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EFFECT OF PARTIAL TRYPTIC PROTEOLYSIS ON THE KINETIC PROPERTIES OF RABBIT MUSCLE GLYCOGEN SYNTHASE

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Summary

The kinetic basis for the changes in the activity of rabbit muscle glycogen synthase I (UDPglucose:glycogen 4α-glucosyltransferase, EC 2.4.1.11) following limited tryptic proteolysis has been investigated. The action of trypsin (0.63 μg/ml) caused a degradation of the native 85 000 dalton subunit to a species of 75 000 daltons and finally of 68 000 daltons. After 6 min of proteolysis, the percent I activity had fallen from 86% to 40% and the total activity (measured in the presence of glucose-6-*P*) by 30%. This corresponded to an enzyme composed primarily (75%) of the 68 000 dalton species and to a lesser extent (24%) of the 75 000 dalton species. The native 85 000 dalton subunit was almost totally absent. The kinetic properties of such tryptically degraded enzyme were compared with the native species: tryptic hydrolysis caused an increase in the $S_{0.5}$ for UDPglucose from 1 mM to 11 mM, and of the $M_{0.5}$ for glucose-6-*P* from 16 μM to 53 μM. This compares with values of 75 mM ($S_{0.5}$) and 340 μM ($M_{0.5}$), respectively, for glycogen synthase D form. Proteolysis also caused changes in the nature of the dependence of reaction rate on UDPglucose concentration, leading to a decrease in the Hill slope. The shape of the Hill plot for glucose-6-*P* variation, however, which was non-linear for the native enzyme, was unchanged by proteolysis. Whatever underlies this complex kinetic behaviour, then, must persist after proteolysis; this might mean, for example, that subunit interactions remained even in the degraded enzyme. The results demonstrate clearly that the kinetic parameters most strongly influenced by tryptic hydrolysis are the apparent affinities for the substrate UDPglucose and the activator glucose-6-*P*. Importantly, however, even extensive proteolysis is less effective than covalent phosphorylation in changing kinetic properties.

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Introduction

Glycogen synthase (UDPglucose:glycogen 4α-glucosyltransferase, EC 2.4.1.11) undergoes a covalent phosphorylation-dephosphorylation cycle [1] that correlates with extensive changes in the kinetic properties of the enzyme. Covalent phosphorylation, which may be catalyzed by cyclic AMP-dependent protein kinase [2], cyclic AMP-independent protein kinase [3] or phosphorylase kinase [4], results in a relative inactivation of synthase: the inclusion of glucose-6-*P*, an activator, however, restores the activity to that of the non-phosphorylated I form. The basis for this activity change resides largely in the decrease in apparent affinity for UDPglucose following phosphorylation [5]. It has been known for some time that the action of trypsin can partially mimic the inactivation of synthase described above [6]. Structurally, partial proteolysis has been reported to involve cleavage near to the C-terminus of the molecule, causing a reduction in the molecular weight of the native 85 000 dalton subunit [7]. Some slightly differing values for the molecular weights of the degradation products have been recorded in different laboratories [7—9]. Changes in activity have been correlated with this proteolytic reaction by monitoring the percent I activity which is simply the ratio of activity measured in the absence of glucose-6-*P* to that in the presence of the sugar phosphate, expressed as a percentage. The present study addresses the more detailed kinetic basis for the inactivation of synthase under defined conditions of proteolysis. We report that tryptic inactivation of synthase correlates primarily with a decrease in the apparent affinity for substrate UDPglucose; concomitantly, the apparent affinity for the activator glucose-6-*P* is also decreased.

Materials and Methods

Purification of glycogen synthase. Glycogen synthase I and D forms were purified by the method of Smith et al. [10], as modified by Takeda et al. [11] using only fresh rabbit muscle. The glycogen synthase samples showed no detectable loss of activity over the period of this work when stored at —45° C.

Assay of glycogen synthase activity. Glycogen synthase activity was measured at 30° C by the method of Thomas et al. [12], based on the incorporation of [U-^{14}C]glucose from UDP[U-^{14}C]glucose into glycogen. For the standard assay, samples of enzyme (20 μl) were added to 40 μl of a solution containing 50 mM Tris-HCl, pH 7.8; 20 mM EDTA; 25 mM KF; 10 mg/ml of glycogen; 6.7 mM UDP[U-^{14}C]glucose (specific radioactivity of 100 cpm/nmol) and, when indicated, 15 mM Na_2SO_4 or 10 mM glucose-6-*P*. In all other cases, the following conditions (taking into account the contributions of the buffer containing the enzyme) were used. The reaction mixture (100 μl) contained 50 mM Tris-HCl, pH 7.8; 12.5 mM EDTA; 1 mM EGTA; 10 mM mercaptoethanol; 7 mg/ml of rabbit liver glycogen; UDP[U-^{14}C]glucose; other additions as indicated and from 50 ng to 100 ng of purified glycogen synthase. The specific activity of the UDPglucose was varied according to the reaction conditions, and up to 60 000 cpm/nmol were used at low UDPglucose concentrations.

The reaction was started by the addition of enzyme, and after an appropriate time (from 5 to 10 min for the I form and from 10 to 15 min for the D

form), 75 μl of the reaction mixture were placed on a filter paper square which was deposited in 66% (v/v) ethanol at 0°C. The filter papers were washed, dried, and counted as for the standard assay [12]. For study of the activation by glucose-6-*P*, UDP[U-^{14}C]glucose was present at a final concentration of 200 μM (specific radioactivity of 7000 cpm/nmol). The reaction mixture also contained 50 mM Tris-HCl, pH 7.8; 12.5 mM EDTA; 1 mM EGTA; 10 mM mercaptoethanol; 7 mg/ml rabbit liver glycogen and from 50 ng to 100 ng of purified glycogen synthase. Reaction blanks were run by placing enzyme onto a filter paper before adding the rest of the reaction mixture and rapidly immersing the paper in 66% (v/v) ethanol at 0°C. Where the concentration or specific activity of the UDP[U-^{14}C]glucose was varied in an experiment, blanks were run to accommodate these conditions. The reaction rate was constant during the consumption of 10—20% of the substrate, and conditions were typically such that less than 10% consumption of UDPglucose occurred. A unit of enzyme activity corresponds to the incorporation of 1 μmol of glucose from UDPglucose into glycogen/min.

Digestion of glycogen synthase I form with trypsin. Glycogen synthase (final concentration 0.63 mg/ml) was incubated at 30°C with trypsin at a ratio of trypsin to synthase of 1 : 1000 (w/w) in a reaction mixture containing 50 mM Tris-HCl, pH 7.0. At various times, aliquots (20 μl) were removed and diluted with 5 ml of 50 mM Tris-HCl buffer (pH 7.8) containing 5 mM EDTA, 5 mM EGTA, 50 mM mercaptoethanol, glycogen (1 mg/ml) and soybean trypsin inhibitor (50 μg/ml). The mixture was incubated at 30°C for a minimum of 20 min before the assay for enzyme activity. This period of incubation allowed the enzyme to reach a level of maximum activity which then remained constant for several hours.

Determination of the molecular weights. Polyacrylamide gel electrophoresis (5% acrylamide) in the presence of sodium dodecyl sulphate was performed according to the method of Weber et al. [13] using samples of 5—15 μg of protein/gel. The determination of the subunit molecular weights of the native subunit and of the proteolytically degraded forms was made using phosphorylase (95 000), bovine serum albumin (68 000), catalase (58 000), ovalbumin (43 000) and glyceraldehyde phosphate dehydrogenase (36 000) as standards. After Coomassie blue staining, the gels were scanned using a Vitatron TLD 100 densitometer.

Other methods. UDP[U-^{14}C]glucose was prepared by the enzymic conversion of [U-^{14}C]glucose (Amersham) into UDPglucose [12]. Using the standard assay conditions described below, more than 98% of the radioactivity in UDP[U-^{14}C]glucose so prepared could be incorporated into glycogen on incubation with purified glycogen synthase. Because relatively high UDPglucose concentrations were used, and because of the serious implications of glucose-6-*P* contamination, UDPglucose (Sigma Chemical Co.) was carefully analyzed for glucose-6-*P* content using glucose-6-*P* dehydrogenase [14]. No glucose-6-*P* was detectable in a 100 mM UDPglucose solution, indicating less than 0.0015% (mol/mol) of the sugar phosphate in the UDPglucose. Human salivary α-amylase was purified as described by Bernfeld [15]. Protein was determined by the method of Lowry et al. [16] and the method of Bradford [17] as modified by Spector [18].

Materials. Rabbit liver glycogen (type III, Sigma Chemical Co.) was purified by passage through a column of AG-501 X-8 (Bio-Rad Co.) ion-exchange resin and precipitation with ethanol before use in enzyme assays [10]. DEAE-cellulose (DE-32) was obtained from Whatman. Trypsin treated with L-1-tosil-amino-2-phenylethylchloromethyl ketone (282 U/mg, Lot TRTPCK 37E833) was purchased from Worthington Biochemical Company. Reagents for disc gel electrophoresis were from Bio-Rad Company. Glucose-6-*P*, UDPglucose, glycogen, Tris, EDTA, EGTA and trypsin inhibitor, were obtained from the Sigma Chemical Company.

Results

Tryptic digestion of glycogen synthase

A prerequisite for the kinetic studies described below was proteolytically degraded glycogen synthase I of defined subunit composition. Thus, the time course of tryptic degradation was carefully followed and the enzyme analyzed both for enzyme activity and, by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate, for changes in the subunit molecular

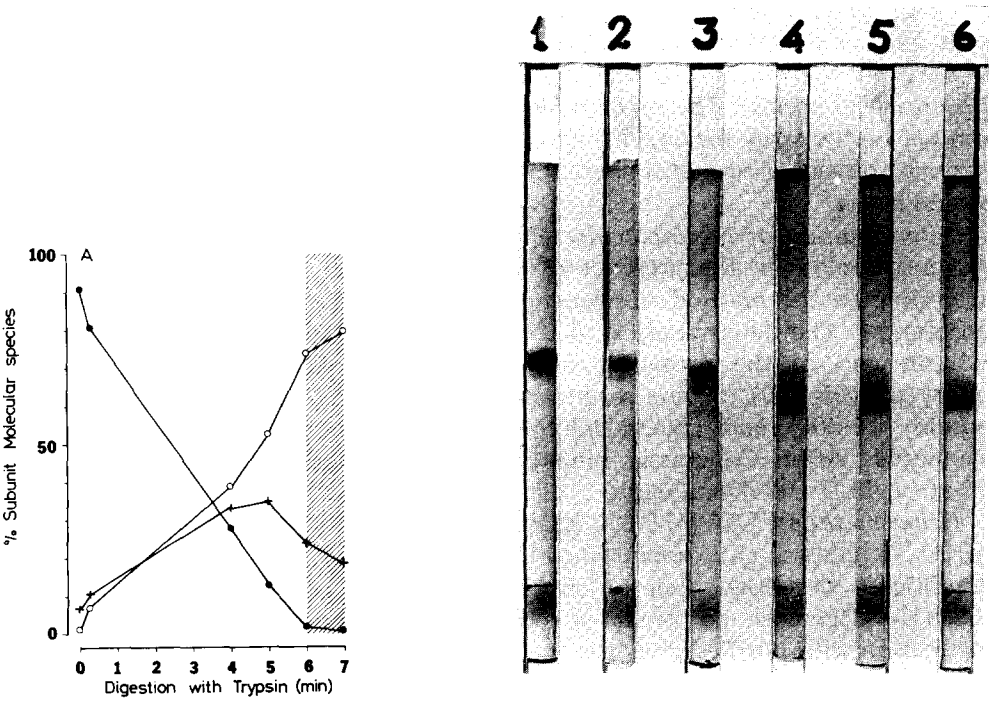

Fig. 1. (A) Time course of tryptic digestion of glycogen synthase. Glycogen synthase (0.63 mg/ml) was digested with trypsin (0.63 μg/ml) and at the indicated times aliquots were removed and diluted with excess trypsin inhibitor. Samples were subjected to polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate and the relative proportions of the different molecular species were determined. •, native 85 000 dalton subunit; +, 78 000 dalton subunit, and ○, 68 000 dalton subunit. The shaded area indicates the composition of the enzyme studied. (B) Gels: 1. Undigested D form (15 μg); 2. I form (inhibitor added before trypsin); 3. I form digested for 15 s; 4. I form digested for 4 min; 5. I form digested for 5 min; 6. I form digested for 6 min. Gels 2—6 contained 7 μg of protein. The band at the bottom corresponds to trypsin inhibitor.

TABLE I

EFFECT OF TRYPSIN ON I AND TOTAL GLYCOGEN SYNTHASE ACTIVITY

The enzyme was incubated with trypsin as in Fig. 1. At the indicated times percent I (in the presence of Na_2SO_4) and total activities were measured. The latter is expressed as a percentage of the activity at zero time. Results are mean ± S.D.

Digestion with trypsin	% I activity with Na_2SO_4	% initial activity with Glc-6-*P*
Inhibitor before trypsin	86 ± 1	100
15 s	86 ± 1	100
4 min	47 ± 4	79 ± 3
5 min	43 ± 4	73 ± 6
6 min	40 ± 5	70 ± 5
7 min	36 ± 5	65 ± 6

weight (Fig. 1). There was a steady decline in the proportion of the native 85 000 dalton subunit, and after 6 min essentially none could be detected. A species of molecular weight 75 000 appeared, reaching a maximum proportion of 35% after 5 min; thereafter, its concentration declined slightly. A 68 000 dalton polypeptide was generated, the proportion of which increased over the whole incubation period. These alterations of the subunit correlated with a decrease in percent I activity from an initial 86% to a minimum of 36%. The total activity of the enzyme (that is, the activity measured in the presence of glucose-6-*P*) fell by 35% over the same period (Table I).

6 min was chosen as a standard incubation time for proteolytic treatment. At this point, the percent I activity was 40% (less than half of the original) whilst the total activity had fallen by only 30%. Also, at this time, the native subunit was no longer detectable and the enzyme was composed primarily (74%) of the 68 000 dalton species, with a 26% contribution of the 75 000 dalton species. Longer times were not used as the loss in total activity became excessive.

Effect of trypsin on the dependence of reaction rate on UDPglucose concentration

The dependence of reaction rate on UDPglucose concentration was determined for both native synthase and enzyme that had been proteolytically cleaved. After incubation with trypsin, proteolysis was terminated by addition of the soybean trypsin inhibitor. Controls (not shown) established that addition of the inhibitor before trypsin led to a UDPglucose saturation curve identical to that of the untreated enzyme; likewise no changes in the subunit composition were noted in this control. Thus, the concentration of inhibitor used was sufficient to inhibit trypsin and its presence did not alter synthase kinetic properties. The results of a representative experiment are shown in Fig. 2. Proteolysis caused a significant increase in the $S_{0.5}$ for UDPglucose (concentration corresponding to half-maximal rate) from a value of 1 mM to approximately 11 mM; the V was reduced to a lesser extent, by about 30%. In addition, the kinetics were modified from an essentially hyperbolic behaviour (Hill slope 1.0) for the native enzyme to more complex kinetics that would correspond formally to negative cooperativity (Hill slope 0.8).

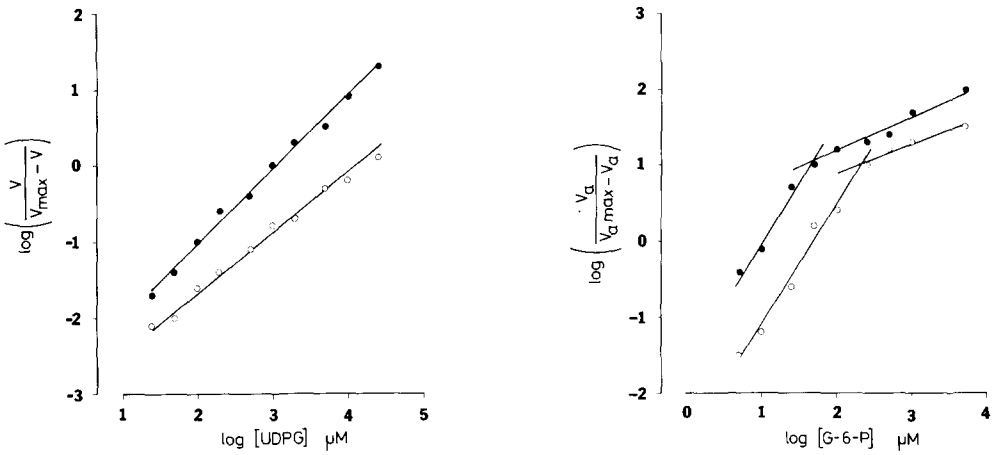

Fig. 2. Effect of proteolysis on UDPglucose saturation curve (Hill plots). Hill plots are shown for the dependence of reaction rate on UDPglucose concentrations of the native (•) and trypsinized glycogen synthase (○). V was estimated from Eadie-Hofstee plots. Trypsinization was performed as indicated in Fig. 1 for 6 min.

Fig. 3. Effect of proteolysis on glucose-6-*P* activation curve (Hill plots). Hill plots are shown for the dependence of reaction rate on glucose-6-*P* concentrations of the native (•) and trypsinized glycogen synthase (○). Glycogen synthase activity was measured as described in Materials and Methods, with 0.2 mM UDPglucose, as a function of glucose-6-*P* concentration. The velocity increase, V_a, caused by glucose-6-*P* (activated rate minus rate in the absence of sugar phosphate) was used in place of velocity. $V_{(max)}$ was calculated from Eadie-Hofstee plots of V_a/[glucose-6-*P*] versus V_a.

Effect of trypsin on the dependence of reaction rate on glucose-6-P concentration

A similar experimental design was applied when the glucose-6-*P* concentration was varied, this time with the UDPglucose concentration 0.2 mM. The kinetics of glucose-6-*P* activation were complex and non-hyperbolic; Hill plots were non-linear with slopes varying from 1.6 at low glucose-6-*P* to 0.4 at high concentration. Similar non-linearity of Hill plots has been reported [5]. Trypsin action had little effect on the shape of the Hill plots but did cause a significant increase in the $M_{0.5}$ for glucose-6-*P* (concentration corresponding to half-maximal activation) from about 16 μM for the native enzyme to 53 μM for tryptically degraded synthase.

Kinetic parameters of the D form

A kinetic analysis of the dependence of reaction rate on UDPglucose concentration and on glucose-6-*P* concentration of a D form preparation was also performed. This D form was obtained by phosphorylation with endogenous protein kinases at an intermediate stage of the purification of the enzyme [11] and had a percent I activity of 22%. The calculated values for the $S_{0.5}$ for UDPglucose and $M_{0.5}$ for glucose-6-*P* (at a UDPglucose concentration of 0.2 mM) of this D form preparation were 75 mM and 340 μM, respectively (data not shown).

A summary of kinetic parameters characterizing the native glycogen synthase I form, the proteolytically degraded I form and the phosphorylated D form are

TABLE II

EFFECT OF PARTIAL PROTEOLYSIS ON KINETIC PARAMETERS OF GLYCOGEN SYNTHASE

$M_{0.5}$, the glucose-6-*P* concentration required for half-maximal activation (0.2 mM UDPglucose). $S_{0.5}$, the UDPglucose concentration required for half-maximal activation. $V_a = V - V_0$ (V_0 = velocity without glucose-6-*P*). *V* was calculated by extrapolation of Eadie-Hofstee plots. It was impossible to determine *V* in the absence of glucose-6-*P* with the D form sample, so $V_{standard}$ is given, the rate measured with the standard assay in the presence of glucose-6-*P* (see Ref. 5). The accompanying $S_{0.5}$ value was calculated from Hill plots, with the assumption that the maximal rate was equal to the value of $V_{standard}$. Results are mean ± S.D. GS, glycogen synthase.

Sample	% I activity with Na_2SO_4	$S_{0.5}$ (mM)	*V*	$M_{0.5}$ (μM)	$V_{a(max)}$
GS I	86 ± 1	1 ± 0.3	33 ± 2	16 ± 4	13 ± 2
GS I_T	39 ± 8	11 ± 3	22 ± 1	53 ± 3	9 ± 2
			$V_{standard}$		
GS D	22 ± 2	75 ± 7	20 ± 1	340 ± 30	11 ± 1

shown in Table II. It is clear that, whilst proteolysis changed properties in the same sense as phosphorylation as reflected in the $S_{0.5}$ for UDPglucose or the $M_{0.5}$ for glucose-6-*P*, phosphorylation was much more potent.

Discussion

The structural effects of trypsin on muscle glycogen synthase reported here are comparable to those described by Takeda and Larner [7], Huang et al. [8] and Soderling [9]. The native 85 000 dalton subunit is degraded by trypsin to two smaller species of 75 000 and 68 000 daltons. The values of molecular weight are somewhat different from those of Takeda and Larner [7] (78 000 and 75 000) but similar to those of Huang et al. [8] (76 000 and 68 000). An important finding of these studies is that the kinetic basis for the proteolytic inactivation of synthase, as followed by the percent I activity, is primarily an increase, of about ten-fold, in the $S_{0.5}$ for UDPglucose. The change in maximal activity was less marked, a decrease of 30%. Proteolysis also correlated with a significant increase in the $M_{0.5}$ for glucose-6-*P*. Extrapolating from these kinetic data, we would suggest that proteolytically cleaved synthase has lost little of its maximal catalytic activity but that the binding of both glucose-6-*P* and UDPglucose has become weaker.

The fact that synthase in these studies displayed non-hyperbolic kinetics towards either glucose-6-*P* or UDPglucose after trypsin action is interesting. For variation of UDPglucose concentration, an operational negative cooperativity was acquired. Two obvious explanations are possible, actual negative cooperativity at the molecular level or else the presence of two kinetically distinguishable species. Since we know that the enzyme contains a mixture of 75 000 dalton and 68 000 dalton subunits, we cannot distinguish these possibilities. However, when glucose-6-*P* concentration was varied, the kinetic behaviour was more complex, involving Hill plots whose slopes varied from 0.4 to 1.6. It is unlikely that heterogeneity of the enzyme can explain such results. Indeed, the precise molecular basis for these non-hyperbolic kinetics is not

known although it has been suggested that the native enzyme has cooperative kinetics associated with its multimeric structure [5]. If this is true, then, proteolytically degraded enzyme, which has essentially full catalytic activity, would also have retained those molecular properties contributing to its cooperative behaviour, such as interactions between subunits.

Qualitatively, trypsin action mimics the covalent phosphorylation of synthase, not only in terms of changes in percent I activity [7,8,9], but also by the more detailed kinetic criteria presented here. However, even the complete destruction of the native subunit caused less extreme changes in kinetic parameters than can be achieved by phosphorylation (Table II). This is a very important point since it emphasizes how powerful are the effects of covalent phosphorylation, which is, of course, thought to mediate the regulation of the enzyme in vivo.

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