Proteomic Investigation of the Binding Agent between Liver Glycogen β Particles

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Supporting Information

ABSTRACT: Glycogen is a highly branched glucose polymer which plays an important role in glucose storage and the maintenance of blood sugar homeostasis. The dimeric protein glycogenin can self-glucosylate to act as a primer for glycogen synthesis, eventually resulting in small (∼20 nm diameter) glycogen β particles with a dimer of glycogenin at their core. In the liver, glycogen is also found in the form of α particles: large bound composites of many β particles. Here, we provide evidence using qualitative and quantitative proteomics and size-exclusion chromatography from healthy rat, mouse, and human liver glycogen that glycogenin is the binding agent linking β particles together into α particles.

1. INTRODUCTION

Glycogen is a complex branched polymer of glucose, containing a small amount of protein,1 with (1→4)-α linear links and (1→6)-α branch points between glucose monomers. Biosynthesis of glycogen starts on the dimeric protein glycogenin, which self-glucosylates at Tyr195 in mouse, rat, and human glycogenin-1 or at Tyr228 in human glycogenin-2 and extends this oligomaltose structure to ∼10 (1→4)-α-linked glucose units.2,3 This oligomaltose primer is then further extended by glycogen synthase, which, together with glycogen branching enzyme, facilitates the formation of a glycogen β particle.4 The liver contains two types of glycogen: small β particles (∼20–50 nm in diameter), which can be bound together to form large α particles (up to hundreds of nanometers in diameter), which exhibit a composite cauliflower-like appearance when viewed under transmission electron microscopy.5 (Note an unfortunate confusing point in nomenclature: the terms “α” and “β” refer both to types of glycosidic linkages in sugars and to types of glycogen particles; the intended meaning can readily be inferred from the context.) In humans, glycogen is most abundant in the liver, which contains both α and β particles,6 and in muscle, which contains β and smaller α particles.5–8 It has been shown that large glycogen α particles release glucose more slowly than β particles (which are small) in the presence of degradative enzymes, presumably because of their lower ratio of surface area to volume.9,10 This suggests that a high abundance of α particles in the liver serves to allow a slow release of glucose, as needed during overnight fasting in diurnal animals, whereas the presence of mainly β and small α particles in muscle is optimal for the fast glucose release required for rapid movement.

Glycogen α particles from the liver of db/db mice, an animal model for type 2 diabetes, are more molecularly fragile than those from healthy mice: they readily break apart into β particles in vitro under conditions that are too mild to break apart α particles from healthy mice.11,12 This suggests that under appropriate conditions in the liver, diabetic liver α particles may also break apart easily into small β particles, which will be more rapidly degraded to glucose.11 This increased fragility of diabetic glycogen could thus be a contributing factor to the poor blood sugar homeostasis characteristic of diabetes, suggesting that the binding of small glycogen β particles together into large glycogen α particles is compromised in diabetic glycogen.8

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It is not known what binds $\beta$ particles together into $\alpha$ particles. Various models have been proposed to explain this process.\textsuperscript{13} It was suggested that disulfide bonding is involved,\textsuperscript{14,15} although later work suggested that this was not the case.\textsuperscript{16,17} It has also been suggested that $\beta$ particles bind together through any of hydrogen bonding and hydrophobic effects,\textsuperscript{18} glycosidic linkages,\textsuperscript{19,20} and a crowding/budding model involving a protein “glue”.\textsuperscript{21} However, the time evolution of the molecular size distribution of both glycogen and phytylglycogen during acid hydrolysis,\textsuperscript{21} and of glycogen particles extracted from formalin-fixed liver and dissolved at high temperature,\textsuperscript{16} are both consistent with a noncovalent protein-mediated linkage (rather than a glycosidic linkage) being the glue binding $\beta$ particles into $\alpha$ particles in the liver.

Here, we apply both qualitative and quantitative mass spectrometry proteomics and molecular size distribution fractionation and determination with size-exclusion chromatography (SEC, a type of gel-permeation chromatography), which separates by molecular size, to investigate the binding agent in liver glycogen samples from mouse, rat, and human liver tissues.

2. RESULTS AND DISCUSSION

2.1. Mass Spectrometric Proteomics of Rat Liver Glycogen. We hypothesized that the glue binding $\beta$ particles into $\alpha$ particles is a protein. To identify this protein, we performed relative quantitative mass spectrometry proteomics of glycogen from the liver of rats sacrificed \( \sim 8 \) h after their last meal. We used size separation by preparative SEC to isolate two separate fractions enriched with $\alpha$ (larger size fraction) or $\beta$ (smaller size fraction) particles (Figure 1b), with the size distribution of unfractionated samples (from analytical SEC) shown in Figure 1a; the variation between individual samples in Figure 1a is typical of that between individual animals, as exemplified elsewhere.\textsuperscript{12} The SEC data are presented as the SEC weight distribution \( w(\log R_g) \), which is the relative weight of molecules with a size (hydrodynamic radius) \( R_g \). We hypothesized that if $\alpha$ particles were bound together by a protein, then this protein would be enriched in the $\alpha$ particle fraction compared with the $\beta$ particle fraction. We performed shotgun mass spectrometry proteomics on replicate SEC-purified glycogen (both $\alpha$- and $\beta$-particle-enriched fractions), and the only protein to be robustly detected in all fractions was glycogenin (Supporting Information Table S1).

To test if glycogenin was enriched in $\alpha$ particles relative to $\beta$ particles, we devised a relative quantitative SWATH-MS proteomics workflow (Supporting Information Figure S1). We used preparative SEC to purify and fractionate rat liver glycogen into $\alpha$- and $\beta$-particle-enriched fractions (no samples were combined), used GOPOD analysis to quantify the glucose-equivalent concentration of each fraction, and normalized the amount of each sample to equivalent glucose content. Analytical SEC showed that these large- and small-size fractions were indeed enriched in $\alpha$ and $\beta$ particles, respectively (Figure 1a,b). Because glycogen, being highly branched, is likely to obstruct diffusion of large molecules such as proteases, we split each sample into two fractions and treated one sample of each with $\alpha$-amylase to digest its glycogen to glucose, thereby making all of the proteins in the sample accessible. Control samples without $\alpha$-amylase treatment would have no change in the accessibility of proteins. Protein that was measured in samples without $\alpha$-amylase digestion was accessible because treatment with trypsin and DTT did not alter the glycogen molecular size distribution (Figure 1c).

The $\alpha$-amylase treatment would release any proteins buried inside glycogen.
particles. All samples were then protease-digested with the addition of equal amounts of trypsin, and peptides were measured with SWATH-MS; all proteins are listed in the Excel files in the Supporting Information. Relative units from the ion currents were used, as we only compared the relative glycogenin abundances in α- and β-particle-enriched fractions. This analytical workflow allowed precise comparison of the relative amount of glycogenin between samples, with normalization both to trypsic self-digest peptides within a sample and to glucose concentrations between samples.

Samples of β-particle-enriched glycogen from rat liver had relatively low levels of glycogenin, which increased by a small but statistically significant amount after α-amylase digestion (Figure 1d). This increase in glycogenin levels was consistent with the generally accepted hypothesis that glycogen particles are initiated from a noncovalently linked glycogenin dimer that remains inaccessible to tryptic digestion.22 This “core initiator” glycogenin would only be accessible after α-amylase digestion. However, most glycogenin is still accessible to trypsins without amylase digestion, indicating that there is a large amount of glycogenin attached to, but not interior to, the glycogen.

We next compared α- and β-particle-enriched glycogen fractions. Both with and without α-amylase digestion, fractions enriched with α particles had significantly more glycogenin than those enriched in β particles. Detection of glycogenin in α-particle-enriched fractions was significantly but not substantially increased after α-amylase digestion, suggesting that glycogenin was also mainly located on the surface of α particles. These results (Figure 1d) confirmed that glycogenin was enriched in α particles relative to β particles and were thereby consistent with glycogenin being the glue between β particles.

How might glycogenin act as both β particle primer and glue? The enzymatic role of glycogenin as a core initiator of glycogen β particles is to build a (1→4)-α glucose oligosaccharide glycosidically linked to glycogenin Y195.23 This activity requires that (1→4)-α glucose oligosaccharide acceptor substrate and uridine diphosphate (UDP) glucose bind at the active site of glycogenin. Binding of (1→4)-α glucose oligosaccharide in the absence of UDP glucose, or with a regulatory inactive enzyme, would allow dimeric glycogenin to act as a lectin, noncovalently linking glycogen β particles together into α particles.

2.2. Mass Spectrometric Proteomics of Mouse and Human Liver Glycogen. To explore and support our postulate of glycogenin being the glue binding β particles together into α particles, we performed similar analyses of mouse and human liver glycogen. As with rats, liver glycogen from mice contained both α and β particles (Figure 2a). Consistent with previous studies,24 human liver glycogen (samples obtained after >8 h of fasting) consisted only of α particles (Figure 2b).

Qualitative proteomic analyses were used to identify the proteins associated with glycogen purified from mouse and human liver samples. As with our analysis of rat glycogen, only glycogenin was robustly detected in all samples (Supporting Information Table S1). We identified glycogenin-2, but not glycogenin-1, in human liver, suggesting that the former is the major form present in that organ. We next used SWATH-MS to analyze the relative glycogenin content in α- and β-particle-enriched fractions. Similar to the result of our analysis of rat glycogen fractions, β-particle-enriched glycogen from mouse liver had relatively low levels of glycogenin, and these levels were significantly increased with α-amylase treatment (Figure 2). Fractions enriched in α particles had higher levels of glycogenin than β-particle-enriched fractions, either with or without α-amylase treatment (Figure 2c). The slight increase in

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**Figure 2.** Proteomic and SEC analyses of mouse and human liver glycogen. (a) Analytical SEC weight distribution of liver glycogen from mice (n = 6) and (b) human (n = 4); SEC signals at 10 nm are ascribed to adventitious small-molecule impurities. Glycogenin content measured by SWATH-MS of liver glycogen from (c) mice and (d) humans after SEC separation into two fractions: Fria, α-particle-enriched; Frβ, β-particle-enriched; and am, pretreated with α-amylase. Values are mean glycogenin abundance normalized to trypsin abundance. Error bars show standard deviation. *P < 0.05.
glycogenin content in α-particle-enriched fractions after α-amylose digestion in the mouse samples (Figure 2c) is not statistically significant but consistent with the majority of glycogenin being surface-accessible, as with rat glycogen (Figure 1d).

We next used SWATH-MS to investigate the glycogenin content of human glycogen, which only contains α particles. This analysis clearly indicated that the glycogenin content significantly increased with α-amylose digestion, with a greater change than seen in the rodent samples (Figure 2d). This indicates that in the human samples, the majority of the glycogenin was inaccessible to trypsin.

It was, at first, surprising that we did not identify previously reported glycogen-associated proteins, including glycogen synthase, glycogen phosphorylase, phosphorylase kinase, and glycogen debranching enzyme. Instead, the only glycogen-associated protein we robustly identified from rat, mouse, and human liver samples was glycogenin. This is probably due to the stringent purification procedure we used to enrich glycogen. Importantly, our purification strategy was able to maintain intact α and β particles. This suggests that the additional glycogen-associated proteins previously observed are associated with but not strongly bound to glycogen and are not required for α-particle assembly. For example, glycogen debranching enzyme is associated with glycogen but is not permanently bound to this glucose polymer.

2.3. Model for α-Particle Binding. Liver glycogen is present as small β particles and large α particles that are agglomerates of β particles. The β particles are built from an oligomaltose primer extended from a glycogen dimer. Using SWATH-MS of liver glycogen from rats, mice, and humans, we identified glycogenin as the only detectable candidate for a protein linking β particles together into large α particles (Figures 1 and 2). On the basis of our data, we propose a new model of glycogen structure in mice, rats, and humans (Figure 3). In this model, β particles are synthesized from self-oligomaltose present on the surface of β particles, which is distinct from the well-understood covalent attachment of oligomaltose to Y195 of glycogenin. Binding of neighboring β particles by many glycogenin dimers would thus provide a stable binding to form α particles.

This proposed mechanism appears to be conserved in rat, mouse, and human glycogen, but with some variation in the precise amounts of glycogenin and in the distribution of α and β particles. However, the physiological regulation of this process is unclear. Glycogen α particles are more fragile in db/db mice than in healthy mice, and it is possible that altered glycogenin interactions play a role in this process.

In this investigation, none of the animals or humans had symptoms of diabetes; applying the same methods to diabetic samples will be the subject of future work.

There is evidence that α particles can be present in muscle glycogen (including cardiac muscle), so although there are functional and structural differences between muscle and liver glycogen, some of the findings on liver glycogen from the present work may also be relevant to muscle glycogen.

3. CONCLUSIONS

We have provided evidence consistent with a model in which the protein glycogenin is the binding glue that joins β particles together to form large α particles in liver glycogen in healthy rats, mice, and humans. This mechanism may provide a novel avenue for pharmacological interventions to improve glucose control in diabetes because glycogen α particles are more fragile in db/db mice than in healthy mice.

4. METHODS

4.1. Animals. Samples were obtained from C57BL/6J +/+ female mice (n = 6), Wistar rats (n = 7), and healthy human liver (n = 4), as described elsewhere. Mice were 8 weeks old when purchased from Hubei Provincial Food and Drug Safety Center (Hubei, China). Mice and rats were kept in a standard specific pathogen-free room with a 12 h dark/light cycle (lights on at 7 am), with the temperature set at 22 ± 1 °C. Animals had ad libitum access to standard chow (6% energy from fat, 14.3 MJ kg⁻¹, Hubei Provincial Center for Disease Control and Prevention) and water. All animals were sedated and humanely euthanized before removal of the liver, which was then excised rapidly, snap-frozen in liquid nitrogen, and stored at −80 °C. This study was carried out in strict accordance with the "Animal Research: Reporting of In Vivo Experiments" (ARRIVE) guidelines. The protocol was approved by the Huazhong University of Science and Technology Tongji Medical College Animal Care and Ethics Committee (IORG no. 0003571). For glycogen obtained from humans, all of whom needed liver surgery, no patients had diabetes or insulin resistance. Briefly, all patients were fasted for at least 8 h before surgery, and tissue was taken for further pathological examination from patients during surgery and was snap-frozen in liquid nitrogen. This conformed to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a priori approval by the Huazhong University of Science and Technology Tongji Medical College Animal Care and Ethics Committee (IORG no. 0003571). Patients gave informed written consent as part of their consent to undergo surgery.

4.2. Glycogen Extraction and Purification. Liver glycogen was prepared and extracted, as previously described. Briefly, 1.5 g of liver tissue was resuspended in...
250 mL of glycogen isolation buffer (50 mM Tris, pH 8, 150 mM NaCl, 2 mM EDTA, 50 mM NaF, and 5 mM sodium pyrophosphate), homogenized, and clarified by centrifugation at 6000g to obtain crude extracts. Supernatants were subsequently ultracentrifuged at 370 000g. Pellets were then separated by centrifugation through a sucrose gradient (37.5% and 75%) at 370 000g to enrich glycogen. Further purification of glycogen followed the method of Tan et al.\textsuperscript{28} using preparative aqueous SEC (as described below) to remove contaminant glycogen-associated proteins not strongly bound to the glycogen.

### 4.3. Size-Exclusion Chromatography

Aqueous SEC was performed, as previously described.\textsuperscript{11} Briefly, all glycogen samples were dissolved in SEC eluent (50 mM NH\textsubscript{4}NO\textsubscript{3} with 0.02% sodium azide) in a thermomixer at 80 °C overnight. Samples (2.2 mL) with 2 mg/mL total glycogen were injected manually into an AF 2000 SEC instrument (Postnova Analytics, Landsberg-Lech, Germany), using a set of preparative columns: SUPREMA precolumn, 30 and 3000 (Polymer Standards Services, PSS, Mainz, Germany) in a column oven at 80 °C at a flow rate of 1.5 mL/min with refractive index detection. The 80 °C temperature is required for efficient solubilization of glycogen. Glycogen α and β particles remained intact after this treatment, meaning that the logic of our analytical workflow is robust. These conditions may well result in loss of less tightly bound proteins, but these are not required for α-particle formation. Samples were collected at appropriate elution times to obtain two size-separated samples, one with more tightly bound proteins, but these are not required for α-particle formation. Samples were collected at appropriate elution times to obtain two size-separated samples, one with more tightly bound proteins, but these are not required for α-particle formation. Samples were collected at appropriate elution times to obtain two size-separated samples, one with more tightly bound proteins, but these are not required for α-particle formation. Samples were collected at appropriate elution times to obtain two size-separated samples, one with more tightly bound proteins, but these are not required for α-particle formation.

### 4.4. Glycogen Content Analysis

Lyophilized glycogen samples from eluents of preparative SEC were dissolved in 250 μL of deionized water, from which an aliquot of 50 μL was taken for analysis using glucose oxidase/peroxidase reagent (GOPOD, Megazyme, Ireland). The reaction mixture (500 μL) [5 μL of amyloglucosidase (Megazyme), 20 μL of glycogen sample, 100 μL of sodium acetate buffer pH 6, and 375 μL of deionized water] was incubated on a thermomixer at 50 °C for 30 min. A 300 μL aliquot was placed in 1 mL of GOPOD and incubated for 30 min at 50 °C on a thermomixer. The absorbance at 510 nm was measured with a UV-1700 spectrophotometer (Shimadzu), and the glycogen content was calculated from a calibration curve created by reacting a range of concentrations of β-glucose with GOPOD reagent. All samples were analyzed in triplicate.

### 4.5. Mass Spectrometry

Samples enriched in α or β particles were diluted to equal glucose-equivalent concentrations in a final volume of 200 μL of 100 mM MOPS buffer pH 7 and separated into two equal aliquots. One microliter of α-amylase (≥1000 units/mg protein, Sigma-Aldrich, diluted 100X) was added to one aliquot, and both were incubated at 37 °C for 1 h. DTTO (Sigma-Aldrich) at a final concentration of 10 mM and 0.5 μg of trypsin (Sigma-Aldrich) were added to each sample and incubated at 37 °C overnight. The resulting peptide solutions were then desalted using C18 ZipTips (Millipore).

Peptides were analyzed by Information Dependent Acquisition and SWATH-MS LC-ESI-MS/MS using a Prominence nanoLC system (Shimadzu) and a TripleTOF 5600 mass spectrometer with a NanoSpray III Interface (SCIEX), as previously described.\textsuperscript{29,30} Sample injection order was randomized. Peptides were identified, as described previously,\textsuperscript{31} using ProteinPilot (SCIEX), searching the UniProt database with parameters: sample type, identification; cysteine alkylation, none; instrument, TripleTOF 5600; species, none; ID focus, biological modifications; enzyme, trypsin; search effort, thorough ID. False discovery rate analysis using ProteinPilot was performed on all. Peptides identified with greater than 99% confidence and with a local false discovery rate of less than 1% were included for further analysis. The ProteinPilot data were used as ion libraries for SWATH analyses.\textsuperscript{32} The abundance of peptides and proteins was measured automatically by PeakView v2.1 software (SCIEX). The accuracy of peak selection by PeakView was manually confirmed in each sample. Statistical comparison of the abundance of glycogenin normalized to trypsin (because the same amount was added to every sample) was performed with MSstats in R, as previously described.\textsuperscript{33} The amount of trypsin used does not affect our quantitative result, as we did not overload the capacity of the LC column or the MS detector in our SWATH method. The mass spectrometry proteomics data have been deposited to the ProteomExchange Consortium via the PRIDE partner repository with the dataset identifier PXD009140 (username: reviewer94902@ebi.ac.uk; password: UCqm45T).\textsuperscript{34}

### ASSOCIATED CONTENT

#### Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsomega.8b00119.

- Proteins identified by LC-MS/MS in glycogen-enriched samples; total glucose content for rat samples; total glucose content for mouse samples; total glucose content for human samples; and overview of mass spectrometry proteomics workflow (PDF)
- Complete information on proteins detected in the proteomic analysis for rat samples (XLSX)
- Complete information on proteins detected in the proteomic analysis for mouse samples (XLSX)
- Complete information on proteins detected in the proteomic analysis for human samples (XLSX)

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**Notes**

The authors declare no competing financial interest.

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