Identification and Characterization of a Critical Region in the Glycogen Synthase from *Escherichia coli*

Alejandra Yep, Miguel A. Ballicora, Mirta N. Sivak, and Jack Preiss†‡

From the Department of Biochemistry and Molecular Biology, Michigan State University, East Lansing, Michigan 48824

Received for publication, November 19, 2003
Published, JBC Papers in Press, December 9, 2003, DOI 10.1074/jbc.M312686200

The cysteine-specific reagent 5,5′-dithiobis(2-nitrobenzoic acid) inactivates the *Escherichia coli* glycogen synthase (Holmes, E., and Preiss, J. (1982) Arch. Biochem. Biophys. 216, 736–740). To find the responsible residue, all cysteines, Cys7, Cys379, and Cys408, were substituted combinatorially by Ser. 5,5′-Dithiobis(2-nitrobenzoic acid) modified and inactivated the enzyme if and only if Cys379 was present and it was prevented by the substrate ADP-glucose (ADP-Glc). Mutations C379S and C379A increased the $S_{0.5}$ for ADP-Glc 40- and 77-fold, whereas the specific activity was decreased 5.8- and 4.3-fold, respectively. Studies of inhibition by glucose 1-phosphate and AMP indicated that Cys379 was involved in the interaction of the enzyme with the phosphoglucose moiety of ADP-Glc. Other mutations, C379T, C379D, and C379I, indicated that this site is intolerant for bulkier side chains. Because Cys379 is in a conserved region, other residues were scanned by mutagenesis. Replacement of Glu377 by Ala and Gln decreased $V_{\text{max}}$ more than 10,000-fold without affecting the apparent affinity for ADP-Glc and glycogen binding. Mutation of Glu377 by Asp decreased $V_{\text{max}}$ only 57-fold indicating that the negative charge of Glu377 is essential for catalysis. The activity of the mutation E377C, on an enzyme form without other Cys, was chemically restored by carbamoylation. Other conserved residues in the region, Ser374 and Gln383, were analyzed by mutagenesis but found not essential. Comparison with the crystal structure of other glycosyltransferases suggests that this conserved region is a loop that is part of the active site. The results of this work indicate that this region is critical for catalysis and substrate binding.

*Escherichia coli* glycogen synthase (EC 2.4.1.21) catalyzes the transfer of a glucosyl unit from ADP-glucose (ADP-Glc) to the non-reducing end of glycogen.

ADP-Glc + 1,4-glucan $\rightarrow$ 1,4-glucosyl-1,4-glucan + ADP

(Eq. 1)

The reaction was described in bacteria in 1964 (1) and its equilibrium is strongly shifted toward the formation of ADP (2). The glycogen synthase from *E. coli* has been purified to homogeneity (2), and some of its properties have been characterized (3–6). Bacterial glycogen synthases and plant starch synthases catalyze the same chemical reaction and share between 30 and 36% identity, suggesting that they could have a common structural and catalytic mechanism. Both of them use ADP-Glc as a glycosyl donor and have molecular masses of 48–55 kDa, whereas the yeast and mammalian glycogen synthases have molecular masses of 70–85 kDa and prefer UDP-glucose. Another feature that differentiates the latter enzymes from bacterial and plant glycogen/starch synthases is their regulation by phosphorylation and allosteric activation by glucose 6-phosphate (7, 8). Malfunction of human glycogen synthase has been associated with several metabolic diseases, such as diabetes mellitus and glycogen storage disease (9, 10). Although there is no obvious sequence similarity between bacterial and mammalian glycogen synthases, it has been proposed that they share a common structure and catalytic mechanism. This was based on prediction of the secondary structure, threading, and hydrophobic cluster analysis (11, 12). Despite the importance of these enzymes, little is known about the structure of the catalytic site. More knowledge of the structure-function relationship in bacterial glycogen synthases could be instrumental in understanding the molecular basis of those disorders.

Few studies have been made to elucidate possible substrate-binding residues, in both bacterial glycogen synthases and plant starch synthases, but the nature of the substrate-binding site remains unclear. Furukawa et al. (13) showed that ADP-Glc protects Lys15 in the *E. coli* glycogen synthase from reaction with pyridoxal phosphate. Replacement of Lys15 by Arg, Gln, or Glu increases the $S_{0.5}$ for ADP-Glc by 7-, 32-, and 46-fold, respectively (13). In the starch synthase IIA from maize endosperm, Arg-specific modification experiments were performed with phenylglyoxal (14). However, the studied Arg residues are not 100% conserved, and, when mutated, no significant shifts in $S_{0.5}$ for ADP-Glc were shown. It has been shown that cysteine-reactive reagents inactivate *E. coli* glycogen synthase, and that this effect could be prevented by the substrates (15). However, the residues in *E. coli* glycogen synthase that are involved in this inactivation have not been identified. DTNB (5,5′-dithiobis(2-nitrobenzoic acid)) has the ability to form mixed disulfide bridges with cysteines, and it has been used to identify the ones involved in catalysis or substrate binding in different enzymes (16–21). In the present study, a combinatorial approach was used to mutate the three cysteines of the *E. coli* glycogen synthase. This strategy enabled us to determine which of those cysteines were responsible for DTNB modification and concurrent loss of activity. In addition, the kinetic properties of the mutants were analyzed to ascertain the functional role of the Cys involved. Because this Cys is in a putative loop with conserved residues that could be interacting with the substrate, we characterized the region by site-directed mutagenesis and chemical modification studies.

*This work was supported in part by Department of Energy Research Grant DE-FG02-93ER20121 and Northern Regional United States Department of Agriculture Grant NC-142. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom correspondence should be addressed. Tel.: 517-353-3137; Fax: 517-353-9334; E-mail: preiss@msu.edu.

‡ The abbreviations used are: ADP-Glc, ADP-glucose; GS, glycogen synthase; DTNB, 5,5′-dithiobis(2-dinitrobenzoic acid); DM1, double mutant C75S,C379S; DM2, double mutant C75S,C408S; DM3, double mutant C379S,C408S; TM, triple mutant C75S,C379S,C408S; TM-E377C, C75S,E377C,C379S,C408S; Bicine, N,N-bis(2-hydroxyethyl)glycine; IAA, iodoacetic acid.
**EXPERIMENTAL PROCEDURES**

**Materials**—[14C]Glucose 1-phosphate was obtained from ICN Pharmaceuticals. ADP-[14C]Glucose was synthesized as previously described (23) in a reaction mixture containing 10 µmol sodium phosphate buffer, 300 µmol NaCl (pH 7.8), and sonicated. After centrifugation, the crude extract was loaded into a nickel-charged NTA-agarose column (nickel-nitrotriacetic acid HisBind® Resin, Novagen) and washed with 20 column volumes of the same buffer plus 10 mM imidazole. His-tagged glycogen synthase was eluted with 5 column volumes of the same buffer plus 250 mM imidazole. The eluted sample was desalted with Econo-Pac 10 DG columns, concentrated, and stored at −80 °C.

**Protein Electrophoresis and Immunoblotting—SDS-PAGE** was performed according to Laemmli (25) using 4–15% Tris-HCl pre-cast gradient gels from Bio-Rad. After electrophoresis, proteins were stained with Coomassie Brilliant Blue R-250 or electrophoreted onto a nitrocellulose membrane. The procedure is a modification of the method of Towbin et al. (26).

**Protein Electrophoresis and Immunoblotting—SDS-PAGE** was performed according to Laemmli (25) using 4–15% Tris-HCl pre-cast gradient gels from Bio-Rad. After electrophoresis, proteins were stained with Coomassie Brilliant Blue R-250 or electrophoreted onto a nitrocellulose membrane. The procedure is a modification of the method of Towbin et al. (26).

**Protein Electrophoresis and Immunoblotting—SDS-PAGE** was performed according to Laemmli (25) using 4–15% Tris-HCl pre-cast gradient gels from Bio-Rad. After electrophoresis, proteins were stained with Coomassie Brilliant Blue R-250 or electrophoreted onto a nitrocellulose membrane. The procedure is a modification of the method of Towbin et al. (26).
Expression and Purification of Wild Type and Mutant Glycogen Synthases—Overexpression of the wild-type recombinant glycogen synthase was performed as described under "Experimental Procedures." In this system, the recombinant enzyme was typically obtained in crude extracts with a specific activity of 70–100 units/mg. This was at least 6000-fold higher than the endogenous activity from BL21(DE3) cells transformed with pET-24a (data not shown). Western blot analysis showed that the expression of the mutant proteins was similar to the wild-type enzyme and all of them were purified to apparent homogeneity determined by SDS-PAGE (data not shown). The mutants with low specific activity were purified using a histidine tag to ensure that no endogenous protein was being co-purified. The expression level of the wild-type enzyme with a C-terminal histidine tag was similar to the wild-type enzyme as determined by Western blotting (data not shown). Controls were performed during the purification procedure to assure that no endogenous glycogen synthase was being co-purified. No glycogen synthase activity (<0.002 units/mg) was detected in the eluates of the nickel-nitrioltriacidic acid resin when we loaded crude extracts of BL21(DE3) cells transformed with pET-24a. The enzymes purified using a histidine tag were homogeneous when subjected to SDS-PAGE (data not shown).

DTNB Inhibition of the E. coli Glycogen Synthase—We replaced Cys7, Cys379, and Cys408 by Ser and measured the activity in the presence of different concentrations of DTNB. The wild-type enzyme was strongly affected by DTNB, with an IC₅₀ of 1.2 ± 0.1 µM. The IC₅₀ for mutants C7S and C408S were 7.8 ± 2.8 and 7.0 ± 1.1 µM, whereas the IC₅₀ for C379S and the triple mutant (TM) increased to 130 ± 30 and 1200 ± 130 µM, respectively. The replacement of Cys379 was the one that made the enzyme most resistant to inactivation by DTNB. However, these results also suggested that DTNB at high concentrations interfere with the glycogen synthesis reaction because the triple mutant, with no cysteines present, was inhibited at high concentrations of DTNB.

The modification by DTNB on single cysteines was studied on double mutants with only one cysteine present (DM1, DM2, and DM3). To reduce nonspecific effects as the ones mentioned above, these studies were performed in two steps, separating the chemical modification by DTNB and the analysis of activity. The enzymes were incubated with DTNB and aliquots were withdrawn at different times to assay the activity, diluting 100-fold the DTNB carried into the assay mixture. With this approach, the wild-type enzyme was rapidly inactivated by DTNB, whereas TM was not affected. The wild-type enzyme had a t₅₀ of inactivation of less than 1 min at 0 °C (Fig. 1) and TM remained active even after 20 min (data not shown). DM2 was inactivated as the wild-type enzyme but DM1 and DM3 were insensitive to DTNB treatment (Fig. 1). Because DM2 has only Cys379 present, this must be the residue responsible for the inactivation by DTNB. The presence of 1 mM ADP-Glc in the incubation mixture prevented the inactivation by DTNB of both the wild-type and DM2. On the other hand, 2.5 mg/ml glycerol did not significantly protect glycogen synthase (Fig. 1).

Inactivation by DTNB seems to be reversible. After a 20-min incubation, treatment with 5 mM dithiothreitol recovered the activity of DM2 and the wild-type enzyme, 95 and 70%, respectively (data not shown). This indicates that in the conditions of this experiment, DTNB only affects the activity through its ability to covalently modify sulfhydryl groups.

Protection of the DTNB Modification—The above mentioned experiments showed that DTNB inactivated the enzyme only if Cys379 was present. To verify that the inactivation proceeded through a chemical modification of this particular residue, the cleavage of DTNB was followed spectrophotometrically in the presence of the different glycogen synthase mutants. First the derivative spectra of DTNB were recorded to increase the sensitivity. As a positive control, 10 µM Cys solution altered the DTNB spectra and a negative peak appeared at 445 nm (Fig. 2). A similar change in the spectrum was obtained by the addition of 10 µM wild-type glycogen synthase, showing that −1 sulphydryl molecule of enzyme reacted with DTNB. ADP-Glc prevented the spectral change, indicating that it protects the enzyme from the modification by DTNB (Fig. 2). A negative control in the presence of the mutant with no Cys (TM) showed that DTNB did not change its spectra, either in the presence or absence of ADP-Glc. Double mutants were analyzed to confirm which Cys was responsible for the spectral changes of DTNB. DM2, which has only Cys379 in its sequence, was the only double mutant that reacted with DTNB as the wild-type (Fig. 2). ADP-Glc also prevented the reaction of DTNB with DM2 (Fig. 2).

Kinetic Characterization of the Cys Mutant Enzymes—The purified wild-type and mutant glycogen synthases were assayed to compare their kinetic parameters. The S₀.₅ for ADP-Glc determined for the wild-type enzyme was 11 µM, which agrees with previously reported data (2) (Table I). The S₀.₅ for C7S and the C408S mutants were not significantly different, whereas the values for C379S and TM increased 38- and 56-fold, respectively. The specific activities of mutants C7S and C408S were 40–60% of that of the wild-type, whereas mutant C379S and TM decreased 6- and 12.5-fold, respectively. DM2 showed a S₀.₅ for ADP-Glc of 18 µM and the specific activity was not significantly different from the wild-type. Conversely, DM1 and DM3 had an S₀.₅ in the same order as the TM, nearly 60-fold higher than the wild-type. DM2 had about 90% of the wild-type enzymatic activity, whereas DM1 and DM3 were 22–24% active. When Cys379 was substituted by Ala or Thr, the S₀.₅ increased 77- and 123-fold, respectively. The substitution of Cys379 by Asp yielded an enzyme with no detectable glycogen

---

Fig. 1. Inactivation of glycogen synthase by incubation with DTNB. The remaining activity of the different enzymes (wild type, DM1, DM2, and DM3) was assayed after different incubation times with 10 µM DTNB as described under "Experimental Procedures." ○, control; ●, 1 mM ADP-Glc; ▲, 2.5 mg/ml rabbit liver glycogen in the incubation mixture. Controls without DTNB verified that the enzymes were stable throughout the experiment (data not shown).
synthesis activity. The S0.5 for glycogen was also determined for the wild-type and the mutant enzymes. The S0.5 of glycogen for different mutants were in a narrow range, from 5-fold lower to 2-fold higher than the wild type.

Inhibition by Glc-1P and AMP—Both Glc-1P and AMP were inhibitors of the wild-type glycogen synthase competing with the substrate ADP-Glc with a KI of 1.15 and 0.17 mM, respectively. When the same parameter was calculated for the mutant C379A, the KI for AMP showed a 2.8-fold increase (Table II), whereas the KI for Glc-1P increased 75-fold with respect to that of the wild type.

Scanning of Essential Residues—As Cys379 is located in a conserved region that is predicted to be a loop (Table III), we decided to study its importance for enzymatic activity. The side chain of each of the 100% conserved residues in that putative

| Glycogen synthase   | ADPGlc S0.5 μM | nH | -Fold | Glycogen S0.5 μM | nH | -Fold | Units/mg | Vmax Relative (mutant/WT) |
|---------------------|----------------|----|-------|------------------|----|-------|----------|--------------------------|
| Wild-type           | 11 ± 2         | 1.18 ± 0.20 | 1.0   | 0.95 ± 0.10      | 0.82 ± 0.04 | 1.0 | 570 ± 37 | 1.00 |
| C7S                 | 15 ± 4         | 1.02 ± 0.12 | 1.4   | 1.72 ± 0.18      | 0.86 ± 0.04 | 1.18 | 237 ± 8  | 0.42 |
| C379S               | 420 ± 15       | 1.36 ± 0.15 | 38    | 1.10 ± 0.15      | 1.36 ± 0.20 | 1.16 | 98 ± 30  | 0.17 |
| C408S               | 13 ± 3         | 1.21 ± 0.27 | 1.2   | 1.30 ± 0.30      | 1.31 ± 0.26 | 1.37 | 365 ± 5  | 0.64 |
| TM (C7S, C379S, C408S) | 620 ± 65        | 1.42 ± 0.19 | 56    | 0.15 ± 0.02      | 1.11 ± 0.20 | 0.16 | 46 ± 4   | 0.08 |
| DM1 (C7S, C379S)    | 610 ± 71       | 0.83 ± 0.07 | 55    | 0.15 ± 0.02      | 0.82 ± 0.10 | 0.16 | 135 ± 12 | 0.24 |
| DM2 (C7S, C408S)    | 18 ± 2         | 0.98 ± 0.06 | 1.6   | 1.65 ± 0.14      | 0.84 ± 0.03 | 1.74 | 500 ± 10 | 0.88 |
| DM3 (C379S, C408S)  | 630 ± 39       | 1.20 ± 0.16 | 57    | 1.50 ± 0.60      | 0.70 ± 0.10 | 1.58 | 126 ± 13 | 0.22 |
| C379A               | 850 ± 32       | 0.95 ± 0.15 | 77    | 0.22 ± 0.03      | 1.11 ± 0.16 | 0.23 | 130 ± 9  | 0.23 |
| C379T               | 1350 ± 76      | 0.70 ± 0.02 | 123   | ND*              | ND |       | 1.5 ± 0.2 | 0.003 |
| C379D               | ND             | ND |     | ND*              | ND |       | <0.01    | <0.0001 |

*ND, not determined.

### Table II

Inhibition of wild-type and C379A mutant with Glc-1P and AMP

| Inhibitor | Wild-type KI μM | C379A KI μM | Increase |
|-----------|-----------------|-------------|----------|
| Glc-1P    | 1.15 ± 0.17     | 86 ± 6      | 75       |
| AMP       | 0.17 ± 0.01     | 0.476 ± 0.025 | 2.8      |
The residues with gray background are 100% conserved. The number before the source is the number of amino acids of the protein, whereas the number before the sequence represents the first aligned amino acid of each protein. The secondary structure prediction of the E. coli sequence is on top of the alignment; E, β-sheet; C, loop; H, helix.

| Accession N° | Bacterial                | Accesion N° | Bacterial                |
|--------------|--------------------------|-------------|--------------------------|
| PO8323       | Escherichia coli         | (477)       | Entercoccus faecalis     |
| P59670       | Agrobacterium tumefaciens| (360)       | Escherichia coli         |
| P59670       | Agrobacterium tumefaciens| (358)       | Pseudomonas aeruginosa   |
| PO8328       | Geobacillus stearothermophilus | (370) | Lactococcus lactis       |
| Q95895       | Mosecola bovis           | (368)       | Pipeobacteriales         |
| Q8VU5        | Anaerobacter sp.         | (378)       | Mycoplasma                |
| Q8XPA1       | Clostridium perfringens  | (376)       | Propionibacteriales       |
| Q9Q955       | Streptococcus pneumonia  | (370)       | Propionibacteriales       |
| Q9R86        | Vibrio cholerae          | (376)       | Propionibacteriales       |
| Q8AYA1       | Chlorobium tepidum       | (384)       | Thermoanaerobacteriales   |

Co-sedimentation with Glycogen—To assess if the presence of a negative charge in position 377 could restore the enzymatic activity of an inactive mutant, we replaced Glu377 with Cys, which is reactive to carbamoylation by IAA. To avoid interference with other Cys, this experiment was performed with the triple mutant. The purified mutant TM-E377C (C7S,E377C,C379S,C408S) was incubated with 1 mM IAA, and aliquots were withdrawn and activity was determined by the assay reaction. Double mutants with only one cysteine per se were used to investigate which sulfhydryl group was responsible for the DTNB-mediated inactivation. When Ser replaced Cys, the enzyme was no longer inactivated by DTNB. On the other hand, ADP-Glc prevented inactivation of the wild-type enzyme or the DM2, both of which contain Cys. The inactivation by DTNB correlates with the modification of one sulfhydryl group per molecule of wild-type glycogen synthase (Fig. 2). The other two cysteines only reacted when the enzyme was previously denatured with 6M guanidine chloride (data not shown). This suggests that Cys7 and Cys108 are most probably buried in the structure of the enzyme. The only double mutant with a sulfhydryl group reactive to DTNB

table III

| Accession N° | Bacterial | accession N° | Bacterial |
|--------------|-----------|-------------|-----------|
| CAB09545     | Zea mays  | (533)       | Zea mays  |
| BAA0796      | Oryza sativa | (525)  | Oryza sativa |
| Plant (granular bound) |           |            |           |
| S07314       | Zea mays  | (473)       | Zea mays  |
| AAN37103     | Oryza sativa | (477)  | Oryza sativa |
| ACO04424     | Arabidopsis thaliana | (476)  | Arabidopsis thaliana |
| AAL3709      | Hordeum vulgare | (471)  | Hordeum vulgare |
| CAA41359     | Solanum tuberosum | (475)  | Solanum tuberosum |

An Essential Loop in the Glycogen Synthase

In this work, we report the results of a chemical modification and mutational analysis of a putative loop that plays a critical role in the glycogen synthase from E. coli. We first found that Cys377 is relevant for the interaction with the substrate ADP-Glc and the analysis was extended to other conserved residues of that area.

The replacement of Glu377 with Cys generated an enzyme with extremely low activity, 2,300-fold less than the TM (Table V). Incubation with 1 mM IAA reactivated the enzyme with extremely low enzymatic activity, 2,300-fold less than that of the TM-E377C enzyme. The purified mutant enzymes were assayed as described under Experimental Procedures.

Experimental Procedures

The mutant enzymes were assayed as described under “Experimental Procedures” to compare their kinetic parameters with the wild type. The specific activity of E377A was 4 orders of magnitude lower than the TM (Table V). Incubation with 1 mM IAA restored the activity of E377A and TM-E377C to the same activity level of the TM (data not shown). As a control, when centrifuged in the absence of glycogen, the wild-type enzyme was found in the supernatant (Fig. 4). The inactive mutants E377A, E377Q, and TM-E377C followed the same pattern of co-sedimentation as the wild type. As a negative control, ADP-glucose phosphorylase from E. coli does not co-sediment with glycogen (Fig. 4).

Kinetic Characterization of Mutants S374A and Q383A—Purified mutants S374A and Q383A were analyzed kinetically (Table VI). Mutant S374A showed no significant differences in the kinetic parameters compared with the wild-type enzyme. The specific activity was comparable, and the apparent affinities for the substrates of the wild-type enzyme. However, the specific activity of mutant Q383A decreased 23-fold. The interaction with the substrates of the Q383A mutant did not seem to be impaired. The apparent affinity for ADP-Glc remained unchanged, whereas that for glycogen was 2 orders of magnitude higher (Table VI).

Table IV

| V_max | ADP-Glc | Glycogen |
|-------|---------|----------|
| Wild-type | His tag | E377A | 0.05 ± 0.01 | 10000 | 70 ± 30 | 1.1 | 27 ± 2 | 1.0 |
| Wild-type | His tag | E377Q | 0.020 ± 0.001 | 25000 | 61 ± 12 | 0.9 | 14 ± 2 | 1.0 |
| Wild-type | His tag | E377D | 10 ± 1 | 57 | 1500 ± 300 | 0.9 | 26 ± 5 | 1.0 |

Discussion

In this work, we report the results of a chemical modification and mutational analysis of a putative loop that plays a critical role in the glycogen synthase from E. coli. We first found that Cys377 is relevant for the interaction with the substrate ADP-Glc and the analysis was extended to other conserved residues of that area.

It has been reported that DTNB inactivates the enzyme, but it had not been determined which cysteine was involved in the process (15). We replaced each of the three Cys by Ser and analyzed the activity in the presence of different concentrations of DTNB to find out the responsible residue. Mutants C379S and TM were clearly the most resistant to inhibition, but DTNB seemed to interfere per se with the catalytic reaction at high concentrations. For that reason, further experiments were performed in two steps, separating the modification by DTNB from the assay reaction. Double mutants with only one cysteine were used to investigate which sulfhydryl group was responsible for the DTNB-mediated inactivation. When Ser replaced Cys797, in any combination, the enzyme was no longer inactivated by DTNB. On the other hand, ADP-Glc prevented inactivation of the wild-type enzyme or the DM2, both of which contain Cys797. The inactivation by DTNB correlates with the modification of one sulfhydryl group per molecule of wild-type glycogen synthase (Fig. 2). The other two cysteines only reacted when the enzyme was previously denatured with 6M guanidine chloride (data not shown). This suggests that Cys7 and Cys108 are most probably buried in the structure of the enzyme. The only double mutant with a sulfhydryl group reactive to DTNB
The TM-E377C His tag was modified by incubation with IAA for 10 min prior to activity determinations, as described under “Experimental Procedures.”

### Table V

| TM (C7S, C379S, C408S) | 46 ± 2 | 1 | 620 ± 65 | 1.4 | 150 ± 20 | 1.1 |
|-------------------------|--------|---|----------|-----|---------|-----|
| TM-E377C His tag        | 0.02 ± 0.01 | 2.3 | 213 ± 74 | 1.1 | 24 ± 2 | 1.0 |
| TM-E377C His tag + IAA  | 50 ± 2 | 0.92 | 810 ± 50 | 0.9 | 70 ± 15 | 1.3 |

### Table VI

| S374A | Q383A |
|-------|-------|
| 490 ± 10 | 600 ± 47 |
| 25 ± 1 | 10 ± 1 |

*Relative to the wild-type enzyme (Table I).*

The substrate ADP-Glc because the $S_{0.5}$ for glycogen was not altered significantly in any of the single mutants (Table I). All the single mutations, such as C7S, C379S, and C408S, slightly affected the catalytic efficiency. They had, respectively, a 2.4-, 5.8-, and 1.6-fold lower specific activity than the wild type. We replaced Cys$^{379}$ by other amino acids to study the effect of different groups at this position on the kinetic properties of the enzyme. We found that C379A and C379T had a 77- and 123-fold lower apparent affinity for ADP-Glc than the wild type (Table I). These significant shifts in the $S_{0.5}$ indicate that Cys$^{379}$ is involved in the interaction with the ADP-Glc or contributing to the proper architecture of its binding site. There does not seem to be an obvious role of this residue in glycogen binding because single mutations did not affect the $S_{0.5}$ for glycogen more than 1.8-fold. Moreover, double mutant DM1 and the triple mutant had a lower $S_{0.5}$ for glycogen than the wild type, whereas the individual mutations showed the opposite effect. It is possible that these mutations caused minor perturbations in the structure that were reflected in those slight kinetic changes.

The $V_{max}$ of the C379S mutant is only six times lower than the wild-type, and is not sufficient to assign a specific catalytic role to Cys$^{379}$. Mutation of Cys$^{379}$ for Ala mainly affected the affinity for ADP-Glc, as the $S_{0.5}$ increased 77-fold and the $V_{max}$ decreased only 4-fold. However, other mutations at this site showed a greater effect on the catalytic efficiency. A mutation to Thr had an even higher $S_{0.5}$ for ADP-Glc (123-fold) but the $V_{max}$ was only 0.3% of the wild-type. It is possible that a steric hindrance is responsible for this effect because the difference between Thr and Ser is only a methyl group. In fact, when a bulkier group such as the Asp group replaced Cys$^{379}$ the activity of the enzyme decreased more than 4 orders of magnitude (Table I). Mutation of Cys$^{379}$ by Leu generated an inactive enzyme, strongly suggesting that the residue is located in or near the substrate site.

Kinetic analysis of the Ser mutant enzymes revealed the importance of Cys$^{379}$, compared with the other cysteines. Replacement of Cys$^{379}$ by Ser decreased the apparent affinity for ADP-Glc 38-fold, whereas mutations on Cys$^7$ and Cys$^{408}$ did not change it significantly (Table I). This effect was specific for the substrate ADP-Glc because the $S_{0.5}$ for glycogen was not altered significantly in any of the single mutants (Table I). All the single mutations, such as C7S, C379S, and C408S, slightly affected the catalytic efficiency. They had, respectively, a 2.4-, 5.8-, and 1.6-fold lower specific activity than the wild type. We replaced Cys$^{379}$ by other amino acids to study the effect of different groups at this position on the kinetic properties of the enzyme. We found that C379A and C379T had a 77- and 123-fold lower apparent affinity for ADP-Glc than the wild type (Table I). These significant shifts in the $S_{0.5}$ indicate that Cys$^{379}$ is involved in the interaction with the ADP-Glc or contributing to the proper architecture of its binding site. There does not seem to be an obvious role of this residue in glycogen binding because single mutations did not affect the $S_{0.5}$ for glycogen more than 1.8-fold. Moreover, double mutant DM1 and the triple mutant had a lower $S_{0.5}$ for glycogen than the wild type, whereas the individual mutations showed the opposite effect. It is possible that these mutations caused minor perturbations in the structure that were reflected in those slight kinetic changes.

The $V_{max}$ of the C379S mutant is only six times lower than the wild-type, and is not sufficient to assign a specific catalytic role to Cys$^{379}$. Mutation of Cys$^{379}$ for Ala mainly affected the affinity for ADP-Glc, as the $S_{0.5}$ increased 77-fold and the $V_{max}$ decreased only 4-fold. However, other mutations at this site showed a greater effect on the catalytic efficiency. A mutation to Thr had an even higher $S_{0.5}$ for ADP-Glc (123-fold) but the $V_{max}$ was only 0.3% of the wild-type. It is possible that a steric hindrance is responsible for this effect because the difference between Thr and Ser is only a methyl group. In fact, when a bulkier group such as the Asp group replaced Cys$^{379}$ the activity of the enzyme decreased more than 4 orders of magnitude (Table I). Mutation of Cys$^{379}$ by Leu generated an inactive unstable form that was proteolyzed in the cell, yielding a polypeptide of 41 kDa (data not shown). Replacement of Cys$^{379}$ by residues of smaller or similar size (Ala, Ser) decreased the affinity for ADP-Glc and only when bigger groups were used (Thr, Asp, and Leu) catalysis was severely affected.

Substrate saturation analysis of the purified mutants indicates that Cys$^{379}$ is involved directly or indirectly in the interaction of the enzyme with ADP-Glc. To further investigate what portion of the ADP-Glc molecule is engaged in this interaction, we studied the affinity of both the enzyme and the mutant C379A for Glc-1P and AMP. Both AMP and Glc-1P can be considered as portions of the molecule of the substrate ADP-Glc. A differential effect on the affinity for these mole-
An Essential Loop in the Glycogen Synthase

8365

An Essential Loop in the Glycogen Synthase

Role of the Putative Loop Bearing Cys$^{379}$—The prediction of the secondary structure of the glycogen synthase from E. coli classifies the enzyme as an α/β protein (38). It has been observed that in enzymes with α/β structures the active sites are constructed with residues in the loops between each strand of a β-sheet and its following helix (39). Particularly, this has been confirmed among the superfamily of glycosyltransferases (38). The region comprised by residues 373–380 is predicted as a loop between a β-sheet and an α-helix (Table III). Taking into account that Cys$^{379}$ was found to be important for the interaction with the substrate, and that other amino acids in this region are highly conserved, our hypothesis was that this putative loop is near the substrate site and could play other important roles for the enzyme. We decided to characterize the functional role of other residues of this area by site-directed mutagenesis.

Replacement of Glu$^{377}$ for Ala or Gln rendered an enzyme with a 10,000- or 25,000-fold decrease in specific activity, respectively. We studied in detail mutants E377A and E377Q and found that, despite being essentially inactive, retained the affinity for Glc-1P in the same magnitude as it did to the apparent affinity for ADP-Glc (75- and 77-fold, respectively; Tables I and II). This indicates that Cys$^{379}$ is important for the interaction of the enzyme with the phosphoglucose moiety of the ADP-Glc and not with the nucleoside portion.

Relationship with Other Glycosyltransferases—Glycosyltransferases have been divided into 65 families based on sequence similarity to founding members with experimentally demonstrated glycosyltransferase activity (40). However, upon analysis of the growing number of crystallographic data, it has been suggested that this separation into families is concealing fundamental structural relationships (41). All these families have been recently grouped into three different folds,
GT-A, GT-B, and GT-C (42). Bacterial glycogen synthases belong to glycosyltransferase family 5 and would have a GT-B fold by several structure prediction analyses (38, 41–43). This family 5 seems to share more structural similarity with the glycosyltransferases from families 3 (eukaryotic glycogen synthases) and 4 (sucrose synthase, trehalose phosphorylase, and mannosyltransferases). From these three families, no three-dimensional structures are available. Family 5 is also structurally related, although more remotely, to family 35 (glycogen, starch, and maltodextrin phosphorylase) and family 20 (trehalose-6-phosphate synthase). Crystal structures from members of these two families are available (34, 44, 45) and have a GT-B fold. This fold comprises two Rossmann-like domains, each composed of a “sandwich” of parallel β-sheets between α-helices (38, 43). All members of families 3, 4, 5, 20, and 35 retain the anomeric configuration of the monosaccharide transferred. The sequence similarities among these families have been discussed, and a conserved negatively charged residue has been localized in a loop that would correspond to the region studied in this work (38, 41).

In this work, we described the first essential catalytic residue found in bacterial glycogen synthases. Despite the extremely low similarity in sequence, prediction analysis of the secondary structure and further alignment can identify this homologous residue among members of other glycosyltransferases that use sugar-nucleotide donors (38). Studies in enzymes of those other families provided evidence of the importance of a negative charge in this region. Mutation E510A of a recombinant fusion of the human muscle glycogen synthase (family 3) with green fluorescent protein reduced the enzymatic activity in crude extracts from 97 to 12 milliunits/mg (12). In that work, it was not possible to accurately determine the decrease of catalytic efficiency because of the very high endogenous activity present in COS-1 cells (12 milliunits/mg). In starch synthases (family 5), this putative loop has also conserved homologous residues to Ser374, Glu377, Cys379, and Gln383. A scanning of acidic residues detected that Glu383 (homologous of Glu377) mutated to Gln was inactive in crude extracts, but no further studies were performed on this mutant (46). The bacterial α-mannosyltransferase AceA (family 4) was mutated in Glu637. The mutant E287A, in a partially purified fusion protein with an S-tag, lost the ability to transfer mannose from GDP-mannose to the acceptor Glcβ1(4)GlcP-P-polyprenol. However, the extent of this reduction was not quantified (47). To assess a residue as catalytic by site-directed mutagenesis is necessary to meet some requirements. It is expected that the reduction in specific activity or $k_{cat}$ should be several orders of magnitude. For that reason, the mutant should be purified in a system without interfering endogenous activity. In addition, there should be evidence that the ability to bind each of the substrates has not been severely altered. Finally, the possibility should be ruled out that the mutant is expressed as a misfolded form; i.e. the original residue plays a structural role in the enzyme rather than a functional role. Despite this negatively charged residue was found important among members of other families, this is the first time that the above mentioned requirements were met to assign a catalytic role in glycosyltransferases that use sugar-nucleotides as donors.

Our results with Glu377 mutants are in good agreement with mutagenesis studies on the maltodextrin phosphorylase. In this family of oligo- or polysaccharide phosphorylases (family 35), a negative charge in this region has been postulated to play a role in catalysis. Despite that the substrates of the phosphorylases are not a sugar-nucleotide, there are many similarities. Not only that they have the GT-B fold (38, 41–43), but also the reverse reaction is chemically equivalent to the bacterial glycogen synthases forward reaction. An α1,4-glucosyl bond is formed upon breakage of a phoshooester bond with the OH1 of α-glucose. The residue with the negative charge, Glu672 in the rabbit muscle glycogen phosphorylase $b$, interacts with OH2 and OH3 of the glucose of the non-reducing end. Glu672 has been postulated, based on the crystal structure, to be involved in binding (44). However, biochemical evidence indicated that the main contribution is catalytic. This conserved residue has been mutated in the E. coli maltodextrin phosphorylase (Glu637) by Asp and Gln and it showed a reduction in $k_{cat}$ of 600- and 900-fold, respectively (48).
Inhibition studies by Glc-1P and AMP on the wild type and mutant C379A indicated that the conserved Cys379 interacts with the phosphoglucose moiety of the ADP-Glc rather than the nucleoside. These results correlate with the prediction of the structure of the E. coli glycogen synthase (38). The Cys379 interacts with the monosaccharide donor (Fig. 5).

The catalytic mechanism of retaining glycosyltransferases has been a challenging problem in enzymology. The strongest mechanistic candidate probably involves a late oxonium-like transition state (34, 50). An important role in the catalytic mechanism of the maltodextrin phosphorylase has been given to Arg266 and Lys269. The former stabilizes the substrate phosphate and the latter positions the phosphate group of the cofactor pyridoxal 5’-phosphate (34, 48, 50). According to crystallographic data, Glu637 partially neutralizes the charge of Lys269. It has been postulated that Glu637 may participate in catalysis through a dual role. First, it would lock the substrate in a more favorable position in the transition state through interaction with 3’-OH of the glucosyl pyranose. Second, it would be involved in the charge network with Lys356 to keep a balanced protonation of the phosphates (48). There is a striking similarity with the structure of the retaining glycosyltransferase trehalose-6-phosphate synthase. It also has two positively charged residues, Arg263 and Lys268, which interact with the α- and β-phosphates of UDP-glucose. The complete catalytic center is almost identical to that of the maltodextrin phosphorylase and those interactions are in a very similar arrangement (34). The difference is that rather than Glu637, trehalose-6-phosphate has Asp362. Because the glycogen synthase, according to a homology model, Glu377 is in a more favorable position in the transition state through interaction with Lys305, in a neighbor loop that also has Arg300 and Lys305 should also be important residues for catalysis, Arg300 and Lys305 should also be important residues for catalysis. These residues are conserved in not only all bacterial glycogen synthases, but also in members of families 3–5 (38). Experiments in the E. coli glycogen synthase showed that mutations to Ala yielded enzymes at least 3 orders of magnitude less active.6 To reveal whether Glu377 works interacting with Lys305 further experiments and crystallographic data will be needed.

Because of the numerous and very important reactions that glycosyltransferases catalyze, members of this superfamily of enzymes are very good candidates for protein design. In this regard, determination of the essential features, defined as a structural rule for the class of proteins that perform a particular function by means of a particular fold, are particularly relevant (51). Of all the conserved residues of this putative loop, Glu377 seems to be the only one that plays an essential function.

6 A. Yep, M. A. Ballicora, and J. Preiss, unpublished results.