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Note

An assay for the transferase activity of glycogen debranching enzyme

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Glycogen debranching enzyme (amylo-1,6-glucosidase/4-α-glucanotransferase, EC 3.2.1.33 + EC.4.1.25) removes the α-(1 → 6) branch points in glycogen phosphorylase limit dextrin by a two step mechanism involving two separate activities. These two activities are found on a single polypeptide chain [1]. The first step is that of a transferase which transfers a maltotriose unit from the four sugar branch to the "main chain" resulting in the elongation of the main chain, with a glucose moiety left attached to it via an α-(1 → 6) linkage. This single D-glucose unit is then hydrolyzed by the glucosidase activity.

Mechanistic studies on this enzyme have been hampered by the absence of convenient, separate assays for each activity. Those previously available have included incorporation of radiolabeled D-glucose into glycogen [2] for the glucosidase activity; complexation of amylopectin with iodine [3], and disproportionation of oligosaccharides [4] for the transferase activity. In recent studies, it has been shown that α-D-glucopyranosyl fluoride (αGF) can be utilized as a substrate for the glucosidase activity [5] and the reaction can be followed simply by monitoring the release of fluoride ion with an ion-selective electrode [6]. No such simple, continuous assay exists for the transferase activity.

The normal reaction catalyzed by the transferase involves the transfer of a maltotriosyl unit from the branch to the main chain, with net retention of anomeric configuration. In view of the success of the glucosidase assay using αGF, and given the ability of other polysaccharide-degrading enzymes such as α-amylase to utilize malto-oligosac-

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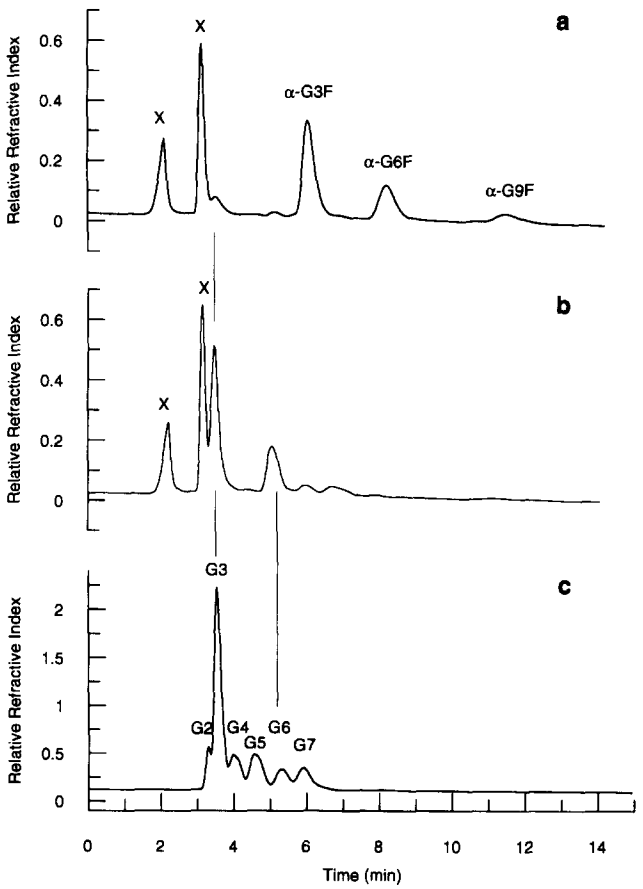

Fig. 1. (a) LC profile of the products of the reaction between αG3F and glycogen debranching enzyme. Chromatographic conditions are provided in the Experimental section (b) LC profile of the hydrolyzed mixture of (a). (c) LC profile of the maltooligosaccharide standards G2–G7. The number on top of the peak indicates the degree of polymerization. In (a) and (b) the peaks marked X at 2 and 3 min are due to NaPi buffer and glycerol (from centricon membrane), respectively.

charyl fluorides as substrates [7], it seemed reasonable to try α-maltotriosyl fluoride (αG3F) as a substrate for glycogen debranching enzyme. Recent developments in the synthesis of α-glycosyl fluorides made this more feasible [8].

αG3F was therefore synthesized essentially according to Jünnemann et al. [8] and tested as a substrate for the debranching enzyme, using the fluoride electrode to monitor reaction. Turnover was indeed observed. In order to confirm that reaction is proceeding via transfer, and not via hydrolysis, and thereby allay any concerns that reaction may be occurring at the glucosidase site rather than the transferase site, the identity of the reaction product was investigated. Results of these experiments can be seen in Fig. 1. The LC trace of the reaction products is shown in Fig. 1(a). Comparison with standard malto-oligosaccharides, Fig. 1(c), reveals that only very small amounts of maltotriose

Table 1
Kinetic parameters for α-maltotriosyl fluoride with glycogen debranching enzyme at various concentrations of glycogen

Glycogen (%)	k_{cat} (min^{-1})	K_m (mM)	k_{cat}/K_m (min^{-1} mM^{-1})
0	31 ± 4	42 ± 10	0.7
0.1	27 ± 1	11 ± 1	2.4
1.0	44 ± 1	11 ± 1	4.0
9.8	80 ± 4	15 ± 2	5.3

(G3) were produced. However, two new peaks of longer retention time, corresponding to longer oligosaccharides, were observed. These presumably correspond to α-maltohexaosyl fluoride (αG6F) (8.5 min) and α-maltononaosyl fluoride (αG9F) (11 min).

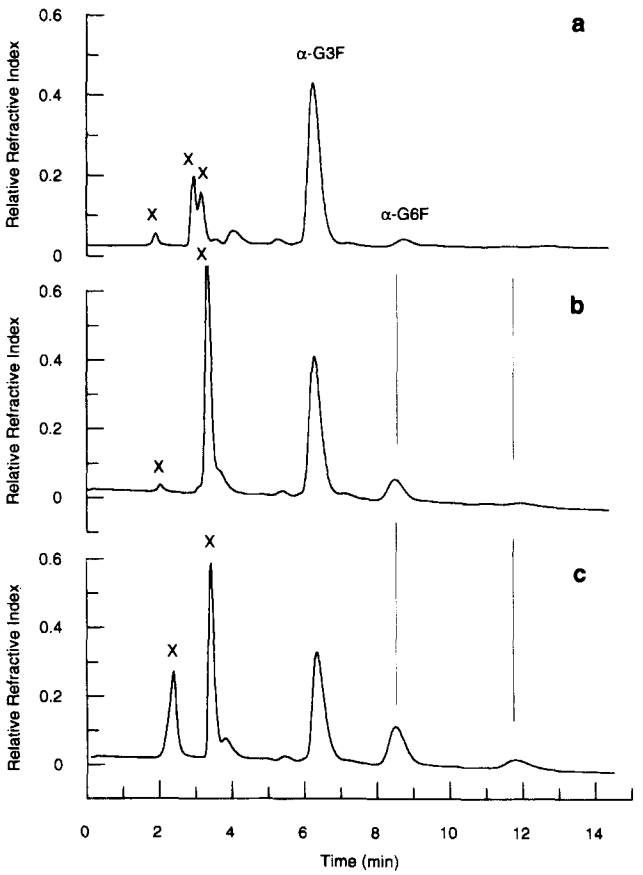

Fig. 2. LC profiles of the products of the reaction between αG3F and glycogen debranching enzyme in the presence of the different glycogen concentrations: (a) 3%; (b) 0.2%; (c) 0%. The peaks marked X at 2 and 3 min are due to NaPi buffer and glycerol (from centricon membrane), respectively. The additional peak in (a) also marked X is due to glucose from the glycogen preparation.

Since no standards of these fluorides were available, the reaction mixture was boiled to hydrolyze the fluorides to the free maltooligosaccharides for which there are standards. The LC profile of this hydrolysis mixture is shown in Fig. 1(b) and clearly reveals the formation of G3 (from the excess αG3F) and maltohexaose (5.2 min), plus a peak likely corresponding to maltononaose. Reaction is therefore clearly occurring at the transferase site.

Rates of reaction were found to increase with substrate concentration. The maximum concentration of αG3F attained (67 mM) was only two times the estimated K_m, thus resulting in large errors (Table 1). However, the value of k_{cat}/K_m (0.7 min^{-1} mM^{-1}) was consistent with the result obtained from the slope of the initial linear portion of the plot. This result was substantially lower than that of $k_{cat}/K_m = 7.4$ min^{-1} mM^{-1} observed for maltopentaose [4]. The effects of inclusion of glycogen into the reaction mixture were investigated to see if this could improve the binding of αG3F. Table 1 shows the Michaelis–Menten parameters determined for reaction in the presence of different concentrations of glycogen. As can be seen, K_m values are lowered upon addition of glycogen, and k_{cat}/K_m values raised as glycogen concentrations increase. There are two possible explanations for this behaviour. One is that the binding of glycogen results in conformational changes which improve the affinity of the enzyme for the αG3F substrate. The other is that glycogen acts as a more effective acceptor of the maltotriosyl moiety than does αG3F itself. This second explanation can be verified by LC product analysis. In the former case, αG6F would be the major product, while in the latter, transfer would occur to glycogen, resulting in elongation of the polymer. The results can be seen in Fig. 2. In the absence of glycogen [Fig. 2(c)], relatively large amounts of αG6F are formed, but as the glycogen concentrations increased to 0.2% [Fig. 2(b)] and 3% [Fig. 2(a)] the amount of αG6F formed decreased substantially despite the fact that 30% of the total fluoride release had occurred, suggesting that the substrate was being transglycosylated onto glycogen. These results confirm that glycogen at higher concentrations is a better acceptor than αG3F.

In conclusion, the use of αG3F in the presence of 0.1–1.0% glycogen provides a convenient, specific assay for the transferase activity of glycogen debranching enzyme. Use of this in conjunction with αGF for the glucosidase activity allows specific, independent monitoring of the two separate activities.

1. Experimental

All chemicals were of analytical reagent grade. Distilled water, purified with a Milli-Q II system, was used for all aqueous solutions. Sodium phosphate (NaPi) and ethylenediaminetetraacetic acid (EDTA) were purchased from Sigma Chemical Co. Maltooligosaccharides were purchased from Boehringer Mannheim. Rabbit liver glycogen (type III) was purchased from Sigma Chemical Co. and was purified with AG-1 × 8 (200–400 mesh, Cl^- form) ion-exchange resin. Glycogen was assayed by the method of Dische [9].

Enzyme. — Rabbit muscle glycogen debranching enzyme was purified as previously described [10].

Substrate. — αG3F was synthesized according to published procedures [8]. The product was analytically pure and the NMR assignments agreed with those made by Jünnemann et al. [8].

Enzyme assay. — To several different concentrations of αG3F (100 mM NaPi, 1 mM EDTA pH 6.0 buffer) was added 5 μL of debranching enzyme (18.3 mg mL^{-1}) to make the final volume 250 μL. The reaction was monitored at 30°C. Release of fluoride ion was followed for ~ 2–5 min up to a maximum of 10% substrate depletion.

Product distribution. — To 200 μL αG3F (8.1 mM) and 40 μL NaPi buffer (50 mM, pH 6.0) was added 10 μL glycogen debranching enzyme (18.3 mg mL^{-1}). The reaction was followed until 30% of the total fluoride ion concentration had been released. The mixture was then filtered at 4°C through a Centricon 10,000 molecular weight cutoff membrane to remove enzyme; 50 μL of the filtrate was analyzed by LC; 100 μL of the filtrate was boiled for 30 min and then 50 μL was analyzed by LC. A standard which contained maltooligosaccharides G2–G7 was used for identifying products.

To 200 μL αG3F (8.1 mM) and 40 μL glycogen (either 1.3% or 19.6% in NaPi buffer) was added 10 μL glycogen debranching enzyme (18.3 mg mL^{-1}). The reactions with glycogen were performed as above.

HPLC system. — The LC system consisted of a Waters high-performance liquid chromatograph equipped with a WISP autosampler, and a Waters refractive index 410 refractometer. A Dextropak column from Waters was used (8 × 100 mm, particle size 4 μm). Water was used as a mobile phase at a flow rate of 1.0 mL min^{-1}. The column was run at ambient temperature (18–25°C).

Assay system. — An Orion combination fluoride ion-selective electrode and a Fisher 9500 pH meter interfaced with a computer were used. The electrode was standardized before use. The nonlinear regression analysis computer program Grafit™ was used for the fitting of all data [11].

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