http://dx.doi.org/10.5012/bkcs.2013.34.2.669

Role of a Highly Conserved and Catalytically Important Glutamate-49 in the *Enterococcus faecalis* Acetolactate Synthase

Mi-Young Lee,[a] Sang-Choon Lee,[a] June-Haeng Cho, Seong Eon Ryu,[†] Bon-Sung Koo,[‡] and Moon-Young Yoon[*]

Department of Chemistry and Research Institute of Natural Sciences, Hanyang University, Seoul 133-791, Korea
[*]E-mail: myyoon@hanyang.ac.kr
[†]Department of Bioengineering, Hanyang University, Seoul 133-791, Korea
[‡]Functional Biomaterial Division, National Academy of Agricultural Science, Rural Development Administration, Suwon 441-857, Korea
Received October 17, 2012, Accepted November 26, 2012

Key Words : Acetolactate synthase, Catabolic, *Enterococcus faecalis*, ThDP-dependent, FAD-independent

Acetolactate synthase (ALS) is a thiamine diphosphate (ThDP)-dependent enzyme that catalyzes the decarboxylation of pyruvate and then condenses the hydroxyethyl moiety with another molecule of pyruvate to give 2-acetolactate (AL). AL is a key metabolic intermediate in various metabolic pathways of microorganisms. In addition, AL can be converted to acetoin, an important physiological metabolite that is excreted by many microorganisms.[1] There are two types of ALSs reported in the literature, anabolic acetohydroxyacid synthase (AHAS) and catabolic ALSs (cALS). The anabolic AHAS is primarily found in plants, fungi, and bacteria, is involved in the biosynthesis of branched-chain amino acids (BCAAs), and contains flavin adenine dinucleotide (FAD), whereas the cALS is found only in some bacteria and is involved in the butanediol fermentation pathway.[1-3] Both of the enzymes are ThDP-dependent and require a divalent metal ion for catalytic activity.[4,5] Despite the similarities of the reactions catalyzed, the cALS can be distinguished from anabolic AHAS by a low optimal pH of about 6.0, FAD-independent functionality, a genetic location within the butanediol operon, and lack of a regulatory subunit.[6-11] It is noteworthy that the structural and functional features of AHAS have been extensively studied, in contrast to those of cALS, for which only limited information is available.[2,3] To date, the only crystal structure of cALS reported is from *Klebsiella pneumonia*,[4] which revealed that the overall structure of *K. pneumonia* ALS is similar to that of AHAS except for the FAD binding region found in AHAS.[4]

The recent discovery of the crystal structures of various ThDP-dependent enzymes, including cALS, has greatly aided the understanding of the overall structural orientation of ThDP and its role in catalysis. As with the other ThDP-dependent enzymes, ThDP is located in the active site of cALS with a unique V-conformation at the subunit-subunit interface, where the diphosphate moiety of ThDP is in contact with one subunit and the aminopyrimidine moiety is in contact with the other subunit.[4] In all of the crystal structures of ThDP-dependent enzymes determined to date, with the exception of glyoxylate carbo-ligase (GCL), a glutamate residue has been found at hydrogen-bonding distance from the N1' atom of the aminopyrimidine ring of the bound ThDP and plays a key role in catalysis (Fig. 1).[12] In addition to ThDP binding, the glutamate interaction with the N1' atom of the ThDP aminopyrimidine moiety is involved in activation of the cofactor for proton exchange in several ThDP-dependent enzymes and induces the formation ofe 1',4'-iminotautomer, which generates the highly reactive ylide required for catalysis.[12] This glutamate residue was found to be highly conserved in almost all of the ThDP-dependent family of enzymes (Fig. 2). Subsequently, the crucial catalytic role of this glutamate residue was extensively studied by site-directed mutagenesis in a few ThDP-dependent enzymes, including AHAS and pyruvate decarboxylase (PDC).[12-15]

Clearly, it is also very important to investigate the extent to which this glutamate is essential in other ThDP-dependent enzymes. In this study we investigated the influence of this catalytically important glutamate residue (Glu-49) on both catalysis and cofactor binding in cALS of *Enterococcus faecalis*. We recently cloned, purified, and characterized the cALS of *E. faecalis*.[16] Site-directed mutagenesis was carried

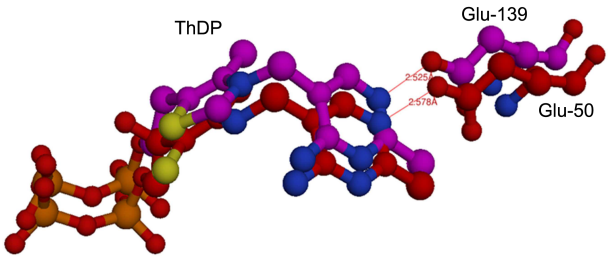

Figure 1. Superimposition of the ThDP of yeast AHAS (purple) and *Z. mobilis* PDC (red) together with the glutamate residue important for ThDP binding and catalysis. The thin red lines represent the hydrogen bonds. The figure was prepared using the coordinates from PDB entries, 1JSC and 1ZPD for AHAS and PDC, respectively.

Abbreviations: AHAS, acetohydroxyacid synthase; cALS, catabolic acetolactate synthase; AL, 2-acetolactate; PDC, pyruvate decarboxylase
[a]Both authors contributed equally

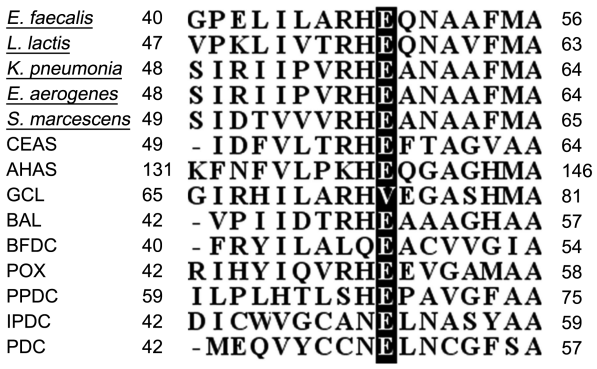

Figure 2. Amino acid sequence alignment of the conserved glutamate residue region of various ALSs and other homologous ThDP-dependent enzymes. The amino acid sequences were aligned using the ClustlW program. The cALS sequences (underlined) used are given with GenBank IDs in parentheses and include *K. pneumonia* (149211), *Serratia marcescens* (296783894), *Enterobacter aerogenes* (336249979), *E. faecalis* (29343247), and *Leuconostoc lactis* (4104436). Subsequently, the other homologous ThDP-dependent enzymes used were AHAS (151946264), pyruvate oxidase (POX; 494458), PDC (4388897), indolepyruvate decarboxylase (IPDC; 18652678), benzoylformate decarboxylase (BFDC; 56965879), benzaldehyde lyase (BAL; 88192045), phenylpyruvate decarboxylase (PPDC; 149242518) and *N*2-(2-carboxyethyl) arginine synthase (CEAS; 254387584).

out and the Glu-49 residue was changed to alanine, glutamine, and aspartate to yield Glu-49-Ala, Glu-49-Gln, and Glu-49-Asp mutations, respectively. Site-directed mutagenesis was performed on the plasmid pET28a carrying *E. faecalis* cALS using the PCR megaprimer method. The primers used are shown in Table 1. The mutant proteins were purified as described previously and the purification stage SDS-PAGE images were shown in Supplementary Figure S1.[17] Activity assays and co-factor characterization were performed using procedures described previously for the wild-type enzyme and the values collected are shown in Table 2. Further, Trp fluorescence quenching studies were carried out with varied ThDP concentrations to investigate the effect of these mutants in the ThDP binding. The wavelength of excitation was 300 nm and the emission was recorded in the range 310-450 nm as described previously.[17]

Table 1. Oligonucleotides used in this study

Primer name	Sequence
Glu-49-Ala F	5'-TTAGCTCGACATGCACAAAATGCA-3'
Glu-49-Ala R	5'-TTAGCTCGACATGGACAAAATGCA-3'
Glu-49-Gln F	5'-TTAGCTCGACATCAACAAAATGCA-3'
Glu-49-Gln R	5'-TGCATTTTGTTGATGTCGACGTAA-3'
Glu-49-Asp F	5'-TTAGCTCGACATGACCAAAATGCA-3'
Glu-49-Asp R	5'-TGCATTTTGGTCATGTCGAGCTAA-3'

*The mutated bases are underlined within the primer sequence.

Table 2. Substrate and co-factor kinetics of wild-type and mutant cALSs

	Specific activity (U/mg)	K_m for pyruvate (mM)	K_c for ThDP (μM)	K_c for Mg^{2+} (mM)	K_D for ThDP (μM)
Wild-type	5.36	1.37	0.031	1.27	220
Glu-49-Ala	0.23	30.58	64.0	0.32	190
Glu-49-Asp	43.97	1.7	8.83	-	155
Glu-49-Gln	0.38	16.24	113.6	0.279	172

The substitution of Glu-49 to a simple non-polar amino acid, alanine caused a significant decrease in the enzyme activity. The mutant Glu-49-Ala had a specific activity of 0.23 U/mg, which is ~4.2% that of the wild-type enzyme (5.36 U/mg) (Table 2). It has also been reported that substitution of this conserved homologous glutamate with alanine in yeast PDC yields a completely inactive protein, where as a ~20-fold decrease in specific activity was observed in *E. coli* AHAS II with a similar mutation.[13,15] The K_m for the enzyme's substrate, pyruvate, was determined to be 30.58 mM, which is ~22-fold higher than that of the wild-type cALS (Table 2). In addition to its role in catalysis, the Glu-49-Ala also had a drastic effect on co-factor ThDP activation. The half-saturating concentration (K_c) for ThDP was determined to be 64 μM (Table 2), which is ~2000-fold higher than that of wild-type cALS (Table 2). A similar observation was made for the *E. coli* AHAS II, where the mutation of glutamate to alanine caused a ~50-fold reduction in affinity for ThDP.[13] However, the Mg^{2+} kinetics were unaltered (Table 2). The activity and co-factor activation results of the Glu-49-Ala cALS mutant were comparable to those observed with mutants of other ThDP-dependent enzymes altered at the homologous glutamate residue. These results further suggest that the Glu-49 of *E. faecalis* ALS plays an important role in the enzyme's substrate catalysis, as it does in other homologous ThDP-dependent enzymes.

The Glu-49-Gln variant, where the side chain of the glutamine residue was modified to one of the same size as that of the wild-type glutamate but unable to carry a charge, showed a specific activity of 0.38 U/mg, which is ~7.0% that of the wild-type enzyme (Table 2). As observed in the cALS, the homologous glutamate alteration to glutamine in other ThDP-dependent enzymes also caused significant 233-fold and 10-fold decreases in activity as compared to the wild-type enzymes in *Zymomonas mobilis* PDC and *E. coli* AHAS II, respectively.[13,14] However, the K_m for the substrate pyruvate was slightly lower than that of the Glu-49-Ala mutant, which was ~11-fold higher than the wild-type cALS (Table 2). As with the Glu-49-Ala variant, the Glu-49-Gln mutant also had a drastic effect on co-factor ThDP activation, with a K_c for ThDP that was ~3600-fold higher than that of wild-type cALS (Table 2). This mutation clearly hampers the requirement of ThDP in cALS and the assay results are also comparable with those from *Z. mobilis* PDC, where the homologous mutant also showed a drastic decrease in affinity for ThDP.[14] As observed with the Glu-49-Ala variant, the Mg^{2+} kinetics were unchanged (Table 2). The Glu-49-Gln variant results for cALS further support a key role for the carboxylate group of the conserved glutamate, which is responsible for hydrogen bonding with the N1' atom of the aminopyrimidine ring of ThDP (Fig. 1).

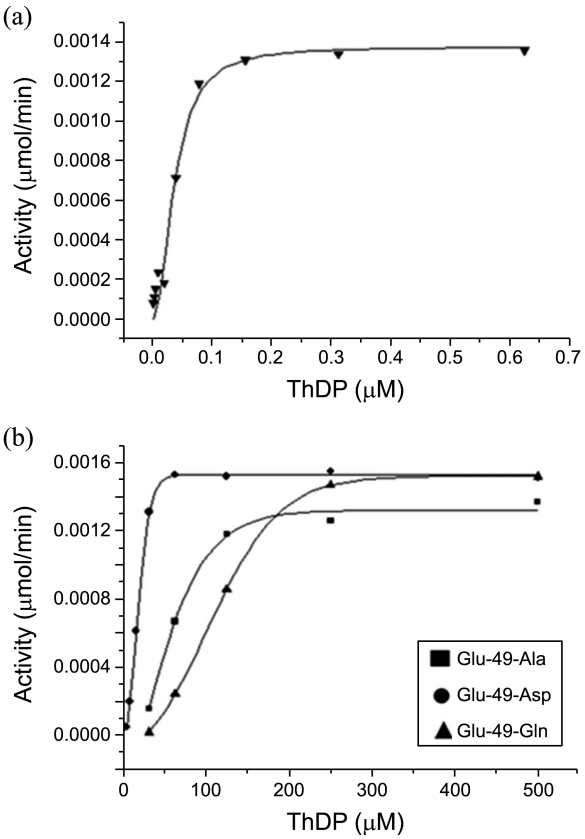

Figure 3. ThDP saturation curves of *E. faecalis* cALS wild-type (a) and mutant proteins (b). The K_c values obtained using these curves are shown in Table 2.

Finally, the Glu-49 was substituted with aspartate, a similarly charged residue with a shorter length. The Glu-49-Asp substitution yielded a fully activated enzyme in the presence of higher concentrations of ThDP (Fig. 3), although it was approximately eight times more active than the wild-type enzyme. In addition, the Glu-49-Asp mutant exhibited normal substrate kinetics, with the K_m for pyruvate equal to that of wild-type cALS (Table 2). In comparison, the homologous glutamate alteration to aspartate in *Z. mobilis* PDC also showed substrate kinetics equal to those of the wild-type enzyme, but the mutation also caused a significant decrease (~3-fold lower than that of wild-type) in the enzyme's specific activity.[14] Moreover, the aspartate substitution significantly affected the activation of ThDP. The K_c for ThDP was determined to be 280-fold higher than that of wild-type cALS (Table 2). A possible explanation for this change in binding is that the shorter length of the substituted aspartate increased the distance between the negatively charged atom of the acidic residue and the N1' atom of the aminopyrimidine ring of ThDP, making hydrogen bonding more challenging. However, according to the Trp fluorescence quenching data, none of the mutants showed significant differences in quenching, where the K_D values obtained of mutant proteins were similar to that of wild-type cALS (Table 2). The results of the Glu-49-Asp substitution further suggest that the distance to the carboxylate group for hydrogen bonding is important for the ThDP binding component of enzyme catalysis.

As described previously, the structural orientation and functional role of the conserved glutamate was widely studied in a few of the ThDP-dependent enzymes using their crystal structures and site-directed mutagenesis.[4,12-15,18,19] Several studies also examined the effect of homologous glutamate mutants on the rate of C2 proton exchange of bound ThDP in other ThDP-dependent enzymes.[13,15] The results of these studies suggest that the glutamate residue has a significant effect on the rate of ionization of ThDP since a significantly decreased exchange rate was observed in glutamate mutants of yeast PDC and *E. coli* AHAS II.[13,15] In summary, the current study further addresses the structural and functional roles of the homologous glutamate in cALS of *E. faecalis*. The results are comparable with other analyses of ThDP-dependent enzymes, which found that the Glu-49-Ala and Glu-49-Gln mutants showed decreased activities and weakened ThDP binding. The structurally and functionally different variant residues, alanine, glutamine, and aspartate, used as substitutions for glutamate, demonstrated the importance of the carboxylate group of glutamate as well as the length of this group from the aminopyrimidine ring of ThDP. The role of Glu-49 in *E. faecalis* cALS appears to be similar to those identified for other ThDP-dependent enzymes; it is essential for catalysis by being located at a hydrogen bonding distance from the N1' atom of the aminopyrimidine ring of ThDP, and this is believed to be an inherent property of most of the ThDP family of enzymes.

Experimental Section

Site-Directed Mutagenesis. Site-directed mutagenesis in *E. faecalis* ALS was performed directly on the pET28a-derived plasmid containing *E. faecalis* ALS cDNA using the site-directed mutagenesis kit. Each reaction contained 100 ng of template DNA, 10 pM of modified forward and reverse primers, and 2.5 mM of each dNTP in 50 μL reaction volumes. Each reaction was performed for 20 cycles of the following program: 95 °C for 30 sec, 55-65 °C for 1 min, and 72 °C for 1 min 20 sec. The primers used for PCR and sequencing are listed in Table 1. DNA sequencing was carried out by the dideoxy chain-termination procedure at Macrogen (Seoul, Korea).

Expression and Purification of Wild-Type and Mutant ALSs. Strains of *E. coli* BL21-DE3 containing the expression vector pET28a-ALS were grown at 37 °C in LB medium containing 50 μg mL^{-1} kanamycin to an A_{600} of 0.7-0.8. CSU expression was induced by adding 0.5 M misopropyl-β-D-thiogalactoside (IPTG). Cells were grown overnight at 18 °C and harvested by centrifugation at 4000 × g for 15 min. The cell lysis and purification of both CSU and the holoenzyme were performed as described previously.[17]

Substrate and Co-Factor Kinetics. The ALS activity was measured as described previously, except for the addition of FAD to the reaction mixture.[16] The final concentrations of enzymes used in the reaction were 0.25, 6, 3, and 0.05 μg/

200 μL of total reaction volume for wild-type, Glu-49-Ala, Glu-49-Gln, and Glu-49-Asp mutants, respectively. The substrate and co-factor kinetics were determined as described previously.[17]

Acknowledgments. This research was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) and Converging Research Center Program funded by the Ministry of Education, Science and Technology (2012R1A1A2008516 and 2012K001445).

References

1. Zijun, X.; Ping, X. *Crit. Rev. Microbiol*. **2007**, *33*, 127.
2. McCourt, J. A.; Duggleby, R. G. *Amino Acids* **2006**, *31*, 173.
3. Gedi, V.; Yoon, M. Y. *FEBS J.* **2012**, *279*, 946.
4. Pang, S. S.; Duggleby, R. G.; Schowen, R. L.; Guddat, L. W. *J. Biol. Chem.* **2004**, *279*, 2242.
5. Gedi, V.; Koo, B. S.; Kim, D. E.; Yoon, M. Y. *Bull. Korean Chem. Soc.* **2010**, *31*, 3782.
6. Störmer, F. C. *J. Biol. Chem.* **1968**, *243*, 3735.
7. Störmer, F. C. *J. Biol. Chem.* **1968**, *243*, 3740.
8. Holtzclaw, W. D.; Chapman, L. F. *J. Bacteriol.* **1975**, *121*, 917.
9. Snoep, J. L.; Teixeira de Mattos, M. J.; Starrenburg, M. J. C.; Hugenholtz, J. *J. Bacteriol.* **1992**, *174*, 4838.
10. Phalip, V.; Schmitt, P.; Diviès, C. *Curr. Microbiol.* **1995**, *31*, 316.
11. Peng, H. L.; Wang, P. Y.; Wu, C. M.; Hwang, D. C.; Chang, H. Y. *Gene.* **1992**, *117*, 125.
12. Shaanan, B.; Chipman, D. M. *FEBS J.* **2009**, *276*, 2447.
13. Bar-Ilan, A.; Balan, V.; Tittmann, K.; Golbik, R.; Vyazmensky, M.; Hubner, G.; Barak, Z.; Chipman, D. M. *Biochemistry* **2001**, *40*, 11946.
14. Candy, J. M.; Koga, J.; Nixon, P. F.; Duggleby, R. G. *Biochem. J.* **1996**, *315*, 745.
15. Killenberg-Jabs, M.; Konig, S.; Eberhardt, I.; Hohmann, S.; Hubner, G. *Biochemistry* **1997**, *36*, 1900.
16. Lee, S. C.; Kim, J.; La, I. J.; Kim, S. K.; Yoon, M. Y. *Enzyme Microb. Technol*. **2013**, *52*, 54.
17. La, I. J.; Karim, M.; Yoon, M. Y. *Bull. Korean Chem. Soc.* **2008**, *29*(9), 1823.
18. Dobritzsch, D.; Konig, S.; Schneider, G.; Lu, G. *J. Biol. Chem.* **1998**, *273*, 20196.
19. Pang, S. S.; Duggleby, R. G.; Guddat, L. W. *J. Mol. Biol.* **2002**, *317*, 249.