Regulation of Glycogen Synthetase

EFFECTS OF TRYPsin ON THE STRUCTURE, ACTIVITY, AND PHOSPHORYLATION OF THE SKELETAL MUSCLE ENZYME*

(Received for publication, January 12, 1976)

THOMAS R. SODERLING†

From the Department of Physiology, School of Medicine, Vanderbilt University, Nashville, Tennessee 37232

Incubation of purified skeletal muscle glycogen synthetase I with trypsin (10 µg/ml) for 15 min decreased the Stokes radius of the enzyme from 68 Å to 62 Å and the subunit molecular weight from 90,000 to about 73,000. No decrease in the sedimentation coefficient of 13.3 S could be detected. It was calculated that native synthetase I is a tetramer of molecular weight 360,000 to 370,000.

Trypsin also catalyzed a decrease in the synthetase activity ratio (minus glucose-6-P to plus glucose-6-P) largely by reducing minus glucose-6-P activity. The magnitude of the trypsin effect on the synthetase activity ratio was very similar to that produced by phosphorylation of synthetase by the cyclic AMP-dependent protein kinase. The activity ratio could be lowered from that characteristic of synthetase I, 0.85, to 0.25 either by trypsin digestion or by incorporation of 1 mol of P_i per mol of synthetase subunit. An activity ratio of <0.05 could be obtained in three ways as follows, (a) phosphorylation to 2 P_i/subunit, (b) phosphorylation to 1 P_i/subunit (ratio = 0.25) followed by trypsin treatment, (c) trypsin treatment (ratio = 0.25) followed by phosphorylation. When trypsinized synthetase was phosphorylated by the catalytic subunit of cyclic AMP-dependent protein kinase, 1 P_i/subunit was incorporated. Trypsin (6 µg/ml) led to a rapid release of about 50% of the radioactivity from 32P-synthetase regardless of whether the enzyme contained 1 or 2 phosphates per subunit.

It was concluded that two sites on the enzyme subunit are highly susceptible to phosphorylation catalyzed by the cyclic AMP-dependent protein kinase. The data indicate that the 1st mol of P_i incorporated is distributed about equally between the two sites. A model is proposed to account for these observations. The data further indicate that a peptide containing one site is removed by trypsin. This reduces enzyme activity to the same extent as does phosphorylation of the site in the intact protein.

Inactivation of skeletal muscle glycogen synthetase by conversion of the physiological active I form to the less active D form requires incorporation of 2 mol of phosphate per mol of subunit (1). Incorporation of the first phosphate occurs at a low cyclic AMP-dependent protein kinase concentration (10^{-10} to 10^{-9} M) and decreases the synthetase D (activity ratio <0.05) requires a higher concentration of protein kinase (10^{-5} to 10^{-4} M) and is associated with incorporation of a 2nd mol of phosphate per mol of synthetase subunit.

Formation of a partially glucose-6-P-dependent form (activity ratio about 0.25) of skeletal muscle synthetase can also be accomplished by limited proteolysis with either trypsin or a Ca^{2+}-dependent protease (2). This is not thought to represent a physiological mechanism since it is irreversible. Belocpitow et al. (3) compared some of the properties of the D form prepared by proteolysis with the D form prepared by partial phosphorylation (activity ratio about 0.2). The enzyme prepared with the Ca^{2+}-dependent protease was less stable to heat and more susceptible to degradation by trypsin than was the phosphorylated form.

Since proteolysis of synthetase mimics to some degree the effects of phosphorylation of the enzyme, the influence of trypsin on the structure, activity, and phosphorylation of synthetase has been investigated in an attempt to understand better the regulation of this enzyme.

EXPERIMENTAL PROCEDURES

Purification, Phosphorylation, and Assay of Glycogen Synthetase—Glycogen synthetase I was purified from rabbit skeletal muscle as described earlier (4) with the modification that potassium phosphate buffer was substituted for the glyceral-P buffer. Only preparations which had a synthetase activity ratio of 0.80 or greater and which exhibited a single peak of activity on sucrose gradient centrifugation were used.
The methodology for the phosphorylation of synthetase I and conversion to the D form as well as for the assay of glycogen synthetase are given elsewhere (11). The synthetase activity ratio is defined as activity measured in the absence of glucose-6-P (and absence of NaSO₄) divided by activity measured in the presence of 4.8 mM glucose-6-P.

Incubation of Glycogen Synthetase with Trypsin—Unless indicated otherwise, limited proteolysis of synthetase was performed for 15 min at 30°C and at pH 7.0 in reaction mixtures containing 0.3 to 1.6 mg/ml of glycogen synthetase, 10 μg/ml of trypsin, 5% sucrose, 25 mM potassium phosphate, 1 mM EDTA, and 20 mM 2-mercaptoethanol. The reaction was terminated by addition of a 10-fold excess of soybean trypsin inhibitor. Soybean trypsin inhibitor did not have any effect on synthetase activity and did not accept any phosphate from [γ-32P]ATP in the presence of protein kinase.

Other Methods—Centrifugation by the method of Martin and Ames (5) on linear 5 to 20% sucrose gradients containing 50 mM potassium phosphate (pH 6.8), 1 mM EDTA, and 40 mM 2-mercaptoethanol was performed for 18 h at 35,000 rpm in a Beckman SW-41 rotor. The molecular weight and frictional coefficient were calculated as described by Siegel and Monty (6). Protein was determined by the method of Lowry et al. (7). Disc gel electrophoresis in the presence of sodium dodecyl sulfate was performed by the method of Shapiro et al. described earlier (8). [T-32P]ATP was prepared by a modification (4) of the method of Glyn and Chappell (9). Phosphorylase b, trypsin, soybean trypsin inhibitor, liver catalase, and phosphofructokinase were purchased from Sigma Chemical Co. ["CIUDP-glucose was from Worthington, pronase from Calbiochem, papain from P-L Biochemicals Inc., and subtilisin from Sigma Chemical Co.

RESULTS

Effects of Trypsin on Physical Properties of Glycogen Synthetase—Rabbit skeletal muscle glycogen synthetase I has been reported to have a sedimentation coefficient of approximately 14 S as determined by sucrose density gradient centrifugation and by sedimentation velocity in the analytical ultracentrifuge (4). The results of Fig. 1A show that native synthetase I (solid line) and synthetase I which had been incubated with 27 μg/ml of trypsin for 10 min (dashed line) had essentially identical profiles on sucrose density gradient centrifugation. In a separate experiment the sedimentation coefficient was estimated to be 13.3 S with the use of phosphorylase b, catalase, phosphorylase a, and phosphorylase kinase as standards. When native and trypsinnized 32P-synthetase were run on sucrose gradients, the results of Fig. 1B were obtained. With native synthetase, 87% of the 32P was accounted for as 32P-synthetase (peak at 9 ml). The small peak of 32P near the top of the gradient probably was [γ-32P]ATP. After trypsin treatment about 40% of the 32P corresponded to 32P-synthetase (peak at 9 ml) while 60% of the 32P remained near the top of the gradient (presumably [T-32P]-peptide, see below). The synthetase in Fig. 1B sedimented further than in Fig. 1A because of different centrifugation conditions (see legend to Fig. 1).

Gel filtration of synthetase I on Sepharose 6B gave rise to a symmetrical peak of activity eluting at V₅⁰/Vₐᵥ = 1.39 where V₅₀ was the elution volume of the synthetase and Vₐᵥ was the void volume of the column. Trypsin-treated synthetase eluted as a skewed peak with a V₅⁰/Vₐᵥ = 1.49. The Stokes radii for native and trypsintreated synthetase are listed in Table I.

With the use of the sedimentation coefficients and Stokes radii as determined above and assuming a partial specific volume of 0.73, the molecular weights and frictional ratios were calculated (6) as listed in Table I. The value for the molecular weight of native synthetase I, 370,000, is in agreement with the suggestion (4) that skeletal muscle synthetase I is a tetramer composed of 90,000 molecular weight subunits (4, 12). The frictional ratio of 1.45 indicates that synthetase I is slightly asymmetric.

Disc gel electrophoresis of synthetase I in the presence of sodium dodecyl sulfate gave rise to a major protein band of molecular weight 90,000 and a minor contaminant of approximately molecular weight 48,000 (Ref. 4 and Fig. 2). When synthetase I was phosphorylated to a level of either 1 or 2 mol of phosphate per mol of synthetase subunit (1), virtually all of the radioactivity was found in the 90,000 synthetase subunit.1 As shown in Fig. 2, limited proteolysis of synthetase by trypsin reduced the subunit molecular weight to approximately 73,000.

Synthetase Activity Ratio after Proteolysis—Incubation of synthetase I with 10 μg/ml of trypsin (Fig. 3) produced a decrease in the activity ratio to the same value (0.24) as obtained with incorporation of 1 mol of 32P per 90,000 g (Ref. 1 and Fig. 7). This decrease in the synthetase activity ratio in response to trypsin occurred within 2 min with no further decrease during the hour incubation. The fall in the activity ratio resulted largely from a decrease in minus glucose-6-P.

1 Unpublished observation.
TABLE I
Physical properties of native and trypsinized synthetase

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Stokes radius</th>
<th>Sedimentation coefficient</th>
<th>Frictional ratio</th>
<th>Subunit molecular weight</th>
<th>Molecular weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>A</td>
<td>$S \times 10^{14}$</td>
<td>1.45</td>
<td>90,000</td>
<td>360,000-370,000</td>
</tr>
<tr>
<td>Trypsin</td>
<td>62</td>
<td>13.3</td>
<td>1.42</td>
<td>73,000</td>
<td>292,000-340,000</td>
</tr>
</tbody>
</table>

Effects of Trypsin on Glycogen Synthetase

The effects of trypsin on glycogen synthetase were investigated, with particular emphasis on the changes in physical properties. The table above summarizes the physical properties of native and trypsinized synthetase.

Trypsin was incubated with synthetase for 15 min, and the effects on synthetase activity and physical properties were monitored. The results showed that trypsin caused a decrease in synthetase activity, with a corresponding increase in molecular weight and sedimentation coefficient. The changes were quantitatively analyzed using gel filtration and sedimentation techniques, and the properties of the enzyme were calculated from the Stokes radius and sedimentation coefficient.

Graphs and figures were used to illustrate the changes in activity and molecular properties over time and concentration of trypsin. The graphs included a time course of trypsin effect on synthetase activity and concentration dependence of trypsin effect.

The changes in synthetase activities and in the activity ratios after incubation for 15 min with varying concentrations of trypsin are shown in Fig. 4. In the absence of trypsin, the synthetase activities were 23 units/ml and 17 units/ml when assayed in the presence and absence of glucose-6-P, respectively. Although 10 µg/ml of trypsin was more effective, even 1 µg/ml produced a significant decrease in the activity ratio. Between 10 and 100 µg/ml of trypsin, there was a large increase in total synthetase activity. It was surprising that incubation of synthetase for 15 min with 1 mg/ml of trypsin only reduced total synthetase activity to 20 units/ml compared to 20 units/ml in the absence of trypsin.

The response of glycogen synthetase to other proteases was determined in the experiment of Fig. 5. Synthetase I was incubated for 15 min with 10, 100, and 1000 µg/ml of each protease. Each of the proteases decreased the activity ratio to varying degrees primarily by elevating total synthetase activity rather than decreasing minus glucose-6-P activity. Although the data of Fig. 5 suggest that 10 µg/ml of papain affected only minus glucose-6-P synthetase activity, additional experiments showed that at 1 min the synthetase activities were 74 and 12 units/ml assayed in the presence and absence of glucose-6-P, respectively. The most striking effects of certain of these proteases are the large increases in total synthetase activity.

Effects of Trypsin on Phosphorylated Synthetase—It was of interest that 1 to 10 µg/ml of trypsin would decrease the...
synthetase activity ratio to only 0.2 to 0.3. However, if the activity ratio was first decreased to this same value by incorporation of about 0.9 mol of P_i/90,000 g, subsequent exposure to trypsin for 15 min lowered the activity ratio to 0.03 (Table II). This activity ratio is the same as that obtained by incorporation of 2 mol of 32P per mol of synthetase subunit (1). Sixty to seventy per cent of the 32P was released as a trichloroacetic acid-soluble species. The increase in total synthetase activity after incorporation of 0.9 mol of P_i/90,000 g (Table II, condition 3) is not normally seen. Phosphorylation does not usually affect total synthetase activity (1, 4).

The time course of the release of 32P (presumably as 32P-peptide) from synthetase by trypsin was investigated.Regardless of whether the initial phosphate content of the synthetase was approximately 1 or 2 mol per 90,000 g, there was a rapid release of about 50% of the phosphate (Fig. 6). The remaining 50% of the 32P-synthetase was quite resistant to this low concentration of trypsin. The release of 32P was blocked by a 10-fold excess of soybean trypsin inhibitor, thus eliminating the possibility of phosphatase contamination of the trypsin.

Phosphorylation of Trypsin-treated Synthetase—The susceptibility of trypsin-treated synthetase to phosphorylation catalyzed by the CAMP-dependent protein kinase was demonstrated by the experiment of Fig. 7. Synthetase I was incubated in the presence or absence of trypsin (8 µg/ml) for 15 min followed by addition of soybean trypsin inhibitor (71 µg/ml). Components of the phosphorylation reaction were then added, and 32P incorporation and synthetase I to D conversion were determined. Incubation of trypsin-treated synthetase (activity ratio of 0.25) with endogenous protein kinase did not result in significant 32P incorporation or I to D conversion (■—■). However, when exogenous protein kinase was added, 1 mol of 32P per mol of subunit was incorporated and the activity ratio declined to 0.05 or less (□—□). These results confirmed previous experiments which showed that incorporation of 1 mol of 32P per 90,000 g was required to decrease the synthetase activity ratio from 0.25 to 0.05 (1). Phosphorylation and I to D conversion of the non-trypsin-incubated synthetase are shown in Fig. 7 for purposes of comparison.

The lack of 32P incorporation and synthetase I to D conversion in the absence of exogenous protein kinase was not due to destruction by trypsin of the protein kinase endogenous to the synthetase preparation. This was determined by measuring 32P incorporation into histone (6 mg/ml) which was added to the phosphorylation reaction (see Fig. 1 of Ref. 1). In fact, there was about 40% more histone kinase activity in the synthetase preparation after the trypsin incubation (data not shown).

![Graph](http://www.jbc.org/)

**Table II**

<table>
<thead>
<tr>
<th>Condition</th>
<th>Treatment</th>
<th>Synthetase activity</th>
<th>Synthetase activity ratio</th>
<th>Mol of 32P per 90,000 g</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>None</td>
<td>0.138 ± 0.006</td>
<td>0.167 ± 0.008</td>
<td>0.83</td>
</tr>
<tr>
<td>2</td>
<td>Trypsin</td>
<td>0.046 ± 0.006</td>
<td>0.200 ± 0.004</td>
<td>0.23</td>
</tr>
<tr>
<td>3</td>
<td>Phosphorylation</td>
<td>0.049 ± 0.002</td>
<td>0.199 ± 0.024</td>
<td>0.24</td>
</tr>
<tr>
<td>4</td>
<td>Phosphorylation, then trypsin</td>
<td>0.006 ± 0.003</td>
<td>0.220 ± 0.011</td>
<td>0.05</td>
</tr>
</tbody>
</table>
Effects of Trypsin on Glycogen Synthetase

The similarities in the synthetase activity ratios after phosphorylation or trypsination of synthetase are striking (Table I and Fig. 7). Trypsin can mimic the effect of incorporation of either the first or second phosphate per subunit. The intermediary form of synthetase, characterized by an activity ratio of about 0.25, must be a stable species of the enzyme since it was formed both by phosphorylation with low protein kinase and by trypsin digestion. This intermediary species of synthetase, regardless of whether it is produced by phosphorylation or trypsin digestion, could not be significantly phosphorylated with 2 x 10^{-10} M protein kinase but did incorporate one phosphate per subunit in the presence of 5 x 10^{-7} M protein kinase (Fig. 7 and Ref. 1).

These effects of trypsin on skeletal muscle synthetase are in contrast to the response of yeast synthetase to proteolysis. The partially glucose-6-P-dependent form of yeast synthetase is characterized by an activity ratio of about 0.26 and phosphate content of about 1.4 mol per subunit (17). Incubation with trypsin produces an increase in the activity ratio to 0.8 to 0.9 primarily through an elevation of minus glucose-6-P activity (18).

Phosphorylation of trypsin-treated skeletal muscle synthetase resulted in incorporation of 1 phosphate per subunit associated with a decrease in the synthetase activity ratio from 0.25 to <0.05 (Fig. 7). This observation is in agreement with the earlier report (1) that a second phosphate per subunit was needed for complete inactivation of synthetase. The experiment of Fig. 7 indicated that a low concentration of trypsin removed only one of the two phosphorylated sites. This conclusion was substantiated by the results of Fig. 6 where only one of the phosphates was lost from ^32P-synthetase containing 2 phosphates per subunit. The fact that trypsin took off about 50% of the ^32P from ^32P-synthetase containing 1 phosphate per subunit implied that the first phosphate which was incorporated was distributed about equally between the two phosphorylation sites.

The results of this study agree with some of the observations just published by Takeda et al. (19, 20). They reported that trypsin digestion removed COOH-terminal peptides giving rise to a 75,000 molecular weight subunit. They also noted that limited trypsination of synthetase I produced a partially glucose-6-P-dependent form of the enzyme. However, they had a 50% loss of total synthetase activity whereas we observed a 20% increase in activity. This difference may relate to the fact that their preparation of synthetase I was a mixture of dimer and tetramer whereas our preparation was all tetramer. Takeda and Larner (20) showed that trypsin removed 1.2 phosphates per subunit from synthetase D containing 3.2 mol of alkali-labile phosphate per subunit. This value of 3 phosphates per subunit of synthetase D was considerably less than the value of six reported by Smith et al. (12). We have shown previously (1) and confirmed in this paper that the tetrameric species of glycogen synthetase, the D form was obtained when 2P_i per subunit were incorporated. Takeda and Larner (20) suggest that the discrepancy between our results and their results on the stoichiometry of phosphorylation may be due to the fact that our synthetase I might contain considerable alkali-labile phosphate. They failed to note, however, that in our initial paper (4) we reported a value of 0.1 to 0.5 mol of alkali-labile P_i per 10^6 g which is similar to their value of 0.2 to 0.9 mol of P_i per 10^6 g (12, 20). It should be noted that we observed phosphorylation in excess of 2 P_i/subunit, but this additional phosphorylation was not associated with any

Fig. 7. Phosphorylation and I to D conversion of native and trypsined synthetase. Synthetase I (1.6 mg/ml) was incubated at 30° for 15 min in the absence or presence of trypsin (8 mg/ml). Soybean trypsin inhibitor (71 μg/ml) was then added. The native and trypsin-digested synthetase were tested at 30° for phosphorylation and I to D trypsin inhibitor (71 &μg/ml) was then added. The native and trypsin-digested synthetase were tested at 30° for phosphorylation and I to D conversion in the presence of endogenous cAMP-dependent protein kinase (10 units/ml) or with added protein kinase catalytic subunit digested synthetase were tested at 30° for phosphorylation and I to D conversion in the presence of endogenous cAMP-dependent protein kinase (10 units/ml) or with added protein kinase catalytic subunit digested synthetase. Synthetase I (72,000 units/ml). Other components of the reaction mixtures were 0.23 mM ATP or 0.23 mM [γ-32P]ATP (0.003 pmol/cpm), 10 mM magnesium acetate, 5 mM cAMP, 10 mM potassium phosphate (pH 7.0), and 10 mM NaF. Symbols: native synthetase with endogenous kinase only (●), or plus catalytic subunit (▲), trypsined synthetase with endogenous kinase only (■) or plus catalytic subunit (○).
Effects of Trypsin on Glycogen Synthetase

change in synthetase activity (1). This additional phosphorylation was more prominent in preparations of synthetase which exhibited multiple peaks of activity on sucrose gradient centrifugation (see discussion of Ref. 1). This may explain the high phosphorylation values obtained by those using preparations of synthetase containing dimeric and tetrameric species.

The following model is proposed to account for the results reported here and elsewhere (1). The two phosphorylation sites which are associated with I to D conversion are about equally susceptible to phosphorylation in synthetase I. Phosphorylation of either site changes the synthetase, perhaps conformationally, making the other site kinetically unfavorable for phosphorylation. Only by greatly increasing the concentration of protein kinase does the rate of phosphorylation of the other site become sufficient to measure. This model predicts that one should obtain 2 phosphopeptides in about equal amounts from synthetase containing 1 P_i per subunit. Additionally, the same 2 phosphopeptides should be produced from synthetase containing 2 P_i per subunit. Synthetase which has been treated first with trypsin and then phosphorylated to 1 P_i per subunit should contain only one of these phosphopeptides. Experiments of this type are being done.

Acknowledgments—The author wishes to express his appreciation to Ms. Martha Bass for her excellent technical assistance and to Drs. Charles R. Park, J. D. Corbin, and J. H. Exton for their helpful suggestions during this investigation and in the preparation of this manuscript.

Note Added in Proof—The results of this study emphasize that investigators studying multiple synthetase kinases must be cautious that their synthetase I to D conversions are not the result of limited proteolysis.

REFERENCES

Regulation of glycogen synthetase. Effects of trypsin on the structure, activity, and phosphorylation of the skeletal muscle enzyme.

T R Soderling


Access the most updated version of this article at http://www.jbc.org/content/251/14/4359

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/251/14/4359.full.html#ref-list-1