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学 位 論 文 要 旨
SUMMARY OF DOCTORAL THESIS

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題目 Title: Molecular engineering of glutamate dehydrogenase from *Bacillus subtilis*.

Glutamate dehydrogenases (GluDHs) are a broadly distributed group of enzymes that catalyze the reversible oxidative deamination of L-glutamate to 2-oxoglutarate (2-OG) and ammonia. The extremely small equilibrium constant of GluDH allows it to act as a useful catalyst in the analysis of amino acids, 2-oxoacids and ammonia which are important tools in clinical chemistry, bioprocess control, and nutrition studies. The special preference of the NAD-dependent GluDH for industrial use is because NAD^+ and NADH are much cheaper than $NADP^+$ and NADPH, respectively.

The diverse nature of bacterium *Bacillus* in nature of its evolutionary sphere is well known. The complete *B. subtilis* genome sequence has been reported to contain two genes *roc G* (also called *yweb*) and *gutB* (also called *ypca*) that expressed GluDHs of 424 and 426 amino acids long peptides, respectively. RocG, the major catabolic GluDH, was cloned, sequenced and expressed at considerable magnitude in *Escherichia coli*. The recombinant Bs-GluDH was purified to homogeneity and has been determined as a hexameric structure (M_r 270 kDa) with strict specificity for 2-OG and L-glutamate requiring NADH and NAD^+ as cofactors, respectively. The enzyme showed low thermostability with T_m = 41ºC due to dissociation of hexamer. To improve thermal stability of this enzyme, we performed error-prone PCR introducing random mutation on cloned GluDH. Two single mutant enzymes Q144R and E27F were isolated from mutant library whose T_m values were 61°C and 49°C, respectively. Furthermore, Q144R has a remarkably high k_{cat} value (452 s^{-1}) for amination reaction at 37ºC which is 1.3 times higher than that of wild type. Thus, Q144R can be used as template gene to modify the substrate specificity of Bs-GluDH for industrial use. Thus, directed evolution offers a powerful approach in widening the thermostability of enzyme with simultaneous increase of activity.

One of the major goals of the studies has been to understand the structural basis of amino acid substrate specificity in amino acid dehydrogenases, and to apply such knowledge to the engineering of novel specificities. The NAD-dependent Bs-GluDH is very specific for 2-OG with K_m 0.65 mM, whereas K_m for OAA was too insignificant to estimate. Changing the substrate specificity from 2-OG to OAA, the residues G82, K80 and M101, predicted to be important involving recognition of substrate, were mutated into two series of mutants using the templates, *rocG* and Q144R, respectively. The k_{cat} values of the constructed single mutants, G82K and M101S are 3.45 and 5.68 s^{-1}, for OAA which are 265 folds and 473 folds higher than those for 2-OG, respectively. On the other hand, thermostable mutant Q144R has also relatively high specificity on 2-OG with K_m 1.22 mM, whereas K_m values of double mutants Q144R/G82K and Q144R/M101S for OAA are 4.29 and 3.48 mM, respectively, compared to their K_m values >100 mM for 2-OG, respectively. The mutant Q144R showed the k_{cat} 452 s^{-1} on 2-OG, whereas Q144R/G82K and Q144R/M101S switch to increased specificity for OAA with k_{cat} values 1.99 and 2.14 s^{-1}, which are 100 and 71 folds higher than those for 2-OG, respectively. Surprisingly, G82K/Q144R and M101S/Q144R double mutants also showed higher magnitude of relative activity on pyruvate which were 350 and 136 folds to those for 2-OG, respectively. Certainly, these two double mutants showed activity in their deamination reactions on alanine. The results involving activity on oxaloacetate of four mutants G82K, M101S, G82K/Q144R and M101S/Q144R indicate that these mutants might have used novel synthetic biochemical pathways to show a comparable activity of both aspartate dehydrogenase and alanine dehydrogenase.