COMMUNICATION

The size of the C-chain maltosaccharide of glycogen: evidence for the presence of only a single branch

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Glycogen is found in mammals and yeast bound to glycogenin forming proteoglycogen. The branched polysaccharide is joined to the protein through the C-chain, a maltosaccharide considered to be 13 glucose units long and double branched as the other branched glycogen B-chains. We described before the isolation of c-glycogenin, the debranched C-chain bound to glycogenin, from muscle proteoglycogen. In this work, the size of the C-chain is analyzed for the first time. The maltosaccharide moiety of c-glycogenin was auto-[14C]glucosylated by a short incubation with UDP-[14C]glucose, and the labeled maltosaccharide was released by heating in 2 M NaOH containing 0.1 M NaBH4 and analyzed by high-performance thin layer chromatography (HPTLC). The results indicate that the C-chain is about half the size of the B-chains, not long enough to be double branched.

Key words: C-chain-bound glycogenin/c-glycogenin/ glycogen structure/glycogenin-bound C-chain/proteoglycogen

Introduction

According to the model for the structure of glycogen (Gunja-Smith et al., 1970; Goldsmith et al., 1982; Meléndez-Hevia et al., 1993; Meléndez et al., 1998, 1999) and the degree of polymerization and branching of glycogen from different species (Meléndez et al., 1997), the molecule is constituted by maltosaccharide chains with an average length of 13 glucose units, joined through α-1,6-linkages forming a branched structure. The structure is composed of three types of chains, non-branched A-chains located at the periphery, branched B-chains containing two branches, and the most internal C-chain on which glucose polymerization and branching initiates (Peat et al., 1952). It is assumed that the C-chain also contains two branches and is 13 glucose units long (Meléndez et al., 1998, 1999), but this has never been determined.

We first showed evidence of protein covalently bound to glycogen and named proteoglycogen the new type of glycogen (Aon and Curtino, 1984). The protein moiety of proteoglycogen, glycogenin (Rodríguez and Whelan, 1985), initiates the glucose polymerization by autoglucosylation to produce the primer for glycogen synthase (Lomako et al., 1988; Pitcher et al., 1988; Smythe and Cohen, 1991). Glycogenin remains bound, through a tyrosine residue (Aon and Curtino, 1985; Smythe et al., 1988), to the C1 position of the glucose residue of the C-chain reducing end (Smythe et al., 1988).

We described a procedure for the isolation of native proteoglycogen from rabbit muscle and of polysaccharide-free glycogenin after exhaustive digestion of proteoglycogen with α-amylase (Carrizo et al., 1997). We also reported the release and isolation of C-chain-bound glycogenin (c-glycogenin) from isoamylolyzed muscle proteoglycogen; the c-glycogenin species was able to autoglucosylate, indicating that it was linked to a maltosaccharide capable of being elongated by autoglucosylation (Romero and Curtino, 2003).

In this work, we analyze the size of the C-chain moiety of c-glycogenin. The C-chain is a maltosaccharide of no more than seven glucose units, which would admit only a single branch, based on the proposed action specificity of the branching enzyme (Manners, 1991; Meléndez et al., 1998, 1999).

Results and discussion

The study by high-performance thin layer chromatography (HPTLC) of the size of the C-chain bound to the tyrosine residue of c-glycogenin required the release of the intact oligosaccharide moiety. This was accomplished by heating in 2 M NaOH containing 0.1 M NaBH4 at 45°C for 14 h, followed by 3 h in a boiling bath. In contrast to acid, heating in alkaline solution has no effect on α-1,4-glucosidic linkages (Somogyi, 1957). Borohydride was included to avoid alkaline degradation of the released maltosaccharides (Edge et al., 1986). No cleavage of glucosidic bonds occurred when standard maltosaccharides were subjected to the alkaline-borohydride treatment under the conditions used (Figure 1). The mobility retardation observed was caused by the reduction of the maltosaccharide aldehyde to alcohol (see Materials and methods).

When the alkaline-borohydride treatment was applied to auto[14C]glucosylated recombinant glycogenin, the addition of 10% trichloroacetic acid after neutralization (see Materials and methods) resulted in no precipitation of labeled material.
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as it was expected for the split of the labeled maltosaccharide from glycogenin (result not shown).

The released oligosaccharide moiety was analyzed by the TLC procedure described for the separation of maltodextrins (Robyt and Mukerjea, 1994). Maltosaccharides of up to 14 glucose units were clearly separated when a mixture of maltosaccharides, released from proteoglycogen by isoamylolysis, was subjected to the TLC procedure (Figure 2A).

It is to be noticed that a C-chain having 13 glucose units would comprise as low as 0.023% of the total glycogen chains, for proteoglycogen with a weight average $M_r$ of $1.0 \times 10^7$. Even if c-glycogenin was isolated from debranched proteoglycogen by passage through a Q Sepharose column (see Materials and methods), a contamination with A- and B-chains should not be excluded. To avoid any interference of the other chains with the identification of the released C-chain, we specifically labeled the maltosaccharide moiety by autoglucosylation of c-glycogenin before the alkaline digestion, and analyzed by HPTLC the size of the labeled material.

In order to analyze the size of the $[^{14}\text{C}]$glucosylated moiety, the autoglucosylated glycogenin species was precipitated with trichloroacetic acid and subjected to treatment with the NaOH–NaBH$_4$ mixture, and the solution neutralized, desalted, and passed through a Q Sepharose column (see Materials and methods). The unbound labeled maltosaccharide eluted with water was mixed with maltosaccharides standards (see Materials and methods) and subjected to HPTLC. The labeled maltosaccharide released by alkaline digestion of recombinant glycogenin, $[^{14}\text{C}]$glucosylated by a 5-min incubation with UDP-$[^{14}\text{C}]$glucose, corresponded to five to eight glucose units (Figure 2B, lane 1). Figure 3 shows that a low level of $[^{14}\text{C}]$glucosylation (~25% of the maximum $[^{14}\text{C}]$glucosylation) is produced by autoglucosylation of the recombinant glycogenin for 5 min. It has been described that recombinant glycogenin is expressed in *Escherichia coli* as the autoglucosylated form, bound to maltosaccharides mainly containing up to seven glucose units (Cao *et al.*, 1993). The polymerization degree of the largest labeled maltosaccharide (Figure 2B, lane 1) is consistent with the incorporation into the recombinant glycogenin species containing bound maltotetraose of no more than one $[^{14}\text{C}]$glucose unit. The other three labeled maltosaccharides observed in Figure 2B, lane 1, would correspond to glycogenin species containing bound maltotetraose, maltopentaose, and maltohexaose, each elongated by the incorporation of one $[^{14}\text{C}]$glucose unit. The autoglucosylation of recombinant glycogenin reaches a plateau after incubation for 40 min (Figure 3), resulting in elongation of the maltosaccharide moiety up to 13 glucose units (Figure 2B, lane 3).
The same kinetics of [14C]glucosylation and low level of 5-min auto[14C]glucosylation was produced by incubation of the isolated c-glycogenin and recombinant glycogenin with UDP-[14C]glucose (Figure 3). To analyze the size of the labeled C-chain moiety of c-glycogenin, it was required that the auto[14C]glucosylation resulted in a minimal elongation. This strongly indicated using a short 5-min incubation with UDP-[14C]glucose. The size of the labeled C-chain, released by the alkaline treatment of the c-glycogenin auto[14C]glucosylated for 5 min (Figure 2B, lane 2), was compared with that of the maltosaccharides released from recombinant glycogenin subjected to low and complete auto[14C]glucosylations (Figure 2B, lanes 1 and 3). As it is shown, the size of the labeled C-chain was shorter than that of the labeled maltosaccharide bound to the maximum autoglucosylated glycogenin. The polymerization degree of the C-chain would be that of the [14C]maltosaccharides observed in the chromatogram (Figure 2B, lane 2) reduced in size by subtraction of at least one glucose unit. Thus, the actual size of the two main labeled bands, which move as maltosaccharides of seven and eight glucose units long, would correspond to C-chains no longer than maltohexaose and a maltoheptaose, respectively. It is to be noticed that a similar size of labeled C-chains might result from even shorter maltosaccharides, because glycogenin bound maltopentaose, maltohexaose or a mixture of both can be [14C]autoglucosylated to maltohexaose and maltopentaose. A C-chain shorter than maltopentaose should not be considered based on the proposed action specificity of the branching enzyme (Manners, 1991; Meléndez et al., 1998, 1999).

A reduction in size of the C-chain during isoamylolysis of proteoglycogen should be discarded because, as it was previously shown (Romero and Curtino, 2003), no split of glucosyl-α-1,4-glucose linkage occurred when [14C]glucosylated c-glycogenin or fully auto[14C]glucosylated glycogenin were subjected to the isoamylolytic digestion. It was also shown (Romero and Curtino, 2003) that no carbohydrate-free glycogenin (apoglycogenin) was released by debranching proteoglycogen under the conditions indicated in Materials and methods.

It is accepted that the branching enzyme detaches a maltoheptaose unit from a chain having at least 11 glucose residues and that this maltoheptaose can be attached to the same chain, provided that it occurs at a distance of at least four residues from other branch (Manners, 1991; Meléndez et al., 1998, 1999). As the C-chain isolated from proteoglycogen is no longer than seven glucose units, we conclude that c-glycogenin is bound to the rest of the polysaccharide through a single branch. A C-chain of at least 10 glucose units should be necessary for admitting two branches as it was proposed before (Meléndez et al., 1998, 1999). How the single branched C-chain might be originated is shown in Figure 4A. Glycogenin-bound maltosaccharide of 13 glucose units can be formed by autoglucosylation, as it was observed in vitro (Figure 2B, lane 3). Then, the branching enzyme would detach six or seven glucoses to be attached in the fifth glucose from the tyrosine residue of glycogenin. Only the single branch bound through α-1,6-linkage to the resulting C-chains of six or seven glucoses would be the acceptor for the next sequence of elongation and branching reactions. This proposed mechanism of formation of the first branch might indicate that both, glycogenin and branching enzyme, contribute to generate the primer for glycogen synthase.

These results show for the first time that the C-chain of glycogen differs from the B-chains in length and number of branches. The size of the C-chain, even if not precisely determined, is only long enough to admit one and not two branches. This is illustrated in Figure 4B, by modification...
of the scheme of the structure of glycogen proposed by Meléndez et al. (1998, 1999). Our work also shows that the maltosaccharide originated by the in vitro complete autoglucosylation of recombinant glycogenin is quite heterogeneous in size, ranging from 6 to 13 units (Figure 2B, lane 3). Further study will be required to elucidate the cause of such different degree of glycogenin autoglucosylation.

Materials and methods

Materials

UDP-[U-14C]glucose (300 mCi/mmol) was purchased from Fundación Instituto Leloir (Buenos Aires, Argentina). Isoamylase from Pseudomonas amylofera, Q Sepharose, glucose, maltose, maltotriose, maltotetraose, maltopentaose, maltotriose, Sephadex G-10, Sephadex G-25, Triton X-114, Triton X-45, leupeptin, pepstatin, N-α-p-tosyl-L-lysine chloromethyl ketone (TLCK), 4-amidinophenylmethylsulfonyl fluoride (APMSF), phenylmethylsulfonyl fluoride (PMSF), isopropylthiogalactopyranoside (IPTG), and dithiothreitol (DTT) were from Sigma-Aldrich (St. Louis, MO). Chitin beads and vector pTYP1 were from New England BioLabs (Beverly, MA). HPTLC plates were from Merck (Darmstadt, Germany). The glycogenin clone was a gift of Dr. P. Roach (Indiana University School of Medicine).

Preparation of proteoglycogen, c-glycogenin, and recombinant glycogenin

Purified proteoglycogen was prepared from rabbit skeletal muscle as described previously (Carrizo et al., 1997). Briefly, the muscle was homogenized, the homogenate centrifuged at 4800 × g and proteoglycogen and membrane vesicles collected by centrifugation of the supernatant at 75,000 × g. The loose vesicles layer covering the proteoglycogen pellet was poured off, the pellet was dissolved in a 1.1% mixture of Triton X-114/Triton X-45 (86.5/13.5) at 4°C, and proteoglycogen was partitioned in the aqueous phase after raising the temperature of the solution to 14°C. The accompanying proteins were precipitated with 3.6 M potassium iodide from the collected aqueous phase before sedimentation and washing of proteoglycogen by centrifugation at 235,000 × g.

C-Glycogenin was released from proteoglycogen (1 mL of a solution 1.6% proteoglycogen glucose) with isoamy- lase (15 µg, 111,300 U) in 15 mM sodium-acetate buffer, pH 6.0, containing 0.02% sodium azide, 0.12 mM TLCK, and 30 µM each of leupeptin, pepstatin, and APMSF (Romero and Curtino, 2003), by incubation for 6 h. The pH of the digested mixture was raised to 7.5 with 0.1 M NaOH and Triton X-100 were added to 60 mM and 0.01%, respectively, and passed through a column of Q Sepharose (0.2 mL) equilibrated with 60 mM NaCl in 20 mM Tris-acetate buffer, pH 7.5, containing 0.01% Triton X-100 (Tris-buffer–detergent). The column was washed with 60 mM NaCl in Tris-buffer–detergent and c-glycogenin was eluted with 0.4 mL of 250 mM NaCl in Tris–buffer–detergent.

Recombinant rabbit muscle glycogenin was prepared as indicated elsewhere (Carrizo et al., 2001). Briefly, the glycogenin cDNA was in frame inserted in the vector pTYB1, upstream of the intein/chitin binding domain sequence. The resultant plasmid (free of mutations in the glycogenin coding region based on DNA sequencing) was transformed into E. coli strain ER2566. Typically, 1 L of culture at an optical density of 0.6–0.8 was induced with 0.3 mM IPTG for 3 h at 30°C. The cells collected by centrifugation were resuspended in 20 mM Tris–HCl buffer, pH 8.0, containing 0.5 M NaCl, 0.1% Triton X-100, 20-µM PMSF, 0.14 mM TLCK and sonicated. The clarified lysate was passed through a chitin column (20 mL) at a flow rate of 0.5 mL/min. The column was then washed with column buffer (20 mM Tris–HCl buffer, pH 8.0/0.5 M NaCl) and then treated with the column buffer containing 50 mM DTT. After 48 h at room temperature, glycogenin was eluted with the column buffer and dialyzed against 20 mM Tris-HCl buffer, pH 7.5, at 4°C to remove excessive DTT.

Preparation of homologous series of maltosaccharides from proteoglycogen

Proteoglycogen was isoamylolyzed overnight in the absence of protease inhibitors, and passed through a Q Sepharose column after the pH of the digested mixture was raised to 7.5 with 0.1 M NaOH. The released maltosaccharides non-retained in the Q Sepharose column were eluted with water, concentrated, and passed through a Sephadex G-25 column. Maltosaccharides fractions of 5–11 and 7–14 glucose units were concentrated, and samples made 70% in ethanol and subjected to HPTLC.

Labeling of the maltosaccharide moieties of c-glycogenin and recombinant glycogenin

Recombinant glycogenin (9.6 µg in 240 µL) and c-glycogenin (900 µL of 250 mM NaCl-eluted fractions from the Q Sepharose column (Romero and Curtino, 2003), corresponding to 9.6 mg of isoamylyzed proteoglycogen) were autol[14C]glucosylated by incubation, for the time indicated in each case, with 8-µM UDP-[14C]glucose as described previously (Romero and Curtino, 2003). The incubation mixtures (the 900 µL mixture vacuum reduced to 100 µL) were made 10% in trichloroacetic acid, cooled in ice for 5 min and centrifuged at 12,000 × g for 3 min. The precipitates were washed twice by centrifugation after sonication in 0.4 mL of cold 5% trichloroacetic acid containing 0.5% phosphotungstic acid. The radioactivity incorporated was measured in a liquid scintillation counter or subjected to alkaline treatment.

Release and isolation of the labeled maltosaccharide moieties of c-glycogenin and recombinant glycogenin

The autol[14C]glucosylated glycogenin species precipitated with trichloroacetic acid was washed with 0.4 mL of acetone and subjected to 2 M NaOH containing 0.1 M NaBH₄ at 45°C for 14 h followed by 3 h in a boiling bath, cooled, neutralized by addition of HCl, and centrifuged at 12,000 × g for 3 min. The supernatant containing the labeled maltosaccharide was desalted by passage through a Sephadex G-10 column (1 × 10 cm), the eluted labeled material was passed through a small column (0.2 mL) of Q Sepharose, and the non-retained labeled material was eluted with

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water, vacuum dried, and dissolved in 70% ethanol. Samples were mixed with glucose, maltotetraose, maltohexaose, and maltoheptaose (1.0 μg each) as internal standards and subjected to HPTLC.

**Thin layer chromatography**

Maltosaccharides were separated on HPTLC plates in the running solvent acetonitrile–ethyl acetate–1-propanol–water (85:20:50:70) described by Robyt and Mukerjea (1994), using a tank designed to obtain highly reproducible chromatograms as described elsewhere (Nores et al., 1994). After running, the plates were air-dried, dipped into 5% H₂SO₄ in ethanol, heated at 120°C for visualization of carbohydrates, and, where indicated, subjected to autoradiography.

The relative mobility (Rₑₑₑₑₑₑ) of commercial maltosaccharides (maltose to maltoheptaose), plotted versus polymerization degree (PD), gave a straight line which was coincident with the straight line obtained from the homologous series of maltosaccharides released from proteoglycogen (Figure 1A).

Reduction of the maltosaccharide aldehyde to alcohol by alkaline–borohydride treatment retarded the mobility in the HPTLC system to that of a maltosaccharide with an extra glucose unit (Figure 1). This was taken into account in order to assess the size of the labeled maltosaccharides by comparison with the mobility of the standard maltosaccharides.

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**Abbreviations**

APMSF, 4-aminophenylmethylsulfonyl fluoride; DTT, dithiothreitol; HPTLC, high-performance thin layer chromatography; TLCK, N-α-p-tosyl-L-lysine chloromethyl ketone.

**References**


