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SITE-DIRECTED MUTAGENIC REPLACEMENT OF GLU-461 WITH GLN IN β-GALACTOSIDASE (*E. coli*): EVIDENCE THAT GLU-461 IS IMPORTANT FOR ACTIVITY

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Glutamic acid 461 of β-galactosidase (*E. coli*) was replaced by gln using site-directed mutagenesis. Kinetic studies on the purified Q461-β-galactosidase showed that it had <0.4% of the wild-type activity (with ONPG as substrate), confirming other studies which have suggested that the negative charge on glu-461 is important for activity. The Km values did not increase, indicating that binding of the substrate was not decreased by this change. Thermal denaturation studies showed Q461-β-galactosidase to be somewhat more susceptible to heat denaturation than the wild-type enzyme.

β-Galactosidase [E.C.3.2.1.23] from *E. coli* has been characterized in terms of its: (a) physical structure (1); (b) amino acid sequence (2); (c) nucleotide sequence (3); (d) mechanism of action (4); and (e) genetic control (5). Studies on residues important for its activity have also been carried out. In particular, Herrchen and Legler (6) were able to identify glu-461 as one of the amino acids that may be important for activity by the use of an active site directed irreversible epoxide inhibitor. In contrast, studies involving chemical modification and inactivation of β-galactosidase activity using water soluble carbodiimides with amino inhibitors, inferred that inactivation was not due to a reaction with a specific group such as glu-461, but with several carboxyl groups (7).

We replaced the wild-type glu-461 codon (GAA) of *lacZ* with a codon for gln (CAA) by site-directed mutagenesis and then studied the kinetics and thermal stability of the resultant purified mutant enzyme. These studies showed that glu-461 is essential for activity.

MATERIALS AND METHODS

Bacterial Strains: (i) BMH71-18 mutL: *K12*, Δ (*lac-pro*), *supE*, *thi/F'*, *proA+B+*, *lacIq*, *lacZ*ΔM15, *mutL*::*Tn*10 - transformed with the mutagenic reaction

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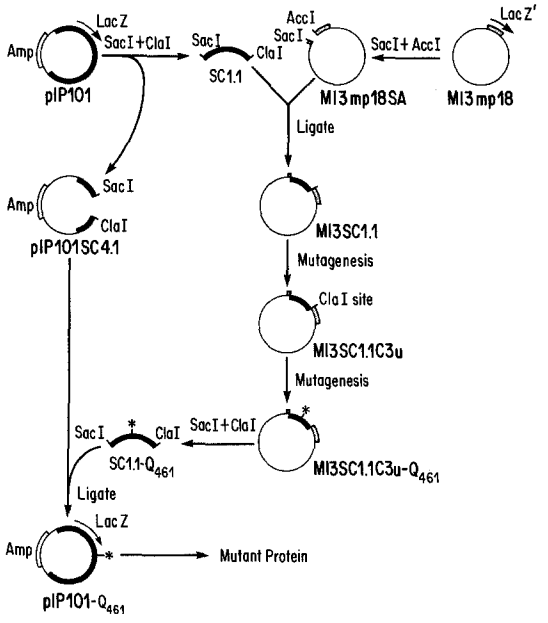

Figure 1: A schematic illustration of the protocol used to create the Q461-β-galactosidase by site-directed mutagenesis.

mixture; (ii) BMH71-18: same as above except is not *mutL::Tn10* - used as lawn cells for plating transformed mut L cells and for the isolation of ss DNA; (iii) TG2:Δ(*lac-pro*), *supE, thi, has D5, recA/F' traD36, proAB, lacIq, lacZ*ΔM15 - used to isolate the replicative form of M13 recombinants; (iv) S185: *his, str,* Δ(*lacz,y,a*), *ptsF,* Δ(*ara c,o*) - transformed with the pIP101-Q461 plasmid for expression of Q461-β-galactosidase.

Chemicals and Enzymes: Restriction and modification enzymes were all purchased from Pharmacia. ^{32}P-γ-ATP was from Amersham while ^{35}S-α-ATP was from NEN. Other chemicals were obtained from Sigma or Fisher (or other similar sources) and were of the purest qualities necessary and available.

Mutagenesis: A scheme summarizing the mutagenesis strategy is presented in Figure 1. A 1.1 Kb fragment from a *lac*Z containing plasmid (pIP101 - obtained from Dr. Müller-Hill, Köln) was digested with Sac I and Cla I and then ligated into M13mp18 which had been digested with Sac I and Acc I to create the mutagenic template (M13SC1.1). The Double Primer method of Carter et. al. (8) was used to change the E461 codon (GAA) to a codon for Q461 (CAA) and also to convert the Cla I/Acc I hybrid site into a Cla I site. The oligonucleotides used to create the mutations were both 17 bases in length and contained only a single base change. Both oligonucleotides as well as the M13 universal primer were obtained from the Regional DNA Synthesis Laboratory at the University of Calgary. After screening for positives using the Dot Blot method and sequencing part of the fragment to ensure that the mutations were present, the 1.1 Kb fragment was ligated back into pIP101 to give the mutant plasmid, pIP101-Q461. This was transformed into the S185 cell line and plated on 2x yeast-tryptone plates in the presence of ampicillin and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside. All of the colonies that grew were white and were screened for the presence of β-galactosidase by electrophoresing sonicated crude extracts on a 7.5-17% SDS-PAGE gradient. A positive colony (one which had a band corresponding to the wild type β-galactosidase band) was selected for purification of Q461-β-galactosidase. The 1.1 Kb fragment containing the Q461 mutation was cloned from the plasmid in this positive colony, back into M13mp18 for nucleotide sequencing of the whole fragment.

Nucleotide Sequencing: All sequencing was done using a Sequenase Dideoxy Sequencing Kit purchased from United States Biochemical Corporation.
Assay: The enzyme was assayed using o-nitrophenyl-ß-galactopyranoside (ONPG) and p-nitrophenyl-ß-D-galactopyranoside (PNPG) as the substrates and measuring the amount of product formed by the absorbance at 420 nm. The assay was done at pH 7.0 and 25°C in TES buffer (30 mM with 140 mM NaCl and 1 mM $MgSO_4$).
Purification of Q461-ß-Galactosidase: Q461-ß-Galactosidase was purified by a modification of the method of Brake et. al. (9). After eluting the enzyme from a DEAE Bio-Gel A column (BioRad), the active fractions were applied to an FPLC Superose-12 column and then to an FPLC Mono Q anion exchange column (Pharmacia). Although the activity was low, there was sufficient enzyme activity present for detection at each step of the purification using ONPG as the substrate and using long assay times.
Kinetic Studies: The activity of the mutant enzyme was measured at various concentrations of ONPG and PNPG and analyzed using the relative weighting scheme of Cornish-Bowden (10). The kinetic constant values (Vm and Km) of Q461-ß-galactosidase were compared to kinetic constant values obtained with the wild-type enzyme (isolated from *E. coli* ML308).
Thermal Stability Studies: The wild-type enzyme and Q461-ß-galactosidase were incubated in a 54°C water bath. Aliquots were removed at various times over a one h period, placed on ice and then measured for residual activity.

RESULTS

Nucleotide Sequence Analysis: The only base change in the entire 1.1 Kb fragment, was the GAA→CAA change at position 461.

Purity of Q461-ß-Galactosidase: The enzyme was purified to >95% purity as determined by SDS-PAGE (reducing) (Figure 2).

Figure 2: A 7.5-17% SDS polyacrylamide electrophoresis gradient gel of purified Q461-ß-galactosidase versus wild-type ß-galactosidase: (a) Q461-ß-galactosidase (overloaded) (b) 1.0 µg of wild-type ß-galactosidase (c) 2.5 µg of wild-type ß-galactosidase (d) 5.0 µg of wild-type ß-galactosidase.

TABLE 1- Kinetic Data For Q461-β-Galactosidase Versus Wild-Type β-Galactosidase

	Wild-Type		Q461	
Substrate	Km (mM)	Vm (μmol/min/mg)	Km (mM)	Vm (μmol/min/mg)
ONPG	0.170 ± 0.01	350 ± 7.8	0.010 ± 0.0013	1.24 ± 0.050
PNPG	0.035 ± 0.001	38 ± 0.4	0.012 ± 0.0012	0.76 ± 0.017

Kinetic Studies: Table 1 shows the Vm and Km values obtained for Q461-β-galactosidase with ONPG and PNPG as substrates. The Vm of the mutant enzyme is 0.4% of the wild-type value with ONPG and about 2% of the wild-type value with PNPG. The Km values were decreased for both substrates.

Thermal Stability: Figure 3 shows the temperature stability of the mutant enzyme versus the wild-type enzyme at 54°C. The activity of Q461-β-galactosidase fell more rapidly than the activity of wild-type β-galactosidase. At 4°C, Q461-β-galactosidase was found to be stable for at least 4 weeks and there was no evidence of any significant loss of activity during incubation at normal assay temperatures (25°C).

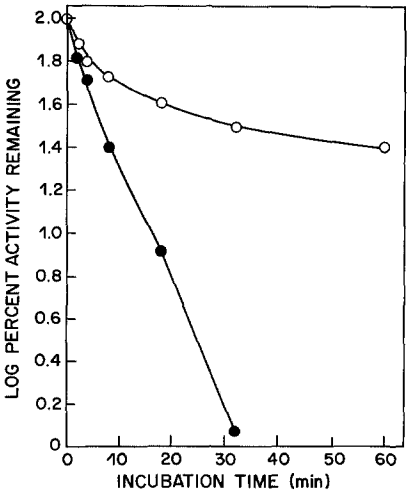

Figure 3: Thermal stability study of Q461-β-galactosidase and wild-type β-galactosidase at 54°C. The open circles represent wild-type β-galactosidase values and the closed circles represent Q461-β-galactosidase values.

DISCUSSION

The Vm values of β-galactosidase, with ONPG and PNPG as substrate, are dependent upon rate constants involved in the catalytic reaction of the enzyme (11). From the Vm values shown in Table 1, it is evident that Q461-β-galactosidase has a very low catalytic efficiency compared to wild-type β-galactosidase for both substrates.

The Km values of Q461-β-galactosidase were also decreased. Because Km is related to the dissociation constant (Ks) for the enzyme-substrate complex, a decrease in Km suggests a decrease in dissociation, and therefore, an increase in substrate binding. Our data, therefore, seem to indicate that Q461-β-galactosidase binds both ONPG and PNPG more strongly than does wild-type ß-galactosidase. One must be careful with such an interpretation, however, since the Km of ß-galactosidase depends on catalytic rate constants as well as on the rate constants for binding and release of substrate (11). Nevertheless, the fact that the Km values reported here are lower, not higher, than the wild-type Km values, means that it is unlikely that substrate binding decreased as a result of the mutation. If anything, the substrate binding was better.

Q461-ß-Galactosidase was somewhat more susceptible to heat inactivation than the wild-type enzyme but it was not dramatically less stable. At normal assay temperatures, Q461-ß-galactosidase did not show any signs of instability and it was stable for a long period of time at 4°C. Thus the activity of Q461-ß-galactosidase is not low because the enzyme is inherently unstable.

A replacement of a glu with a gln residue changes the charge on the side chain from negative to neutral without significantly affecting the size of the side chain. This study, therefore, strongly supports the Herrchen and Legler study (6) which suggested that the negative charge supplied by glu-461 is essential for ß-galactosidase activity.

REFERENCES

1. Karlsson, U., Koorajian, S., Zabin, I., Sjostrand, F.S. and Miller, A. (1964) J. Ultrastructure Res. 10, 457-469.
2. Fowler, A.V. and Zabin, I. (1978) J. Biol. Chem. 253, 5521-5525.

3. Kalnins, A., Otto, K., Ruther, U., and Müller-Hill, B., (1983) EMBO 2, 593-597.
4. Sinnott, M.L. (1978) FEBS Letters 94, 1-9.
5. Miller, J.H. and Reznikoff, W.S. (1978) The Operon, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, U.S.A.
6. Herrchen, M. and Legler, G. (1984) Eur. J. Biochem. 138, 527-531.
7. Gaunt, M.T. and Huber, R.E. (1987) Int. J. Biochem. 19, 47-52.
8. Carter, P., Bedouelle, H., Waye, M.M.Y. and Winter, G. (1985) Oligonucleotide Site-Directed Mutagenesis in M13, Anglian Biotechnology Limited, Colchester, Essex, England.
9. Brake, A.J., Fowler, A.V., Zabin, I., Kania, J. and Müller-Hill, B. (1978) Proc. Natl. Acad. Sci. 75, 4824-4827.
10. Cornish-Bowden, A. (1976) Principles of Enzyme Kinetics, Butterworths, London, England.
11. Tenu, J.-P., Viratelle, O.M., Garnier, J., and Yon, J., (1971) Eur. J. Biochem. 20, 363-370.