosphorylated by glycogen synthase kinase-2, and this suggested that the two kinases phosphorylated different sites on the enzyme [1]. The amino acid sequences at the two sites phosphorylated by cyclic AMP-dependent protein kinase have been determined [2–4]. In this paper, we show that the endogenous glycogen synthase kinase-2 activity, preferentially phosphorylates a serine residue distinct from either of the sites labelled by cyclic AMP-dependent protein kinase. In conjunction with the information presented in [4], it is concluded that this serine is located seven residues from the N-terminus of the polypeptide chain.

2. Materials and methods

Glycogen synthase *a* was purified to near homogeneity [5] following modifications described in [6]. The protein inhibitor of cyclic AMP-dependent protein kinase was partially purified as a byproduct of the preparation of protein phosphatase inhibitor-1 [7]. The phosphorylation of glycogen synthase by endogenous glycogen synthase kinase-2, was carried out at 25°C, pH 7.0 in the following incubation: glycogen synthase 1.0 mg/ml, sodium glycerophosphate 10.0 mM, EDTA 0.4 mM, ethylene glycol bis (2 aminoethylether) *N,N'* tetracetic acid 0.1 mM, protein kinase inhibitor in excess of that required to inhibit the endogenous cyclic AMP-dependent protein kinase completely, magnesium acetate 3.0 mM, and [γ-^{32}P]ATP 1.0 mM. The stoichiometry of phosphorylation was calculated using an absorbance index, $A^{1\%}_{280\ nm}$, of 13.4 and a subunit mol. wt 88 000 [5].

Electrophoresis and ascending chromatography were carried out on thin layer cellulose sheets (Eastman no. 13255). Electrophoretic mobilities (EM) were expressed relative to aspartic acid at pH 6.5 (pyridine/acetic acid/water, 50:2:450) and chromatographic mobilities (CM) relative to the solvent front (butanol/pyridine/acetic acid/water, 30:20:6:24). Phosphopeptides were detected by staining the sheets with 0.001% fluorescamine or by autoradiography, and eluted by extracting the cellulose with 1.0 M ammonium hydroxide. Amino acid sequences were determined by a microdansyl

Edman procedure [8]. Digestion of proteins and peptides with proteinases was carried out at 37°C in 100 mM ammonium bicarbonate.

3. Results

3.1. *Isolation of tryptic and chymotryptic phosphopeptides*

The phosphorylation of glycogen synthase by endogenous glycogen synthase kinase-2, reached a plateau after incubation for about 120 min, at a level which was usually in the range 0.3–0.5 molecules of phosphate incorporated per subunit. Incubations were terminated by addition of 0.1 vol. 100% trichloroacetic acid, allowed to stand on ice for 10 min, and centrifuged at 20 000 × *g* for 10 min. The supernatant was discarded and the precipitate was washed twice with 5% trichloroacetic acid and twice with water. The precipitate was resuspended at 5 mg/ml in 100 mM ammonium bicarbonate containing 0.1 mg/ml trypsin (Worthington TPCK treated) or 0.3 mg/ml chymotrypsin (Worthington 3X crystallised) and digested for 3 h (trypsin) or 6 h (chymotrypsin), with occasional shaking. The suspensions were centrifuged at 20 000 × *g* for 10 min, and the clear supernatant which contained > 95% of the ^{32}P radioactivity was lyophilised. The freeze-dried material was redissolved in 100 mM ammonium bicarbonate and subjected to gel filtration on Sephadex G-50 (Pharmacia), equilibrated in the same solvent. Typical elution profiles are shown in fig.1 and 2. A single major peak of ^{32}P radioactivity was observed following digestion with either trypsin ($v/v_o = 1.8$) or chymotrypsin ($v/v_o = 2.0$). These peaks termed T1 and C1 respectively (fig.1,2) were pooled, lyophilised and further purified by ascending chromatography. In both cases the major fluorescamine-

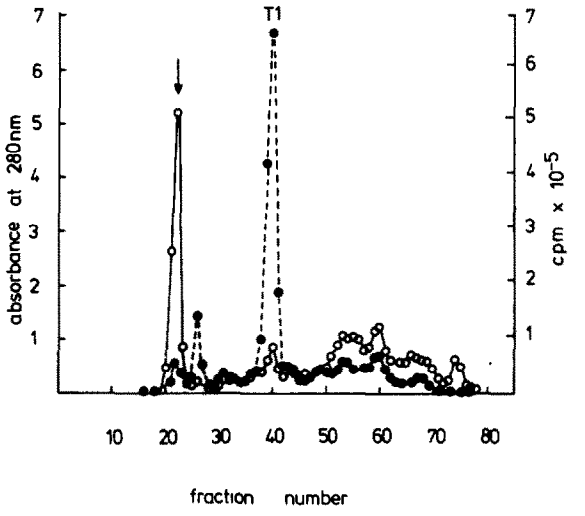

Fig.1. Gel filtration of peptide T1. ^{32}P-Labelled glycogen synthase (100 mg) containing 0.33 molecules of phosphate per subunit was digested with trypsin and the digest was chromatographed on Sephadex G-50 Superfine (150 × 1.5 cm) equilibrated in 100 mM NH_4HCO_3. (•–––•) ^{32}P radioactivity (Cerenkov counting); (o–o) $A_{280\ nm}$. The arrow denotes the position of the void volume, v_o. The flow rate was 10 ml/h and 3.8 ml fractions were collected. Fractions 38–41, which were pooled, corresponded to 33% of the radioactivity applied to the column.

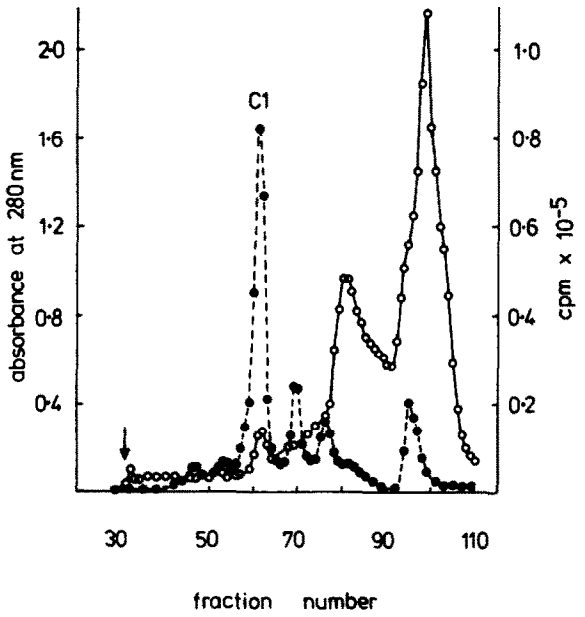

Fig.2. Gel filtration of peptide C1. ^{32}P-Labelled glycogen synthase (44 mg) containing 0.45 molecules of phosphate per subunit was digested with chymotrypsin and the digest was chromatographed on Sephadex G-50 Superfine (150 × 1.5 cm) equilibrated in 100 mM NH_4HCO_3. (•–––•) ^{32}P radioactivity (Cerenkov counting); (o–o) $A_{280\ nm}$. The arrow denotes the position of the void volume, v_o. The flow rate was 10 ml/h and 2.5 ml fractions were collected.

staining peptide was found to coincide with the ^{32}P radioactivity. The peptides were eluted and their purity was indicated by the finding of a single N-terminal amino acid; threonine in the case of peptide T1, and serine in the case of peptide C1. The amino acid analyses of these peptides are given in table 1. The phosphate content of the peptides was only about half that of the amino acids present as single residues. The most likely explanation for this result is that the procedure copurified both the phosphorylated and unphosphorylated forms of these peptides. The high proportion of hydrophobic amino acids is consistent with the chromatographic mobility of the peptides and the composition of C1 is consistent with the view that it is contained within peptide T1.

The dansyl Edman procedure yielded the following N-terminal sequences:

Table 1
Amino acid compositions of the tryptic and chymotryptic phosphopeptides

Amino acid	Peptide T1	Peptide C1
Aspartic acid	5.58 (6)	4.00 (4)
Threonine	1.27 (1)	
Serine	3.62 (4)	3.81 (4)
Glutamic acid	6.25 (6)	4.05 (4)
Proline	1.09 (1)	1.05 (1)
Glycine	1.67 (1–2)	1.35 (1)
Alanine	2.22 (2)	
Valine	3.80 (4)	1.77 (2)
Isoleucine	0.40	
Leucine	4.99 (5)	4.00 (4)
Phenylalanine	2.00 (2)	1.87 (2)
Lysine	0.84 (1)	
Arginine	0.23	
Tryptophan	+ (+)	+ (+)
^{32}P-phosphate	0.46 (1)	0.48 (1)
Total	33–34	23
N-Terminus	Thr	Ser

Hydrolyses were carried out in 6 N HCl + 0.01 M phenol for 20 h, and the analyses were done at 110°C on a Beckman Multichrom Analyser. Tryptophan was determined by a spot test using the Ehrlich reagent. Serine and threonine were corrected for 10% and 5% destruction, respectively, and valine and isoleucine were corrected assuming the hydrolysis of Val–X and Ile–X bonds was only 90% complete. The compositions are normalised to leucine, and impurities below 0.2 mol are omitted

T1 Thr–Leu–Ser–Val–Ser–Ser–Leu

C1 Ser–Val–Ser–Ser–Leu–Pro–Gly

3.2. *Location of the phosphoserine*

We have reported that the phenylthiohydantoin derivative of phosphoserine is unstable and rapidly undergoes hydrolysis to inorganic phosphate [2]. The phosphoserine can therefore be located by carrying out electrophoresis at pH 6.5, before and after each cycle of the Edman degradation.

Peptide C1 (200 nmol) was digested with staphylococcal proteinase (0.1 mg/ml) for 6 h, and subjected to electrophoresis at pH 6.5. The single ^{32}P-labelled peptide, termed S1 (EM = +0.35), was eluted, and its N-terminal sequence was shown to be:

Ser(P)–Val–Ser–Ser–Leu–Pro–Gly–

More than 90% of the radioactivity attached to the phosphopeptide was converted to inorganic phosphate after the first step of the Edman degradation. Coupled with the identification of serine as the first residue, this indicated that the N-terminal serine and no other residue in the peptide was phosphorylated. The sequence of the first six residues of S1 were identical to C1, and the first residue of C1 was also shown to be phosphoserine by the same procedure.

When the peptide T1 was subjected to successive Edman degradations, the ^{32}P radioactivity comigrated with the fluorescamine-staining peptide material for the first two rounds of the Edman degradation, and inorganic phosphate appeared only after the third cycle. It is therefore concluded that the sequence phosphorylated by the endogenous glycogen synthase kinase-2 is:

Thr–Leu–Ser–(P)–Val–Ser–Ser–Leu–Pro–

3.3. *The phosphoserine is located at residue 7 from the N-terminus of glycogen synthase*

Following the sequence determination, we noticed that the first four residues, Thr–Leu–Ser–Val–, corresponded to residue 5–8 from the N-terminus of glycogen synthase reported by Takeda et al. [9] suggesting that the phosphoserine might be located at residue 7 from the N-terminus of the polypeptide chain. However they reported that residue 4 from the

N-terminus was serine [9], whereas it would be expected to be lysine or arginine, since T1 is a tryptic phosphopeptide. The sequence reported by Takeda et al. [9], has been confirmed and extended by Huang and Krebs [4] except that these workers have found residue 4 to be arginine and not serine. Furthermore, the phosphopeptide sequence that we have determined corresponds exactly to residues 5–13 from the N-terminus determined by Huang and Krebs [4]. This confirms that the phosphoserine is indeed located at residue 7. The amino acid sequence in this region is therefore:

$$\overset{1}{\text{Pro}}\text{–Leu–Ser–Arg–}\overset{5}{\text{Thr}}\text{–Leu–}\overset{7}{\text{Ser(P)}}\text{–Val–Ser–}\overset{10}{\text{Ser}}\text{–Leu–Pro–Gly–}$$

4. Discussion

The results described here show that the endogenous glycogen synthase kinase-2 activity in purified glycogen synthase, initially phosphorylates a single serine residue which is distinct from both the sites phosphorylated by cyclic AMP-dependent protein kinase. We suggest that the site phosphorylated by glycogen synthase kinase-2 be termed site-2, and that the two sites phosphorylated by cyclic AMP-dependent protein kinase be termed site-1a and -1b, termed site-2 and site-1, respectively in [2].

The finding that site-2 is only seven residues from the N-terminus of glycogen synthase, which comprises nearly 800 residues, is of interest since the site on glycogen phosphorylase, phosphorylated by phosphorylase kinase is also very close to the N-terminus (residue-14 out of 841 residues) [12]. In the following paper we report that glycogen synthase kinase-2 activity is strongly stimulated by the calcium-dependent regulator protein in the presence of calcium ions [10]. Thus, two protein kinases whose activities are activated by calcium ions phosphorylate similar regions of their substrate proteins. The proximity of site-2 to the N-terminus of glycogen synthase should facilitate investigations of the phosphorylation of this site in vivo.

The amino acid sequences at a number of the sites phosphorylated by cyclic AMP-dependent protein kinase have been determined, and the striking feature common to all these sites is the presence of at least two adjacent basic amino acids just N-terminal to the residue that this phosphorylated [12]. Furthermore, elegant studies using synthetic peptides related to the phosphorylation site on L-type pyruvate kinase have shown that if either of the two adjacent arginines is substituted by a neutral amino acid, the rate of phosphorylation is reduced drastically [13,14]. This finding may explain why site-2 in glycogen synthase is not phosphorylated at a significant rate by cyclic AMP-dependent protein kinase. Nevertheless, it is interesting that there is an arginine residue in site-2 three amino acids N-terminal to the phosphoserine. It will be important to apply the synthetic peptide approach to this sequence in order to see whether this arginine plays any role in determining the substrate specificity of glycogen synthase kinase-2.

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