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Identification of the C-terminus of Rabbit Skeletal Muscle Glycogen Synthase

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The primary structure of a tryptic peptide containing one of the phosphorylation sites on rabbit skeletal muscle glycogen synthase (site 1b) has been redetermined and shown to correspond to the C-terminus of the protein. The sequence is :-SNSVDTSSLSTPSEPLSSAPSLGEERN.

Glycogen synthase (E.C.2.4.11) is regulated by multisite phosphorylation. The enzyme can be phosphorylated in vitro on a minimum of 10 serine residues by at least 7 protein kinases [1]. Phosphorylation also occurs at multiple sites in vivo, increasing in response to adrenaline and decreasing in response to insulin [reviewed in 2,3]. The phosphorylation sites are clustered within the polypeptide chain near the N-terminus and C-terminus of the molecule [4]. While analysing the structure of glycogen synthase by fast atom bombardment mass spectrometry (FABMS), we found that the molecular mass of the tryptic peptide containing site-1b (the phosphoserine residue closest to the C-terminus [4]) did not correspond to the value expected from the published sequence [5]. Reinvestigation of the primary structure, reported here, has led to the identification of the C-terminus of the protein.

MATERIALS AND METHODS

The peptide containing site-1b was isolated by brief tryptic digestion of native glycogen synthase and gel-filtration on Sephadex G-50 Superfine as described in ref 6, followed by chromatography on a Vydac C_{18} reverse-phase HPLC column using a linear gradient of water/acetonitrile in 0.1%(v/v) trifluoroacetic acid. The tryptic peptide was eluted at 30% acetonitrile.

Chymotryptic subdigestion was carried out for 4h at 37°C in 50mM ammonium acetate (pH 8.4), at an enzyme:substrate weight ratio of 1:50. Subdigestion

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with S. aureus V8 proteinase was performed in an identical manner except that the enzyme:substrate weight ratio was 1:20. Reactions were terminated by lyophilisation.

Primary structure analysis was performed using a Beckman 890D Sequencer as in ref 7. FAB mass spectra were taken on a Kratos MS 50 mass spectrometer equipped with an Ion Tech FAB gun operating with xenon at 8kV with a current of 40μA. Samples (3-5nmoles) were dissolved in 1% acetic acid (5μl) and dried on the probe tip in vacuo. A 50:50:1 mixture of thioglycerol:diglycerol:HCl (2μl) was added and mixed thoroughly with the sample before insertion into the ion source.

RESULTS AND DISCUSSION

The tryptic peptide containing site-1b was analysed on a Beckman 890D Sequencer and yielded a single N-terminal sequence SNSVDTSSL, identical to that reported previously [5,6]. The complete primary structure of the peptide was reported to be SNSVDTSSLSPPTESLSSAPLGEQDR [5], which should generate a molecular ion (MH^+) of 2660Da when subjected to FABMS in the positive ion mode. Surprisingly, however, FABMS yielded two molecular ions with masses (MH^+) of 2633Da and 2747Da, these species being present in similar amounts. This observation, in conjunction with the N-terminal sequence information, indicated that the preparation was a mixture of two components, one of which contained an additional asparagine residue (mass 114Da) at its C-terminus. These observations indicated that the asparagine residue must correspond to the C-terminus of glycogen synthase, since trypsin does not cleave asn-Xaa peptide bonds. This idea was confirmed by digesting 440μg of glycogen synthase with 8μg of carboxypeptidase A for 3h at 37°C in 0.2ml of 0.2M N-methylmorpholine acetate pH 8.5, which released 0.5mol asparagine per mol glycogen synthase subunit. No asparagine was released in control incubations lacking either glycogen synthase or carboxypeptidase A (not shown). The presence of two molecular ions at 2633Da and 2747Da is explained by partial cleavage of an arg-asn bond, since trypsin has very weak exopeptidase activity.

The mass of 2747Da was 87Da greater than the value of 2660Da calculated from the previously reported primary structure, indicating the presence of an additional serine residue. The tryptic peptide was therefore subjected to an extended Sequencer run which allowed the first 25 residues to be assigned

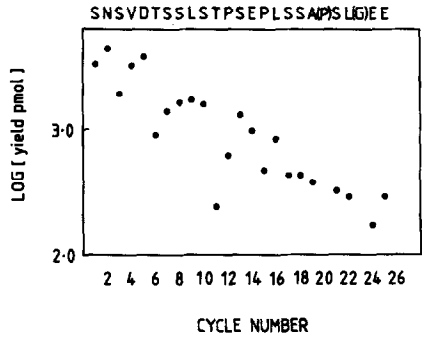

Fig 1. Sequencer analysis of the tryptic peptide containing site-1b. The graph shows a plot of log (yield pmol) (o) against cycle number. The peptide (10nmoles) was introduced into the spinning cup. Residues 20 and 23 (in parentheses) could be assigned qualitatively, but not quantitatively.

unambiguously (Fig 1). The analysis not only identified the additional serine at residue-21, but corrected further errors in the original sequence at residues 11,13,15 and 25 (residue 24 in the original sequence). The complete primary structure of the peptide, consistent with FABMS, is:

SNSVDTSSLSTPSEPLSSAPSLGEERN.

The revised structure was confirmed by subdigestion with chymotrypsin and (separately) with S.aureus V8 proteinase, followed by FABMS. Under the digestion conditions employed, chymotrypsin caused partial cleavage of all three leu-Xaa bonds, while V8 proteinase produced partial cleavage of the glu-pro and glu-arg bonds. Cleavage of the glu-arg bond established that residue 25 was glutamic acid, and not glutamine as assigned previously (5). The molecular masses of the peptides PLSSAPSLGEE (MH^+ = 1086), PLSSAPSLGEER (MH^+ = 1242), PLSSAPSLGEERN (MH^+ = 1356), SSAPSLGEER (MH^+ = 1032), SSAPSLGEERN (MH^+ = 1146) and STPSEPLSSAPSL (MH^+ = 1272) were consistent with the new assignment of proline at residue 15, the additional serine at residue 21, and the proposed structure at the C-terminus.

The amino acid sequence of the 124 residue C-terminal cyanogen bromide fragment of glycogen synthase is given in Fig. 2. The calculated molecular mass (13.67kDa) is far lower than the value of 24-28kDa (depending on phosphorylation state) estimated by SDS polyacrylamide gel electrophoresis [4]. Abnormally low binding of detergent, perhaps due to the small numbers of hydrophobic and lysine residues, may explain the slow migration of this

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1         10        20        *   *   *  40 *   *   50        60
ALAKAFPDHFTYEPHEADATQGYRYPRPASVPPSPSLSRHSSPHQSEDEEEPRDGLPEEDGERYDEDEE

70        80        *  90        *        110       120
AAKDRRNIRAPQWPRRASCTSSSGGSKRSNSVDTSSLSTPSEPLSSAPSLGEERN
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Fig 2. Complete primary structure of the C-terminal cyanogen bromide fragment of rabbit skeletal muscle glycogen synthase.
The asterisks denote serine residues phosphorylated in vitro [reviewed in refs 2 and 3].

peptide. The molecular mass of the peptide comprising residues 24 to 124 (11.1kDa), which is generated by brief chymotryptic attack of native glycogen synthase, is very similar to the value of 11.6kDa determined by sedimentation equilibrium centrifugation [8]. Knowledge of the correct primary structure in this region will be important for further analysis of the in vivo phosphorylation state of glycogen synthase, as well as for DNA cloning studies.

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