Processivity and Subcellular Localization of Glycogen Synthase Depend on a Non-catalytic High Affinity Glycogen-binding Site*§

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Adelaida Díaz1,2, Carlos Martínez-Pons1,3, Ignacio Fita4, Juan C. Ferrer1, and Joan J. Guinovart1,3*†§

From the 1Institute for Research in Biomedicine, the 2Institut de Biologia Molecular de Barcelona, Consejo Superior de Investigaciones Científicas, and the 3Departament de Bioquímica i Biologia Molecular, Universitat de Barcelona, 08028 Barcelona and 4CIBER de Diabetes y Enfermedades Metabólicas, 08017 Barcelona, Spain

Glycogen synthase, a central enzyme in glucose metabolism, catalyzes the successive addition of α-1,4-linked glucose residues to the non-reducing end of a growing glycogen molecule. A non-catalytic glycogen-binding site, identified by x-ray crystallography on the surface of the glycogen synthase from the archaeon Pyrococcus abyssi, has been found to be functionally conserved in the eukaryotic enzymes. The disruption of this binding site in both the archaeal and the human muscle glycogen synthases has a large impact when glycogen is the acceptor substrate. Instead, the catalytic efficiency remains essentially unchanged when small oligosaccharides are used as substrates. Mutants of the human muscle enzyme with reduced affinity for glycogen also show an altered intracellular distribution and a marked decrease in their capacity to drive glycogen accumulation in vivo. The presence of a high affinity glycogen-binding site away from the active center explains not only the long-recognized strong binding of glycogen synthase to glycogen but also the processivity and the intracellular localization of the enzyme. These observations demonstrate that the glycogen-binding site is a critical regulatory element responsible for the in vivo catalytic efficiency of GS.

Glycogen is a polymer of α-1,4- and α-1,6-linked glucose residues that is synthesized in the cytoplasm of living organisms of the three domains: archaea, bacteria, and eukarya. It is an energy reserve compound whose design is optimized to permit the storage of large amounts of glucose without causing a significant increase in the osmolarity of the cell and the rapid mobilization of glucose units when energy demand is high (1). The conservation of this metabolic pathway during evolution points to its crucial role in the carbon/energy metabolism of the cell. In higher eukaryota, glycogen synthesis and utilization is fundamental for global glucose homeostasis. Polymerization is performed by glycogen synthase (GS), which catalyzes the formation of α-1,4-glycosidic bonds using UDP-Glc or ADP-Glc as the glucosyl donor, whereas the branching enzyme is responsible for introducing the α-1,6-linked branches every 11–14 glucose residues. The corresponding catabolic enzymes are glycogen phosphorylase (GP), which catalyzes the phosphorolysis of the polymer, and the debranching enzyme, responsible for eliminating the branching points.

In eukaryotic organisms, glycogen synthase activity, which in most cases is rate-limiting for the synthesis of the polysaccharide, is highly regulated by phosphorylation and allosteric activation by glucose 6-phosphate. How this regulation is brought about in yeast GS-2 has been unveiled very recently through the determination of the x-ray structures of the free and the glucose 6-phosphate-bound enzyme (2). Human muscle glycogen synthase (HMGS) has nine serine residues, the phosphorylation of which leads to the inactivation of the enzyme (3). This enzyme also shows a glycogen-dependent subcellular localization; in cells containing glycogen, it is found in the cytoplasm, bound to the polysaccharide, but when glycogen deposits are exhausted, it translocates to the nucleus (4). It has been proposed that glycogen acts as a cytosolic retention factor for HMGS (5) and for yeast GS (6).

Many of the enzymes that are involved in the glycogen metabolism possess, in addition to their catalytic sites, distinct non-catalytic carbohydrate binding modules (CBMs) or specific glycogen-binding sites (7), which provide these enzymes with high affinity for the polysaccharide. Thus, both the glycogen branching and the debranching (8) enzymes have CBMs appendaged to the N- and C-terminal regions, respectively. The CBM present in glycogen branching enzyme is similar to that found in several glycosidases and has been defined as a glycogen binding module (9). In GP, a glycogen-binding site was identified from crystallographic binding studies with maltopentaose.

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The atomic coordinates and structure factors (code 3L01) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1–S5.

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3 Recipient of Predoctoral Fellowship AP2005-3078 from the Ministerio de Educación.
4 To whom correspondence should be addressed: Baldori i Reixac 10, 08028 Barcelona, Spain. Tel.: 34–934037163; E-mail: guinovart@irbbarcelona.org.

5 The abbreviations used are: GS, glycogen synthase; EcGS, E. coli GS; PaGS, P. abyssi GP, glycogen phosphorylase; HMGS, human muscle glycogen synthase; CBM, carbohydrate binding module; TRITC, tetramethylrhodamine; PaGSm, monomeric PaGS; FRAP, fluorescence recovery after photobleaching; saHMGS, superactive HMGS.
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and maltoheptaose, which also bind to the catalytic site and act as substrates (10). The precise role of this site, called the “glycogen storage site,” is not fully understood. In addition to the enzymes directly responsible for glycogen synthesis and degradation, other proteins with regulatory functions on these pathways are also known to bind to this polysaccharide. For example, AMP-activated protein kinase, a known sensor of the AMP/ATP levels, also acts as glycogen sensor through a CBM present in its β-subunit (11). Protein phosphatase 1, the primary phosphatase acting on the key phosphorylation sites of GS, also in close association with GP, has several glycogen targeting subunits, each containing a CBM in its respective sequence (12). Laforin, a protein identified in studies of Lafora disease that is involved in a novel regulatory mechanism of glycogen deposition through the control of GS levels (13), also holds a CBM that allows the enzyme to bind to glycogen (14).

It is now generally accepted that the glycogen particle is a complex organelle. The polysaccharide is not only the substrate and product of the proteins that participate in its synthesis and degradation but also is the scaffold upon which enzymes involved in glycogen metabolism, proteins involved in their regulation and other proteins (15), establish intricate interrelations. A crucial aspect of the coordinated regulation of glycogen metabolism is precisely the capacity of these enzymes to remain bound to the polysaccharide. Curiously, however, no CBM or glycogen-binding site has been described for GS. Recently, an inactive mutant of Escherichia coli GS (EcGS) crystallized in the presence of maltopentaose and maltohexaose was shown to bind malto-oligosaccharides at the acceptor substrate-binding site plus three other sites at the N-terminal domain surface (16). In this report we identify and structurally characterize a glycogen-binding site present on the surface of the GS from Pyrococcus abyssi (PaGS), a homotrimeric protein (17) in which each subunit exhibits the characteristic GT-B fold: two Rossmann-fold domains with the catalytic center located in a deep cleft between them (18–20). We show that this glycogen-binding site is conserved in eukaryotic GSs; furthermore, we analyzed its functional role and demonstrate that disruption of this binding site in GS from human muscle alters its intracellular distribution and, more importantly, has a profound effect on its efficiency to synthesize glycogen both in vitro and in vivo.

EXPERIMENTAL PROCEDURES

Expression Construct—The full-length PaGS cDNA (21) was cloned into the Ndel/Sall sites of pCold I (TaKaRa) (22), an expression vector that adds an N-terminal His₆ tag to the expressed protein. The monomeric form of PaGS, lacking the 11 C-terminal residues (Gly-427—Leu-437), was constructed (QuikChange, Stratagene) by replacing Gly-427 by a stop codon. A T426A mutation was also introduced to generate an inactive mutant of PaGS. The Y174A mutant of PaGS was obtained by co-crystallization with 10 mM UDP-glucose and 10 mM maltohexaose using the sitting drop vapor diffusion method at 4 °C. The drops were prepared with a robot (Cartesian) in 96-well crystallization plates by the mixture of enzyme (0.1 μl) at 5 mg/ml with the reservoir solution (0.1 μl) containing 2.2 mM ammonium sulfate and 0.2 M potassium chloride. Crystals appeared after 12 days and continued to grow for about 2 months. Crystals were flash-cooled by immersion in liquid nitrogen using 20% glycerol in mother solution as cryoprotectant.

Production of PaGS—HEK293A cells (Invitrogen) were seeded on five 150-mm diameter culture dishes and transfected with pDEST/N-SF-TAP_HMGS constructs (WT, Y239A, or Y242A). The first day post-transfection, the medium was changed to complete DMEM supplemented with 10% FBS containing 10 mM UDP-glucose and 10 mM maltohexaose. The cells were harvested by centrifugation at 6000 × g and resuspended in 50 mM Tris- HCl, pH 7.4, containing 1 mM PMSF and 5 mM 2-mercaptoethanol. Cell lysis was performed by a freezing-thawing cycle after treating the suspension with 1 mg/ml lysozyme at 4 °C during 1 h. DNase I (0.05 mg/ml) and 1 mM magnesium chloride were added to the homogenate, and after 1 h of stirring, the soluble fraction was separated by centrifugation at 15,000 × g and filtered through a 0.22-μm membrane (Millipore). Cell extract was loaded onto a nickel affinity column (HisTrap HP, GE Healthcare) equilibrated with 50 mM Tris-HCl, pH 7.4, plus 5 mM 2-mercaptoethanol at room temperature. The column was washed, and the recombinant protein was eluted with a linear gradient of 0–150 mM imidazole. Fractions with the enzyme were concentrated with Centriprep YM-30 (Millipore) and loaded on a Mono Q 5/50 GL anion exchange column (GE Healthcare). The enzyme was eluted with a linear gradient of 0–1 M NaCl, concentrated and loaded on a Superdex 200 gel filtration column (GE Healthcare). Column fractions were analyzed by SDS-PAGE, and fractions with the highest concentration of enzyme were pooled and concentrated (supplemental Fig. S1A). The protein concentration of purified enzymes was determined with the Bradford dye reagent (Bio-Rad) (23). The identity of the recombinant enzymes and the absence of post-translational modifications were confirmed by sequencing of the N terminus and by electrospray mass spectrometry. Crystals from the monomeric PaGS were obtained by co-crystallization with 10 mM UDP-glucose and 10 mM maltohexaose using the sitting drop vapor diffusion method at 4 °C. The drops were prepared with a robot (Cartesian) in 96-well crystallization plates by the mixture of enzyme (0.1 μl) at 5 mg/ml with the reservoir solution (0.1 μl) containing 2.2 mM ammonium sulfate and 0.2 M potassium chloride. Crystals appeared after 12 days and continued to grow for about 2 months. Crystals were flash-cooled by immersion in liquid nitrogen using 20% glycerol in mother solution as cryoprotectant.
replaced by fresh Dulbecco’s modified Eagle’s medium (DMEM) cell culture medium supplemented as before. On the second day post-transfection, the medium was replaced by supplemented DMEM but with 1% fetal bovine serum (FBS) and without glucose, and cells were left to incubate overnight. The following day recombinant enzymes were purified using a variation of a previously described method (24). Cell culture medium was removed, and 1 ml of cold lysis buffer (30 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.5% Nonidet P-40, 1 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride, 25 mM okadaic acid, 10 µg/ml leupeptin, 10 µg/ml aprotinin, and 10 µg/ml pepstatin, 20% glycerol) was added per dish. Cells were collected using cell scrapers and incubated in lysis buffer for 20 min in an orbital shaker at 4 °C and low speed. The extract was then centrifuged for 10 min at 5000 g. The supernatant was then collected and passed through a 0.45-µm filter. Next, 400 µl of Strep-Tactin Superflow Resin (IBA Tool) was added to the extract, and it was incubated overnight at 4 °C using an overhead tumbler. The following day cell extract with resin was centrifuged at 1000 × g and 4 °C for 5 min. The resin was transferred to an Eppendorf tube, where it was washed 5 times using 1 ml of cold wash buffer (30 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% Nonidet P-40, 20% glycerol) and transferred to a microspin column (GE Healthcare). The resin was washed five times using 1 ml of cold TBSG buffer (30 mM Tris-HCl, pH 7.4, 150 mM NaCl, 20% glycerol). The resin was then incubated for 10 min with elution buffer (2.5 mM desthiobiotin, 30 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 20% glycerol), and protein was eluted. Finally, we increased the glycerol and NaCl content up to 50% and 1 M, respectively, to prevent protein aggregation. Protein was flash-frozen with liquid nitrogen and stored at −80 °C. Purified HMGs were quantified following the method described by Bradford (23) and analyzed by SDS-PAGE (supplemental Fig. S1B).

Crystal Structure Determination—Monomeric PaGS was co-cryrstallized with UDP-glucose and maltotetraose by the vapor diffusion method. A diffraction data set collected from a flash-cooled crystal at the BM16 beam line of the European Synchrotron Radiation Facility was integrated with MOSFLM and scaled with SCALa of the CCP4 program suite (25).

The structure was determined by molecular replacement using the available coordinates of trimeric PaGS (17). The search, performed with MOLREP (25), was done first using the PaGS N-terminal domain (residues 1–217) and then completed with the C-terminal domain (residues 218–413). The model was improved by rigid body refinement and alternating cycles of automatic and manual refinement to a final Rcryst of 19.8% (Rfree of 22.8%, calculated with 5% of the data randomly selected) at 2.6 Å resolution (Table 1). Solvent molecules were generated automatically with ARP/wARP (25) and visually checked with COOT (26). At the end TLS (translation/libration/screw) by subunits and domains was performed. The final structure displayed good stereochemistry, as analyzed by PROCHECK (27).

Structural figures were made with PyMOL (DeLano Scientific LLC, San Carlos, CA).

Glycogen Synthase Activity Assays—GS activity was determined at several temperatures using two methods: (i) a radioactive method that measures the incorporation of radiolabeled glucose onto glycogen (28) and (ii) a fluorescent method that measures the amount of UDP released in the reaction via its conversion to ADP by the action of nucleoside 5′-diphosphate kinase. Measurements were performed in 384-multiwell plates containing reaction buffer (33 mM Tris-HCl, pH 7.4, 16.6 mM KCl, 10 mM MgCl₂, 1 mM EGTA, 200 µM ATP, 4.5 mM UDP-glucose, 6.7 mM glucose 6-phosphate, 1 unit/µl nucleoside 5′-diphosphate kinase), purified GS, and 50 mM maltotetraose as acceptor substrate. After 10 min at 37 °C, the resultant ADP was measured using ADP Hunter Plus kit (Discoverx).

Co-sedimentation with Glycogen—The procedure is a modification of a previously published protocol (29). 4 µg of purified PaGSmon or the Y174A mutant were incubated in 0.4 ml of co-sedimentation buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% (v/v) 2-mercaptoethanol, 0.1 mg/ml bovine serum albumin) with or without 10 mg/ml rabbit liver glycogen at 4 °C for 30 min. After centrifugation at 100,000 × g for 90 min, the supernatant was collected, and the pellet fraction was incubated for 1 h with 200 µl of co-sedimentation buffer supplemented with 22 units/ml amylase at 30 °C. 10 µl of each fraction was subjected to SDS-PAGE, and the resultant gel was stained with InstaBlue (Expedeon).

Transfection—HEK293A cells were transfected using polyethyleneimine (Polysciences). For each 150-mm diameter culture dish we used a mixture of 20 µg of plasmidic DNA with 175.5 µl of 1 mg/ml polyethylenimine in a total volume of 2.5 ml of 150 mM NaCl. The mixture was incubated for 10 min at room temperature and then added to the cell culture dish containing 20 ml DMEM supplemented with 2 mM l-glutamine, 25 µM non-essential amino acids, 20 µM β-mercaptoethanol, 100 U/ml penicillin, 100 µg/ml streptomycin, and 5% FBS. 

TABLE 1

| Data collection       | P2,2,2,1 |   |
|-----------------------|----------|---|
| Space group           |          |   |
| a (Å)                 | 103.6    |   |
| b (Å)                 | 119.0    |   |
| c (Å)                 | 141.1    |   |
| Resolution (Å)        | 25.0-2.6 |   |
| Rsym (%)              | 19.8     |   |
| Rfree (%)             | 22.8     |   |
| Number of atoms       |          |   |
| Protein               | 6788     |   |
| Maltotetraose         | 90       |   |
| Glycogen              | 24       |   |
| Potassium             | 6        |   |
| Chloride              | 6        |   |
| Water                 | 97       |   |
| B-factors (Å²)        |          |   |
| Protein               | 16.4     |   |
| Maltotetraose         | 88.8     |   |
| Glycogen              | 78.2     |   |
| Potassium             | 77.6     |   |
| Chloride              | 64.7     |   |
| Water                 | 37.4     |   |
| Root mean square distance | 0.022     |   |
| Bond angles (degrees) |          |   |
| Protein               | 1.98     |   |

Values in parentheses are for the highest resolution shell.

* Rmerge = Σ[Σ(hkl) - (f(hkl))/Σ(f(hkl))].

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mm D-glucose, 10% (v/v) FBS, 100 units/ml penicillin, and 100 mg/ml streptomycin. Cells were left to transfect overnight. HeLa cells were transfected using Lipofectamine 2000 (Invitrogen) following the manufacturer’s protocol.

**Immunocytochemistry**—HeLa cells were seeded on glass coverslips, transfected, and left overnight with DMEM supplemented as usual. At 24 h post-transfection, cells were fixed using 4% paraformaldehyde in PBS for 20 min and rinsed three times with PBS. After fixation, they were then incubated with NaBH₄ (1 mg/ml) for 10 min and permeabilized for 20 min with PBS containing 0.2% (v/v) Triton X-100. Blocking and incubation with the primary and secondary antibodies were carried out as previously described (5). Coverslips were washed, air-dried, and mounted onto glass slides using Mowiol as mounting medium. A monoclonal antibody against glycogen (a gift from O. Baba, Tokyo Medical and Dental University) was used (30), and tetrarmethylrhodamine (TRITC)-conjugated goat anti-mouse IgM was used as a secondary antibody (Chemicon). Nuclei were stained with DAPI (Sigma). Images from the resulting preparations were obtained under a Leica SP2 Spectral microscope.

**Fluorescence Recovery after Photobleaching (FRAP)**—For FRAP experiments, a monolayer of HeLa cells was grown on a MatTek glass-bottomed dish and transfected with either the wild-type or Y242A GFP-HMGS construct. At 24 h post-transfection, FRAP experiments were performed using a Leica SP5 microscope. During the experiment cells were kept at 37 °C with 5% CO₂ in supplemented DMEM medium. A 63×1.4 oil objective lens and a 488-nm argon laser line were used to acquire images of 512 x 512 pixels at 1000 Hz, with line averaging x4, 4-airy units pinhole opening, and a minimum acquisition rate of 1.1 s/frame. To assess the affinity of the HMGS constructs for glycogen, a whole cytoplasmic glycogen particle and the correspondent bound GFP-HMGS were photobleached. The typical FRAP sequence was acquired as follows: 10 pre-bleaching images at 1.1 s/frame (relative laser power 5%), one bleaching scan (relative laser power 100%, scan time about 1.1 s), and post-bleaching acquisition of 100 images at 1.1 s/frame followed by a slower rate acquisition of 50 images every 5 s. Images were corrected for cell compartment movement and rotation using ImageJ (W. Rasband, NIH) complemented with the specific algorithm plug-in “StackReg” (31). Fluorescence intensity was quantified in FRAP and in up to six control regions of interest. Region of interest data were background-subtracted, and FRAP curves were “double-normalized” as described elsewhere (32). FRAP curves were successfully fitted with double exponential fitting for the GFP-HMGS Y242A construct using IgorPRO6 software complemented with the K_FRAPcalc version 9 procedure (Kota Miura, EMBL-Heidelberg, Germany).

**Glycogen Measurement**—HeLa cells were seeded on 60-mm dishes and transfected. At 24 h post-transfection, culture medium was replaced by DMEM without glucose but with 1% (v/v) FBS, and cells were incubated overnight. The following day some dishes were flash-frozen in liquid nitrogen to zero-time values. Then medium was replaced by DMEM supplemented as previously but with 25 mm D-glucose. After 2-h incubation, dishes were flash-frozen in liquid nitrogen. Glycogen content was determined using an amyloglucosidase-based assay, as described elsewhere (33).

**Electrophoresis and Immunoblotting**—HeLa cells were seeded on 60-mm dishes and transfected. At 24 h post-transfection, culture medium was replaced by supplemented DMEM but without glucose and with 1% (v/v) FBS, and cells were incubated overnight. The following day cells were flash-frozen in liquid nitrogen and scraped using 100 μl of ice-cold homogenization buffer consisting of 10 mM Tris-HCl, pH 7.4, 150 mM potassium fluoride, 15 mM EDTA, 15 mM 2-mercaptoethanol, 0.6 M sucrose, 1 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride, 25 mM okadaic acid, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 10 μg/ml pepstatin. The extracts were passed 10 times through a 25-gauge needle. Protein concentration was measured following the method described by Bradford (23) using a Bio-Rad assay reagent. 20 μg of each homogenate was resolved by 10% SDS-PAGE, transferred onto a PVDF membrane, and probed with a rabbit antibody against GFP (Immunocontact) and a mouse monoclonal antibody against actin (Sigma). Secondary antibodies conjugated to horseradish peroxidase against rabbit (GE Healthcare) or mouse (Dako Cytomation) immunoglobulins were used. Immunoreactive bands were visualized using an ECL Plus kit (GE Healthcare) following the manufacturer’s instructions.

**RESULTS**

**The Monomeric Form of PaGS**—The PaGS trimer is stabilized almost exclusively by the interactions, mainly hydrophobic, between the 11 C-terminal residues (Gly-427—Leu-437) (supplemental Fig. S2) of one subunit and the residues that line a cavity in the N domain of the neighboring subunit. Removal of these interactions in a PaGS variant lacking these 11 C-terminal residues resulted in a monomeric protein (PaGSmon) (Fig. 1A), as indicated by gel filtration analysis. The lower complexity of PaGSmon possibly facilitated its recombinant expression, which gave a 10-fold higher yield than that of the wild type and was instrumental in the attainment of complexes of the enzyme with its substrates (see below). At 37 °C, PaGSmon exhibited a similar specific activity to that of the wild-type trimeric enzyme, as measured by a radioactive method that uses glycogen as the acceptor substrate (28) (Table 2 and supplemental Fig. S1A). However, although the specific activity of wild-type PaGS increased by 38-fold at 80 °C (the optimal temperature for the enzyme), that of PaGSmon increased only 7-fold. This difference is possibly due to the reduced thermal stability of the monomeric variant, caused by exposure to the solvent of the hydrophobic cavity in the N-terminal domain that participates in the oligomerization interactions.

The structure of crystals of PaGSmon obtained in the presence of UDP-glucose and maltohexaose was solved by molecular replacement and refined at a resolution of 2.6 Å (Table 1). The crystal asymmetric unit contained two subunits, each including residues Met-1 to Ala-426, which like the wild-type enzyme, have only residue Asp-128 in a disallowed region of the Ramachandran plot and a disulfide bridge between Cys-221 and Cys-350. The crystallographic structure confirmed the monomeric organization of this PaGS variant, which is otherwise practically identical to the open conformation reported for subunit A in the trimeric enzyme (17), with a root mean square distance of only 0.6 Å. Although the enzyme was crystallized in...
the presence of UDP-glucose and maltohexaose, the active site showed electron density that could not be attributed to these substrates but to two glycero molecules and to one Cl\textsuperscript{−} ion and one K\textsuperscript{+} ion (Fig. 1A and supplemental Fig. S3). Superposition with the structure of the inactive E377A variant of EcGS in complex with maltotriose (16) (PDB code 3CX4) at the acceptor-binding site showed that the glycerol molecules found in PaGSmon are in almost the same position as the maltotriose molecule bound to EcGS and that the glycerol oxygen atoms are arranged in the same way as those of maltotriose (supplemental Fig. S3B). Moreover, the Cl\textsuperscript{−} and K\textsuperscript{+} ions found in the PaGSmon structure occupy similar positions to those of the phosphate groups from the ADP in the EcGS complex. Both Cl\textsuperscript{−} and K\textsuperscript{+} ions are bound to the C-terminal domain face of the active site, whereas glycero molecules bind on the N-terminal domain face, mimicking the binding of the acceptor oligosaccharide molecule but without triggering the closed conformation of the enzyme.

A Novel Glycogen-binding Site of PaGS—Interestingly, despite the absence of bound maltohexaose in the active site, the electron density map showed four glucose units of a maltohexaose molecule bound to the surface of the N-terminal domain of PaGSmon (Fig. 2A), away from the active site (over 20 Å from the essential His-151). This external binding site is composed of residues from the entrance of strand β8 and from the loops α5/β10 and α6/αβ, which make six hydrogen bonds with the bound glucose units, three to the backbone of Leu-171, Asp-176, and Asp-178, and three to the side chains of Lys-157 and Thr-182 (Fig. 1, B and C). Binding is also stabilized by stacking interactions with Pro-173, His-181, Tyr-185, and in particular with the side chain of Tyr-174, which acts as a reel around which the four glucose units coil. No crystal packing interactions appear to influence the binding of the glucose residues, which have exactly the same organization in the two subunits of the crystal asymmetric unit.

When superimposed onto the structure of the trimeric wild-type PaGS, the oligosaccharide-binding sites of the three PaGSmon subunits cluster close to the molecular three-fold axis in the upper face of the trimer (supplemental Fig. S2). Superposition of the structures of malto-oligosaccharide-bound PaGSmon, EcGS (16), and rabbit muscle GP (10) reveal that the three enzymes bind the malto-oligosaccharides in structurally equivalent positions (Fig. 3, A and B). In these enzymes, the binding geometry of the malto-oligosaccharides and the precise location of the binding sites within the respective N-terminal domains are more conserved than the amino acids that comprise these sites (Figs. 1C and 2A–C). Another oligosaccharide binding site has been reported in EcGS (16). Although this site is structurally conserved in PaGS,
we did not observe electron density attributable to an oligosaccharide bound to the PaGSmon molecule.

We next performed experiments of co-sedimentation with glycogen. PaGSmon and a mutant in which Tyr-174 had been replaced by an Ala residue (Y174A PaGSmon) were ultracentrifuged in the presence of 10 mg/ml rabbit liver glycogen. In these conditions, PaGSmon was found mainly in the pellet, as determined by SDS-PAGE, whereas the Y174A variant was essentially in the supernatant. As a control, both enzymes were detected in the supernatant when centrifuged in the absence of glycogen (Fig. 4A). This result together with the structure of the oligosaccharide-PaGSmon complex strongly suggests that the affinity of this non-catalytic glycogen-binding site for the polysaccharide is greater than that of the acceptor-binding site in the catalytic cleft of the enzyme. We attempted the crystallization of Y174A PaGSmon in the presence of maltohexaose and/or UDP-glucose in several conditions, but we were unable to obtain crystals of any complex of the mutant enzyme.

Although Y174A PaGSmon was obtained with the same purity as PaGSmon (supplemental Fig. S1A), their specific activities differed substantially as determined by the radioactive method that measures the incorporation of [14C]glucose from labeled UDP-glucose into glycogen (28). At 37 °C Y174A PaGSmon was 4 times less active than PaGSmon, and this difference increased up to 14 times at 80 °C (Table 2). To determine whether the effect of the Y174A mutation on the PaGS activity is due to the loss of catalytic competence of the enzyme or rather to a change in the way it specifically interacts with glycogen, we developed a new fluorescent method to evaluate the glycosyltransferase activity of PaGS that uses small oligosaccharides as acceptor substrates. This method (validated as shown in supplemental Fig. S4) measures UDP released from UDP-glucose by the GS-catalyzed reaction using maltohexaose as the glucose acceptor. This substrate is too short to interact simultaneously with the acceptor site at the catalytic cleft and with the non-catalytic glycogen-binding site, although separate molecules can. PaGSmon and Y174A PaGSmon displayed similar specific activities when 50 mM maltohexaose was used as the acceptor substrate (Table 2), indicating that the active site of the enzyme is not affected by the Y174A mutation.

The Glycogen-binding Site of Human Muscle GS—The central residue of the PaGS glycogen-binding site, Tyr-174, is not conserved in bacterial GSs but could be sequence-aligned (Fig. 1C) with a Tyr residue found in all eukaryotic GSs analyzed, in agreement with the phylogenetic relatedness between archaeal and eukaryotic GSs (34). Furthermore, in the eukaryotic enzymes, this Tyr residue, Tyr-239 in HMGS, is located within a highly conserved region that in the recently available yeast GS structure (2) superimposes well with the external binding site of PaGS (Figs. 2D and 3C).
To check whether Tyr-239 is part of a glycogen-binding site in HMGS, we generated two variants of the enzyme in which Tyr-239 or the nearby conserved Tyr-242 were replaced by an Ala residue. Both variants together with the wild-type HMGS were expressed in HEK293A from plasmids that fuse one FLAG and two Strep-Tag II epitopes at the N terminus of the coding region of the enzymes (24). Recombinant enzymes were purified as described under “Experimental Procedures” (supplemental Fig. S1B), and their specific activities were measured by the radioactive method using glycogen as the acceptor molecule. The Y239A and Y242A variants showed 55 and 59% that of the wild-type HMGS specific activity, respectively (Table 3). Also, using the radioactive method, we measured the initial reaction rates of the wild-type HMGS and of the Y239A and Y242A mutants for varying concentrations of glycogen. The data were fitted to the Michaelis-Menten equation (supplemental Fig. S5), and the apparent \( K_m \) values for glycogen of the three enzymes were calculated. The Y242A mutation increased 3.5-fold the \( K_m \) of the enzyme for glycogen, whereas the Y239A mutation resulted in a more than a 10-fold increment (Table 3). When the specific activity was measured using the fluorescent method and maltohexaose as the glycosyl acceptor, the wild-type HMGS and Y239A variant provided very similar values, whereas the Y242A mutant showed an approximate 25% decrease in specific activity. Taken together, these results suggest that the mutations introduced in HMGS do not significantly alter the glycosyltransferase catalytic activity of the enzyme but, rather, change its affinity for glycogen, thereby indicating that the region around residue 239 is a non-catalytic glycogen-anchoring site of HMGS.

Intracellular Localization of the HMGS Glycogen-binding Mutants—To study the effect of the mutations on the intracellular distribution of the enzyme, we generated the Y239A and Y242A variants of HMGS N-terminally fused to GFP and trans-
fected the constructs into HeLa cells. Confocal microscopy of cells fixed 24 h after transfection showed that GFP-HMGS was located exclusively in the cytoplasm of the cells and excluded from the nucleus. Fluorescent signal concentrated on aggregates that were also labeled with a specific anti-glycogen antibody (30) (Fig. 4B). In contrast, the Y239A mutant was distributed between the cytoplasm and the nucleus. In this compartment the variant enzyme exhibited a speckled pattern, whereas in the cytoplasm it gave a diffuse signal that did not co-localize with glycogen deposits. The Y242A mutant showed an intermediate behavior; most of the green fluorescent signal was found in the cytoplasm, and it co-localized with the glycogen staining, but nuclei also showed a diffuse fluorescent signal. These results suggest that the Y242A mutant has an impaired capacity to bind glycogen, whereas the Y239A mutation drastically decreases the affinity of the glycogen-anchoring site for the polysaccharide.

We performed FRAP analysis to assess the in vivo affinity of wild-type HMGS and the Y242A mutant for glycogen (Fig. 5). It was not possible to estimate the affinity of the Y239A mutant using this method because it did not co-localize with glycogen granules. The recovery after photobleaching of the GFP-HMGS bound to a single glycogen granule was ~30 times slower than that of the Y242A variant. FRAP curves for Y242A GFP-HMGS gave a recovery half-time (τ₁/₂) of 13.2 ± 6.1 s (n = 14 cells) and a mobile fraction (A) of 78 ± 7%, whereas the estimated value of τ₁/₂ for GFP-HMGS was 430 ± 190 s (n = 8 cells). The A value for GFP-HMGS could not be accurately calculated due to the very slow recovery process. The increased exchange rate between the free and glycogen-bound fraction of the Y242A variant of HMGS suggests that its affinity for glycogen is considerably reduced.

In Vivo Glycogen Synthesis—We examined the effect of Y239A and Y242A mutations on the capacity of HMGS to drive glycogen accumulation in vivo. Transient expression of wild-type HMGS in cultured mammalian cells largely results in multiple phosphorylation and, therefore, inactivation of the enzyme. To circumvent the effects of phosphorylation on the catalytic activity of the transiently expressed enzymes, we generated the Y239A and Y242A variants of a superactive form of HMGS fused to GFP (GFP-saHMGS). The GFP-saHMGS construct codes for a protein in which all known phosphorylatable Ser residues have been changed to Ala and produces a highly active form of HMGS, which cannot be inactivated by phosphorylation (5). HeLa cells were transfected with the constructs encoding for soluble GFP or with each one of the GFP-saHMGS forms: wild-type and the Y239A or Y242A variants. Twenty-four hours after transfection, cells were incubated overnight without glucose followed by a 2-h incubation in the presence of 25 mM glucose, and then total glycogen was measured (Fig. 6A).

Similar levels of the three GFP-fusion proteins were expressed as determined by Western blot (Fig. 6B). Differences in glycogen content were very small in the cells incubated overnight without glucose. However, after 2 h in the presence of the monosaccharide, cells transfected with GFP-saHMGS accumulated 4 times more excess glycogen over the GFP-expressing control cells than the GFP-saHMGS Y239A and 2 times more than the Y242A variant.

**DISCUSSION**

The high affinity of GS for glycogen, its substrate, and product has long been recognized. It constitutes a key factor in classical schemes designed for the purification of the enzyme from natural sources (35). This high affinity also explains at least in part the intracellular distribution of eukaryotic GSs, for which
glycogen has been proposed to act as a cytoplasmic retention factor (5, 6). Here we describe and characterize an oligosaccharide-binding site on the surface of the archaeal GS from *P. abyssi* and show that this site binds glycogen with high affinity. Crystallographic analysis of the complex between maltotetraose and a monomeric form of *PaGS* shows that the oligosaccharide binds at the N-terminal domain of the enzyme, curling around the lateral chain of Tyr-174. The location of the glycogen-binding site within the overall structure of *PaGS* is similar to one of the malto-oligosaccharide-binding sites recently described for *E. coli* (16) and, most interestingly, to the glycogen-storage site in GP (10). The similar overall fold and active site architecture of GS and GP have been reported previously (18), and the presence of an analogous glycogen-binding site further strengthens the notion that these two enzymes descend from a common ancestor.

The glycogen-binding site is functionally conserved in eukaryotic GSs. The central residue of the glycogen-binding site in *PaGS*, Tyr-174, aligns with an invariant Tyr residue in the eukaryotic enzymes, Tyr-239 in HMGS, which in turn is found within a well conserved region. In both the archaeal and the human GSs, the glycogen-binding site has higher affinity for the polysaccharide than that of the acceptor site in the catalytic cleft and is fundamental in determining the interaction between glycogen and these enzymes. Thus, although wild-type *PaGS* co-sediments with glycogen, the Y174A variant does not. Although HMGS presents a characteristic cytoplasmatic distribution in the cellular interior and co-localizes with glycogen, the Y239A variant is distributed between the cytoplasm and the nucleus of the cells and does not co-localize with the polysaccharide. The much faster exchange rate between the glycogen-bound and the free fractions of the Y242A variant of HMGS, as determined in the FRAP experiments, and the somewhat altered subcellular distribution of this variant also indicate that this region is responsible for the high affinity of the enzyme for glycogen. It is noteworthy that, although amino acid identity between archaeal and eukaryotic GSs in this region is very low, the glycogen-binding site is functionally conserved in these enzymes.

Disruption of the capacity of GS to interact with glycogen through this site has a substantial effect on the catalytic efficiency of the enzyme when glycogen, the natural substrate, is the glucosyl acceptor. However, when maltotetraose is used as substrate, the activity of the glycogen-binding site mutants of *PaGS* and HMGS is essentially the same as that of the corresponding wild-type enzymes, thereby indicating that these mutations do not adversely affect the overall folding or the catalytic site of the enzymes. Furthermore, this observation also implies that the binding of an oligosaccharide to the glycogen-binding site does not have an allosteric activating effect on the enzymes. If this were the case, the activity of wild-type *PaGS* with maltotetraose should be higher than that of the Y174A variant, as the glycogen-binding site would be occupied by the oligosaccharide in the wild-type enzyme but not in the mutant. Thus, this site apparently serves to maintain GS permanently bound to glycogen, a large branched molecule that can simultaneously interact with the glycogen-binding and the catalytic sites of the enzyme. This has direct consequences on the kinetic parameters of the GS-catalyzed reaction. The apparent *Kₘ* for glycogen of wild-type HMGS is ~14 times lower than the value obtained for the Y239A variant. The Y242A mutant, which shows some intermediate affinity for the polysaccharide at the glycogen-binding site as determined by the subcellular localization and the FRAP experiments, presents an intermediate *Kₘ* value only three times higher than that of the wild-type enzyme. Interestingly, the specific activities of wild-type HMGS and *PaGS* are ~2 and 14 times higher than those of the corresponding glycogen binding mutants. This observation suggests that binding of the enzyme to glycogen through the high affinity site not only has the effect of increasing the local concentration of substrate seen by the enzyme, thus lowering the apparent *Kₘ*, but also makes the GS-catalyzed glucosyl transfer reaction faster, probably by adequately orienting the enzyme with respect to the glucose moieties at the non-reducing ends of the glycogen polymer.

For an enzyme-catalyzed reaction to proceed at a fast rate, the enzyme must be able to quickly release the reaction products, which therefore must not bind too tightly to the catalytic site. For GS and, in general, for enzymes acting on polymeric substrates, this poses a problem because the product of the reaction and the substrate are essentially the same. The way nature has found to overcome this problem in GS and possibly in GP, due to the similar architecture of their respective catalytic sites, consists of using a non-catalytic high affinity glycogen has been described for similar to one of the malto-oligosaccharide-binding sites in eukaryotic GSs. The central residue of the glycogen-binding site in GP (10). The similar overall fold and active site architecture of GS and GP have been reported previously (18), and the presence of an analogous glycogen-binding site further strengthens the notion that these two enzymes descend from a common ancestor. Although HMGS presents a characteristic cytoplasmatic distribution in the cellular interior and co-localizes with glycogen, the Y239A variant is distributed between the cytoplasm and the nucleus of the cells and does not co-localize with the polysaccharide. The much faster exchange rate between the glycogen-bound and the free fractions of the Y242A variant of HMGS, as determined in the FRAP experiments, and the somewhat altered subcellular distribution of this variant also indicate that this region is responsible for the high affinity of the enzyme for glycogen. It is noteworthy that, although amino acid identity between archaeal and eukaryotic GSs in this region is very low, the glycogen-binding site is functionally conserved in these enzymes.
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gen-binding site that compensates for the relatively low affinity that the catalytic site displays for glycogen. This high affinity site makes GS a processive enzyme that remains attached to its polymeric substrate and performs multiple rounds of catalysis without dissociating while still being able to quickly release the product from the active site after each round of catalysis. The advantage of a processive HMGS over a non-processive enzyme is shown in the experiments with cultured cells. There is a correlation between the affinity for glycogen and the capacity to drive the accumulation of the polysaccharide in vivo such that an HMGS with an intact glycogen-binding site makes, after a 2-h incubation with glucose, twice or four times more excess glycogen over the control cells than the Y242A or the Y239A variants, respectively. Because the enzymes used in this experiment had the phosphorylation sites that regulate the catalytic activity mutated into non-phosphorylatable residues, this effect cannot be attributed to different activation states of the variants but rather to the tightness of the binding to glycogen and the processivity of the enzyme. However, although we do not address this issue in the present work, the capacity of GS to remain bound to glycogen undoubtedly has an impact on the regulatory mechanisms that control the enzyme levels (13) or its activity. The regulatory enzymes involved in these mechanisms, such as laforin or PP1, possess carbohydrate binding modules that also direct them to the glycogen molecule (12, 14).

Taken together, our findings offer new insights into the structural bases and functional role of the long-recognized affinity of GS for its substrate. The presence of a high affinity glycogen-anchoring site confers the enzyme properties that are seminal with regard to its catalytic efficiency, its intracellular behavior, and possibly, its regulation within the glycogen granule. These observations also invite a revision of the relevance of non-catalytic binding sites in the functioning of other enzymes involved in glycogen metabolism.

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