Requirements for Catalysis in Mammalian Glycogenin*

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Glycogenin is a glycosyltransferase that functions as the autocatalytic initiator for the synthesis of glycogen in eukaryotic organisms. Prior structural work identified the determinants responsible for the recognition and binding of UDP-glucose and the catalytic manganese ion and implicated two aspartic acid residues in the reaction mechanism for self-glucosylation. We examined the effects of substituting asparagine and serine for the aspartic acid residues at positions 159 and 162. We also examined whether the truncation of the protein at residue 270 (Δ270) was compatible with its structural integrity and its functional role as the initiator for glycogen synthesis. The truncated form of the enzyme was indistinguishable from the wild-type enzyme by all measures of activity and could support glycogen accumulation in a glycogenin-deficient yeast strain. Substitution of aspartate 159 by either serine or asparagine eliminated self-glucosylation and reduced trans-glucosylation activity by at least 260-fold but only reduced UDP-glucose hydrolytic activity by 4–14-fold. Substitution of aspartate 162 by either serine or asparagine eliminated self-glucosylation activity and reduced UDP-glucose hydrolytic activity by at least 190-fold. The trans-glucosylation of maltose was reduced to undetectable levels in the asparagine 162 mutant, whereas the serine 162 enzyme showed only an 18–30-fold reduction in its ability to trans-glucosylate maltose. These data support a role for aspartate 162 in the chemical step for the glucosyltransferase reaction and a role for aspartate 159 in binding and activating the acceptor molecule.

Within a cell, glycogen functions as a reserve of glucose when metabolic demand for glucose outpaces the ability of the cell to obtain it from extracellular sources (1). Glycogen itself is a branched polymer of glucose composed of a series of α-1,4-glycosidic linkages with branch points occurring, on average, every 10–13 glucose residues through the formation of α-1,6-glycosidic linkages (1). The synthesis of glycogen is similar to that of other complex biologic polymers in that its synthesis has distinct initiation and elongation stages. The elongation stage of glycogen synthesis is catalyzed by glycogen synthase in concert with the branching enzyme. The initiation stage of glycogen synthesis is catalyzed in an autocatalytic manner by glycogenin (2, 3).

The reaction catalyzed by glycogenin can be defined as autocatalytic, in that a solution of glycogenin, in the presence of UDP-glucose and Mn²⁺ cations, will catalyze the formation of short glucose polymers covalently attached to a tyrosine hydroxyl group on the surface of the enzyme (4). The site of attachment has been identified as Tyr-194 in mammalian muscle forms of glycogenin (5). Expression of glycogenin in Escherichia coli defective in UDP-glucose production yields glycogenin devoid of covalently attached glucose (6), whereas addition of UDP-glucose to such a glycogenin preparation or expression of glycogenin in E. coli competent for UDP-glucose production yields a population of glycogenin molecules with differing lengths of covalently attached glucosyl chains. Glycogenin continues to transfer glucose residues to this growing nascent glycogen chain until the chain length approaches ~10 residues, and past this length the efficiency of transfer decreases (4, 7–8).

Glycogenin-like molecules have been found in organisms as diverse as yeast and humans (4). A study of the conserved residues based on these sequences has identified seven regions of conserved sequence or sequence domains (4). However, only the first four sequence domains are found easily by sequence alignment in most of the known glycogenins. In terms of catalytically functional amino acids, there are relatively few universally conserved residues. Within domain I, a conserved Tyr occurs at the position homologous to residue 14 in rabbit muscle glycogenin. Within domain II, conserved Arg and Lys residues occur at positions 75 and 85, respectively. The DXD motif that is conserved throughout much of the glycosyltransferase family occurs at positions 100–102. Domain III contains a conserved Asn at position 132, conserved acidic residues at 159 and 162, and a conserved Gln residue at 163. Within conserved sequence domain IV, conserved His, Lys, and Trp occur at positions 211, 217, and 219, respectively. Experiments performed with both the yeast and mammalian forms of glycogenin show that the consensus sequence “WE(X)₂(D)Y” in sequence domain VII is involved in interactions with glycogen synthase and would appear to facilitate bulk glycogen synthesis by directly associating the priming enzyme with the major synthetic enzyme (9). Previous mutagenesis has demonstrated that the conserved Lys within conserved domain II is catalytically essential (10). Tyr-194, although necessary for self-glucosylation (11), is not catalytically essential such that a Phe-194 mutant enzyme can glucosylate, in trans, alternative acceptor molecules (12) and glycogenin subunits that retain Tyr-194 (10).

Glycogenin is a member of the glycosyltransferase family 8, a diverse set of glycosyltransferases, which includes among other enzymes lipopolysaccharide glucosyltransferases. Eight distinct members of the glycosyltransferase superfamly have had their three-dimensional structures solved (13–20). These

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1 J. Zhou, unpublished observations.
glycosyltransferases share a similar nucleotide binding fold of ~120 residues comprising a four-stranded α + β domain that is responsible for the majority of the interactions with the UDP moiety (21, 22). In contrast, the remainder of the structures is fairly dissimilar, undoubtedly reflecting their differences in catalytic chemistry, nucleotide sugar, and acceptor molecule specificities. Four of these structures belong to the inverting class of glycosyltransferases and are expected to differ from glycogenin in their chemical and catalytic properties. However, four structures belong to the retaining class of glycosyltransferases and are expected to utilize common catalytic strategies and, therefore, active site residues. Similar to what has been shown for retaining glycosidoses (23, 24), retaining glycosyltransferases may follow a two-step catalytic strategy involving a covalent enzyme-substrate intermediate with inverted stereochemistry, followed by a second attack at the C-1 atom of the sugar ring that yields the final product and retention of configuration (Scheme 1). However, conflicting data exist as to the identity of the catalytic nucleophile in these structures.

In this study we sought to examine the catalytic contributions of two key residues within the substrate-binding and active sites of rabbit glycogenin identified previously from structural analyses as potential catalytic residues. Specifically, the catalytic contributions of Asp-159 and Asp-162 were assessed by site-directed mutagenesis, as well as the functional consequences of truncation of the polypeptide chain at residue 270. We report here the characterization of these mutant enzymes by both steady-state kinetics and direct structure determination, and we compare the results with those of the wild-type enzyme.

**EXPERIMENTAL PROCEDURES**

**Mutagenesis, Expression, and Purification—**All mutants were generated using the QuickChange kit (Stratagene). In all cases, prior to expression and purification, the complete coding region was resequenced to confirm that only the desired mutation(s) were incorporated. The Δ270 construct was created through the introduction of a four-bp deletion at positions 271 and 272 of the wild-type rabbit muscle glycogenin cDNA, thereby eliminating the C-terminal 63 amino acids. Recombinant His$_6$-tagged glycogenin was purified using procedures published previously (10). Briefly, BL21 (DE3) cells transformed with the appropriate pET-28a plasmid were grown at 37 °C until the cells reached an absorbance of 0.7 at 600 nm, at which time isopropyl β-thiogalactopyranoside was added to a final concentration of 0.4 mM. The cells were then grown at 30 °C for an additional 4 h. Alternatively, following induction of gene expression by isopropyl β-thiogalactopyranoside, the incubation temperature could be reduced to 18 °C, and the cells were allowed to grow for an additional 16 h. The bacterial cells were collected by centrifugation and resuspended in 50 mM Na-HEPES, pH 7.5, 1 mM Na-EDTA, 1 mM benzamidine, and 1 mM dithiothreitol. Lysis of the cell suspensions was performed using a French press operated at 1000 pounds/square inch. A high speed supernatant was obtained by centrifugation, and this material was loaded onto a nickel-nitrilotriacetic acid column (Qiagen) and washed to base line with 50 mM Na-HEPES, pH 7.5, 5 mM dithiothreitol, 2 mM benzamidine, and 1 mM Na-EDTA and applied to the Q-Sepharose column. The column was washed to base line with the above buffer, and then the bound protein was eluted by using a linear gradient from 0 to 0.5 M NaCl in a volume equal to 20 column volumes. Purity was monitored using SDS-PAGE analysis. For crystallization, the protein eluted from the Q-Sepharose column was buffer exchanged into 15 mM Na-HEPES, pH 7.5, 1 mM dithiothreitol, and concentrated to 9 mg/ml.

**Molecular Mass Determination by Gel Filtration—**Estimates for the solution molecular mass for the glycogenin enzymes were determined on a Superose-12 column (Amersham Biosciences) equilibrated in phosphate-buffered saline. All enzymes were concentrated between 1.5 and 1.8 mg/ml (17–20 μl). A total volume of 0.2 ml (3–3.6 μg) was injected onto the 24-ml column operated at 0.3 ml/min for analysis. The column was standardized using blue dextran, aldolase (158,000 daltons), albumin (67,000 daltons), ovalbumin (43,000 daltons), chymotrypsin (20,000 daltons), and ribonuclease (13,700 daltons).

**Enzymatic Assay—**The ability of wild-type and mutated glycogenins to catalyze self-glucosylation was assessed by using an assay containing 0.6 to 1.8 μM enzyme in 50 mM Na-HEPES, pH 7.5, 5 mM MnCl$_2$, 2 mM dithiothreitol, and 30 μM UDP-[U-14C]glucose in a total volume of 10 μl, which was incubated for either 1 or 2.5 min at 30 °C. Assays in which the concentration of UDP-glucose was increased (50–515 μM) were modified by the addition of cold UDP-glucose to the reaction mix and quantitated after accounting for the altered specific activity of the substrate. An aliquot (5 μl) removed from the reaction was spotted onto PS1 chromatography paper, which was then washed three times (30 min each wash) in 0.5% phosphoric acid and then in ethanol. The dried paper was counted in a liquid scintillation counter. The ability of wild-type and mutated glycogenins to catalyze the transfer of glucose to the alternative acceptor, maltose, was determined by incubating glycogenin (1–4 μM) for 2.5 min at 30 °C in 50 mM Na-HEPES, pH 7.5, 5 mM MnCl$_2$, 2 mM dithiothreitol, and 30 μM UDP-[U-14C]glucose containing 50 mM maltose (Sigma) as the glucose acceptor in a total volume of 10 μl. The reaction was terminated by the addition of a final concentration of 2% (v/v) glacial acetic acid. An aliquot (5 μl) from the reaction mixture was then spotted on silica gel thin layer plates (Whatman PE SILG UV) and subjected to ascending chromatography in a mobile phase containing acetonitrile:acetic acid:ethanol:water in the ratio 65:5:10:20. The plate was then dried and subjected to autoradiography. Quantitation of the individual reaction species was performed by excising the radioactive products from the TLC plates, followed by scintillation measurements of the excised species. A similar assay procedure and mixture, but without added maltose, was utilized to assess the ability of the purified glycogenin enzymes to catalyze the nonproductive hydrolysis of UDP-glucose to free glucose and UDP.

**Glycogen Accumulation in S. cerevisiae—**The 194F mutant form of human glycogenin-1 was modified with an N-terminal FLAG tag for monitoring protein expression level and sublocalized into the yeast expression plasmid p425Gal1 (25). The Δ271–103A mutant form of human glycogenin-1 was constructed by a combination of site-directed mutagenesis to produce the 103A mutation and restriction digestion with NdeI to truncate the cDNA at codon position 271. The resulting construct was then modified with an N-terminal V5 tag for monitoring protein expression level and sublocalized into the yeast expression plasmid p426GPD (20). Wild-type human glycogenin-1 was sublocalized into the p426GPD plasmid to use as a complementation control. CEOexpression from doubly transformed cells could then be obtained using a yeast strain that required functional selection for both Leu$^+$ and Ura$^+$. Such a strain was derived from EG328-1A (25) and therefore labeled 5g12glg2. The Δ271–103A protein expression was prepared in the following manner. GLG1 and GLG2 were disrupted with the TRPI gene by using a PCR-based strategy. pTRPI was carried on yeast plasmid YEp13 (26) to create templates to fragment that had the TRPI gene flanked by 45 nucleotides of GLG1 or GLG2. The PCR products were transformed into yeast to create the strains with the desired gene disruptions. J22-a (MATa trpl1 leu2 uro3-52 glg1-2::TRPI) was derived from EG328-1A (MATa trpl1 leu2 uro3-52::PGLG1, J22-b (MATa trpl1 thr4 uro3-52 glg2::TRPI) was derived from DH4-101 (MATa trpl1 thr4 uro3-52) by disrupting GLG1. J22-a and J22-b mating created the diploid J25-a.
Enzyme                      | Self-glucosylation | Trans-glucosylation | UDP-glucose hydrolysis |
|--------------------------|-------------------|--------------------|-----------------------|
| Wild-type (glucosylated) | 0.32 ± 0.09       | 2.1 ± 0.1          | 1.01 ± 0.30           |
| Wild-type (non-glucosylated) | 0.84 ± 0.30     | 1.6 ± 0.1          | 0.39 ± 0.03           |
| 162S                      | <0.005            | 0.07 ± 0.02        | 0.003 ± 0.001         |
| 159N                      | <0.005            | <0.001             | <0.001                |
| 158S                      | <0.005            | 0.005 ± 0.001      | 0.074 ± 0.002         |
| 150N                      | 0.006 ± 0.007     | 0.008 ± 0.001      | 0.082 ± 0.030         |
| Δ270                     | 0.34 ± 0.08       | 2.1 ± 0.1          | 0.95 ± 0.10           |
| Δ270–162S                | <0.005            | 0.12 ± 0.05        | 0.005 ± 0.001         |
| Δ270–162N                | <0.005            | <0.001             | <0.001                |
| Δ270–159S                | <0.005            | <0.001             | 0.23 ± 0.15           |

The abbreviations used are: MES, 4-morpholinethanesulfonic acid; r.m.s.d., root mean square deviation.

**RESULTS**

Purification and Characterization of Δ270 and Enzymes with Mutations at Positions 159 and 162—Truncation of the full-length glycogenin molecule at residue 270 resulted in a fully active enzyme (Δ270) whose specific activity for self- or trans-glucosylation was indistinguishable from the full-length enzyme (Table I). Mutation of Asp-159 or Asp-162 to either Ser or Asn resulted in stable enzymes that could readily be purified for enzymatic study. These mutants migrated on SDS-PAGE faster than the respective active parent enzymes, suggesting reduced self-glucosylation in E. coli (not shown). Consistent with this observation, the mobility of the full-length mutant enzymes on SDS-PAGE was identical to wild-type enzyme expressed and purified from an E. coli strain defective in UDP-glucose production (6). The wild-type glucosylated glycogenin, wild-type nonglucosylated glycogenin, and the full-length 159S and full-length 162S, Δ270–159S, and Δ270–162S were all determined by gel filtration to exist as >95% dimer (103,000 daltons for the full-length enzymes or 58,000 daltons for the Δ270 enzymes) in solution with less than 5% of a tetrameric species present (176,900 daltons for full-length enzyme or 102,000 daltons for the Δ270 enzymes). However, the full-length 159N, 162N, and the Δ270–162N enzymes appeared to exist as both tetrameric and dimeric species in solution, with approximately twice the concentration of tetramer versus dimer under the conditions of our gel filtration analysis. No monomeric or higher order species were detected for any of the enzymes analyzed by this method.

**Enzymatic Activity Measurements**—The 159S, 159N, 162N, and 162S mutant glycogenin enzymes possess self-glucosylation activities below the limit of detection of the assay. Increasing the concentration of UDP-glucose in the assay from 30 to 515 μM or increasing the incubation time did not change the results (Table I). The 162N mutant possessed undetectable activity for the trans-glucosylation of maltose and the hydrolysis of UDP-glucose to free glucose under the conditions of the assay. The 162S mutant was between 18- and 30-fold less active for the trans-glucosylation of maltose and the hydrolysis of UDP-glucose, depending on whether the activity measurements were performed on the full-length 162S enzyme or on the Δ270–162S enzyme. The trans-glucosylation activity of the 159S enzyme was reduced to undetectable levels, whereas its activity for the hydrolysis of UDP-glucose was reduced only 4–14-fold, depending on whether the mutation was in the full-length enzyme or the Δ270 enzyme. The ability of the 159N enzyme to catalyze the trans-glucosylation of maltose was reduced by 260-fold, but similar to the 159S enzyme, the hydrolysis of UDP-glucose was only reduced 12-fold.

accomplished by using the program CNS (version 1.1) (33). Visual inspection and manual correction of the resulting model structures were accomplished by using the program package O (34).
For the wild-type glucosylated form of glycogenin, an analysis of the reaction products obtained from the trans-glucosylation assay showed that 93% of the transferred glucose molecules appeared in maltotriose, 6% were attached to glycogenin, and 1% was liberated as free glucose. In the hydrolytic reaction, 63% of the transferred glucose molecules were found as free glucose and 37% were attached to glycogenin. The non-glucosylated form of the wild-type enzyme had less hydrolytic activity relative to its self-glucosylation activity. The products of the hydrolytic assay showed that 31.5% of the transferred glucose appeared as free glucose, and 68.5% were attached to glycogenin. In contrast, the trans-glucosylation of maltose by the non-glucosylated enzyme showed results nearly identical to that of the glucosylated form of glycogenin, with 5.5% of the transferred glucose molecules attached to glycogenin, 94.5% appeared in maltotriose, and no detectable free glucose. There was also no evidence for the formation of maltotetrose during the time course of the trans-glucosylation assays.

Rescue of Glycogen Accumulation in Yeast—We examined the ability of the Δ270 construct of glycogenin to support glycogen accumulation in an indirect manner. Because the Δ270 enzyme was capable of both self- and trans-glucosylation reactions, we could not be sure that any glycogen accumulation was not because of the ability of the Δ270 enzyme to trans-glucosylate an alternative acceptor molecule in yeast. We have shown previously that the Phe-194 mutant of glycogenin was incapable of self-glucosylation but could glucosylate alternative acceptor molecules, including catalytically inactive glycogenin molecules (10). The yeast Saccharomyces cerevisiae contains two distinct genes for glycogenins, and double knock-out strains cannot accumulate measurable levels of glycogen (9). Transformation of glg1Δglg2 strains of yeast with native mammalian forms of glycogenin can rescue this glycogen accumulation defect (Fig. 1A). In contrast, glg1Δglg2 strains of yeast transformed only with the Phe-194 mutant form of glycogenin do not accumulate significant amounts of glycogen (Fig. 1B). For this experiment, we created a truncated form of the human glycogenin-1 gene in which the manganese ligand Asp-103 was mutated to alanine (Δ271–103A). We knew from prior assays that this protein was devoid of both self- and trans-glucosylation activities. Expression of this mutant alone could not rescue the glycogen accumulation defect in glg1Δglg2 yeast (not shown). However, when both the Phe-194 and Δ271–103A mutant glycogenins were transformed into glg1Δglg2 yeast cells, glycogen accumulation was restored, albeit at slightly lower levels than are obtained with wild-type glycogenin (Fig. 1C). This result demonstrates that the Δ271–103A acts as a glucose acceptor for the Phe-194 mutant and is able to support glycogen synthesis in the yeast cell.

Structures—The three-dimensional structures for the Δ270 enzyme complexed with UDP and Mn2+ and the full-length apo-form of the 159N enzyme, the apo- and UDP-glucose/Mn2+ bound forms of 159S, and the apo- and UDP-glucose/Mn2+ bound forms of 162S have been solved to resolutions between 2.6 and 1.98 Å (Table I). All structures, except the Δ270 crystals, have a monomer within the asymmetric unit with the biological dimer created by one of the crystallographic dyad axes in the unit cell. For those structures solved in the I222 space group, the additional dyad axes form what is likely to be a nonphysiologic back-to-back tetramer mediated by crystallographic contacts with a sulfate ion precisely located at the intersection of the three dyad axes in this space group (“a special position”). This interaction was also observed in the wild-type enzyme crystallized in this same space group (20). In the Δ270 structure, the biological dimer comprises the asymmetric unit, and the additional dyad axis in the P63 space group forms a front-to-front type of tetramer that may have implications for one mode of self-glucosylation (Fig. 2a). Most interestingly, the orientation of the monomers along their respective dimer axes in these two space groups differs by 6.5°, suggesting that the dimer interaction surface in glycogenin is a flexible one (Fig. 2b).

At the subunit level, the structures of all the mutant enzymes were nearly identical to the wild-type enzyme structure (r.m.s.d. values for C-α atoms between 0.3 and 0.4 Å). The overall structures of the respective mutant enzymes were identical to each other (r.m.s.d. values for C-α atoms <0.2 Å for each comparison set). The mode of interaction between the enzyme and the bound substrate molecules was also unchanged from that described in previous work (20). All structures maintained the same non-proline cis-peptide bond between residues 118 and 119 (20). The side chains for the substituted residues at positions 159 and 162 were clearly visible in the electron density maps (Fig. 3). Compared with wild-type enzymes, all structures exhibited electron density for additional residues in the disordered loop between positions 233 and 240. In the 159S/N and 162S mutant structures, this would appear to be mediated by contact with the side chain of an ordered Tyr-194 residue. In the Δ270 structure, the increased order is probably because of the formation of the face-to-face tetramer (Fig. 2).

As with the full-length wild-type enzyme, which crystallized in the same space group and with similar cell dimensions (20), none of the full-length mutant structures exhibited any electron density for residues 265–333. One point of difference in these structures was that the electron density for residues 190–195 was stronger in the mutant enzymes such that the side chain of Tyr-194 can now be visualized. Prior wild-type structures, as well as the Δ270 structure reported here, exhibited no electron density for the side chain of Tyr-194, and the density for the main chain atoms in this region was not always continuous. The helix in which the residue lies is also shifted from the position seen in the wild-type structure (Fig. 4). This shift in the position of the helix was likely because of the new packing interactions contributed by the now more ordered side chain of Tyr-194.

The 162S enzyme showed the least change in active site structure when compared with the wild-type enzyme, whereas both the 159S and 159N structures exhibited local changes in structure associated with the differences in the hydrogen bonding capabilities of these residues (Fig. 3). Both the 159S and 159N structures exhibited discrete disordering of Tyr-196,
which normally formed a hydrogen bond with Asp-159 across
the dimer interface (20). In addition, the 159S enzyme exhib-
ted an increase in the conformational flexibility for residues
157–160, as evidenced by higher B-factors and weaker main
chain electron density (Fig. 3). This was not entirely corrected
upon UDP-glucose binding. In both the 159S and 162S UDP-
glucose/Mn2\textsuperscript{2+}/H11001
bound structures, an ordered water molecule
was located 4 Å from the C-1 atom of the glucose moiety. It was
held in position through interactions with the main chain am-
ide nitrogen of residue 162 and either the side chain carboxyl
oxygen of Asp-162 or Asp-159, depending on the position of
the mutation (Fig. 5). A water molecule was bound in a similar
location in the wild-type UDP-glucose structure and formed
hydrogen bonds with both the main chain nitrogen of Asp-162
and its side chain carboxylate (20).

**DISCUSSION**

Prior investigation of glycogenin has established its critical
role in the initiation of glycogen synthesis (2, 3). In this study,
we sought to assess the effects of mutations at residues within
the active site that were designed to establish the chemical
requirements for glucosyl transfer. We also examined the struc-
tural and functional effects of truncating the glycogenin enzyme.
The position of UDP-glucose is also shown for context to the active site. Figure was prepared using the programs MOLSCRIPT (38) and Raster3D (39, 40).

We solved the three-dimensional structures of four of the mutant constructs, both in the presence and absence of bound nucleotide. All structures are highly similar to the wild-type enzyme, except that the mutations at positions 159 and 162, which resulted in nonglucosylated enzymes, affected the positioning of the α-helix that contains the site of self-glucosylation, Tyr-194. Because the space group and thus the crystal-packing contacts are the same for these mutants and for the wild-type enzyme, it is likely that this shift in helical position reflects the actual position of the helix prior to self-glucosylation and that the position observed in the wild-type enzyme reflects the position of the helix following self-glucosylation. This is further supported by the structure of the Δ270 enzyme, which like the wild-type enzyme is glucosylated and exhibits the same helical position and disordering of Tyr-194 despite distinctively different crystal packing contacts.

Although the mutations have minimal impact on the three-dimensional structures of the enzymes, two of the mutations, 159N and 162N, had an impact on the oligomerization of the enzyme in solution. All enzymes existed as a mixture of tetramer and dimer in solution under the conditions of our gel filtration experiments. For the 159S and 162S forms, more than 95% of the enzyme is present as a dimeric species in solution. However, for both the 162N and 159N enzymes, greater than 66% of the enzyme was present as a tetrameric species in solution, with the remaining fraction existing as a dimeric species. The fact that all enzyme samples, including the wild-type enzyme, contain both species suggests that the mutations are not creating novel interactions but rather are altering the equilibrium between pre-existing states. We do not believe that this altered equilibrium significantly impacts the enzymatic properties reported here, because both the 159S and 159N enzymes had similar enzymatic characteristics, similar three-dimensional structures, but quite different dimer-tetramer distributions. Prior work has shown that when glucogenin concentration in the assay is varied, half-maximal activity is achieved when the concentration is in the low micromolar range (10). Initially, it was thought that catalysis occurred within a dimer and that this concentration dependence might represent the dissociation of the dimers into inactive monomers (10). Alternatively, based on the gel filtration data presented here, the concentration dependence might reflect an association between dimers in solution to form a Michaelis complex, such as the tetramer observed in the Δ270 structure. Further exploration of this phenomenon is clearly warranted.

Previous work has shown that glucogenin, purified as recombinant protein from bacteria or from tissue sources, is susceptible to proteolysis and yields a predominant species approximately 6 kilodaltons smaller than the intact enzyme (35). This proteolytic fragment was shown to be active for self-glucosylation and was suggested to represent the N-terminal catalytic fragment of the enzyme (35). Our own structural investigations are consistent with this finding, because none of our full-length structures exhibit interpretable electron density past residue 265, despite the presence of these residues in the crystals (20). We found that glucogenin truncated at residue 270 produced a stable and active enzyme with properties similar to the wild-type enzyme for all enzyme reactions investigated. The $K_m$ for UDP-glucose was not adversely affected. Thus, at least for the glucosyltransferase reaction, the C-terminal domain does not significantly contribute to the activity of the enzyme.

An important question remains: what effect do the C-terminal 62 residues have on glucogenin function? We report here that the structural scaffold of a similarly truncated but functionally inactive form of human glucogenin-1 (Δ271–103A) was able to rescue glucogen accumulation in a glg1glg2 yeast strain when cotransfected with the Phe-194 form of glucogenin-1. The Phe-194 form of glucogenin is incapable of self-glucosylation and cannot by itself rescue the glucogen accumulation defect in glg1glg2 yeast strains. The Phe-194 enzyme has been shown to catalyze the addition of glucosyl chains to other molecules of glucogenin (10), and so the addition of glucosyl chains to the Δ271–103A form of glucogenin by the Phe-194 enzyme is the most reasonable explanation for the rescue of the glucogenin accumulation defect. Consequently, yeast glucogen synthase is apparently capable of recognizing the truncated but glucosylated form of glucogenin as its substrate for further glucogen synthesis. Qualitative analysis of the level of glucogen accumulation showed that the truncated form of human glucogenin-1 accumulated less glucogen when compared with full-length glucogenin-1 (Fig. 1). This suggests that although not essential for self-glucosylation and glucogen accumulation, the C-terminal domain of glucogenin may augment the level of glucogen that can be attained in vivo.

A surprising aspect of this study was the extent to which both the full-length and Δ270 enzymes catalyzed the hydrolysis of UDP-glucose to UDP and glucose. For the glucosylated forms...
of glycogenin, the $k_{cat}$ value for this reaction was $\sim 3$ times that for self-glucosylation and $\sim 50\%$ the rate for the trans-glucosylation of maltose (Table I). Previous work has shown that the trans-glucosylation reaction proceeds with a greater $k_{cat}$ value than self-glucosylation, and this work is consistent with prior results (12, 36). However, it is interesting that the trans-glucosylation reaction appears to suppress both the hydrolytic reaction and the intrinsic self-glucosylation reaction. Because the assays use the same preparation of glycogenin, it suggests that the addition of the exogenous acceptor molecule to the assay components increases the efficiency of the transglycosylase reaction. We feel that this result is a consequence of utilizing glycogenin that has already added an average of four to five glucose residues to itself during expression in E. coli. Prior work has shown that the efficiency of glucosyl transfer decreases with increasing glucose chain length attached to Tyr-194 (7). The decreased efficiency might be due to an inability to stably bind the longer chains of glucose in the acceptor site. Consequently, it is possible that the hydrolytic reaction gains in apparent efficiency because of decreased occupancy of the acceptor site. Addition of maltose to such preparations of glycogenin apparently restores the native efficiency of the transglycosylase reaction by effectively increasing the occupancy of the acceptor site. This hypothesis is supported by the data from the nonglycosylated enzyme purified from E. coli deficient in UDP-glucose. The nonglycosylated form of the enzyme had less hydrolytic activity relative to its self-glucosylation activity, whereas the transglucosylation activity of the nonglycosylated form of glycogenin was identical to the glucosylated forms.

The precise mechanism by which retaining glycosyltransferases catalyze their reaction has been a subject of controversy. One view is that, like retaining glycosidases, retaining glycosyltransferases catalyzes the enzymatic transfer via a double nucleophilic substitution reaction (double $S_{2}$ reaction) in which a covalent intermediate between an active site nucleophile and the donor sugar is formed with inverted stereochemistry, followed by transfer of the donor sugar to the acceptor substrate with subsequent re-inversion of the stereochemistry at the anomeric carbon (23). Recent data from mutagenic and structural studies on $\alpha$-1,3-galactosyltransferase and also LgtC appear to support an $S_{n}$ mechanism that lacks a covalent enzyme-glycol intermediate (37). Based on sequence conservation among glycogenin enzymes and among retaining glycosyltransferases, we have suggested previously that Asp-162 could function as the catalytic nucleophile for a double-displacement mechanism (20). However, we could not rule out Asp-159 as the putative nucleophile because it is also a conserved residue among glycogenins, although not in all glycosyltransferases. Both Asp-159 and Asp-162 are approximately equidistant from the C-1 atom of the bound UDP-glucose molecule in our structures.

To test the respective roles of these two potential catalytic residues, each residue was substituted with either Asn or Ser. The rationale for these choices was that Asn is isosteric but chemically inert for nucleophilic attack or general acid/base catalysis and that Ser lacks general acid/base capability but could function as a weak nucleophile. All four mutations yielded enzymes that lacked detectable self-glucosylation activity and thus provide little mechanistic insight. However, measurement of the ability of each mutant enzyme to transfer glucose to maltose as the acceptor or the ability of the enzyme to catalyze the futile hydrolytic formation of free glucose from UDP-glucose proved more informative. Both mutations at position 162 decreased UDP-glucose hydrolytic activity by at least 190-fold (Table I). The 162N mutant enzyme showed a greater than 1000-fold decrease in both the UDP-glucose hydrolysis and glucosyltransferase activities, such that any activity is below the ability of our assays to detect. The 162S mutant exhibited UDP hydrolysis activity that was 190–300-fold lower than the wild-type enzyme and glucosyltransferase activity that was 20–30-fold lower than wild-type enzyme, depending on the background of the mutations (2270 or full-length, respectively). The differences in the behavior of the 162S and 162N mutant can be related to the chemical character of their substituted side chains. The side chain of Asn is chemically inert as a nucleophile under the conditions of the assay; consequently, the 162N mutant had the lowest activity in all assays because, in a double nucleophilic displacement mechanism, both reactions require the participation of a nucleophile. Most interestingly, the 162S mutant did possess measurable hydrolytic and glucosyltransferase activity, suggesting that the Ser side chain can be activated to serve as a weak nucleophile in the glycogenin active site. The juxtaposition of Lys-85 to the Ser-162 side chain (Fig. 3) could influence the environment of this residue to lower the pK$_a$ value of the hydroxyl group and consequently produce a reasonably good nucleophile. The fact that the hydrolytic and transglycosylation reactions are differentially affected could be related to the fact that acceptor binding may further desolvate the active site and thereby increase the nucleophilic character of the Ser residue for the transglycosylation reaction. Previous work has shown the mutation of Lys-85 to Gln inactivated glycogenin for glucosyltransferase activity, suggesting that the pairing of the protonated amino group (Lys-85) and the deprotonated putative nucleophile at position 162 is essential for catalysis.

The mutations at position 159 reduced glucosyltransferase activity for maltose by at least 260-fold but only reduced hydrolytic activity between 4- and 14-fold, depending on the background of the mutation. These data, combined with the data for 162S and 162N, suggest that the 159N/S mutants were deficient not in the initial breakage of the phosphoryl-carbon bond but in activating the acceptor molecule (either maltose or water). The position of Asp-159, in the context of other glycosyltransferases, is consistent with participation in acceptor binding and activation (13–19, 37). Consequently, we suggest that Asp-159 is involved in both binding and activation of the acceptor molecule. In principle, the side chains of both Ser and Asn could form a hydrogen bond with the acceptor molecule to correctly position it for catalysis but then cannot facilitate the deprotonation of the 4′-OH because of their ineffectiveness in general acid/base chemistry. The fact that the 159S enzyme was devoid of transglycosylation activity and the 159N enzyme possessed low, but measurable, transferase activity suggests that hydrogen bonding capability alone is insufficient for full functionality. This conclusion is based on the interactions contributed by residue 159 to the local structure of the active site. The side chain of residue 159 caps the peptide nitrogen atom of the adjacent glycine residue in all structures we have determined (Fig. 3). However, the side chains of both Asp and Asn can form hydrogen bonds to an acceptor molecule with the opposing carboxyl oxygen or the opposing amide nitrogen, respectively (Fig. 3). The unbranched and smaller side chain of Ser cannot perform both functions, and so the 159S mutant enzyme must either sacrifice interactions with the acceptor molecule or the structural integrity of this important area of the enzyme. In either case, activity for the glucosyltransferase reaction is more severely affected in the 159S enzyme. That glucosyltransferase activity in the 159N enzyme was reduced 260-fold suggests that hydrogen bonding capability is secondary to the ability of the residue to accept a proton and thereby activate the acceptor molecule.
Our data cannot address the issue of whether the reaction goes through an \( S_{2,2} \) or \( S_{3,1} \) mechanism. However, the roles of these two residues do not change significantly if in fact the \( S_{3,1} \) mechanism is the correct interpretation. In an \( S_{3,1} \) mechanism, Asp-162 would facilitate breakage of the carbon–phosphoryl bond by electrostatic stabilization of the oxocarbenium intermediate, whereas Asp-159 would position and activate the incoming acceptor molecule.

In conclusion, we have shown that neither truncation of mammalian glycogenin nor the selected substitution of key catalytic residues within the substrate binding and active sites results in large changes in the tertiary structure of the catalytic core of the protein. The most significant changes in structure were associated with lack of glucosylation of Tyr-194 that results from the substitutions of Asp-159 and Asp-162, whereas only limited and local changes to the structure were observed at the actual site of the mutations. The mutations at positions 159 and 162 also appear to have little effect on the structure of the ground-state enzyme-UDP-gluco complex, suggesting that the catalytic effects of these substitutions are manifested either in the formation of the ternary complex with the acceptor molecule or during the actual glucosyl transfer reaction. Substitutions at both 159 and 162 are associated with a lack of self-glucosylation activity as well as severe reductions in the trans-glucosylation of maltose or the hydrolysis of UDP-glucose. The differential effects of the mutation on these distinct reactions support a direct role for Asp-162 in the chemical step of the reaction and a lack of glucosylation of Tyr-194 that results from the substitutions of Asp-159 and Asp-162, whereas only limited and local changes to the structure were observed at the actual site of the mutations.