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The substrate specificity of isoamylase and the preparation of apo-glycogenin*

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ABSTRACT

A new facet of the specificity of the glycogen-debranching enzyme, isoamylase, namely, the hydrolysis of a carbohydrate-amino acid linkage, is described. This bond joins the terminal, reducing-end D-glucose unit of glycogen to the hydroxyl group of tyrosine in glycogenin, the primer protein for glycogen biogenesis. The specificity was further defined by demonstrating that 4-nitrophenyl α-maltotrioside and higher homologs also act as substrates. The splitting of the glycogen–glycogenin bond by isoamylase indicates the α-anomeric configuration of the terminal D-glucose unit. It also provides a means of preparing apo-glycogenin. Pullulanase, a somewhat similar starch- and glycogen-debranching enzyme, does not split these new isoamylase substrates, permitting the 4-nitrophenyl saccharides to be used in distinguishing between isoamylase and pullulanase.

INTRODUCTION

Isoamylase and pullulanase are enzymes of major importance in the study of starch and glycogen, and their products of degradation. Both enzymes hydrolyze the (1→6)-branch linkages of amylaceous polysaccharides but with important differences. Pullulanase readily debranches amylopectin and alpha-limit dextrins derived therefrom[1]. It has little or no action on glycogen, unless the (1→4)-bonds are simultaneously hydrolyzed, for example, by beta-amylase[1]. Isoamylase readily and totally debranches both amylopectin and glycogen, making it the enzyme of choice for the study of the molecular profiles of the unit chains set free[2].

In terms of oligosaccharide substrates, neither enzyme will split a single α-D-glucose unit attached by a (1→6)-bond to a maltosaccharide[1]. Pullulanase will split α-maltose linked (1→6) to maltosaccharides. Isoamylase requires three D-glucose units in the form of α-maltotriose, or a longer chain, and shares this type of substrate with pullulanase, provided that the (1→6)-bond is at a point of branching; pullulanase does not require that its substrate be branched[1–3].

In further distinction between these enzymes, we now report that isoamylase, but not pullulanase, will split the glycosidic bond linking an α-maltosaccharide (maltotriose or larger) to an aromatic aglycon, which may be tyrosine in the self-glucosylating primer for glycogen synthesis (glycogenin) or 4-nitrophenol as in 4-nitrophenyl α-maltotrio-

* Dedicated to Professor David Manners

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side. This finding has provided new structural information on glycogenin, as well as enabling the carbohydrate-free protein to be prepared for metabolic studies.

EXPERIMENTAL

Materials. — 4-Nitrophenyl α-D-glucopyranoside, α-maltoside, and higher homologs were purchased from Boehringer. Highly purified *Pseudomonas* isoamylase was a gift from Hayashibara Biochemical Laboratories, Hiroshima, Japan. Pullulanase and all other reagents were purchased from Sigma Chem. Co, St Louis, MO.

Muscle self-glucosylating protein (SGP) having M_r 37 kDa was prepared as described by Lomako *et al.*[4]. The M_r-42-kDa form of SGP arises as a by-product of this preparation[5]. Self-glucosylation with UDP-D-[^{14}C]glucose was carried out as described by Lomako et al.[4].

Methods. — Digestion of oligosaccharide substrates with the debranching enzymes was carried out for isoamylase in 1-mL digests at pH 4.5 and room temperature in 100mM sodium acetate buffer with an enzyme concentration of 10 μg/mL and 16mM substrate. Hydrolysis of the 4-nitrophenyl saccharides was monitored by addition of 0.1M Tris·HCl buffer, pH 9.5 (1.9 mL) to 0.1 mL of digest and measurement of absorption at 405 nm. Hydrolysis of muscle glycogen (10 mg/mL) was carried out similarly with measurement of the reducing power formed as described by Nelson[6]. Pullulanase (100 μg/mL) was similarly incubated with 16 mM 4-nitrophenyl saccharides in 100mM ammonium acetate, pH 7.0.

For the digestion of ^{14}C-glucosylated SGP by trypsin, the purified SGP was allowed to self-glucosylate with UDP-D-[^{14}C]glucose[4]. Unreacted UDP-D-glucose was removed on a small column of Sephadex G-25. The labeled SGP (20 000 c.p.m.) was digested at 37° in a 150-μL digest with TPCK-treated trypsin (100 μg) for 24 h, and the enzyme was renewed for another 24 h and then fractionated on Bio-Gel P4 (0.8 × 90 cm) in 50mM NH_4HCO_3, 1-mL fractions being collected. The ^{14}C-containing fractions were freeze-dried and incubated in 100mM sodium acetate buffer (1 mL; pH 4.5) overnight at room temperature with isoamylase (50 μg) and then returned for fractionation on the same Bio-Gel P4 column.

To demonstrate inhibition of the hydrolysis of ^{14}C-labelled SGP by glycogen, the SGP (28×10^3 c.p.m.) was incubated with isoamylase as described above, in the absence and presence of muscle glycogen (1 mg/mL) at room temperature. Portions of the digests (50 μL) were removed at intervals to determine the amount of ^{14}C precipitated by 1 mL of 10% trichloroacetic acid.

RESULTS AND DISCUSSION

Specificity of isoamylase. — The results to be reported arose from an accidental observation. We have reported the purification to homogeneity of a self-glucosylating protein (SGP) from rabbit muscle[4] that appears to be identical with a protein, glycogenin, that we had earlier shown to be covalently bonded to muscle glycogen in a 1:1 molar

proportion[7] *via* the novel glucosyl–tyrosine bond[8,9]. SGP is, we consider, the primer on which glycogen is built[4,7].

SGP as isolated contains two or more (1→4)-linked α-D-glucose units[4]. After self-glucosylation, the longest maltosaccharide chain detected was maltooctaose[10]. SGP, we now know, arises from the autolysis of a low-molecular-weight form of glycogen, proglycogen[11,12]. Its formation is accompanied by a second self-glucosylating protein having M_r 42 kDa (p42), which we were able to separate from the M_r 37 kDa (p37) form in the last stages of purification on h.p.l.c.[11]. Radioautographs of the [^{14}C]glucosylated M_r-37-kDa and 42-kDa species, separated by sodium dodecylsulfate–poly(acrylamide) gel electrophoresis were reported by Lomako *et al.*[12]. When treated with alpha-amylase, p42 was converted into p37[11,12]. Therefore p42 seems to be glycogenin carrying carbohydrate units additional to those in p37. To determine the nature of this additional carbohydrate component, [^{14}C]glucosylated p42 was treated with the glycogen-debranching enzyme isoamylase[2], [^{14}C]glucosylated p37 being the control. Surprisingly p37, as well as p42, lost its ^{14}C-label. Examination of the products released from ^{4}C-labeled p37 showed this to be mainly ^{14}C-labeled maltoheptaose and maltooctaose. Since maltooctaose represents the fully glucosylated saccharide chain of SGP (p37)[10], isoamylase had apparently split the glycosidic linkage between maltooctaose and Tyr-194 in glycogenin to which the saccharide is linked[13]. This observation suggested a new specificity for isoamylase, namely that its hydrolytic action is not confined

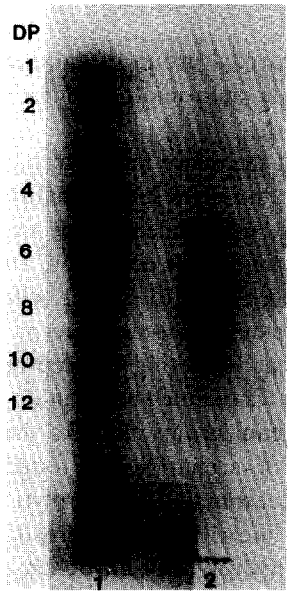

Fig. 1. Radioautography of the products of isoamylase action on [^{14}C]-glucosylated SGP. The products were separated by t.l.c. on silica gel in ethanol–water–butanol (5:4:5): DP, degree of polymerization. Lane 1, ^{14}C-labeled proglycogen[11] was partly hydrolyzed with acid[10] to provide ^{14}C-labeled-D-glucose and maltosaccharides as reference standards. Lane 2, ^{14}C-labeled SGP was treated with isoamylase as described in the Experimental section.

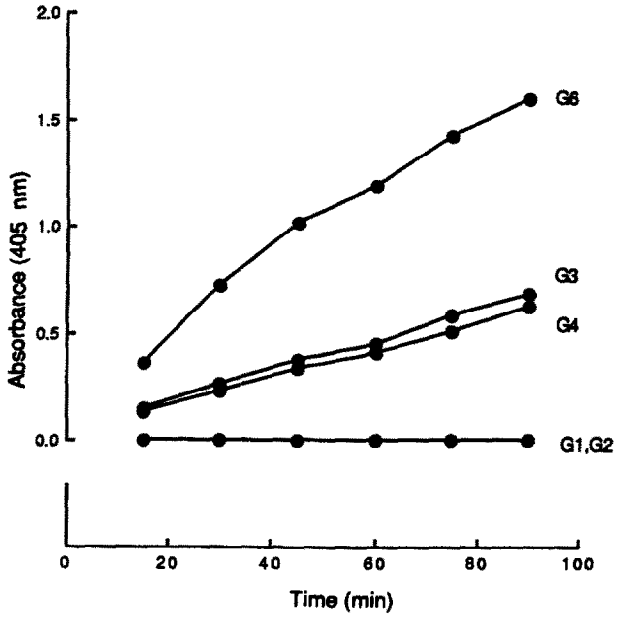

Fig. 2. Hydrolysis of 4-nitrophenyl saccharides by isoamylase: G1 and G2, G3, etc. are respectively 4-nitrophenyl α-D-glucopyranoside, α-maltoside, etc. Experimental conditions are described in the Experimental section.

to splitting an α-maltosaccharide linked to the primary hydroxyl group of another D-glucose unit, but is less restrictive, being able to hydrolyze an α-maltosaccharide linked to a noncarbohydrate component.

New substrates for isoamylase. — The observation that the major maltosaccharide released from D-[^{14}C]glucosylated SGP (Fig. 1) was the same as the largest maltosaccharide released by acid hydrolysis, *i.e.*, maltooctaose[10], was evidence that the enzyme was splitting the maltooctaosyl–tyrosine bond. To confirm this deduction, an experiment was devised based on the fact that we had been able to inhibit self-glucosylation by SGP with 4-nitrophenyl α-D-glucopyranoside and α-maltosaccharides[5]. The inhibition proved to be the result of the added saccharide acting as acceptor of transferred D-glucosyl residues. 4-Nitrophenyl α-maltoside was a much better inhibitor (acceptor) than maltose suggesting that the 4-nitrophenol group mimicks the tyrosine residue in glycogenin. Accordingly, this same group of saccharides was tested with isoamylase and results were obtained in line with our reasoning that hydrolytic removal of the 4-nitrophenol group might be observed, and in line with what we already know of isoamylase action on model saccharides. The enzyme hydrolyzed 4-nitrophenyl α-malto-trioside, -tetraoside, and -hexaoside by splitting the bond to 4-nitrophenol (Fig. 2). This was shown by the release of the aglycon (absorbance at 405 nm). Isoamylase did not hydrolyze the α-D-glucopyranoside or α-maltoside (Fig. 2), in line with the observation that α-maltotriose is the smallest saccharide that isoamylase will split from model saccharides[1,2].

The K_m values of isoamylase for these new substrates were not measured, but the rate at which the bond in 4-nitrophenyl maltotrioside is split, relative to the hexaoside and to the branch linkages of glycogen, was measured; the ratio is 1:3:454. The trioside and tetraoside were hydrolyzed at equal rates (Fig. 2). When the other debranching enzyme, pullulanase, was incubated with the 4-nitrophenyl saccharides, no hydrolysis occurred.

These observations permit the following conclusions: (a) The specificity of the isoamylase is directed primarily to the maltosaccharide that it splits from its attachment, and the attachment need not be a carbohydrate. Probably many other substrates can be devised for this enzyme, consisting of α-maltotriose or a larger maltosaccharide linked to the hydroxyl group of a suitable acceptor. (b) Substrates of defined structure have now been found, which make it possible to distinguish between isoamylase and pullulanase; previously glycogen was the only substrate degraded by isoamylase and not by pullulanase. Since experiments to characterize a debranching enzyme will usually be carried out in a crude system, where interfering enzymes may be present, it is advantageous to test for isoamylase with an oligosaccharide substrate instead of glycogen. (c) The new isoamylase substrates are commercially available as essentially pure entities. They are prepared by incubating cyclodextrin glucanotransferase (EC 2.4.1.19) with cyclodextrin and 4-nitrophenol. (d) The observations are also important for our knowledge of the structure and enzymology of glycogenin, as noted below.

Linkage of carbohydrate to glycogenin. — Previously, partial acid hydrolysates were used to determine the maximum length of maltosaccharide in self-glucosylated

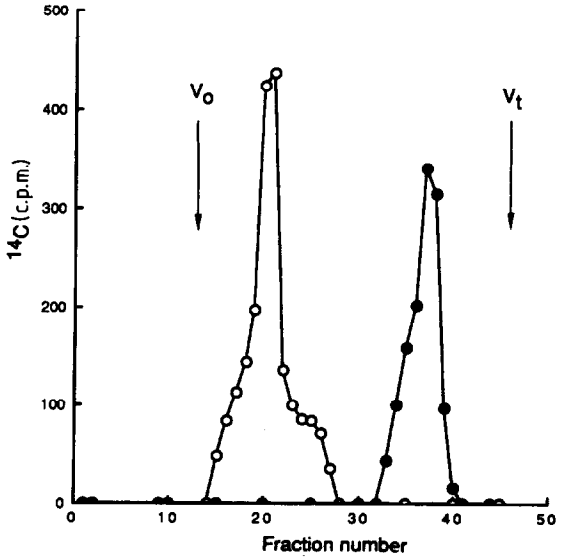

Fig. 3. Hydrolysis by isoamylase of the D-[^{14}C]glucosylated tryptic fragment of D-[^{14}C]glucosylated SGP: (-O-O-) Fractionation of the tryptic peptide on Bio-Gel P4. (-●-●-) After treatment of the ^{14}C-labeled tryptic peptide with isoamylase, the digest was again passed through Bio-Gel P4. Experimental conditions are described in the Experimental section.

SGP, since no method of selectively cleaving the maltosaccharide–tyrosine bond was available. Maltooctaose was found to be the longest maltosaccharide present in the hydrolysate[10]. Now, it is possible to specifically cleave the glucosyl–tyrosine bond in order to investigate the nature of the maltosaccharides attached to the tyrosine residue. The result was not a precise one, as maltooctaose was present together with an almost equal amount of maltoheptaose, some nonamer and decamer, and compounds having a smaller mol.wt. than that of the heptaose (Fig. 1). Self-glucosylation had been allowed to proceed to apparent completion and the experiment was repeated several times showing that the chain lengthening does not precisely cease at maltooctaose. A possible complicating factor could be a trace of α-D-glucosidase in the SGP, which is certainly present in crude preparations.

Another feature of the release of maltosaccharides from SGP was its incomplete extent. In several experiments, this did not proceed beyond 60%. ^{14}C-Labeled material that was precipitable with 10% trichloroacetic acid always remained on the starting line after t.l.c. of the maltosaccharide products (Fig. 1). Since SGP forms oligomers[4], incomplete hydrolysis might be the result of the steric hindrance afforded by the substrate molecules. Thus, labeled p37 was hydrolyzed with trypsin and the action of isoamylase on the product was tested. SGP was allowed to undergo self-glucosylation with UDP-D-[^{14}C]glucose and then treated with trypsin. A ^{14}C-labeled peptide was obtained and separated on Bio-Gel P4 (Fig. 3). This was treated with isoamylase and

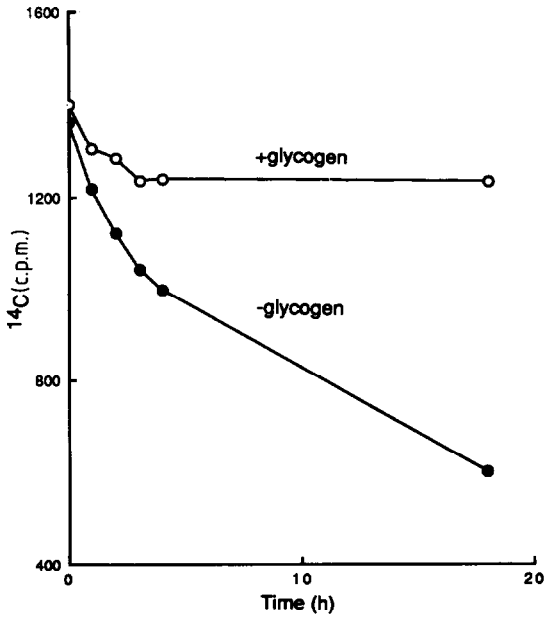

Fig. 4. Inhibition by glycogen of the hydrolysis of D-[^{14}C]glucosylated SGP by isoamylase. The control digest contained isoamylase and D-[^{14}C]glucosylated SGP. The digest labeled " + glycogen" contained, in addition, 1 mg/mL of muscle glycogen. The amounts of ^{14}C precipitable by 10% trichloroacetic acid were measured at various time intervals. Experimental conditions are described in the Experimental section.

refractionated again on the same sieve. The result (Fig. 3) demonstrated complete hydrolysis of the carbohydrate–tryptic bond. It was concluded that steric hindrance was the reason for the incomplete isoamylolysis of labeled SGP and that total hydrolysis of the maltosaccharide–tryosine bond occurs with the tryptic peptide.

Although the preparation of isoamylase was highly purified and the hydrolysis of the glycogen–glycogenin occurred optimally at the characteristic acidic pH optimum of 4.5 for isoamylase, an experiment was devised to demonstrate that it was the glycogen-debranching activity of the isoamylase preparation, and not an unrelated activity, that was involved in splitting the glycogen–tyrosine bond in D-[^{14}C]glucosylated SGP. This was shown by a competition experiment in which the addition of glycogen initially retarded, and then completely inhibited the release of ^{14}C from the SGP by isoamylase (Fig. 4).

The hydrolysis of the glucosyl–tyrosine bond is of importance for two reasons. First, based on the known specificity of isoamylase in hydrolyzing α-D-glucosidic bonds, we may conclude that it is an α-D-glucosyl residue that is linked to glycogenin. The anomeric configuration of this residue was the one missing piece of information preventing the full structural description of the carbohydrate–protein linkage region in SGP and glycogenin. Secondly, the hydrolysis of SGP by isoamylase allows the preparation of carbohydrate-free glycogenin (apo-glycogenin). A method of preparing this substance has been sought in order to use it as a substrate to try to learn how the first D-glucosyl residue or residues become attached to SGP. All samples of SGP and glycogenin isolated by ourselves and others have been found to contain carbohydrate[4,14]. It was established that when UDP-D-[^{14}]glucose was added after hydrolysis with isoamylase, autoglucosylation of apo-glycogenin did not occur.

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