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Cloning, Expression, and Nucleotide Sequence of a Mutant *glgC* Gene from *Escherichia coli* B

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A mutant *glgC* gene contained in a 10.9-kb *Pst*I fragment was cloned from the *Escherichia coli* B strain SG5 via colony hybridization by using a wild-type *glgC* probe. The altered allosteric properties of the expressed ADPglucose synthetase were found to result from the conversion of proline to serine at amino acid residue 295.

ADPglucose synthetase (EC 2.7.7.27), which catalyzes the conversion of Glc-1-P and ATP to ADP glucose and PP_i, is an important regulatory enzyme in both bacterial glycogen biosynthesis (22, 25) and plant starch biosynthesis (23, 24). In *Escherichia coli*, the main effectors of this allosteric enzyme are the activator fructose-1,6-bisphosphate (FBP) and the inhibitor AMP (22, 25).

A combination of chemical modification and site-directed mutagenesis studies have identified the presence of key lysine residues at positions 39 and 195 involved in the activator site (8, 20, 21) and active site (13, 16, 21) of this 431-amino-acid enzyme. In addition, there is a tyrosine residue at position 114 apparently involved in substrate and activator binding (15, 16, 21). Allosteric mutants of ADPglucose synthetase are readily available and are useful tools in the identification and characterization of important functional regions. The *E. coli* 618 mutant enzyme, which has a higher apparent affinity for FBP and a lower apparent affinity for AMP than the wild-type enzyme does, was found to have a single mutation at position 336 converting glycine to aspartate (13a, 14). A similar enzyme from mutant strain CL1136 was found to have a mutation at position 67 which converted arginine to cysteine (9).

The mutant enzyme from the glycogen-overproducing *E. coli* B strain SG5 also has a higher affinity for FBP and a lower affinity for AMP than the wild-type enzyme does (10, 11). The purified enzyme from SG5 has the same molecular weight, first 27 N-terminal amino acids, and C terminus as the wild-type enzyme (4), indicating that the dramatic allosteric change might also be due to a single-amino-acid mutation. This report describes the cloning, expression, and sequencing of the mutant *glgC* gene from SG5.

Cloning of the SG5 mutant *glgC* gene. Standard protocols were used in all general DNA and microbe manipulations (27). Table 1 describes all relevant bacterial strains and plasmids. Chromosomal DNA from mutant strain SG5 was isolated as previously described (19), cut with *Pst*I, and ligated into the *Pst*I site of pBR322. The ligated DNA was then transformed into *E. coli* HB101. The SG5 library was screened by colony hybridization with a probe constructed from the wild-type *glgC* gene (from pOP12; Table 1) by the random priming method of Feinberg and Vogelstein (7). Screening of the library was performed as previously described (9, 18). A total of 2.5×10^3 colonies were screened. Although HB101 has its own *glgC* gene, the intensity of signal from colony hybridization corresponding to the multicopy plasmid was much greater than the signal corresponding to the HB101 cells alone. Two colonies showed a positive hybridization signal and were found to contain the identical plasmid designated pPD5 (Table 1). Figure 1 shows partial

TABLE 1. Bacterial strains and plasmids

Bacterial strain or plasmid	Relevant genotypic or phenotypic characteristic(s)	Reference
E. coli strains		
HB101	*supE44 hsdS20*(r_B^- m_B^-) *recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1*	27
MV1193	Δ(*lac-proAB*) *rpsL thi endA spcB15 hsdR4* Δ(*srl-recA*)*306*::Tn*10*(Tet^r)	27
DH5α	*supE44 hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	27
G6MD3	Δ(*mal-asd*) (*glg*)	29
Plasmids		
pBR322	Ap^r Tet^r	2
pOP12	Ap^s Tet^r, pBR322 plus 10.9-kb insert at *Pst*I (Ap) site containing *asd* and *glg* genes	19
pPD5	Ap^s Tet^r, pBR322 plus 10-kb insert from mutant strain SG5 containing *asd* and *glg* genes	This work
pUC118, pUC119	Ap^r	17
pCJ1	Ap^r, pUC119 plus 1.9-kb *Hin*cII fragment (*glgC*) from pPD5	This work
pCJ2	Ap^r, pUC118 plus 1.3-kb *Hin*cII-*Hin*dIII fragment from pCJ1	This work
pCJ3	Ap^r, pUC119 plus 0.6-kb *Hin*dIII-*Hin*cII fragment from pCJ1	This work

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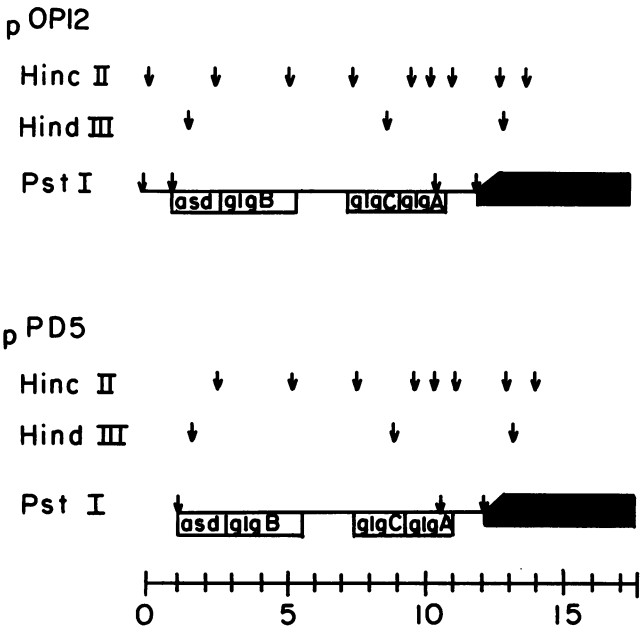

FIG. 1. Restriction maps of pOP12 and pPD5. The arrows indicate the restriction sites, and the black portion of the plasmid represents the pBR322 vector. The scale shows kilobases.

restriction maps for pPD5 and pOP12. All fragment sizes are in agreement with the proposed orientation of pPD5.

Expression and partial purification of the mutant ADPglucose synthetase from pPD5. Plasmid pPD5 was transformed into the *glg asd* mutant G6MD3 (Table 1) (29) for expression. The transformed G6MD3 strain no longer required diaminopimelic acid for growth because of cotransformation of the neighboring *asd* gene (Table 1; Fig. 1). Extraction and partial purification of the mutant enzyme were done as previously described (4, 10) and included sonication, heating, ammonium sulfate precipitation, and DEAE anion-exchange chromatography. During purification, the activity of ADPglucose synthetase was measured in the pyrophosphorolysis direction as previously described (26), using $^{32}PP_i$. The enzyme in the crude sonicate had a specific activity of 1 U/mg, which is about 10-fold higher than the value reported for the parent SG5 strain (4) and is probably due to the multicopy plasmid. After DEAE chromatography, the enzyme had a specific activity of 50 U/mg, or fivefold greater than the value previously obtained at this step (4), which represents about 50% purity.

Kinetic analysis of the mutant ADPglucose synthetase from pPD5. The enzyme pooled from the DEAE chromatography step was suitable for kinetic analysis in the synthesis direction (26; forming ADP glucose from [^{14}C]glucose-1-phosphate), as there were no significant interfering reactions and the reaction rate was linear versus enzyme concentration and time in the range used. This result was expected, as the kinetic parameters of the homogenous enzyme (4) had been found to be identical to those of far less purified enzyme (10). Table 2 presents the kinetic parameters for the activator, inhibitor, and substrates for the mutant ADPglucose synthetase from pPD5 and the parameters calculated for the wild-type enzyme, using the same conditions and reagents for a direct comparison. Data were analyzed using nonlinear least-square fitting to a modified Michaelis-Menten equation accounting for the Hill number, maximum rate of reaction (V_{max}), concentration of substrate that gives 50% of maximum velocity ($S_{0.5}$), concentration of activator that gives 50% of maximum activation ($A_{0.5}$), and concentration of inhibitor that gives 50% of maximum inhibition ($I_{0.5}$) with the use of a computer program (3). The kinetic parameters obtained were in good agreement with those found by using direct Hill plots (6). The $A_{0.5}$ value for FBP is twofold lower for the mutant enzyme from pPD5 than for the wild-type enzyme, and the $I_{0.5}$ value for AMP is ca. twofold higher for the enzyme from pPD5 than for the wild-type enzyme (10). In addition, the mutant's affinities for substrates and Mg^{2+} were higher than those of the wild-type enzyme in the absence of FBP. Maximal activation by FBP was 13.4-fold

TABLE 2. Kinetic parameters of mutant and wild-type ADPglucose synthetase

Ligand	Enzyme[a]	Kinetic parameter of enzyme[b] (mean ± SEM)					
		Without FBP			With 1.5 mM FBP		
		$A_{0.5}$	$S_{0.5}$	Hill no.	$I_{0.5}$	$S_{0.5}$	Hill no.
FBP	Mutant	18.3 ± 0.9 μM (22 μM)		1.6 ± 0.1 (1.8)			
	Wild type	42.2 ± 1.7 μM (68 μM)		1.7 ± 0.1 (2)			
AMP	Mutant				185 ± 15 μM (170 μM)		1.6 ± 0.3 (1.5)
	Wild type				94 ± 7 μM (105 μM)		1.5 ± 0.2 (1.7)
ATP	Mutant		0.9 ± 0.1 mM (1.0 mM)	2.4 ± 0.3 (1.8)		0.27 ± 0.02 mM (0.24 mM)	1.6 ± 0.2 (1.4)
	Wild type		2.2 ± 0.7 mM (1.3 mM)	1.6 ± 0.3 (1.8)		0.29 ± 0.02 mM (0.38 mM)	2.2 ± 0.3 (1.8)
Mg^{2+}[c]	Mutant		5.6 ± 0.2 mM (11 mM)	5.0 ± 0.9 (4)		2.5 ± 0.1 mM (2 mM)	4.9 ± 0.5 (4.6)
	Wild type		8.2 ± 0.4 mM (12.3 mM)	3.7 ± 0.4 (4.5)		2.5 ± 0.2 mM (2.3 mM)	4.4 ± 0.8 (4.8)
Glc-1-P[d]	Mutant		22.5 ± 2.7 μM (39 μM)	0.7 ± 0.1 (0.7)		34.1 ± 3.4 μM (43 μM)	1.1 ± 0.1 (1)
	Wild type[e]		132 ± 10 μM (120 μM)	0.5 ± 0.03 (0.6)		27.3 ± 2.1 μM (36 μM)	1.1 ± 0.1 (1)

[a] The mutant enzyme is from pPD5, and the wild-type enzyme is from *E. coli* B.

[b] Kinetic parameter abbreviations: $A_{0.5}$, concentration of activator that gives 50% of maximum activation; $S_{0.5}$, concentration of substrate that gives 50% of maximum velocity; $I_{0.5}$, concentration of inhibitor that gives 50% of maximum inhibition. The values in parentheses are the values for SG5 and wild-type enzymes from reference 10.

[c] Mg^{2+} assay mixtures in the absence of FBP contained 5 mM ATP, rather than 7.5 mM ATP (10).

[d] Glc-1-P assay mixtures in the absence of FBP contained 5 mM ATP and 12 mM Mg^{2+}, rather than 7.5 mM ATP and 25 mM Mg^{2+}.

[e] In the absence of FBP, the maximum rate of metabolism was estimated by extrapolation from a Lineweaver-Burk plot.

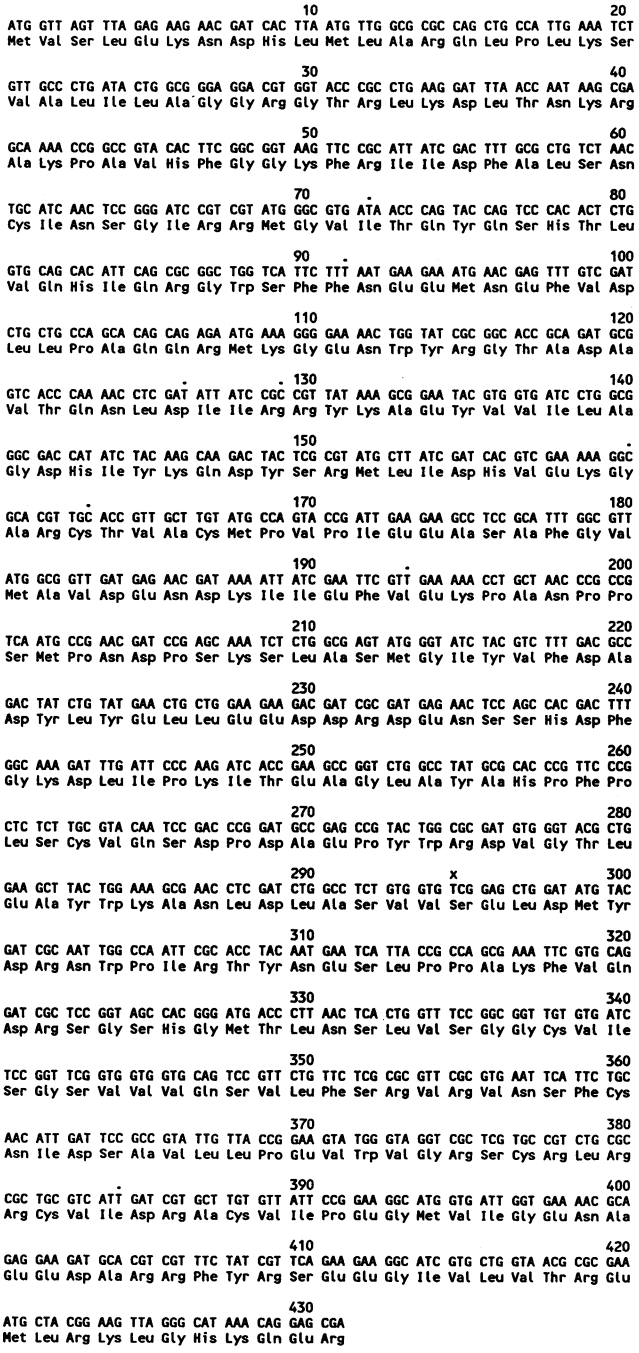

FIG. 2. Nucleotide (antisense) and amino acid sequences of ADPglucose synthetase from pPD5. The numbering refers to the amino acid sequence. Dots over bases indicate non-codon-changing base differences from the wild-type enzyme, whereas the letter X over the first base of residue 295 depicts a codon change (proline to serine).

for the pPD5 enzyme and 34.4-fold for the wild-type enzyme, which is very consistent with previous data for the two enzymes (10). This analysis verifies that the pPD5 enzyme is identical to that from SG5; no changes were introduced in the cloning step.

Subcloning and sequencing of the mutant ADPglucose synthetase gene. The recombinant plasmid pPD5 was digested with *Hin*cII and the 1.9-kb fragment corresponding to the *glgC* gene (19) was blunt end ligated into the *Hin*cII site of pUC119. Upon transformation into strain MV1193, only the reverse orientation (with respect to the *lac* promoter) of *glgC* could be isolated (pCJ1 [Table 1]). Problems with maintaining certain intact *glgC* genes in both orientations on pUC plasmids have been encountered before (unpublished results). To circumvent this problem, the forward orientation was subcloned in two parts by utilizing the unique *Hin*dIII site in *glgC*. The *glgC* fragment from pCJ1 was isolated and cut with *Hin*dIII, and the resulting fragments of ca. 1.3 (5′ end) and 0.6 (3′ end) kb were ligated into pUC118 and pUC119, yielding pCJ2 and pCJ3, respectively (Table 1). The dideoxy sequencing method (28) with Sequenase (U.S. Biochemical Corp.) was used to sequence the entire coding regions of both strands with oligonucleotide primers derived from the wild-type sequence (19). Single-stranded DNA templates were prepared by using helper phage M13K07 (31). Some double-stranded sequencing was also performed; for our system, sequencing using plasmid templates from strain DH5α was the most successful.

Figure 2 shows the antisense strand nucleotide and deduced amino acid sequences of the pPD5 *glgC* gene. Dots appearing over bases indicate non-codon-changing base differences from the previously sequenced K-12 wild-type *glgC* (1, 14). These are the same non-codon-changing differences seen with the previously cloned *E. coli* B mutant CL1136 (9), indicating small differences between the two *E. coli* strains. One-codon difference was found between the pPD5 (SG5) and wild-type genes: at amino acid position 295, proline was converted to serine by a CCG-to-TCG change as seen on the antisense strand, changing the total number of prolines and serines to 20 and 31, respectively. Such a change is in agreement with amino acid composition data of highly purified SG5 and wild-type enzymes (4, 12) where the small differences detected involved these amino acids (for SG5, 21.5 ± 1.4 prolines and 32.5 ± 1.4 serines; for the wild type, 23.2 ± 1.4 prolines and 28.3 ± 2.4 serines). The changed proline position has been shown to be highly conserved in alignment of ADPglucose synthetases from 10 different sources (30).

The SG5 enzyme is most similar to the mutant enzyme from *E. coli* K-12 strain 618 (13a, 14). Both enzymes contain mutations in the C-terminal half and behave kinetically as if partially activated in the absence of FBP. The effect of the substitution for the SG5 enzyme may be mediated by changes in the environment around certain lysine and arginine residues involved in binding of ligands, as the mutant enzyme exhibits less sensitivity to inactivation by trinitrobenzenesulfonate and phenylglyoxal (4, 5). Changes in contacts between subunits might be involved, as the SG5 enzyme aggregates to high-molecular-weight forms in the presence of FBP (4).

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