EFFECT OF PROTEASES ON THE STRUCTURE AND ACTIVITY OF RABBIT SKELETAL MUSCLE GLYCOGEN SYNTHETASE

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1. Introduction

Rabbit skeletal muscle glycogen synthetase exists in two forms, the non-phosphorylated, glucose 6-phosphate-independent I-form and the phosphorylated, glucose 6-phosphate-dependent D-form. The conversion of the I-form to the D-form can be effected by phosphorylation catalyzed by either cyclic AMP-dependent [1,2] or cyclic AMP-independent [3–5] protein kinases and by limited proteolytic digestion [6–9], whereas conversion brought about by phosphorylation can be reversed by the action of phosphoprotein phosphatase(s), that due to proteases is irreversible. Takeda and Larner [8] and Soderling [9] reported that the conversion of synthetase I to synthetase D by trypsin is accompanied by a decrease in the molecular weight from 90 000 to about 75 000. Takeda and Larner [8] further reported that the action of trypsin on synthetase is restricted to the COOH-terminal part of the molecule.

This report is concerned with further examination of the effect of proteolysis digestion on glycogen synthetase structure and activity using subtilisin as well as trypsin for this purpose. A striking feature of the effect of subtilisin is a marked activation of the enzyme that occurs during the early part of the reaction as seen by activity determinations carried out in the presence or absence of glucose 6-phosphate. In the course of this work the amino acid sequence of the NH_2-terminal portion of the synthetase was determined and found to be slightly different from that reported [10]. It was noted that low levels of either trypsin or subtilisin cause some modification of the NH_2-terminal region of the synthetase.

2. Materials and methods

Glycogen synthetase I was isolated from rabbit skeletal muscle as previously described [2,11]. Partial hydrolysis of the synthetase by trypsin and subtilisin was carried out at 30°C in buffer containing 50 mM Tris–HCl (pH 7.5), 2 mM EDTA, 45 mM β-mercaptoethanol and 10% sucrose at 1–2 mg protein/ml and at substrate-to-trypsin or substrate-to-subtilisin ratios ranging from 5000:1 to 100:1 (w/w). For measurement of synthetase activity after partial proteolytic hydrolysis, the reaction was stopped by diisopropylfluorophosphate (DFP) and immediately diluted 50-fold in cold buffer containing 50 mM Tris–HCl (pH 7.5), 1 mM EDTA, 0.25 mg/ml bovine serum albumin, 5 mg/ml glycogen and 45 mM β-mercaptoethanol. Synthetase activity was assayed at 30°C by the method of Thomas et al. [12] in the presence and absence of glucose 6-phosphate. The final concentration of synthetase in the assay ranged from 10–20 μg/ml. Specific activity is defined as the incorporation of 1 μmol glucose from UDP-glucose into glycogen per min per mg protein under the assay condition. For samples used in sodium dodecyl sulfate electrophoresis, the reaction was stopped by adding an equal volume of glacial acetic acid to the reaction mixture, which was frozen and lyophilized. Sodium dodecyl sulfate gel electrophoresis was performed in 7.5% polyacrylamide according to Weber and Osborn [13].

To prepare the samples for NH_2-terminal sequence determinations, the protease digestion was terminated by adding concentrated trichloroacetic acid to a final concentration of 10%. The mixture was cooled on ice

for 30 min and centrifuged. The pellet was collected and washed several times with cold 5% trichloroacetic acid, then extracted several times with ether for removal of trichloroacetic acid and lyophilized. NH_2-terminal sequence analyses of the intact and trypsin- and subtilisin-digested synthetase were performed using the Beckman 890 C sequencer with the standard protein program and 0.1 M Quadrol as buffer. Fractions from the sequencer were converted to the phenylthiohydantoin by heating at 80°C for 10 min in 1 N HCl [14]. The PTH derivatives were extracted with ethyl acetate and were identified by a combination of:

(i) Gas chromatography;
(ii) Thin-layer chromatography on polyamide sheets [15];
(iii) Amino acid analysis after regeneration at 150°C for 16 h in 6 N HCl.

PTH-Arg, which stays in aqueous phase, was identified by:

(i) Sakaguchi stain [16] and phenanthrenequinone spray [17] after pH 1.9 high-voltage paper electrophoresis with standard PTH-Arg as reference;
(ii) Amino acid analysis after regeneration as in (iii) above.

3. Results

3.1. *NH_2-terminal amino acid sequence of glycogen synthetase*

The NH_2-terminal amino acid sequence of rabbit skeletal muscle glycogen synthetase was determined to be:

Pro–Leu–Ser–Arg–Thr–Leu–Ser–Val–Ser–Ser–Leu–Pro–Gly–Leu–Glu

differing from that reported by Takeda et al. [10] in that residue 4 is arginine rather than serine.

3.2. *Limited digestion of glycogen synthetase with trypsin*

It has been reported [6,8,9] that glycogen synthetase I can be converted to a glucose 6-phosphate-dependent form by limited digestion with trypsin. This was confirmed here as in shown in fig.1 in which trypsin was used at a synthetase-to-trypsin ratio (w/w) of 1000:1. With varying synthetase-to-trypsin ratios ranging from 100:1 to 5000:1 essentially the same results as shown in fig.1 were obtained except that with lower ratios, the conversion to the glucose 6-phosphate-dependent form was faster. It will be noted that there was some increase in activity as measured in the presence of glucose 6-phosphate during the first 10 min. This was followed by a gradual decay to approximately the original activity seen at 30 min. The activity assayed in the absence of glucose 6-phosphate dropped rapidly but almost leveled off within 10 min. It can be seen in fig.2 that the loss of synthetase I activity corresponds to a transformation of the enzyme from an original 90 000 mol. wt species to an approx. 75 000 mol. wt species. The NH_2-terminal sequence of the trypsinized

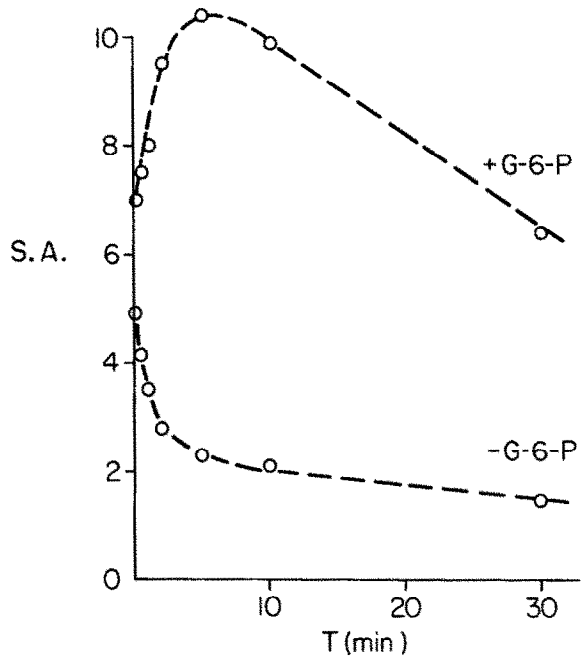

Fig.1. Time course of trypsin digestion of glycogen synthetase. Synthetase I (2 mg/ml) was incubated at 30°C with trypsin (2 μg/ml) for the indicated times. The reaction was stopped with DFP and diluted in a buffer containing 5 mg/ml glycogen, 0.25 mg/ml BSA, 50 mM Tris–HCl buffer (pH 7.5), 1 mM EDTA and 45 mM β-mercaptoethanol, and assayed for synthetase activity in the presence and absence of glucose 6-phosphate. The synthetase activity was expressed as specific activity (S.A.).

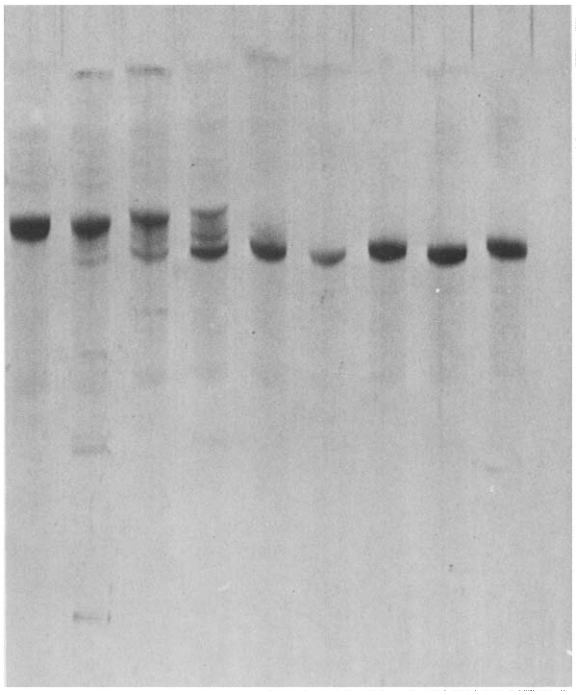

Fig.2. Sodium dodecyl sulfate disc gel electrophoresis of glycogen synthetase during limited tryptic digestion. Synthetase I was incubated with trypsin as in fig.1 for the indicated times. The digestion was stopped by adding glacial acetic acid to the reaction mixtures which were lyophilized immediately. Electrophoresis was carried out in 7.5% polyacrylamide gels according to Weber and Osborn [13].

(1000:1, w/w, 10 min) synthetase was found to be:

Thr–Leu–Ser–Val–Ser–Ser–Leu–Pro–Gly–Leu–Glu

i.e., a tetrapeptide, Pro–Leu–Ser–Arg, is missing as compared with the NH_2-terminal sequence of untreated synthetase. This transformation would be expected from the specificity of trypsin. The result presented here is different from that reported by Takeda and Larner [8] who reported no change in the amino-terminal portion of the synthetase due to mild treatment with trypsin. The reason for the difference is not clear at the present time, but the tryptic peptide reported by Rylatt and Cohen [18], which also has 4 residues deleted from the NH_2-terminal, supports the result that NH_2-terminal sequence of synthetase and partially-trypsin-digested synthetase is different.

3.3. *Limited digestion of glycogen synthetase with subtilisin*

The result of limited digestion of glycogen synthetase with subtilisin was strikingly different from that of trypsin digestion although both were carried out under the same conditions. Whereas the major effect of trypsin was conversion of synthetase I to a glucose 6-phosphate-dependent form, subtilisin activated synthetase activity both in the presence and absence of glucose 6-phosphate when the synthetase-to-subtilisin ratio was more than 1000:1 as is shown in fig.3. The plus glucose 6-phosphate activity remained high even after 1 h incubation, whereas the minus glucose 6-phosphate activity decayed after

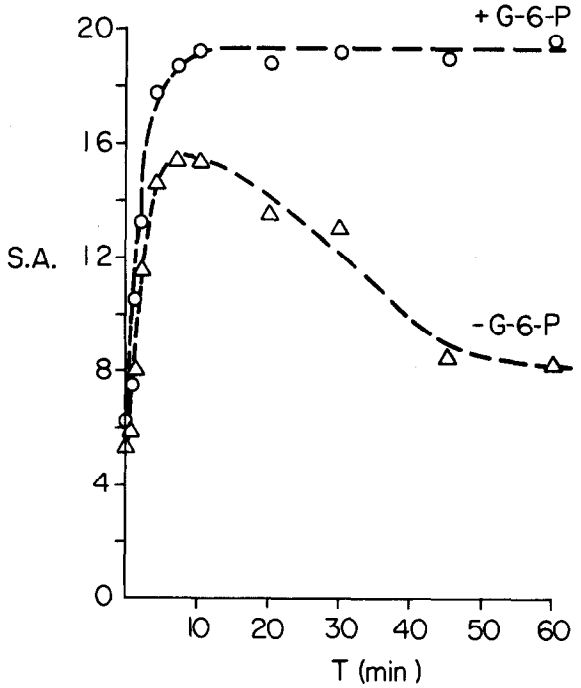

Fig.3. Time course of subtilisin digestion of glycogen synthetase. The conditions for the digestion and activity assay were identical to that in fig.1 except that subtilisin at a synthetase to subtilisin ratio (w/w) of 5000:1 was used.

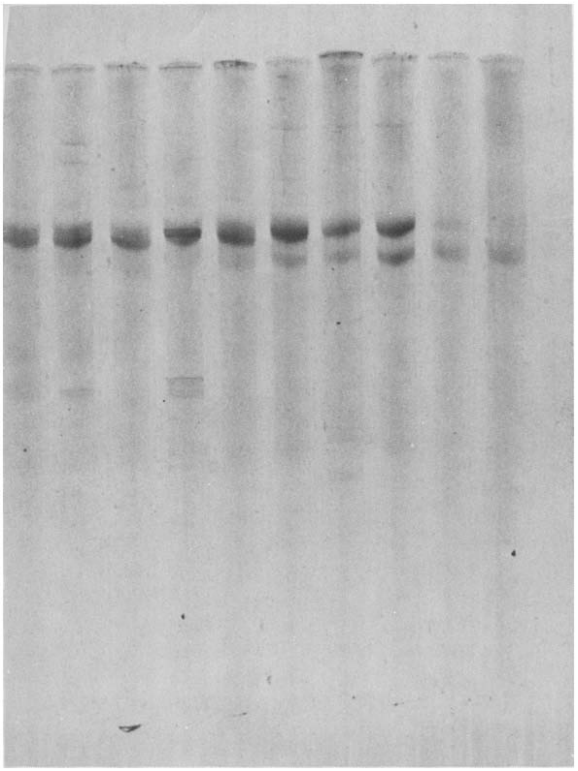

Fig.4. Sodium dodecyl sulfate disc gel electrophoresis of synthetase during limited subtilisin digestion. Glycogen synthetase I was incubated with subtilisin at a synthetase-to-subtilisin ratio of 5000:1 (w/w) at 30°C for the indicated times under identical conditions to those of fig.3. Electrophoresis was performed as in fig.2.

10 min. This latter change corresponded to the appearance of a smaller molecular-weight species of the synthetase (fig.4). At a synthetase-to-subtilisin ratio less than 500:1 (w/w) (not illustrated), the loss of activity as measured in the absence of glucose 6-phosphate was accentuated but the glucose 6-phosphate-dependent activity remained high. When the NH_2-terminal sequence was analyzed on subtilisin-digested glycogen synthetase (either with 1000: 1 or 5000:1 for 10 min) the major sequence was as follows:

Ser–Val–Ser–Ser–Leu–Pro–Gly–Leu–Glu

This is 6 residues shorter than the NH_2-terminal of synthetase itself.

4. Discussion

In this study we extended the NH_2-terminal sequence of glycogen synthetase somewhat beyond that reported in [10]. One difference was noted in that it was found that residue 4 from NH_2-terminal of synthetase is arginine instead of serine. Limited digestion of synthetase I by trypsin and subtilisin differed in their effects on the activity and in the structural changes that resulted. Tryptic digestion, as reported [6,8,9] mimics the phosphorylation by cyclic AMP-dependent [1,2] and cyclic AMP-independent kinases [3–5] by converting glycogen synthetase I to a glucose 6-phosphate-dependent form. As shown in fig.2, there is a bond or bonds extremely susceptible to tryptic digestion in the COOH-terminal part of synthetase molecule which, when split, results in the conversion of the 90 000 mol. wt subunit to an approx. 75 000 mol. wt species within minutes under a wide range of trypsin concentrations. We also found in the present study that the tetrapeptide, Pro–Leu–Ser–Arg, is missing from the NH_2-terminal of the synthetase molecule after limited tryptic digestion. This finding is in contrast to the previous report by Takeda and Larner [10] who found that there was no change in the NH_2-terminal sequence caused by this treatment. The data presented in this paper, together with the report by Rylatt and Cohen [18] shows that arginine is the 4th residue from the NH_2-terminal of the synthetase molecule.

Limited subtilisin digestion elevated glycogen synthetase activity assayed in the presence or absence of glucose 6-phosphate (fig.3). With prolonged digestion or with higher subtilisin concentrations, glycogen synthetase-I was converted to a glucose 6-phosphate-dependent form, but activity assayed in the presence of glucose 6-phosphate remained elevated. The NH_2-terminal-sequence analysis of subtilisin-treated synthetase showed that there were 6 residues missing as compared with the original synthetase. The sodium dodecyl sulfate–disc gel electrophoresis

patterns shown in fig.4 suggest that subtilisin cleaves the NH_2-terminus of synthetase-I and this is followed by cleavage in the COOH-terminal of the molecule. The conversion of synthetase-I to a glucose 6-phosphate-dependent form seems to correlate with the appearance of the lower molecular species, as it does with tryptic digestion. The results presented here suggest that both the NH_2-terminal and the COOH-terminal of the synthetase molecule are susceptible to tryptic and subtilisin cleavage. They also suggest that the COOH-terminal of synthetase is responsible for the conversion of synthetase-I to the D-form. Cleavage of the NH_2-terminal may be responsible for the activation of synthetase.

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