

Tyrosine-194 of glycogenin undergoes autocatalytic glucosylation but is not essential for catalytic function and activity

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Received 17 February 1994

Abstract

Glycogenin is the protein primer for glycogen synthesis. By autocatalytic transglucosylation from UDPglucose, it creates a malto-octaose chain attached to its Tyr-194. It has been uncertain whether the autocatalysis includes the addition of the first glucose residue to Tyr-194. We now show this to be the case. However, we also demonstrate, contrary to a claim by others, that Tyr-194 is not necessary for the catalytic function and activity of glycogenin.

Key words: Glycogenin; Transglucosylation; *n*-Dodecyl β -maltoside

1. Introduction

Glycogenin is the autocatalytic protein that primes glycogen synthesis [1,2]. Transglucosylation from UDPglucose creates a malto-octaose priming chain linked to Tyr-194 of the protein [3,4]. Here we report on the role of Tyr-194 in the autocatalytic function of the enzyme. The first glucose residue of the malto-octaose is bound to the tyrosine. The second and subsequent glucose residues are bound to glucose. While it was clear that the formation of the glucose–glucose bonds was autocatalytic [1,2], it was not clear whether this also applied to the glucosylation of the tyrosine or whether a separate transglucosylase was involved. Here we demonstrate that the homogenous recombinant protein does glucosylate Tyr-194. The question may also be asked, and has been asked by Cao et al. [5], whether Tyr-194 is necessary for the catalytic function of glycogenin. To probe this question they studied the behavior of Tyr→Phe-194 and Tyr→Thr-194 mutant recombinant proteins. They found both to be incapable of self-glucosylation, nor would the Phe-194 mutant protein glucosylate a 30-residue synthetic peptide corresponding to the sequence of glycogenin around Tyr-194. It was concluded that ‘tyrosine-194 is essential for function (and) activity’.

Here we report a contrary finding. We have engineered the same Phe-194 and Thr-194 mutants. Both are

catalytically active and both can glucosylate an alternative acceptor. Tyr-194 is therefore not essential for function and activity.

2. Materials and methods

2.1. Materials

All biochemicals were from Sigma or Fischer Scientific Co. unless otherwise stated. UDP[¹⁴C]glucose was from NEN Products. Crystalline isoamylase was a gift from Hayashibara Biochemical Laboratories, Hiroshima, Japan. Reagents for SDS-PAGE were from Bio-Rad. Biochromic acid for protein determination with bovine serum albumin as standard was obtained from Pierce. The Lambda Zap II rabbit muscle cDNA library was obtained from Stratagene. The Genius system (Boehringer-Mannheim) was used for chemiluminescent detection during screening of the cDNA library. Sep-Pak C₁₈ cartridges were from Millipore.

2.2. Assays for glycogenin

In the experiments with isoamylase, to determine the manner in which the first glucose residue is added to glycogenin, the degree of [¹⁴C]glucosylation of glycogenin was determined by allowing the protein to self-glucosylate at room temperature in the presence of UDP[¹⁴C]glucose and Mn²⁺, precipitating in 10% trichloroacetic acid after 20 min and counting the ¹⁴C radioactivity in a scintillation counter [1]. Using this assay it was determined that the Phe-194 and Thr-194 mutants were unable to self-glucosylate to any detectable degree.

When testing the Phe-194 and Thr-194 mutants for their ability to use an alternative glucose acceptor, in comparison with the wild-type protein, we employed *n*-dodecyl β -maltoside (DBM) as the acceptor using the glycogenin assay method developed by Manzella et al. [6]. The radioglucosylated DBM so formed was isolated for counting of radioactivity by absorption to and desorption from Sep-Pak C₁₈ cartridges as in [6]. The degree of transglucosylation achieved by the three homogeneous recombinant proteins as reported in Fig. 1 was measured using 1, 2, 4 and 10 ng of wild-type and Phe-194 mutant and 5, 7.5 and 10 ng of Thr-194 mutant glycogenin. The results shown are the averages of 2 measurements at each protein concentration, measured after 1 h at 37°C. The activities of the three proteins, measured during their purification, were also determined with DBM. The values shown in Table 1 for the homogeneous proteins are based on the activities for

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Abbreviation: DBM, *n*-dodecyl β -maltoside

2 ng of wild-type and Phe-194 mutant and 5 ng of Thr-194 mutant. The amounts of enzyme assayed in the lysates and after DEAE–Sephacel were not greater than that corresponding to 2 or 5 ng of the pure proteins.

2.3. Cloning and mutagenesis of glycogenin cDNA and expression of recombinant proteins

Glycogenin cDNA was isolated from a rabbit muscle cDNA library constructed on Lambda ZAP II phage by the standard plaque hybridization technique, with detection of positive clones by chemiluminescence. The screening probe was generated by PCR amplification utilizing degenerate primers and the cDNA library as a template. The mutagenesis was achieved by the 'megaprimer' method [8]. The sub-cloning of the cDNAs into expression vectors and the expression of the proteins were done essentially as described by Viscupic et al. [7] and Studier et al. [9]. The sequence of the wild-type cDNA was identical to that reported by Viscupic et al. [7].

2.4. Purification of the wild-type and mutant forms of glycogenin

E. coli BL21/DE3 cells were transformed with the expression constructs, cultured in M9ZB medium and induced essentially as in [7]. The cell pellets from 40-ml bacterial cultures expressing each protein were resuspended in 2 ml of lysis buffer (50 mM HEPES, pH 7.5, 2 mM CHAPS, 1 mM EDTA, 5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 0.1 mM *N*-*p*-tosyl-L-lysine chloromethyl ketone, 0.15 mM pepstatin, 0.2 mM leupeptin, 2 mM benzamidin and 0.5 mg/ml lysozyme), sonicated and centrifuged at 12,000 rpm for 20 min. The supernatants were applied to DEAE–Sephacel columns (30 ml) equilibrated with 50 mM HEPES, 2 mM CHAPS (HC). Elution was achieved with an ascending gradient (150 ml) of NaCl (from 0 to 0.6 M) in HC. The fractions (1.5 ml each) containing the recombinant proteins, as detected by self-glucosylating activity in the case of the wild-type and by SDS-PAGE analysis in the case of the mutant proteins, were pooled. After addition of ammonium sulfate to 20% saturation, the samples (5 ml) were subjected to hydrophobic chromatography on a phenyl–Sephacel CL-4B column (8 ml), equilibrated with HC containing 0.15 M NaCl and ammonium sulfate at 20% saturation (starting buffer). The glycogenin proteins were desorbed with HC (100 ml); this elution was preceded by a descending gradient of starting buffer to HC (150 ml). Fractions of 3 ml each were collected. Wild-type glycogenin was detected by self-glucosylation and then SDS-PAGE and the mutant proteins by SDS-PAGE after first measuring the optical density of the particular fraction at 280 nm to detect protein. Homogeneity by SDS-PAGE was the criterion used in combining the final products.

The transglucosylase activities of the recombinant proteins, relative to each other, and at stages in the purification, were measured in the original lysates and in the pooled fractions by [14 C]glucosylation of DBM (Table 1).

2.5. Deglycosylation of wild-type glycogenin by isoamylase

Purified wild-type glycogenin (6.8 pmol) was incubated for 1 h at room temperature with 5 μ M unlabelled UDPglucose and 5 mM Mn^{2+} in 50 mM Tris-HCl, pH 7.4. This was to allow autoglucosylation to become complete. To the 50 μ l digest was added 50 μ l of 200 mM sodium acetate, pH 4.5, including isoamylase (120 U). After 3 h the pH was returned to 7.4 with 2 M Tris-HCl. Mn^{2+} was added to 5 mM and UDP[14 C]glucose to 4 μ M. Self-glucosylation was allowed to occur during 20 min when an equal volume of 20% trichloroacetic acid was added to precipitate the glucosylated protein for scintillation counting. The results of five such experiments are shown in Table 2, compared with the 14 C incorporated into glycogenin treated identically other than with isoamylase.

In a second series of experiments, purified wild-type glycogenin (20 pmol) was incubated overnight at room temperature with isoamylase (60 U) in 100 mM sodium acetate, pH 7.0 (50 μ l). Then a mixture (50 μ l) of 100 mM Tris HCl buffer, pH 7.4, 10 mM Mn^{2+} and 4 μ M UDP[14 C]glucose was added and incubated for 20 min. One half of the digest was precipitated with trichloroacetic acid for counting of 14 C radioactivity. The other half was heated at 100°C for 1 min to inactivate glycogenin and isoamylase. 1 M sodium acetate buffer, pH 7.0, and 1 M calcium chloride were added to give final concentrations of 200 mM and 10 mM respectively, together with crystalline pancreatic α -amylase (20 U), followed by another 10 U after 10 h. After incubation overnight,

the 14 C radioactivity in the trichloroacetic acid-insoluble residue was counted. An identical parallel experiment was performed save for the inclusion of isoamylase. The results from four such pairs of digests are shown in Table 3.

3. Results and discussion

3.1. Purification of recombinant proteins

Induced lysates of *E. coli* transformed with expression plasmids containing the cDNA for rabbit muscle glycogenin and the Phe-194 and Thr-194 mutants, contained 8–9 mg of soluble protein/40 ml (Table 1). In a two-step column procedure, each protein was purified to homogeneity in yields from 25% (Phe-194) to 30% (Thr-194) and 50% (wild-type) (Table 1). The degree of purification achieved was 2.5. Therefore glycogenin and the mutants constituted 40% of the soluble protein in the lysates.

3.2. Transglucosylase activity of wild-type glycogenin and the Phe-194 and Thr-194 mutants

The recombinant glycogenins under comparison were wild-type, Phe-194 and Thr-194 mutant proteins based on the sequence for rabbit muscle glycogenin and expressed in *E. coli*. Each was purified to homogeneity.

We confirmed the report by Cao et al. [5], who have also prepared the same three proteins, that the recombinant wild-type enzyme as isolated is already glucosylated and undergoes additional Mn^{2+} -dependent self-glucosylation while the Phe-194 mutant does not, nor, as also reported by Cao et al. [5], does the Thr-194 mutant, indicating that the alternative hydroxyl group afforded by Thr is not a substitute for that in Tyr.

We posed the question of whether the mutants retained transglucosylase activity or whether, as concluded by Cao et al. [5], Tyr-194 is essential for this function. Previously we noted that maltose is inhibitory to self-glucosylation, an observation that led us to the discovery that several *p*-nitrophenyl α -maltosaccharides, notably the maltoside, as well as the α -glucoside, inhibit self-glucosylation in the sense that they act as alternative acceptors of the transferred α -glucose [10]. This type of alternative acceptor clearly provided an appropriate means of testing whether the mutants could transglucosylate. Recently Manzella et al. [6] described an even more effective alternative acceptor, DBM. That this should be such an efficient acceptor was unexpected. It had been thought that the much greater inhibition (because of being a competing acceptor) by *p*-nitrophenyl α -maltoside vs. maltose was because in the former the maltose is entirely in the α -form and is linked to an aromatic aglycone which might be mimicking Tyr-194 [10]. DBM, however, conforms to neither criterion.

All three recombinant proteins transglucosylated DBM (Table 1, Fig. 1). The concentration–activity curves showed upward trends at higher enzyme concentrations. At the lower concentrations the wild-type and

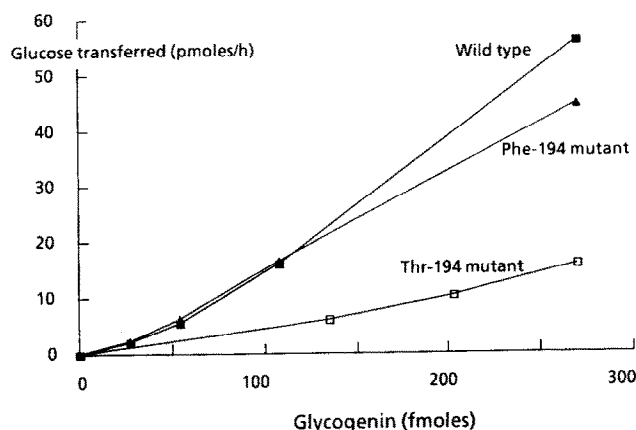


Fig. 1. The rates of [^{14}C]transglucosylation of dodecyl β -maltoside by recombinant glycogenins utilizing UDP[^{14}C]glucose. For conditions, see section 2.

Phe-194 mutant proteins had identical activities. The activity of the Thr-194 mutant was 50% of the others (Table 1). Clearly the Phe- and Thr-194 mutants are very effective as transglucosylases.

Elsewhere we will report a detailed comparison of how the three recombinant proteins also use the p-nitrophenyl α -saccharides as acceptors. The two mutant proteins transglucosylate these acceptors with an interesting specificity difference from the wild type.

We have, therefore, demonstrated that the Phe-194 and Thr-194 mutants of glycogenin are active transglucosylases and, contrary to the report by Cao et al. [5], Tyr-194 is not necessary for transglucosylase activity in this protein. The function of the Tyr appears to be largely confined to accepting transferred α -glucose residues. We may note that the evidence offered by Cao et al. [5] for the alleged catalytic inactivity of the Phe-194

mutant was its inability to glucosylate a synthetic 30-unit peptide corresponding to the region around Tyr-194 in glycogenin. However, since the wild-type glycogenin also failed to glucosylate the peptide, the conclusion drawn by Cao et al. [5] is invalid.

3.3. Glucosylation of Tyr-194 of glycogenin

As already noted by Viscupic et al. [7], wild-type recombinant glycogenin is obtained in an already glucosylated form. Therefore all the glucose residues, including the first, joined to Tyr-194, are added autocatalytically or, if not, then *E. coli* must contain a transglucosylase that adds the first glucose to Tyr-194.

To explore this question, we made use of a property we recently discovered in isoamylase, the glycogen-debranching enzyme [11]. Isoamylase hydrolyses not only the 1,6-interchain glucosidic linkages of glycogen, but, in addition, the bond between the terminal glucose and Tyr-194 of glycogenin.

Accordingly, we treated the already glucosylated wild-type glycogenin with isoamylase in order to create carbohydrate-free protein. This was then incubated with UDPglucose, in comparison with glycogenin not treated with isoamylase. If glycogenin can glucosylate its Tyr-194, then the isoamylase-treated protein should undergo additional glucosylation.

The experiment presented a technical problem. Isoamylase has a pH optimum of 4.5 at which pH glycogenin is inactivated. We discovered, however, that if the isoamylase treatment at pH 4.5 was confined to 3 h at room temperature, some activity was retained by the glycogenin as seen on return to pH 7.4 and addition of UDP[^{14}C]glucose. The results in Table 2, a comparison of the relative degrees of [^{14}C]glucosylation of glycogenin held at pH 4.5 with and without treatment with isoamylase, show clearly that exposure to isoamylase enabled a

Table 1
Purification of recombinant glycogenin (wild-type) and two mutant proteins (Phe and Thr mutants)

	Total activity ($\mu\text{mol glucose/min}$)	Total protein (mg)	Specific activity ($\mu\text{mol/glucose/min/mg protein}$)	Yield (%)	Purification (fold)
Wild-type					
Lysate	9.4	8.9	1.1	100	1.0
DEAE-Seph.	5.8	3.0	1.9	61	1.7
Phenyl-Seph.	4.6	1.7	2.7	49	2.5
Phe mutant					
Lysate	11	8.2	1.3	100	1.0
DEAE-Seph.	7.6	2.8	2.7	69	2.1
Phenyl-Seph.	2.8	0.9	3.1	25	2.4
Thr mutant					
Lysate	5.0	8.9	0.56	100	1.0
DEAE-Seph.	3.5	2.6	1.3	70	2.3
Phenyl-Seph.	1.5	1.1	1.4	30	2.5

After purification on DEAE-Sephacrose and phenyl-Sephacrose, each protein was homogeneous as seen by SDS-PAGE.

Table 2

Effect of isoamylase treatment on the degree of self-glucosylation of wild-type recombinant glycogenin

¹⁴ C]Glucose incorporated into glycogenin (mmol/mol glycogenin)		
	Untreated	Treated
	71	836
	89	680
	49	806
	52	720
	47	810
Average	62 ± 18	770 ± 67

The results depict the degree to which wild-type glycogenin was able to glucosylate itself after treatment at pH 4.5 in the presence of isoamylase ('Treated') to remove pre-existing maltosaccharide chains, and after incubation at pH 4.5 in the absence of isoamylase ('Untreated'). Shown are the results of five pairs of experiments. For conditions, see section 2.

much greater (more than 12-fold) degree of re-glucosylation to occur (Table 2). Each sample, prior to treatment at pH 4.5, had been exposed to unlabelled UDPglucose. Therefore no re-glucosylation was expected, and little occurred, in the absence of isoamylase.

It could be argued that α -amylase, a common enzyme impurity, would have the same effect as isoamylase. We already had evidence that the crystalline isoamylase employed did not contain this impurity. This evidence was the absence of maltose, a characteristic product of α -amylolysis, from the maltosaccharide chains released by treatment of glycogenin with isoamylase [11].

We nevertheless resorted to an additional experiment to test for α -amylase. This was based on the knowledge that when native glycogenin, isolated in an already glucosylated form, is allowed to self-glucosylate with UDP[¹⁴C]glucose, most of the ¹⁴C label thereby added can be removed by α -amylase [1,12,13]. This is because α -amylase can shorten the maltosaccharide chains of glycogenin to glucose and maltose residues, where the average length of the chains in the glycogenin specimen em-

ployed was already six glucose residues. However, if α -amylase was used to treat glycogenin in which every glucose residue in the maltosaccharide chains was ¹⁴C-labelled, including that attached to Tyr-194, then some [¹⁴C]glucose (as α -amylase-resistant glucose or maltose residues) must remain attached to the protein.

Accordingly, we allowed glycogenin to undergo self-[¹⁴C]glucosylation after treatment with isoamylase. In this experiment we kept the glycogenin at pH 7.0 during treatment with isoamylase, at which pH the latter is feebly active. Then, after incubating with UDP[¹⁴C]glucose, the glycogenin preparation was treated with α -amylase and the [¹⁴C]glucose remaining with the protein was measured. An identical sample was treated in the same way except for the omission of isoamylase. Once again (see Table 3), treatment of glycogenin with isoamylase, vs. no treatment, allowed more subsequent ¹⁴C-glucosylation to occur (cf. Table 2). (In this experiment there was no preincubation with unlabelled UDPglucose.) There was also significantly more α -amylase-resistant [¹⁴C]glucose than when isoamylase pre-treatment was not applied (Table 3). An α -amylase impurity could not be responsible for the presence of the additional α -amylase-resistant [¹⁴C]glucose in isoamylase-treated glycogenin, otherwise there would be no difference in the amounts of ¹⁴C remaining after α -amylase treatment.

We may conclude that isoamylase treatment of glycogenin had, as expected, created the carbohydrate-free protein and that, on being offered UDP[¹⁴C]glucose, the protein had proceeded to re-glucosylate itself, including the first glucose residue, joined to Tyr-194.

Given the knowledge that isoamylase can split both glucosyl-glucose and glucosyl-tyrosine bonds [11], the results of the test for such dual specificity in the case of the auto-glucosylation of glycogenin are not unexpected. Nevertheless, the knowledge now gained, that glycogenin is entirely self-sufficient for its own glucosylation, is very important because it eliminates a previous area of uncertainty at the beginning of glycogen synthesis, namely the addition of the very first glucose residue.

Table 3

Enhancement of self-glucosylation of glycogenin by pretreatment with isoamylase: test for contamination by α -amylase

¹⁴ C]Glucose incorporated into glycogenin (mol/mol glycogenin)				
Untreated with isoamylase		Treated with isoamylase		
Before α -amylase treatment	After α -amylase treatment	Before α -amylase treatment	After α -amylase treatment	
0.86	0.04	1.54	0.18	
0.89	0.13	1.60	0.43	
0.70	0.08	1.34	0.29	
0.90	0.14	1.45	0.39	
Average	0.84 ± 0.09	0.10 ± 0.05	1.48 ± 0.11	0.32 ± 0.11

The results depict the extent of self-[¹⁴C]glucosylation by wild-type glycogenin, treated and not treated with isoamylase at pH 7.0, followed by treatment of the [¹⁴C]glucosylated protein with α -amylase to learn how much ¹⁴C was resistant to α -amylase. These are the results of four sets of experiments. For conditions, see section 2.

Acknowledgements: This work was supported by a grant from the National Institutes of Health (DK 37500). We are grateful to Dr. L. Roden and his colleagues for making available to us the manuscript of [6] prior to its publication. We thank Erik Lagzdins and John Rodriguez for carrying out some of the initial experiments with the DBM assay.

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