Note

Altering the Substrate Specificity of Glutamate Dehydrogenase from Bacillus subtilis by Site-Directed Mutagenesis

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Received April 18, 2005; Accepted May 31, 2005

The Lys80, Gly82 and Met101 residues of glutamate dehydrogenase from Bacillus subtilis were mutated into a series of single mutants. The wild-type enzyme was highly specific for 2-oxoglutarate, whereas G82K and M101S dramatically switched to increased specificity for oxaloacetate with $k_{cat}$ values 3.45 and 5.68 s$^{-1}$, which were 265-fold and 473-fold higher respectively than those for 2-oxoglutarate.

Key words: glutamate dehydrogenase; Bacillus subtilis; substrate specificity

Glutamate dehydrogenase (GluDH) is one of the enzymes that offer information concerning enzymological properties and relationships between structure and function. The extremely small equilibrium constant of GluDH allows it to act as a useful catalyst in the analysis of amino acids, 2-oxo acids, and ammonia, which are important tools in clinical chemistry, bioprocess control, and nutrition studies.1) One of the major goals of our studies has been to understand the structural basis of substrate specificity in GluDH from Bacillus subtilis (Bs-GluDH), and to apply that knowledge to the engineering of novel substrate specificities. Among the amino acid dehydrogenase super family, the amino acid specificity of GluDH, which catalyzes the reversible oxidative deamination of L-glutamate to 2-oxoglutarate (2-OG) and ammonia, is relatively strict.2,3)

Analysis of enzyme homology with other GluDHs has shown that the residues conserved among the GluDH families, regardless of their quaternary structure, are clustered in the three-dimensional structure, emphasizing the remarkably similar layout of the glutamate binding site and the active site pocket.4,5) In the context of crystal structure information on the Clostridium symbiosum GluDH–glutamate complex (Cs-GluDH),6,7) the $\varepsilon$-amino group of Lys80 (Cs-GluDH Lys89) and side chain hydroxyl group of Ser358 (Cs-GluDH Ser380) form salt bridge and hydrogen bond interactions respectively with the $\gamma$-carboxylic group of L-glutamate (Fig. 1). The three-dimensional structure model information on Bs-GluDH suggests that apart from those residues, Gly82 and Met101 residues lie close to the binding site for 2-OG in Bs-GluDH (Fig. 1). Residue Gly82 is conserved in the GluDH family whereas the equivalent residue in Cs-GluDH is Gln110 for Met101. In this study, which aimed at altering the substrate specificity of Bs-GluDH from 2-OG to oxaloacetate (OAA), we chose K80, G82, and M101 residues and constructed a series of single mutant enzymes that form electrostatic interactions or hydrogen bonds with oxygen atoms of the $\beta$-carboxylic group of OAA. Here we report that among the constructed single mutant enzymes, G82K and M101S dramatically shifted their specificity and showed preferentially good activity for OAA, the probable substrate for L-aspartate production in the reductive amination reaction.

The rocG gene encoding NAD$^+$-dependent Bs-GluDH has been cloned and expressed at considerable magnitude in Escherichia coli.8) Site-directed mutagenesis was introduced into the rocG gene with a Mutan Super Express kit (Takara, Ohtsu, Japan).9) The dideoxynucleotide chain-termination method was employed for sequencing using an ABI PRISM 3100-Avant Genetic Analyzer (Boston, MA, U.S.A.) and sequence data were analyzed using GENETYX-SV/RC 7.0 software (Software Development, Tokyo, Japan). The mutant enzymes and synthetic primers used were K80R, 5'-CGGTCC-GACCGTGCCGGGCGT-3', G82K, 5'-CAAAGGGGAAAGTGTTGGC-3', M101K, 5'-CCATTGGAACGCTCAA-3', and M101S 5'-CCATTTGGAAGCTACGCTCAA-3'. The transformants were grown at 37°C for 20 h in

Abbreviations: GluDH, glutamate dehydrogenase; 2-OG, 2-oxoglutarate; IPTG, isopropyl thio-β-D-galactoside; OAA, oxaloacetate
LB medium containing 0.005% ampicillin and 1 mM IPTG. The cells were suspended in potassium phosphate (KP) buffer, pH 7.3, and subjected to French press (Ohtake, Tokyo, Japan) two times at a pressure of 1500 kg/cm$^2$. After ultracentrifugation ($181\times 000$/g, 1 h), the recombinant enzyme was purified from the supernatant according to a procedure used for the native enzyme. In each step of purification, expression and purity were checked by SDS–PAGE (12%) under the conditions described by Laemmli. All mutant enzymes were purified to homogeneity, and exhibited a single band with subunit $M_r$ similar to that of wild-type enzyme ($M_r 46,000$) on SDS–PAGE (data not shown).

Homology models for the 3D structures of the wild-type and mutant enzymes were built using the same method. Table 1 shows the substrate specificity of the wild-type and mutant enzymes on a number of 2-oxo acids as their relative activities as well as the specific activities for 2-OG. The wild-type enzyme exhibited negligible activity on OAA as well as on pyruvate in relation to its considerably higher activity on 2-OG. On the other hand, among the five single mutant enzymes, G82K and M101S had 280-fold and 495-fold higher preferences respectively for OAA over 2-OG, while those enzymes showed very low specific activities for 2-OG compared with that of the wild-type enzyme. Those mutant enzymes took pyruvate and aromatic 2-oxoacids as the substrates of second-order preference. G82K and M101S showed 23-fold and 15-fold higher reactivity respectively for pyruvate, and 34-fold and 21-fold higher reactivity respectively for aromatic 2-oxoacid than their activities for 2-OG. Thus the mutation at those ascribed positions somewhat broadened the specificity of G82K and M101S. But, regardless of the context of relative activity, the specific activities of mutant enzymes compared to the wild-type on the various 2-oxo acids listed in Table 1 were not drastically altered as to those of their magnitude of activities for OAA from 2-OG. Thus, the preferences for OAA of both mutant enzymes, G82K and M101S were distinctly superior to those for other 2-oxo acids.

The reactivities of three other single mutant enzymes, G82R, K80R, and M101K, were not found to be significant compared to those of G82K and M101S on the 2-oxo acids listed in Table 1. Because only G82K and M101S showed substantially high reactivity on

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**Table 1.** Substrate Specificity of Single Mutant Enzymes in Reductive Amination Reaction

<table>
<thead>
<tr>
<th>2-Oxo acids</th>
<th>Wild type$^a$</th>
<th>G82K$^a$</th>
<th>G82R$^a$</th>
<th>K80R$^a$</th>
<th>M101K$^a$</th>
<th>M101S$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-OG</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>(440$^b$)</td>
<td>(0.026$^b$)</td>
<td>(0.019$^b$)</td>
<td>(0.014$^b$)</td>
<td>(0.012$^b$)</td>
<td>(0.019$^b$)</td>
<td></td>
</tr>
<tr>
<td>OAA</td>
<td>0.23</td>
<td>28,000</td>
<td>ND$^c$</td>
<td>208</td>
<td>117</td>
<td>49,500</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>0.11</td>
<td>2,270</td>
<td>91</td>
<td>216</td>
<td>126</td>
<td>1,530</td>
</tr>
<tr>
<td>$\beta$-Phenylpyruvate</td>
<td>6.00</td>
<td>1,080</td>
<td>145</td>
<td>188</td>
<td>69</td>
<td>2,050</td>
</tr>
<tr>
<td>2-Oxo-3-methylvalerate</td>
<td>0.05</td>
<td>731</td>
<td>129</td>
<td>225</td>
<td>215</td>
<td>132</td>
</tr>
<tr>
<td>2-Oxo-iso-valerate</td>
<td>2.70</td>
<td>26</td>
<td>356</td>
<td>408</td>
<td>96</td>
<td>289</td>
</tr>
<tr>
<td>2-Oxo-iso-caproate</td>
<td>0.10</td>
<td>73</td>
<td>213</td>
<td>191</td>
<td>54</td>
<td>236</td>
</tr>
<tr>
<td>2-Ketohexanoate</td>
<td>ND$^c$</td>
<td>1,850</td>
<td>502</td>
<td>279</td>
<td>129</td>
<td>432</td>
</tr>
<tr>
<td>$p$-Hydroxyphenylpyruvate</td>
<td>0.05</td>
<td>3,430</td>
<td>329</td>
<td>182</td>
<td>31</td>
<td>121</td>
</tr>
</tbody>
</table>

$^a$Relative activity: One Hundred percent corresponds to the specific activity for 2-OG of each enzyme.

$^b$Specific activity: units mg protein$^{-1}$.

$^c$ND, not detectable.
OAA, we determined the kinetic parameters of those two mutants. The steady-state kinetic parameters of the wild-type and the mutant enzymes are shown in Table 2. G82K showed a $k_{\text{cat}}$ value of 3.45 s$^{-1}$ for OAA compared to its $k_{\text{cat}}$ value of 0.013 s$^{-1}$ for 2-OG. The former value was 265 fold higher than the latter one, and was 1.2% of that of the wild-type enzyme for 2-OG. The $K_m$ values of G82K for OAA and NADH were 4.16 and 0.095 mM respectively, whereas for 2-OG the $K_m$ was greater than 100 mM indicating that the G82K mutant showed binding affinity good enough to endorse dehydrogenation catalysis of OAA but not of 2-OG. Similarly, the $k_{\text{cat}}$ value of M101S for OAA was 5.68 s$^{-1}$, which is 1.7% of that of wild-type enzyme for 2-OG, whereas the $k_{\text{cat}}$ value for 2-OG was 0.012 s$^{-1}$, showing that about a 473-fold higher catalytic shift had been achieved for OAA. The $K_m$ values of M101S for OAA, NADH, and 2-OG were 2.27, 0.091, and greater than 100 mM respectively. Therefore, the estimated catalytic efficiency, $k_{\text{cat}}/K_m$ values of G82K and M101S for OAA were 0.829 and 2.50 s$^{-1}$-mM$^{-1}$ respectively. Although these $k_{\text{cat}}/K_m$ values were 0.16 and 0.47% of that of wild-type enzyme for 2-OG (529 s$^{-1}$-mM$^{-1}$), the use of a large amount of mutant enzymes gave comparable activities of aspartate dehydrogenase (AspDH). Furthermore, in the oxidative deamination reactions, the single mutants G82K and M101S showed specific activity on L-aspartate 0.39 and 0.47% of that of wild-type enzyme with its natural substrate, 2-OG. It might be more fruitful to combine molecular evolutionary engineering with specific selection to achieve further subtle improvements in catalytic capability. In addition, we are making progress in resolving the crystal structure of these single mutant enzymes and hope to identify the details of the structural basis of their substrate specificities to OAA.

### Acknowledgments

This work was supported in part by a Grant-in-Aid for Scientific Research (C) (no. 15580079) from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

### References

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