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Properties of carbohydrate-free recombinant glycogenin expressed in an *Escherichia coli* mutant lacking UDP-glucose pyrophosphorylase activity

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Abstract Glycogenin, the self-glucosylating primer for glycogen synthesis, is expressed in wild-type *E. coli* as a recombinant protein in an already partly glucosylated form, owing to the presence of its substrate, UDP-glucose. By using an *E. coli* mutant strain lacking in UDP-glucose pyrophosphorylase activity, we have succeeded in expressing carbohydrate-free glycogenin (apo-glycogenin) in good yield. When provided with UDPxylose, it autocatalytically adds 1 xylose residue. With UDP-glucose, an average of 8 glucose residues are added. However, release of the self-synthesized maltosaccharide chains with isoamylase reveals them to be a mixture. Chains as long as 11 glucose residues (maltoundecaose) are present. The ability of recombinant apo-glycogenin to self-glucosylate is further proof that a separate enzyme is not needed for the addition of the first glucose residue to Tyr-194 of the protein.

Key words: Glycogenin; Apo-glycogenin; Carbohydrate-free glycogenin

1. Introduction

Glycogenin is the self-glucosylating protein that primes glycogen synthesis and remains as a covalently bound part of the glycogen molecule (for reviews see [1,2]). It does not occur in muscle in the free state and is obtained, enzymically active, by degradation of the glycogen. The product still carries tyrosine-bound maltosaccharide, representing the originating chain (C-chain) of the glycogen molecule. When the recombinant enzyme is expressed in *E. coli*, the enzyme also carries a maltosaccharide chain [3,4]. This is because *E. coli* contains the glycogenin substrate, UDP-glucose, synthesized by UDP-glucose pyrophosphorylase. The autocatalysis includes the ability to glucosylate Tyr-194, as well as to add the next several 1,4-linked α-glucose units that constitute the maltosaccharide primer chain for further elongation by glycogen synthase [5]. Therefore, once expressed in the *E. coli* cell, the glycogenin proceeds to glucosylate itself.

In order to study the properties of glycogenin further, including X-ray crystallographic analysis, there is a need to obtain the enzyme in a carbohydrate-free state (apo-glycogenin). Until now, the only available procedure made use of the ability of the glycogen debranching enzyme, isoamylase, to split the linkage between the glycogen C-chain and Tyr-194 [5,6]. This procedure is unsatisfactory because the wide difference in pH optima between isoamylase and glycogenin (over 3 pH units) results in the partial inactivation of the latter. Also, isoamylase will only remove the C-chain if it is at least 3 glucose units in length.

We have overcome this problem by employing an expression system for rabbit muscle glycogenin in an *E. coli* strain that lacks UDP-glucose pyrophosphorylase activity, and have studied some of the properties of the carbohydrate-free product, which is synthesized in good yield in a fully active form.

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2. Materials and methods

2.1. Materials

E. coli CGSC 4954 was obtained from the *E. coli* Genetic Stock Center, Department of Biology, Yale University, PO Box 6666, New Haven, CT 06511–8155, USA. It is a galactose-minus strain lacking UDP-glucose pyrophosphorylase activity, the assignment of its lesion being galU106. Isoamylase was a gift from Hayashibara Biochemical Laboratories, Hiroshima, Japan. Glucose oxidase, peroxidase and crystalline pancreatic trypstin (180 μg/mg) were obtained from Boehringer-Mannheim. Radioactive UDP-glucose and UDP-xylose were purchased from American Radiolabeled Chemicals. Sep-Pak C_{18} cartridges were from Millipore. Bio-Rex RG 501-X8 resin was a Bio-Rad product. Costar microtitration plates were used. Except as noted, all other reagents came from Sigma.

2.2. Preparation of E. coli CGSC 4954 for expression of glycogenin cloned into a pET vector

The cloning of rabbit muscle glycogenin cDNA and its subcloning into a pET-11d expression vector are reported elsewhere [4,5]. A lysogenization kit (Novagen) for site-specific integration of a T7 RNA polymerase gene-containing prophage, λDE3, into the *E. coli* CGSC 4954 chromosome was used to generate a lysogenized host capable of expressing target genes cloned in pET vectors under the control of the T7 promoter. Lysogenization and verification of CGSC 4954-λDE3 lysogens were done according to the manufacturer's guidelines. The lysogenic bacterial clone chosen for expression was designated CGSC 4954-1.

2.3. Expression and purification of apo-glycogenin

The recombinant pET-11d vector containing the wild-type glycogenin cDNA, pET-R [4], was transformed into *E. coli* CGSC 4954-1. Cultures of transformed bacteria were started-up by inoculation at high dilution of growth medium (12 g of tryptone, 24 g of yeast, 10 g of NaCl, 0.25 g of Tris-base and 4 ml of glycerol per 1 of water) supplemented with ampicillin at 200 μg/ml and grown at 27°C for 12 h. The temperature was then raised to 37°C. When the culture reached an optical density of 0.6 (1 cm), induction was started by addition of isopropyl β-D-thiogalactopyranoside to 1 mM. Fermentation was allowed to proceed for 3 h before the cells were harvested by centrifugation (4000 rpm for 15 min). The same two-step purification procedure reported for the wild-type, Phe-194 and Thr-194 mutant forms of glycogenin was used to obtain homogeneous protein [5].

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2.4. Immunoblotting

The procedure used by Lomako et al. [7] was followed, using a polyclonal antibody generated with native rabbit-muscle glycogenin as antigen.

2.5. Self-glucosylation reactions

The self-glucosylation reaction mixtures (100 μl each) contained 50 mM Tris-HCl, pH 7.4, 5 mM Mn^{2+}, and 20 μM UDP[^{14}C]glucose or UDP[^{14}C]xylose. When the glucose donor was used, the reaction was incubated at room temperature for 2 h before TCA precipitation and counting (see [7]). Reactions with the xylose donor were incubated for 2 h, 4 h and overnight at room temperature before TCA precipitation and counting. The results reported for xylose incorporation correspond to overnight incubations. Initial reaction rates were obtained with 20 μM radiolabeled UDP-glucose after 5 min.

2.6. Glucose determination

A highly sensitive glucose oxidase assay [8] capable of detecting amounts of glucose down to 20 ng was used to determine whether glycogenin expressed in the UDP-glucose pyrophosphorylase-deficient *E. coli* strain was glucosylated. If the protein contained a minimum of one glucose unit per molecule, 100 μg would yield 0.5 μg of glucose, an amount well within the range of the assay, and detectable after acid hydrolysis (Table 1). For this reason, 100 μg of purified protein, ± 0.5 μg of added glucose, along with 100 μg of Phe-194 glycogenin mutant (negative control) were subjected to acid hydrolysis in a PICO-TAG Work Station (Waters) and subsequent glucose determination. Galactose (50 μg) was added to each protein sample to minimize the loss of glucose in hot acid. After addition of the galactose, the solutions were dried in Kimax hydrolysis tubes under speed vacuum in a concentrator (Savant), and 2 M trifluoroacetic acid (200 μl) was pippeted into the bottom of a reaction vial where the tubes were placed. In order partially to evaporate the acid, the capped reaction vial was installed into the PICO-TAG Work Station and a vacuum (1–2 Torr) was applied. The vacuum valve was then closed and the vial placed in an oven at 100°C for 4 h. The residues in the hydrolysis tubes were vacuum dried, resuspended in water (20 μl) and transferred to microtitration plates for the glucose determination assay. Water was pippeted into a separate row of wells to serve as a reagent blank. Stock solutions containing 0.5 μg and 1 μg glucose/20 μl were used as standards. The glucose oxidase reagent (100 μl) consisting of 0.1 M citrate-sodium citrate buffer, pH 6.0, containing 15 U of glucose oxidase/ml, 2.5 U of peroxidase/ml, 0.05% bovine serum albumin and 0.07% 3,3′, 5,5′-tetramethylbenzidine, was added to each well, and the plate stored at 37°C for 10 min. After adding 100 μl of 0.9 M H_2SO_4 to each well, the color produced was analyzed at 450 nm in a microplate reader (SLT-Labinstruments).

2.7. Debranching of fully [^{14}C]glucosylated apo-glycogenin and analysis of the released maltosaccharide chains

Approximately 90 pmol of purified protein were allowed to self-radioglucosylate for 2 h at room temperature. The labeled protein was then separated from the other constituents of the reaction mixture in an 8 ml Sephadex G25 column, in 25 mM ammonium bicarbonate, pH 8.0, 2 mM CHAPS. The label emerged in two 400 μl fractions which were lyophilyzed, resuspended in water (80 μl) and subjected to trypsinolysis with enzyme (0.165 μg) in 0.5 M ammonium bicarbonate pH 8.5 (20 μl) at 37°C for 4 h, when 0.1 M phenylmethylsulfonyl fluoride in propane-2-ol (1 μl) was added. The 100 μl digest was then taken to 200 μl with the addition of sodium acetate buffer, pH 4.5, to a final acetate concentration of 0.1 M, plus 180 U of isoamylase. After an overnight incubation at room temperature, the released ^{14}C-labeled oligosaccharides were desalted and purified by passage through a mixed-bed resin column (0.5 ml, Bio-Rex RG501-X8) connected to a Sep-Pak C_{18} reverse-phase cartridge prewetted with acetonitrile. Elution was accomplished with 5 ml of water in 0.5 ml fractions in which the ^{14}C-lebeled oligosaccharides were detected by scintillation counting and combined. After lyophylization, the radiolabeled material was dissolved in 30 μl of water, of which 10 μl were chromatographed on Whatman 3MM paper in butanol:pyridine:water (6:4:3, by vol.) A mixture of glucose, maltose, maltotriose, maltotetraose, maltopentaose and maltoheptaose was used for reference. After eight solvent ascents, the chomatogram was dried and exposed to Kodak X-OMAT AR film for 1 week. Standards were revealed by alkaline silver nitrate [9] (see Fig. 3).

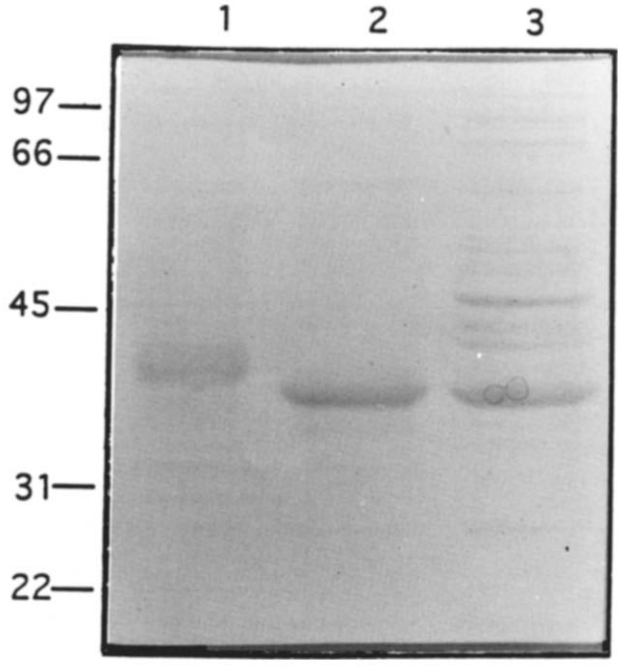

Fig. 1. SDS-PAGE analysis of glycogenins expressed in BL21/DE3 and CGSC 4954 *E. coli* hosts, with proteins revealed by staining with Coomassie blue. Lane 1, wild-type glycogenin expressed in the BL21/DE3 UDP-glucose-synthesizing host. Lane 2, Phe-194 mutant glycogenin expressed in the same host. Lane 3, wild-type glycogenin expressed in the CGSC 4954, UDP-glucose-deficient host.

3. Results and discussion

3.1. Preparation and properties of apo-glycogenin

The strain of *E. coli* used as the host for the expression of glycogenin is one known to be lacking in UDP-glucose pyrophosphorylase activity [10]. This does not affect glycogen synthesis because the nucleoside diphosphate precursor for *E. coli* glycogen is ADP-glucose [11]. Nor is glycogenin known to be the primer for *E. coli* glycogen synthesis.

We used the same expression system as for the synthesis of the recombinant wild-type, Phe-194 and Thr-194 mutant glycogenins [4,5]. There the level of expression was excellent and a two-step, 2.5-fold purification process is sufficient to obtain homogeneous products in overall yields from 25% (Phe-194) to 49% (wild-type). The same procedure works equally well for the recombinant protein from the *E. coli* CGSC 4954-1 strain. We did not quantitate the recovery of apo-glycogenin (the yield of pure protein is about 4 mg/100 ml of medium) but the Coomassie blue stain of the lysate after SDS-PAGE (Fig. 1, lane 3) reveals that a protein band in the expected position for carbohydrate-free glycogenin was the most prominent component, comparable in amount to the wild-type enzyme [4,5]. We can refer to the expected position because, for comparison, the wild-type (Fig. 1, lane 1) and Phe-194 mutant (Fig. 1, lane 2) recombinant proteins were also subjected to SDS-PAGE. The wild-type enzyme, already carrying carbohydrate which would increase its mass by approx. 1 kDa, is seen to migrate noticeably slower than the presumed carbohydrate-free Phe-194 protein, which cannot glucosylate itself [4,5,12]. The major protein band in the *E. coli* CGSC 4954-1 lysate migrates with the

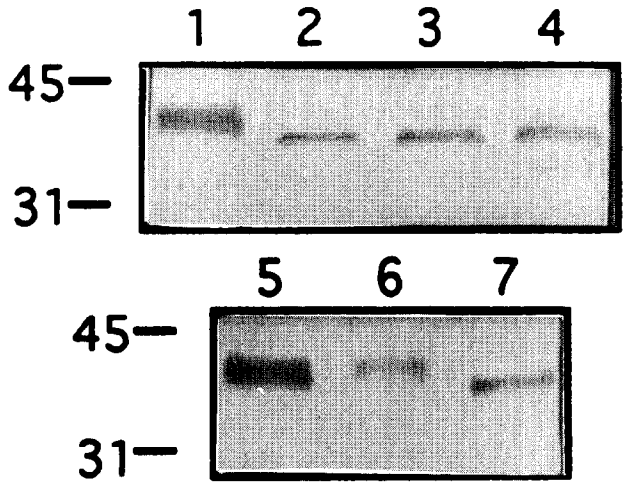

Fig. 2. Immunoblotting of recombinant glycogenins after SDS-PAGE. 1, wild-type; 2, 7 apo-glycogenin before self-glucosylation; 3, Phe-194 mutant protein; 4, Thr-194 mutant protein; 5, wild-type; 6, apo-glycogenin after self-glucosylation. The numbers at the left correspond to the positions occupied by standards with masses of 45 and 31 kDa.

Phe-194 mutant protein (Fig. 1, lanes 2,3). When purified to homogeneity and examined by immunoblotting with the same reference proteins, the differences in M_r were maintained (Fig. 2). The wild-type enzyme (Fig. 2, lanes 1,5) is seen to have a higher M_r than apo-glycogenin (Fig. 2, lane 2) or the Phe-194 (Fig. 2, lane 3) and Thr-194 (Fig. 2, lane 4) mutant proteins [4,5]. Also the wild-type band is more diffuse because it is only incompletely glucosylated (see below) and there is heterogeneity between the maltosaccharide chains. This is noted also in the lower panel where the apo-glycogenin is seen to increase in M_r after self-glucosylation (Fig. 2, lanes 6 vs. 7) and the more fully glucosylated enzyme gives a sharper band than does the incompletely glucosylated wild-type enzyme.

We devised a microchemical analytical procedure to carry out what we believe to be the first quantitative carbohydrate analysis of enzymically active glycogenin. This is based on a glucose oxidase assay capable of the accurate determination of submicrogram amounts of glucose [8]. Hydrolysis of the protein was carried out with 2 M trifluoroacetic acid vapor at 100°C for 4 h, followed by enzymic determination of any glucose set free. Conditions were established for accurate assessment by hydrolysing bovine serum albumin, maltopentaose and glucose in test mixtures, with galactose added to all samples to diminish the destruction of glucose by acid (which was compensated for by putting glucose standards through the same procedure). The hydrolysis conditions were judged sufficient to hydrolyse bound glucose by checking that they caused complete hydrolysis of maltopentaose.

Trial experiments revealed that the same amount of glucose hydrolyzed in presence of different proteins gave rise to different absorbancies in the glucose oxidase assay. We decided to use as the reference the Phe-194 mutant protein corresponding to rabbit-muscle glycogenin [4,5]. It cannot glucosylate itself and carbohydrate is not known to be linked anywhere else in the protein. In Table 1 we show the actual $A_{450\,nm}$ in the ELISA reader, corresponding to 100 μg of Phe-194 enzyme vs. 100 μg of apo-glycogenin. (The absorbances, relative to water, were negative.) The slight difference in absorbance between the two protein samples corresponded to a glucose content in the apo-glycogenin of 0.06 molecular proportions. Clearly, within the limits of experimental error, and assuming that the Phe-194 mutant protein was carbohydrate-free, the apo-glycogenin was also carbohydrate-free. Therefore we now have the capability, for the first time, to synthesize carbohydrate-free glycogenin in quantity.

The homogeneous, carbohydrate-free glycogenin underwent self-glucosylation in presence of UDP-glucose and Mn^{2+}. This was a direct demonstration that the addition of the first glucose residue, to Tyr-194, is autocatalytic, presumably by the same active center that adds a further seven glucose-to-glucose residues in α-1,4-linkage (cf. [5]). It is because the chemistry of the first glucosidic bond to glycogenin is different from the others that there has been hesitancy in assuming that its formation would also be autocatalytic, but the probability of this being the case was heightened when we found that isoamylase, which hydrolyses the interglucosidic branch points of glycogen, also hydrolyses the glycogen–tyrosine bond [6]. It was with the use of isoamylase that we recently generated carbohydrate-free glycogenin and demonstrated its autocatalytic capability of reglucosylating itself [5]. But, as noted above, this is not a satisfactory method to obtain a fully active, homogeneous carbohydrate-free product.

3.2. *Stoichiometry of self-glycosylation by apo-glycogenin*

The apo-glycogenin readily underwent self-glucosylation and incorporated 7.67 ± 0.098 (3 measurements) molecular proportions of glucose. This is close to the expected 8, corresponding to malto-octaose, which we earlier identified as the longest saccharide released by mild acid hydrolysis of native muscle glycogenin [6,13]. With the same enzyme preparation, we had also shown that UDP-xylose, an alternative substrate [14,15], can be added only once, that is, neither UDP-glucose nor UDP-xylose can be used to donate to a maltosaccharide chain already capped by xylose [16]. Apo-glycogenin proceeded to add 0.95 ± 0.006 molecular proportions of xylose (3 observations). This was, of course, the exactly predictable result but could not have been relied on in advance because the previous observations of xylose addition had been with glycogenin already carrying maltosaccharide chains. Here we were observing the addition of xylose, not to glucose, but to Tyr-194.

Another aspect of the ability to glucosylate Tyr-194 directly

Table 1
Analysis of recombinant glycogenins for glucose content

	Δ absorbance at 450 nm
1. Apo-glycogenin (100 μg)	−0.115 ± 0.027 (4)
2. Apo-glycogenin (100 μg) + free glucose (0.5 μg)	0.103 ± 0.005 (2)
3. Phe-194 mutant protein (100 μg)	−0.128 ± 0.017 (4)
4. Free glucose (0.5 μg) alone	0.326 ± 0.012 (2)

Apo-glycogenin (± added glucose) and the Phe-194 mutant glycogenin were analyzed for their possible content of bound glucose, released by acid hydrolysis. Figures in parentheses are the numbers of measurements. The glucose determination method [8] employed a mixture of glucose oxidase and peroxidase, with 3,3′, 5,5′-tetramenthylbenzidine as chromophore (see section 2.6). Samples 1–3 were subjected to acid hydrolysis. Sample 4 was an untreated glucose standard. Relative to water, the protein samples (nos. 1,3) gave negative readings. The absorbance difference between samples 2 and 1 (0.218), corresponding to the inclusion of 0.5 μg of glucose in sample 2, v. 0.326 for the glucose standard, reflects the loss of glucose caused by the hot acid treatment.

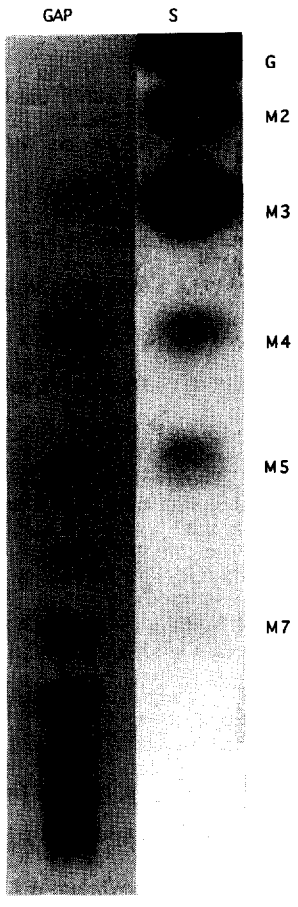

Fig. 3. Radioautograph of maltosaccharides released from apo-glycogenin after self-glucosylation with UDP[^{14}C]glucose, followed by trypsinolysis, hydrolysis with isoamylase, and separation by ascending paper chromatography. Details are in section 2. Glucose (G) and maltosaccharide (M2 = maltose, etc.) standards (silver nitrate strain) are on the right(S) and isoamylase-treated glucosylated apo-glycogenin on the left (GAP).

was that of determining the rate of addition of the first glucose residue. This might occur at a rate quite different from that of subsequent glucoses. We made the comparison between already glucosylated wild-type recombinant glycogenin and apo-glycogenin and observed that the latter self-glucosylated at a rate of 0.45 mol of glucose/mol of enzyme/min. The rate for the already partly glucosylated wild-type enzyme was 0.17 mol/mol/min and it took up, in total, 3.6 mol of glucose/mole of enzyme vs. 7.7 for apo-glycogenin. Therefore the rate of addition of the first glucose residue to glycogenin is not limiting to the overall rate of glucosylation.

3.3. *Upper limit of length of the maltosaccharide chain of glycogenin*

Taken together, the self-addition of approximately eight glucose residues or one xylose residue to homogeneous apo-glycogenin further confirms that the addition of the first sugar residue to Tyr-194 does not require the action of a separate enzyme, a possibility that was once considered [2]. The glucose-to-tyrosine transferring activity is evidently contained within glycogenin and is most likely the same active center that carries out glucose-to-glucose transfer.

We proceeded to examine the lengths of the maltosaccharide chains of fully ^{14}C-glucosylated apo-glycogenin, using isoamylase to split the bonds to tyrosine [5,6]. The products were separated by paper chromatography and a radioautograph prepared (Fig. 3). It becomes clear from this experiment, what had previously been suspected when native glycogenin was similarly treated, that 8 glucose residues is not a fixed and upper limit to the self-growth of the primer chain. In isoamylase-treated native glycogenin we had seen maltosaccharides with 4–10 glucose residues, 7 and 8 predominating [6]. Some maltopentaose was also present. In the present case we see 7–11 glucose residues as the major products, in about equal amounts. We should note that the qualitative comparison of spot intensities as a means of judging relative amounts will be more valid in this experiment because, since we were using apo-glycogenin, all glucose residues were radiolabelled. Previously, using already glucosylated native glycogenin, the amount of ^{14}C introduced into the chains was dependent on their initial and, probably, variable length.

We may conclude that glycogenin has the capability of lengthening its maltosaccharide chain to 11 (or even higher) rather than the 8 glucose residues previously supposed [6,12], but that elongation sometimes does not proceed to completion. Connected with this would be whether the chain elongation is multichain or single chain, a possibility beyond the scope of this report. A further relevant factor is whether the self-glucosylation is intra- or inter-molecular. Other workers have concluded that the reaction is intramolecular, based on first-order kinetics when the enzyme concentration is varied [11,17]; but Cohen et al. [18] reported the muscle enzyme to be a dimer, which renders questionable the evidence from kinetics. We have also reported that glycogenin is oligomeric [7] and, since glycogenin can transglucosylate other acceptor substrates, have suggested that the oligomerization may have a purpose, that is, to facilitate intermolecular transglucosylation [19]. More recently, we have demonstrated that the supposed first-order kinetics break down at high dilutions of glycogenin [4]. The rate of decay with dilution is suggestive of the dissociation of an interactive complex. What the variability of maltosaccharide chain length in Fig. 3 may represent is the result of association–dissociation of glycogenin monomers. Dissociation when, say, the chain had reached 8 glucose units would release monomeric glycogenin which might well have difficulty in re-uniting with another monomer, for further glycosylation.

Another consideration is the presence, after isoamylase treatment, of minor amounts of smaller oligosaccharides, even a trace of glucose (Fig. 3). Isoamylase does not release glucose or maltose, at least when in 1,6-glucosidic linkage. Were these minor products the result of an amylase contaminant? The isoamylase preparation was amylase-free [6]. The apo-glycogenin, although purified, might be accompanied by a trace of hydrolase from the host *E. coli*. Another possibility is related to our report that glycogenin hydrolyses UDP-glucose to glucose at a rate significant compared with that at which it self-glucosylates [4]. It might hydrolyse its own primer chain. Yet another possibility is that the pancreatic trypsin used to hydrolyse the glycogenin before isoamylase treatment was contaminated with α-amylase. (The trypsin treatment is necessary in order to facilitate isoamylase action [6].) Whatever the reason for the traces of glucose and small saccharides, the main conclusions still stand.

We have noted that when recombinant wild-type or apo-glycogenin is allowed to self-glucosylate with UDP[^{14}C]glucose, the reaction does not reach a plateau. Instead the amount of protein-bound ^{14}C begins slowly to decrease, suggesting hydrolysis of the maltosaccharide chain (results not shown). In the presence of excess UDP-glucose this would not be expected because the enzyme should reglucosylate itself, but, eventually, the concomitant hydrolysis of the glucose donor substrate will defeat the ability of the enzyme to reglucosylate itself.

Aspects such as this, and the reason for the range of saccharides seen after isoamylase action, must wait on attempts to learn whether the glycosidase and protease activities are inherent within the recombinant enzymes or are the results of contaminants.

In conclusion, recombinant apo-glycogenin can be expressed in good yield in *E. coli* in a readily purifiable, fully active condition. The means are now open, which we are pursuing, to crystallize and study the three-dimensional structure of the apo-enzyme, as well as to determine the locations of the important functional sites. A means of doing this has been presented by the so-far unpublished observation that partial trypsinolysis of glycogenin gives rise to still-active degradation products that self-glucosylate but which have a mass only half that of the native enzyme. Truncated proteins will be expressed when structural information from the tryptic fragments is to hand.

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