Glycogen Synthase Sensitivity to Glucose-6-P Is Important for Controlling Glycogen Accumulation in Saccharomyces cerevisiae

Bartholomew A. Pederson†, Wayne A. Wilson, and Peter J. Roach‡

From the Department of Biochemistry and Molecular Biology, Indiana University School of Medicine and the Indiana University Center for Diabetes Research, Indianapolis, Indiana 46202

Glycogen is a storage form of glucose utilized as an energy reserve by many organisms. Glycogen synthase, which is essential for synthesizing this glucose polymer, is regulated by both covalent phosphorylation and the concentration of glucose-6-P. With the yeast glycogen synthase Gsy2p, we recently identified two mutants, R579A/581A/582A and R586A/588A/591A, in which multiple arginine residues were mutated to alanine that were completely insensitive to activation by glucose-6-P in vitro (Pederson, B. A., Cheng, C., Wilson, W. A., and Roach, P. J. (2000) J. Biol. Chem. 275, 27753-27761). We report here the expression of these mutants in Saccharomyces cerevisiae and, as expected from our findings in vitro, they were not activated by glucose-6-P. The R579A/R581A/R582A mutant, which is also resistant to inhibition by phosphorylation, caused hyperaccumulation of glycogen. In contrast, the mutant R586A/R588A/R591A, which retains the ability to be inactivated by phosphorylation, resulted in lower glycogen accumulation when compared with wild-type cells. When intracellular glucose-6-P levels were increased by mutating the PFK2 gene, glycogen storage due to the wild-type enzyme was increased, whereas that associated with R579A/R581A/R582A was not greatly changed. This is the first direct demonstration that activation of glycogen synthase by glucose-6-P in vitro is necessary for normal glycogen accumulation.

Many organisms utilize glucose as a preferred nutrient (1). After entering a cell through glucose transporters, it is converted to glucose-6-P, which can then be processed through several metabolic pathways, depending on the cell type. Glucose-6-P can enter glycolysis, the pentose phosphate pathway, or the glycogenic pathway. In the last mentioned pathway, glucose-6-P is converted via UDP-glucose to glycogen, which serves as a glucose and energy reserve (2, 3). Consistent with its placement at a metabolic branch point, glucose-6-P has been viewed as an important metabolic regulator (2, 3). It is an allosteric inhibitor of glycogen phosphorylase (5, 6) and an allosteric activator of glycogen synthase (3).

In the budding yeast Saccharomyces cerevisiae, glycogen is synthesized when the cells sense nutritional deprivation (7). Yeast has two genes, GSY1 and GSY2, that encode glycogen synthases, of which Gsy2p is the predominant nutritionally regulated form (8). Disruption of both glycogen synthase genes results in cells that are unable to synthesize glycogen (8). Glycogen synthase activity is reduced by covalent phosphorylation at up to three sites and, as noted, activated by glucose-6-P (3). The best characterized yeast glycogen synthase kinase is the cyclin-dependent protein kinase Pho85p, acting with the Pcl8/10p cyclins (9). We recently proposed a three-state model (10), based on the effects of Pho85p/Pcl10p phosphorylation on enzyme kinetic properties, to describe the regulation of glycogen synthase. According to this model, the lowest activity state, I, corresponds to phosphorylated glycogen synthase in the absence of glucose-6-P. The intermediate state, II, corresponds to the dephosphorylated enzyme in the absence of glucose-6-P, and the highest activity state, III, corresponds to enzyme in the presence of glucose-6-P and is independent of the phosphorylation state. In these same studies we used scanning mutagenesis, converting Arg and Lys residues to Ala, to identify several mutant forms of Gsy2p in which activation by glucose-6-P was modified (10). Two of these mutants (R579A/R581A/R582A and R586A/R588A/R591A) were mutated in a 13-residue segment that is highly conserved in glycogen synthases from different species and may be involved in a conformational transition related to the control of activity. Both mutants are completely resistant to glucose-6-P-induced increases in enzyme activity. However, although phosphorylation of R586 (R586A/R588A/R591) inactivated the enzyme normally, it had little effect on R579 (R579A/R581A/R582A) (Table I). By expressing the above glycogen synthase mutants in S. cerevisiae, we sought to assess the importance of allosteric activation by glucose-6-P on glycogen accumulation in cells. In summary, we provide the first direct evidence that impairment of glucose-6-P activation has a negative impact on glycogen synthesis in living cells.

EXPERIMENTAL PROCEDURES

Strains and Media—Standard bacterial and yeast culture conditions and techniques for manipulation were used. The yeast strains used were DH5-72 (MATa trpl1 leu2 uro3 thr4 pfk2::URA3), DH3 (MATa trpl1 leu2 uro3 gsy1::LEU2 gsy2::URA3), EG328-1A (MATa trpl1 leu2 uro3::URA3 (provided by K. Tatchell), and BP1 (MATa trpl1 leu2 uro3 gsy1::LEU2 gsy2::URA3 pfk2::URA3). Rich medium contains 1% (w/v) yeast extract, 2% (w/v) Bacto-peptone, and 2% (w/v) glucose. Synthetic complete medium (SC) contains 0.67% (w/v) yeast nitrogen base, 2%...
(w/v) glucose, and complete supplement mix (Bio 101 Inc.). Synthetic selective medium contains 0.87% (w/v) yeast nitrogen base, 2% (w/v) glucose, and complete supplement mix (Bio 101 Inc.) lacking tryptophan, adenine, leucine, or uracil as appropriate. Plasmids were maintained in Escherichia coli strain DH5a.

Yeast Expression Vector Construction—GSY2 wild-type and mutant coding sequences contained in the pET28a vector (10) were excised with NdeI and XhoI and ligated into the high copy number plasmid pBluescript SK+ (11) digested with SmaI and XhoI. All mutants were confirmed by sequencing.

Enzyme Assays— Aliquots (~1 × 10⁷) of cells grown in SC-Trp medium to the stationary phase were used to inoculate 25-ml liquid cultures of SC-Trp. The cultures were incubated at 30 °C and, at the indicated times, an aliquot (0.5 ml) of culture was collected for determining cell density, then another aliquot (1 ml) was collected, and cells were harvested by centrifugation for subsequent glycogen determination measurements. Synthetic complete medium (25 ml) lacking tryptophan was inoculated with yeast at a density to reach saturation after growth at 30 °C for 24 h, at which time the cells (5 ml) were harvested by centrifugation (1,500 × g in a clinical centrifuge for 1 min at room temperature). The cell pellet was frozen on dry ice and stored at −80 °C until it was analyzed for glycogen synthase protein expression and enzyme activity. Frozen cells were thawed on ice and resuspended in 300 μl of homogenization buffer (50 mM Tris-HCl, 1 mM EDTA, 5 mM dithiothreitol, 50 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 0.1 mM Nα-p-tosyl-L-lysine chloromethyl ketone, 5 mM benzamidine, 0.25 μg/ml leupeptin, and 0.5 μg/ml apro tinin, pH 7.4). Glass beads were used to break the cells as described previously (12). This extract was used for glycogen synthase assays and Western blot analysis.

Glycogen synthase activity was determined by measuring the incorporation of glucose from UDP-[1-14C]glucose into glycogen by the method of Thomas et al. (13) as described by Hardy et al. (12). Activity was measured both in the absence and presence of 7.2 mM glucose-6-P. Total activity is defined as that measured in the presence of this metabolite. The 1+/− glucose-6-P activity ratio is defined as the glycogen synthase activity measured in the absence divided by activity measured in the presence of 7.2 mM glucose-6-P under standard conditions. This ratio provides a kinetic index of the relative phosphorylation state of glycogen synthase, with a low value being indicative of a highly phosphorylated enzyme (reviewed in Ref. 14). Reactions were at 30 °C, typically for 10–15 min. Rabbit liver glycogen (Sigma-Aldrich) was deionized by passage through a MBD-22 (Resintech, Inc.) mixed bed exchanger prior to use.

Glycogen Determination—For analysis of glycogen accumulation in cells grown on plates, aliquots (5 μl) of yeast culture were spotted onto SC-Trp plates, and cells were grown for the indicated time before detection of glycogen by exposing plates to iodine vapor (15).

Quantitative determination of the glycogen content of yeast cells was as described previously (16). Briefly, frozen cells (as described above) were resuspended with 200 μl of 20% KOH and boiled for 1 h in a water bath with occasional shaking. Samples were cooled on ice for 2 min followed by the addition of 150 μl of 5 M HCl to neutralize samples. Glycogen was precipitated with 1 ml of ice cold 95% (v/v) ethanol and then pelleted by centrifugation at 17,500 × g for 10 min. The pellet was washed twice with 66% (v/v) ethanol, dried, and re-suspended in 400 μl of 50 mM sodium acetate and 5 mM CaCl₂ (pH 5.0) followed by digestion with 30 μg of amylglucosidase and 2 μg of α-amylase at 37 °C overnight. The glucose released was quantitated as described previously by Hardy et al. (12). The glycogen concentration was normalized to cell number.

Western blot analysis for detecting Gsy2p used an antibody raised against a Gsy2p peptide (17). Enhanced chemiluminescence (Amersham Biosciences) and horseradish peroxidase-conjugated antibody (Sigma) were used for detection. Protein was measured by the method of Bradford (18) using bovine serum albumin as a standard. Densitometry was used to quantitate relative signal intensities on Western blots.

RESULTS

Our previous mutagenesis of Gsy2p had yielded two mutants that, from analysis of recombinant protein in vitro, were impaired in their control by glucose-6-P (Table I). The objective of the present study was to exploit these mutations to judge the importance of glucose-6-P control for glycogen accumulation in vivo. To this end, we needed yeast strains lacking endogenous glycogen synthase to avoid the possible formation of hetero-meric glycogen synthase complexes containing wild-type and mutant subunits that would complicate interpretation. We utilized two strains: (i) gsy1 gsy2, in which both glycogen synthase genes are deleted; and (ii) psh2 gsy1 gsy2, which additionally lacks the β-subunit of 6-phosphofructo-1-kinase (encoded by PFK2). Both strains allow a plasmid-born GSY2 gene to be the only source of glycogen synthase. The latter strain has elevated glucose-6-P levels due to a block in glycolysis (19–21) and provides a way to judge the effect of increasing the glucose-6-P concentration in vivo.

Expression of these mutants, as well as of wild-type GSY2 and control vector, in gsy1 gsy2 cells was compared with wild-type yeast in terms of glycogen synthase protein level, activity, and glycogen accumulation (Fig. 1). With the control vector, the null phenotype of the host strain was reflected in undetectable levels of glycogen synthase activity and of glycogen. Transformation with plasmid encoding the wild-type glycogen synthase GSY2 resulted in a 7-fold increase in total glycogen synthase activity as compared with the parent wild-type yeast (EG328-
strain, the total glycogen synthase activity was 
With wild-type Gsy2p overexpressed in the 
changes in the glycogen synthase phosphorylation state. 
glucose-6-P promotes glycogen synthesis without significant 
mutant strain. Expression of the R579 
activity was measured in the absence (open bars) or presence (filled bars) of glucose-6-P as described under “Experimental Procedures.” Data are shown for two independent experiments. B, glycogen 
activity of R579, which can be inactivated by phosphorylation, was 
affected by phosphorylation, whereas the other (R586) could 
activity ratio was calculated by dividing glycogen synthase activity measured in 
the absence of glucose-6-P by activity measured in the presence of the 
metabolite. Data are the average of two independent experiments. D, glycogen synthase expression as determined from densitometric quantification of Western blot of glycogen synthase expression (inset). Relative intensity is the average of two independent experiments.

The two mutant forms of glycogen synthase were insensitive to glucose-6-P activation when assayed in yeast extracts, as expected from their behavior as recombinant proteins produced in E. coli, and they had −/+glucose-6-P activity ratios ≥1 (Fig. 1C). For R586, glucose-6-P may in fact have caused a modest inhibition of activity (Fig. 1A). In terms of expression level, the R579 protein was present at ~50% of the level of plasmid-encoded wild-type Gsy2p as judged by Western analysis (Fig. 1D) with just ~25% of the activity in the presence of glucose-6-P. This result is consistent with the known effect of the mutation to decrease the $V_{\text{max}}$ of the enzyme (10). Nonetheless, cells expressing this mutant accumulated almost twice as much glycogen as those with a wild-type GSY2 plasmid. A similar finding was obtained if glycogen was determined by staining colonies grown on solid media with iodine vapor (Fig. 2). These results fit with the mutated Gsy2p being completely insensitive to control by phosphorylation or glucose-6-P binding 
in vivo, hence leading to overaccumulation of glycogen despite the lowered glycogen synthase activity. Cells expressing 
the R586 mutant had a similar amount of Gsy2p protein as cells expressing wild-type enzyme, but the activity was 
only 10–15% of wild-type measured in the presence of glucose-
6-P (Fig. 1). This observation is consistent with the mutant 
protein still being able to be inactivated by phosphorylation. The decreased activity correlated with reduced glycogen 
accumulation (Figs. 1 and 2), suggesting that the insensitivity to glucose-6-P limited the ability of the cells to 
synthesize glycogen.

The same plasmids were expressed in the pfk2 gsy1 gsy2 strain to assess how increased glucose-6-P in vivo affected glycogen synthesis (Figs. 2 and 3). The activity of wild-type Gsy2p was ~40% lower in this background, with little change in the −/+ glucose-6-P activity ratio, compared with the gsy1 gsy2 strain. Still, the glycogen was increased by ~2-fold in the pfk2 gsy1 gsy2 mutant, suggesting that the elevated glucose-6-P promotes glycogen synthesis without significant changes in the glycogen synthase phosphorylation state. With wild-type Gsy2p overexpressed in the pfk2 gsy1 gsy2 strain, the total glycogen synthase activity was ~4-fold that in the wild-type EG328-1A strain, with a lower activity ratio but a 2–3-fold increase in glycogen accumulation over the wild-type strain (Fig. 3), again essentially duplicating the phenotype of a pfk2 mutant strain. Expression of the R579 and R586 mutants resulted in similar levels of protein as the wild-type Gsy2p (Fig. 3D). The total activity of the R579 mutant was ~50% that of the wild-type Gsy2p, and glycogen was only slightly elevated over that associated with 
expression of wild-type Gsy2p. Thus, the uncontrolled R579 mutant 
and the wild-type enzyme, hyperactivated by glucose-6-P, led to similar degrees of glycogen hyperaccumulation. The activity of R586, which can be inactivated by phosphorylation, was lower by ~75% compared with wild-type Gsy2p and ~40% 
compared with R579, and this translated into a ~50% decrease in glycogen storage. Therefore, control of this mutant 
enzyme by phosphorylation was able to exert some check on glycogen accumulation in the face of elevated glucose-6-P.

**DISCUSSION**

The objective of this study was to assess the contribution of glucose-6-P activation of glycogen synthase to control glycogen accumulation in yeast cells. It has been proposed for years that glucose-6-P is a physiological regulator of glycogen synthase based on biochemical studies in vitro. As for all controls by ligand binding, however, actual proof of the role in vivo is difficult, and for glycogen synthase there was no such evidence. We had identified mutants of yeast Gsy2p that were insensitive 
to activation by glucose-6-P. One mutant (R579) was also unaffected by phosphorylation, whereas the other (R586) could still be inactivated. The mutations, which are in a highly conserved short stretch of glycogen synthase, appear to influence transitions between different activity states of glycogen synthase (10). In our three-state model, both mutants would ba-
sally be in state II but are unable to reach the most active state, III, normally achieved in the presence of glucose-6-P. R579 would be irreversibly locked into state II, whereas R586 can adopt the least active state, I, following phosphorylation. When corresponding mutations were made in rabbit muscle glycogen synthase (23), similar results were obtained, implying that the arginine residues important for activation by glucose-6-P are conserved. Anderson and Tatchell (24) also analyzed two mutations in this same region of Gsy2p and found that one of the mutant enzymes, E590K, had a significantly increased activity ratio in vivo. The R579 and R586 mutants provide a telling means for testing the effect of disabling glucose-6-P control, alone or in combination with crippled phosphorylation control, on glycogen synthesis in vivo.

An important, albeit expected, outcome of the present study was the finding that when the R579 and R586 mutants were expressed in yeast lacking endogenous glycogen synthase, their activity in yeast extracts was indeed not increased by glucose-6-P, just like the recombinant proteins produced in E. coli. This result was critical to the intent of the study. Our primary conclusion is that the ability of Gsy2p to be activated by glucose-6-P is needed for normal glycogen accumulation in yeast. Additional loss of phosphorylation control, in the completely uncontrolled R579 mutant, leads to glycogen hyperaccumulation. When increased cellular glucose-6-P concentration is engineered in the pfk2 gsy1 gsy2 strain, the expression of wild-type Gsy2p correlates with elevated glycogen accumulation, basically reproducing the phenotype of pfk2 mutants as documented previously (21). Expression of the R579 mutant in this strain caused a similar level of glycogen storage, as did wild-type enzyme, and only a modest increase in glycogen as compared with its expression in cells with normal glucose-6-P levels. From these results we can conclude that the cellular glucose-6-P level has relatively little impact on glycogen accumulation mediated by R579. The R586 mutant, lacking activation by glucose-6-P but still turned off by phosphorylation, accumulates the least glycogen in either background. However, comparing the glycogen levels in the presence and absence of PFK2, there is a clear increase in the pfk2 strain when the glucose-6-P is elevated.

This last point suggests that glucose-6-P can affect glycogen accumulation independently of activating glycogen synthase, and, in fact, this is consistent with a number of previous observations. Especially when glucose-6-P is elevated, as in pfk (19–21) or pgi1 (25) mutants, there is the possibility that impairment of glycolysis forces glucose into glycogen by mass action via elevation of UDP-glucose levels. This may be the explanation for the increased glycogen accumulation by the R579 mutant when PFK2 is null. Glucose-6-P has also been implicated as an activator of glycogen synthase phosphatase (26) and an inhibitor of glycogen synthase kinase (21), most likely via glucose-6-P-induced changes to glycogen synthase. Arguing against a glucose-6-P-mediated change in glycogen synthase phosphorylation state is the very low \(-/+)\) glucose-6-P activity ratio of wild-type Gsy2p in both the gsy1 gsy2 and pfk2 gsy1 gsy2 strains. This indicates that the phosphorylation state of glycogen synthase has not been altered substantially by the higher levels of glucose-6-P found in the latter strain. For this mechanism to be relevant for mutants R579 and R586, it would require that the glucose-6-P-mediated changes in glycogen synthase that increase activity be distinct from the conformational changes affecting phosphorylation-dephosphorylation. Though not impossible, we consider this eventuality unlikely. Another glucose-6-P interaction is the inhibition of glycogen phosphorylase through the stimulation of phosphorylase dephosphorylation (27). Although such control may indeed occur in vivo, it is unlikely to explain the differences in behavior of cells expressing the two different mutant Gsy2p enzymes.

Though not the intent, the results of this study raise an interesting point regarding the use of overexpression of metabolic enzymes in cells to measure flux control coefficients in metabolic control analysis (28, 29). In metabolic control analysis, the fractional change in flux through a pathway as a function of changing the amount of enzyme defines the flux control coefficient (28), and flux-determining enzymes would have a high value (up to 1 in a linear pathway). With an intrinsically regulated enzyme like glycogen synthase, however, we see that 7-fold overexpression of Gsy2p (wild-type Gsy2p in gsy1 gsy2 versus wild-type in Fig. 1) causes but a modest increase in glycogen accumulation, presumably reflecting the regulatory capacity of the cell to avoid excessive glycogen storage. A similar observation was made for muscle glycogen synthase expressed in COS cells (22). In the case of the unregulated R579 mutant, the result is a striking increase in glycogen accumulation (R579 in gsy1 gsy2 versus wild-type in Fig. 1). Which then is the more appropriate measure of the ability of the Gsy2p to control the flux to glycogen? While accepting that our analyses were poorly designed in terms of attempting metabolic control analysis, we would argue that, at least in its basic formulation, a meaningful flux control coefficient is not experimentally accessible for an enzyme that undergoes regulation, such as by phosphorylation.

The importance of phosphorylation of glycogen synthase for yeast glycogen accumulation has been shown by site-directed mutagenesis (17) and by a genetic screen (24). Elimination of even a single phosphorylation site caused glycogen hyperaccumulation. The role of glucose-6-P had been inferred from biochemical studies of yeast glycogen synthase and mutants, such as those noted above, that lead to aberrant accumulation of metabolites. The results of this investigation give the first direct evidence for glucose-6-P-mediated control of glycogen synthase in vivo, demonstrating that, without this positive input, glycogen storage is impaired as compared with wild-type enzyme. Additional loss of control by phosphorylation, however, leads to an uncontrolled enzyme and hyperaccumulation of the polysaccharide.

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Molecular characterization of *Saccharomyces cerevisiae* Δ^3^-Δ^2^-enoyl-CoA isomerase.

Brian V. Geisbrecht, Dai Zhu, Kerstin Schulz, Katja Nau, James C. Morrell, Michael Geraghty, Horst Schulz, Ralf Erdmann, and Stephen J. Gould

Page 33187: It has come to our attention that the image in Fig. 4B of this paper, which shows the distribution of a GFP-Ylr284C fusion protein in a pex8 mutant of *S. cerevisiae*, is identical to an image that was published in another paper by our laboratory as representing a GFP-Ygl184C fusion protein (1). Based on this error we retract this image. However, we do not retract our observation that the GFP-Ylr284C fusion protein is located in punctate, peroxisome-like structures in the wild-type strain but not in the pex8 strain, as this observation was based on the examination of hundreds of cells from each strain and not on the erroneous image in Fig. 4. Moreover, we have recently repeated the experiments reported in the flawed papers and have found that the GFP fusions to Ylr284C and Ygl184C behave as we reported previously. Finally, we have tested our conclusion that Ylr284C is a peroxisomal protein by co-expressing it with CFP-PTS1 in wild-type yeast (Fig. 8 below). These experiments show co-localization between the peroxisomal matrix marker, CFP-PTS1, and YFP-Ylr284C, confirming our prior conclusion that the Ylr284C protein is targeted to peroxisomes.

FIG. 8. The fusion protein YFP-Ylr284C co-localizes with the peroxisomal matrix protein CFP-PTS1. An *S. cerevisiae* strain (BY4733 (2)) expressing CFP-PTS1 from the constitutive PGK1 promoter and YFP-Ylr284C from the galactose-inducible GAL1 promoter (3) was grown overnight in minimal medium containing galactose as sole carbon source, fixed, and then examined by confocal fluorescence microscopy and by phase-contrast microscopy using a Zeiss 510 Meta microscopy apparatus. The distribution of CFP-PTS1 (A), the distribution of YFP-Ylr284C (B), and a merge of the CFP-PTS1 (green) and YFP-Ylr284C (red) fluorescence patterns with a phase contrast image of the same cells (C).

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Differential voltage-dependent K⁺ channel responses during proliferation and activation in macrophages.

Rubén Vicente, Artur Escalada, Mireia Coma, Gemma Fuster, Ester Sánchez-Tilló, Carmen López-Iglesias, Concepción Soler, Carles Solsona, Antonio Celada, and Antonio Felipe

Page 46312, under “Results”: Lines 36 and 37 from the top. Because margatoxin and ShK-Dap22 were 10 times more concentrated than originally described, the IC₅₀ values for inhibition were ~50 and ~30 pM for MgTx and ShK-Dap22, respectively.

Glycogen synthase sensitivity to glucose-6-P is important for controlling glycogen accumulation in Saccharomyces cerevisiae.

Bartholomew A. Pederson, Wayne A. Wilson, and Peter J. Roach

Page 13764, Abstract and Introduction: R579A/R581A/R582A, used to designate a mutant form of glycogen synthase, should be replaced with R579A/R580A/R582A throughout.