Glutamate-459 Is Important for *Escherichia coli* Branching Enzyme Activity†

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ABSTRACT: The branching enzyme belongs to the amylolytic family, a group of enzymes that cleave and/or transfer chains of glucan. The amylolytic enzymes are homologous and all contain four conserved regions, proposed to contain the active site. By primary structure analysis, a conserved position unique to branching enzymes has been identified. This residue, which is either Asp or Glu, depending on the species, is located immediately after the putative catalytic Glu-458 (*Escherichia coli* numbering). Branching enzymes differ from other amylolytic enzymes in having this acid pair, and we asked if this motif could be essential for branching enzyme action.We used site-directed mutagenesis of the Glu-459 residue in the *E. coli* branching enzyme in order to determine the significance of the conserved Asp/Glu in branching enzymes. A substitution of Glu-459 to Asp resulted in increased specific activity compared to wild-type, suggesting that the mutation had created a more efficient enzyme. Changing Glu-459 to Ala, Lys, or Gln lowered the specific activities and altered the preferred substrate from amylose to amylopectin.

Branching enzymes (BE)[1] catalyze the formation of α-1,6-glucosidic linkages of glycogen in bacteria (*1*) and of starch in plants (*2*). The enzyme hence plays an important role in the biosynthesis of starch and glycogen (*2*).

Branching enzymes from a variety of sources have been identified and described, including *Escherichia coli* (*3*−*5*), maize (*6*−*8*), and rice endosperm (*9*, *10*).

Homology between the branching enzymes and other amylolytic enzymes was first described by Romeo et al. (*11*). Baba et al. (*12*) showed that branching enzymes belong to the family of amylolytic enzymes, as they contain four highly conserved regions also found in α-amylase, pullulanase, isoamylase, and cyclodextrin glucanotransferases. Jespersen et al. (*13*) predicted all of the above enzymes to fold into a $(\alpha/\beta)_8$ barrel similar to that observed in the crystal structures of α-amylases (*14*−*16*) and cyclodextrin glucanotransferases (*17*, *18*). Site-directed mutagenesis studies of the active center of neopullulanase (*19*) suggested that the catalytic mechanism of the amylolytic enzymes was similar (*20*). Further resemblance between branching enzymes and other amylolytic enzymes was established by Kuriki et al. (*21*), who concluded that three of the conserved residues located in the regions defined by Baba et al. (*12*) were involved in the active site of the branching enzymes, as seen in α-amylases (*14*) and cyclodextrin glucanotransferases (*17*).

α-(1,4)-glucan linkages are cleaved by the branching enzyme, α-amylase, pullulanase, and cyclodextrin glucanotransferase, whereas α-(1,6)-glucano-α-(1,4)-glucan linkages are cleaved by isoamylase and also pullulanase. Following α-(1,4) cleavage, the branching enzyme forms an α-(1,6)-glucano-α-(1,4)-glucan linkage and the cyclodextrin glucanotransferase forms a cyclic or linear α-(1,4)-glucanopyranosyl chain. Even though the members of the amylolytic family may share structural features and regions of conserved amino acids, they catalyze different reactions; this poses the question of which residues are responsible for the distinct catalytic properties. This report describes studies on a conserved amino acid in branching enzymes located after the invariant Glu situated in region 3 [as first pointed out by Jespersen et al. (*13*)], forming an acid pair near the proposed catalytic site. This acid pair is not observed in the other members of the amylolytic family. We investigate the properties of the constructed *E. coli* branching enzyme (BE) mutants E459D, E459A, E459K, and E459Q and conclude that both the efficiency of catalysis and the preferred substrate for branching activity are affected.

MATERIALS AND METHODS

E. coli BE Expression Vector. H. P. Guan kindly provided the plasmid pEXSB, the construction of which has been described previously (*5*). The pEXSB plasmid is a pET-23d derived plasmid containing the origin p15A, T7 promotor, T7 terminator, kanamycin resistance, and the *E. coli* glgB gene encoding the *E. coli* BE.

Construction of Mutants. The pEXSB plasmid purified from Epicurian Coli XL2-Blue cells (Stratagene Corp., La Jolla, CA) was used as dsDNA template in site-directed mutagenesis (Quickchange site-directed mutagenesis kit, Stratagene Corp.). Oligonucleotides were synthesized using an Applied Biosystems model 380A DNA synthesizer (Macro-molecular Facility, Department of Biochemistry, Michigan State University). The mutations were confirmed by DNA sequencing, and secondary mutations were ruled out by sequencing the entire gene for all constructions. DNA sequencing was done by the dideoxy chain-terminating

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[1] Abbreviations: BE, branching enzyme; mBE, maize endosperm branching enzyme. WT, wild-type; c.l., chain length; dp, degree of polymerization; HPAEC, high-performance anion exchange chromatography; PAD, pulsed amperometric detector.

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Escherichia coli	454	VTMA**E**EST	Baecker *et al.* (*4*)
Bacillus stearothermophilus	347	LMIA**E**DST	Takata *et al.* (*29*)
Synechococcus	489	LSIA**E**EST	Kiel *et al.* (*30*)
Streptomyces aureofaciens	489	VTIA**E**EST	Homerova *et al.* (*31*)
Arabidopsis II	533	IVVG**E**DVS	Fisher *et al.* (*32*)
Maize I	465	TVVA**E**DVS	Baba *et al.* (*12*)
Maize II	498	VTIG**E**DVS	Fisher *et al.* (*33*)
Rice I	460	TIVA**E**DVS	Mizuno *et al.* (*34*)

FIGURE 1: Partial sequence alignment of several branching enzymes from various organisms. The conserved Glu constituing region 3 in the amylolytic family as defined by Baba et al. (*12*) is indicated in bold. The shaded residues are the *E. coli* E459 and equivalent residues in the alignment.

method (*22*) using the dsDNA as template. The primers used were the T7 universal primers or synthetic 21-mer oligonucleotides.

Expression of Wild-Type (WT) and Mutant E. coli BE. An overnight culture of the transformed cells (BL21[DE3]) carrying the recombinant plasmid encoding the gene for WT BE or the constructed mutants was diluted 1:20 (v/v) in fresh Luria broth medium containing 50 μg mL^{-1} kanamycin. The cells were grown at 37 °C to $A_{600} = 0.5$ and allowed to cool to 25 °C. Flasks were transferred to a shaker at room temperature, and T7 RNA polymerase expression was induced by adding isopropyl-D-thiogalactoside to 0.5 mM. After 2 h incubation, endogenous expression was turned off by adding rifampicin to 150 μg mL^{-1}. Incubation was continued for 16 h at room temperature, and cells were then harvested in a refrigerated centrifuge.

Purification of E. coli BE Wild-Type and Mutants. The cell pastes obtained were about 1.5 g per 1 L of culture. The purification of the *E. coli* BE WT and the mutants was performed as described by Guan et al. (*5*). Protein concentration was measured with the BCA protein assay reagent (*23*), using BSA as the standard.

Assay of BE Activity. BE activity was measured by three different assays as described by Guan and Preiss (*8*).

Assay A. The phosphorylase A stimulation assay is based on the stimulation by BE of the synthesis of α-D-glucan from α-D-Glc-1-P catalyzed by rabbit phosphorylase a (*24*). Reaction mixtures contained, in a final volume of 100 μL, 100 mM citrate (pH 7.0), 10 mM AMP, 0.2 mg phosphorylase a (Sigma Chemical Co.), and 50 mM D-[14C]-Glc-1-P (50 DPM nmol^{-1}) and were initiated by adding an appropiate amount of BE. One unit is defined as 1 μmol of Glc incorporated into α-D-glucan per min at 30 °C.

Assay B. The branching linkage assay determines the number of branching points introduced by BE into the substrate, reduced amylose (*25*). Substrates were prepared by the reduction of enzymatically synthesized amylose [AS-320, degree of polymerization (dp) 1815] as described by Takeda et al. (*25*). Reaction volumes contained 25 mM MOPS (pH 7.5) and an appropriate amount of substrate in a final volume of 100 μL. The reaction was initiated by adding BE. One unit is defined as 1 μmol of branching linkages formed per minute at 30 °C.

Assay C. The iodine stain assay is based on measuring the decrease in absorbance of the glucan−iodine complex resulting from the branching of the substrate, amylose (potato type III [dp 1176, chain length (c.l.) 884]), or amylopectin (corn, Sigma Chemical Co.) (*6*, *8*). Reaction mixtures contained 50 mM citrate (pH 7.0) and 0.1 mg substrate in a final volume of 500 μL. The reaction was initiated by the addition of an appropriate amount of enzyme. One unit is defined as the decrease in absorbance of 1.0 per min at 30 °C.

Analysis of the Chain Length Distribution by High-Performance Anion Exchange Chromatography (HPAEC). Reduced amylose (5 mg, AS-320, dp 1815) was incubated in 25 mM MOPS, pH 7.5 (500 μL) at 30 °C with BE (5 mU of *E. coli* BE WT and mutants by assay B). After 1 and 4 h, the reaction in a 200 μL aliquot was terminated by boiling for 2 min. The synthesized α-glucan was then debranched by adding 1 M acetate buffer (pH 3.5, 20 μL) and isoamylase (10 μL, 1180 U/ μL). After 90 min at 45 °C the solution was boiled for 5 min. The solution was filtered through a 0.22 μm membrane and 25 μL samples injected in the HPAEC (BioLC, Dionex, Sunnyvale, CA), using a CarboPac PA-1 column (250 × 4 mm). Eluent A was 150 mM sodium hydroxide; eluent B was 150 mM sodium hydroxide containing 500 mM sodium acetate. The gradient program was the following: 25% eluent B (75% eluent A) at time 0, 45% at 15 min, 60% at 45 min, 70% at 80 min, and 80% at 100 min, with a flow rate of 0.3 μL min^{-1}. The standard was 25 μL of 5 mg μL^{-1} maltodextrin (dp 1−20, Aldrich, WI).

RESULTS

Figure 1 shows a partial sequence alignment of several branching enzymes from a variety of bacteria and plants. Only the sequences in the vicinity of region 3, conserved in the amylolytic family as defined by Baba et al. (*12*), are shown. The residue immediately following E458 (*E. coli* BE numbering) in region 3 is a conserved acid in all branching enzymes, a fact first pointed out by Jespersen et al. (*13*). For prokaryotes this acid is mostly Glu, while higher plants have Asp in this position. This residue, Glu-459 (*E. coli* BE numbering) was targeted for site-directed mutagenesis studies; the mutants E459A, E459D, E459K, and E459Q were constructed as described above.

The BL21(DE3) strain used for expression of the *E. coli* BE WT and mutants is *glgB*+. This poses the problem that the recorded activity from the constructed mutants potentially could arise from the endogenous *E. coli* BE. Therefore, we used rifampicin during the induction to inhibit endogenous transcription. Rifampicin is a potent inhibitor of bacterial

Table 1: Specific Activities of WT *E. coli* BE and Mutant BE Glu-459[a]

		specific activities (U/mg)				
assay		WT	E459D	E459A	E459K	E459Q
phosphorylase a,	**A**	1009	1862	312	66	126
branching linkage,	**B**	1.1	1.8	0.21	<0.01	0.16
iodine stain,	**C**					
with amylose,	$\mathbf{C_1}$	95	139	11	1.1	10
with amylopectin,	$\mathbf{C_2}$	55	77	22	3.3	14.5
ratio of activity						
	A/B	926	1064	1521	>6000	800
	$\mathbf{A/C_1}$	10.6	13.4	29.4	58	12.6
	$\mathbf{A/C_2}$	18.4	24.4	14.3	20	8.7
	$\mathbf{C_1/C_2}$	0.58	0.55	2.1	2.9	1.5

[a] C_1 and C_2 denote the iodine staining assay with amylose and amylopectin as substrates, respectively.

RNA polymerases (*26*), but does not affect the T7 RNA polymerase that transcribes the recombinant plasmid. An immunoblot with 0.2 μg crude extract of *E. coli* BE WT and the mutants showed for each, one distinct band, while the negative control (BL21[DE3] carrying plasmid pET23d) even when loaded with excess crude extract, i.e., 5 μg or even 15 μg, showed no bands (data not shown). Furthermore, the negative control lacked any detectable BE activity in the crude extract. These observations indicate rifampicin blocked the expression of endogenous branching enzyme, and the detected BE activity arose solely from the expressed mutant plasmids. When compared to the original method published by Guan et al. (*5*), this method increased the specific activity of the crude extract 3-fold due to less endogenous expression (data not illustrated).

The WT *E. coli* BE and the four constructed mutants were purified to near homogeneity to compare their properties. All the purified proteins showed only one band of about 85 kDa on a SDS−PAGE gel when 1 μg was loaded on the gel. The specific activity of the WT enzyme in the phosphorylase A assay was 1009 U mg^{-1} (Table 1) compared to 1111 U mg^{-1} obtained by Guan et al. (*5*).

Table 1 compares the specific activities of the *E. coli* BE WT and the E459D, E459D, E459K, and E459Q mutants. Interestingly, the E459D showed a higher specific activity when compared to the WT in all assays (185%, 160%, 145%, and 140% relative to WT activity in the phosphorylase A assay, branching linkage assay, iodine assay with amylose, and iodine assay with amylopectin, respectively).

Table 1 includes the ratios of activity for the three different assays. Guan and Preiss (*8*) showed that these ratios are useful for differentiation of the properties of branching enzymes. The WT and E459D have, essentially, similar ratios of activity, indicating that the properties of the E459D mutant are similar to the WT. The other mutants show lower specific activities as compared to WT, the E459K being the less active especially in the branching linkage assay. The C_2/C_1 ratio increases from a 0.58 in the WT to 2.1, 2.9, and 1.5 in the E459A, E459K, and E459Q, respectively. In other words, these mutations altered the substrate preference from amylose to amylopectin.

The kinetic properties of *E. coli* BE had not been studied previously, and a full characterization was needed for this study. Using reduced amylose AS-320 as substrate, we investigated the kinetic parameters of *E. coli* BE WT and the E459D mutant. The computed K_m values displayed in Table 2 show a lower K_m (11.7 μM) for the WT as compared to that of the E459D mutant (23.5 μM). The specific activities (V_{max} per mg of protein) were 3.7 U/mg for the WT and 5.9 U/mg for the E459D. The estimated specific activities as well as the high K_m of E459D are approximate due to the low solubility of amylose that does not allow the use of high concentrations of substrate. On this basis, the indicated specific activity of the E459D mutant is a low estimate.

Table 2: Kinetic Parameters of WT *E. coli* and Mutant E459D BEs[a]

	WT	E459D
specific activity (μmol × min^{-1} mg^{-1})	3.7 ± 0.2	5.9 ± 0.8
K_m (μM)	11.7 ± 0.7	23.5 ± 5.6
K_{cat}/K_m (s^{-1} M^{-1})	4.5×10^5	3.5×10^5

[a] Activity was measured by the branching linkage assay (assay B) using reduced amylose AS-320 as substrate.

The K_m and specific activities for the maize branching enzymes have been published previously with assays using AS-320 as substrate (*27*). The reported K_m values were 59 μM for both mBEI and mBEII, but due to a computational error in the amount of substrate, these numbers are off by a factor of 7. The corrected values for K_m are 8.4 μM for both mBEI and mBEII. The specific activity was 3.3 U/mg for mBEI and 0.62 U/mg for mBEII. Therefore, the kinetic parameters of *E. coli* BE WT closely resemble the mBEI. Fersht (*28*) defined the substrate specificity as K_{cat}/K_m. This ratio is similar for WT and E459D (4.5×10^5 s^{-1} M^{-1} and 3.5×10^5 s^{-1} M^{-1}, respectively, Table 2).

Modification of the structure of reduced amylose AS-320 by BE was analyzed for the WT and all mutants except E459K, due to its low activity. Figure 2 shows the HPAEC pulsed amperiometric detector (PAD) response chromatograms of WT and E459D after 4 h of branching as described in Materials and Methods. The mutant enzymes E459A and E459Q yielded similar results to the WT (data not illustrated). The only significant difference in the chromatograms in Figure 2 is the presence of a dp 4 peak in the chain length distribution of E459D which is not observed for WT enzyme. Therefore the HPAEC chromatogram was rerun with 1.5 μg of maltotetraose included in the sample to test if the retention time of the maltotetrose corresponded to that of the observed peak. This increased the proposed dp 4 peak 2-fold in size and we concluded the E459D does differ from WT in transferring this particular chain length. A similar study of the *E. coli* BE WT was done recently by Guan et al. (*5*) where they registered dp 4 and even dp 3, however, without presenting the corresponding PAD chromatograms. The relative peak area is plotted in Figure 3 and is based on all PAD response chromatograms, and the figure shows that there is little difference in the branching products of the enzymes tested, the most commonly transferred chain length being 11.

Since the focus of our study is a conserved acid in the branching enzymes, we studied the activity over a pH range for the *E. coli* BE WT and E459A (Figure 4); the effect of pH on *E. coli* BE activity had not been reported previously. Figure 4 indicates that the activity profile of the WT and E459A are similar over the pH range tested, with an optimum near pH 7.7. The reported pH optimum for the maize

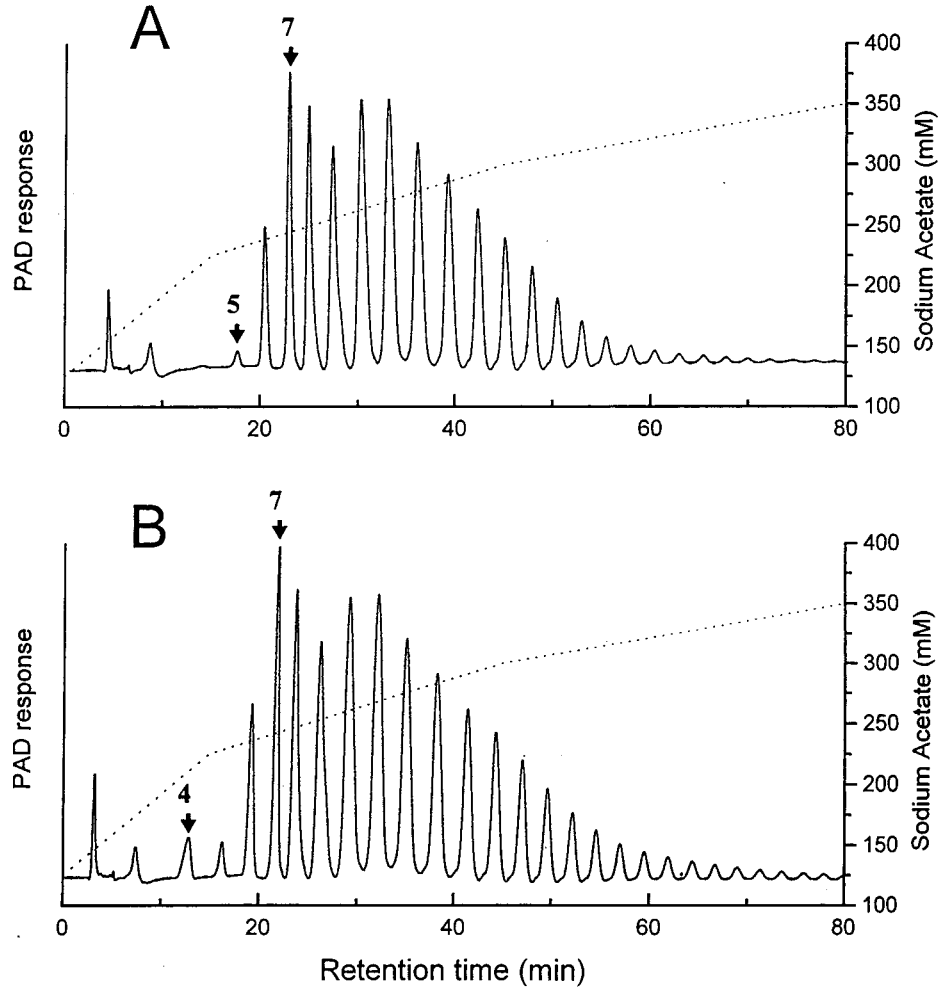

FIGURE 2: HPAEC analysis of the debranched α-glucan formed by action of *E. coli* BE WT on reduced amylose AS-320 (A) and of E459D (B). Number on peak indicates the corresponding c.l. The sodium acetate gradient is superposed on chromatogram.

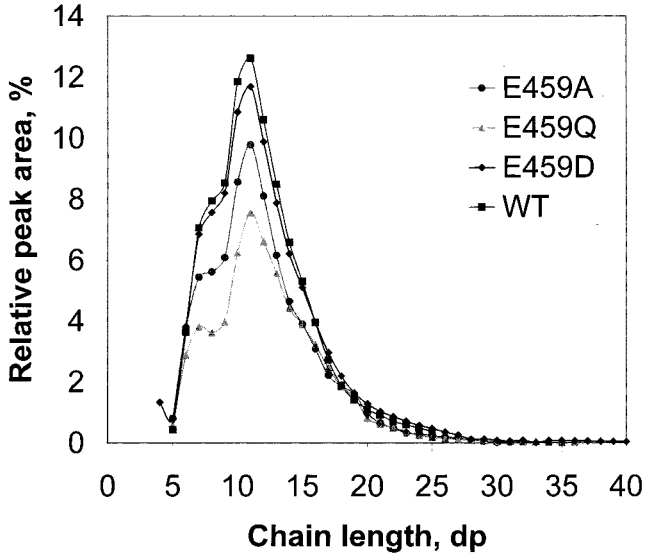

FIGURE 3: Relative peak areas of the transferred glucan chains. Sum of dp 1−40 was taken as 100%.

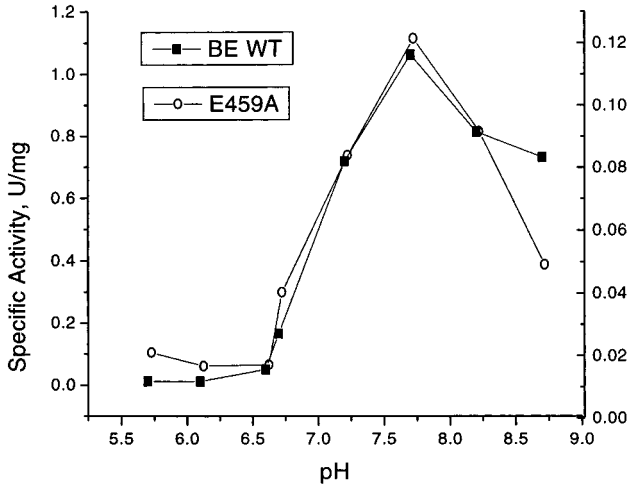

FIGURE 4: pH profile of *E. coli* BE WT and E459A mutant. Left Y-axis is specific activity for WT and right Y-axis for E459A as defined by the branching linkage assay (assay b).

branching enzyme isoforms is 7.5 (*25*). If the Glu-459 were involved in acid−base catalysis, a substitution would create a shift in the relative activity at a particular pH, but this is not the case. Thus, a role of Glu-459 as destabilizing the deprotonated form of the neighboring Glu-458, proposed to be a catalytic residue, can be ruled out.

CONCLUSION

On the basis of results presented here, we conclude that Glu-459, located in the conserved region 3, is important for the function of the *E. coli* BE. When Glu-459 is changed to other amino acids, a significant change in the properties of the enzyme is seen with respect to specific activities, kinetic parameters, and substrate specificity. The conservative

change from Glu to Asp even led to higher specific activities compared to the WT in all three assays available.

It is worth noting that all higher plant branching enzymes (as well as a few from prokaryotes) have an Asp at this position. It would be interesting to study the consequences of the reciprocal conservative substitution at this position in a plant branching enzyme.

Glu-459 is not likely to be involved in chain transfer, as the HPAEC-PAD chromatograms were virtually identical for all constructed mutants. It is also evident that Glu-459 is not an essential catalytic residue, as some activity remained even when it was substituted with an amino acid of opposite charge (E459K). However, Glu-459 could be involved in catalysis of a step which is not rate limiting, based on our observations of altered specific activity and substrate preference. The pH profiles of the WT and the E459A mutant rules out the possibility that Glu-459 is involved in acid−base catalysis as the pH profiles as the profiles can be superimposed.

A three-dimensional structure is required before we can assign a specific role for Glu-459, and in turn, the reason for this residue being a conserved acid in all known branching enzymes.

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