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Escherichia coli Arabinose Isomerase and *Staphylococcus aureus* Tagatose-6-Phosphate Isomerase: Which is a Better Template for Directed Evolution of Non-Natural Substrate Isomerization?

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Metallic and non-metallic isomerases can be used to produce commercially important monosaccharides. To determine which category of isomerase is more suitable as a template for directed evolution to improve enzymes for galactose isomerization, L-arabinose isomerase from *Escherichia coli* (ECAI; E.C. 5.3.1.4) and tagatose-6-phosphate isomerase from *Staphylococcus aureus* (SATI; E.C. 5.3.1.26) were chosen as models of a metallic and non-metallic isomerase, respectively. Random mutations were introduced into the genes encoding ECAI and SATI at the same rate, resulting in the generation of 515 mutants of each isomerase. The isomerization activity of each of the mutants toward a non-natural substrate (galactose) was then measured. With an average mutation rate of 0.2 mutations/kb, 47.5% of the mutated ECAIs showed an increase in activity compared with wild-type ECAI, and the remaining 52.5% showed a decrease in activity. Among the mutated SATIs, 58.6% showed an increase in activity, whereas 41.4% showed a decrease in activity. Mutant clones showing a significant change in relative activity were sequenced and specific increases in activity were measured. The maximum increase in activity achieved by mutation of ECAI was 130%, and that for SATI was 190%. Based on these results, the characteristics of the different isomerases are discussed in terms of their usefulness for directed evolution of non-natural substrate isomerization.

Keywords: Arabinose, tagatose-6-phosphate, isomerase, directed evolution, metal ion

Isomerases mediate the interconversion of aldose and ketose sugars, thus providing a means of producing commercially valuable monosaccharides. Fructose, an isomer of glucose, has been commercially manufactured from glucose using molecularly evolved D-xylose isomerase, which naturally interconverts xylose and xylulose *in vivo* [2]. Tagatose is a sugar substitute used as a sweetener. Many studies on the development of isomerases for tagatose production by directed evolution have been conducted. In particular, an arabinose isomerase has been used to mediate the conversion between L-arabinose and L-ribulose *in vivo*, and D-tagatose has been manufactured from D-galactose *in vitro* [5, 13]. Xylose and arabinose isomerases have been reported to be metalloenzymes harboring divalent cations such as magnesium, manganese, and cobalt in their structures [1, 8]. These ions play a role in stabilizing intermediates during the reaction, the mechanism of which is known as hydride transfer (Fig. 1A). Glucose-6-phosphate and tagatose-6-phosphate isomerases, on the other hand, are categorized differently [14]. These enzymes do not possess metal ions. Since the intermediates of non-metallic isomerases have been shown to form enediol products, their reactions are said to proceed by an enediol mechanism (Fig. 1B).

Directed evolution has become a powerful tool to improve the characteristics of enzymes. Directed evolution of metallic arabinose isomerases has enhanced tagatose production by achieving a faster turnover rate [6] and modifying of optimal conditions [11]; however, directed evolution of non-metallic isomerases for the tagatose production has not yet been reported. A comparison of metallic and non-metallic isomerases as templates for directed evolution should enable us to take advantage of this tool for improving enzymes for tagatose production.

In this study, we chose L-arabinose isomerase from *Escherichia coli* (ECAI; E.C. 5.3.1.4) and tagatose-6-phosphate isomerase from *Staphylococcus aureus* (SATI; E.C. 5.3.1.26) as models of a metallic and a non-metallic isomerase, respectively. Trends in galactose isomerization

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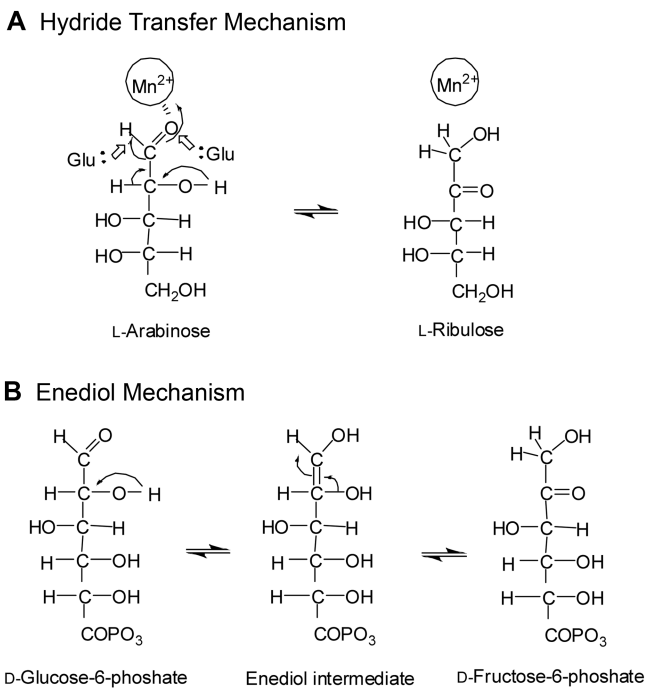

Fig. 1. Proposed hydride transfer mechanism of a metallic isomerase (**A**) and the enediol mechanism of a non-metallic isomerase (**B**).

activities were analyzed after introducing random mutations in both enzymes at the same mutation rate. Opportunities to acquire enzymes for galactose isomerization by directed evolution are discussed based on the results of this analysis.

Escherichia coli ER2566 (New England BioLabs Inc., Ipswich, MA, U.S.A.) was used for the construction of plasmids and enzyme expression. The L-arabinose isomerase gene (*araA*) was amplified by PCR using the oligonucleotides TTGAATTCATGACGATTTTTGATAATTAT (*Eco*RI site underlined) and TTGGTACCTTAGCGACGAAACCCGTA (*Kpn*I site underlined) based on the genome sequence of *E. coli* K-12 substrain W3110 (KCTC 2223; GenBank AC_000091). The resulting 1.5-kb DNA fragment was ligated into *Eco*RI–*Kpn*I-digested pTrc99A (AP Biotech Inc., Uppsala, Sweden) after excision from the T-vector, which yielded pTrc-araA. The plasmid pTrc-lacAB, which harbors the tagatose-6-phosphate isomerase structural gene (*lacAB*) from *Staphylococcus aureus* (GenBank NC_002745), was from our laboratory stock [10]. Plasmids pTrc-araA and pTrc-lacAB were used as templates for PCR-mutagenesis of metallic and non-metallic isomerases, respectively.

To introduce random mutations, error-prone PCR was carried out using a Diversity PCR Random Mutagenesis Kit (ClonTech Laboratories, Palo Alto, CA, U.S.A.) according to the manufacturer's protocol. The pTrc-araA and pTrc-lacAB templates were amplified with the oligonucleotides of M13/pUC-rev and pBAD-rev. The mutated *araA* and *lacAB* PCR products (1.5 kb and 0.9 kb, respectively) were digested with *Eco*RI–*Kpn*I and *Nco*I–*Bam*HI, respectively, and then subcloned into similarly digested pTrc99A and transformed into *E. coli* ER2566 by electroporation (BTX ECM; Harvard Apparatus, Holliston, MS, U.S.A.). Oligonucleotide construction and DNA sequencing were performed at a facility of Macrogen Co. (Seoul, Korea).

Cells were grown on agar plates containing Luria–Bertani (LB) medium with ampicillin (50 μg/ml) at 37°C. Colonies were transferred to 96-well plates containing 200 μl of LB–ampicillin and a master plate. The 96-well plates were incubated at 37°C and 900 rpm overnight to allow growth of cells. Ten μl samples of culture were transferred to another 96-well plate containing 200 μl of fresh induction medium (LB–ampicillin–2 mM lactose) and incubated at 37°C and 900 rpm for 6 h to induce protein expression. Cell growth was measured on a multiplate reader (Microplate Reader 550; Bio-Rad, Richmond, VA, U.S.A.) at 600 nm. Eighty μl of induced cells were mixed with 20 μl of lysis buffer [0.1% Triton X-100 and 1 mM phenylmethylsulfonyl fluoride (PMSF) in MacIlvain buffer; pH 7.5] and incubated at 37°C and 400 rpm for 30 min to lyse cells. One hundred μl of 100 mM galactose solution was added and the 96-well plate was incubated at 37°C and 400 rpm for 2 h to allow galactose isomerization. Tagatose formation in the reaction mixture was visualized by adding 170 μl of a H_2SO_4/cysteine/carbazole solution and measuring the OD at 560 nm [4]. The relative activity of the mutated isomerase was expressed as the ratio of enzyme activity ($OD_{560\ nm}$/$OD_{600\ nm}$·min.) compared with that of wild-type isomerase.

The specific activity of selected mutants was determined as described above except for cell lysis. For the preparation of an enzyme solution, actively growing cells expressing the selected mutant enzyme were harvested by centrifugation of 1 ml of culture broth and disrupted using a sonicator (Vibracell Sonics & Materials Inc., Danbury, CT, U.S.A.) set at 30 W for 1 min at 1-s intervals on ice. After removing the cell debris by centrifugation, the supernatant was used for analysis of protein content and enzyme activity [3].

The protein concentration of the enzyme solution was estimated with a Protein Assay Kit (Bio-Rad, La Jolla, CA, U.S.A.) using bovine serum albumin as a standard. Enzyme expression was confirmed by 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and visualized by staining with Coomassie Brilliant Blue.

Random mutations were introduced into ECAI and SATI at the same rate, and the activity against a non-natural substrate of the resulting mutants was analyzed (Fig. 2). Accordingly, 47.5% (270/515) of mutated ECAIs showed an increase in galactose isomerization activity compared with wild-type ECAI, whereas 52.5% (243/515) showed a decrease. The number of mutants showing a significant increase in activity (more than 20%) was 17, whereas that showing a significant decrease (more than 20%) was 21. On the other hand, 58.6% of mutated SATI showed an

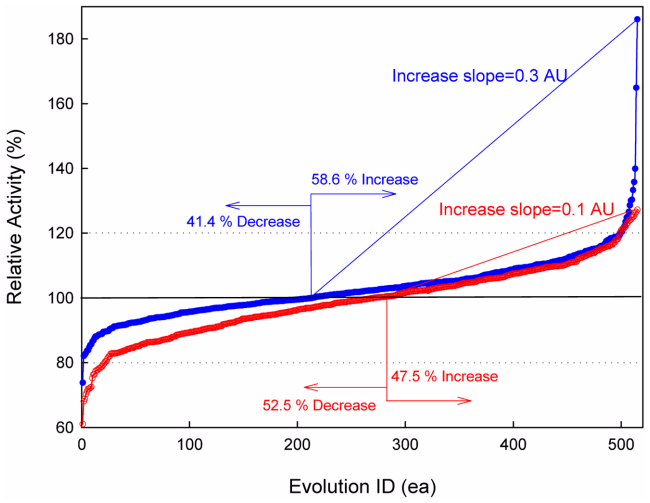

Fig. 2. Changes in the isomerization of a non-natural substrate by ECAI and SATI by introducing random mutations.
Results for ECAI are shown in red and in those for SATI are in blue. The rate of increase in activity (slope) was calculated as % increase/number of mutants and was expressed in arbitrary units (au). The dotted lines indicate the 20% error-range based on the relative activity of the wild type.

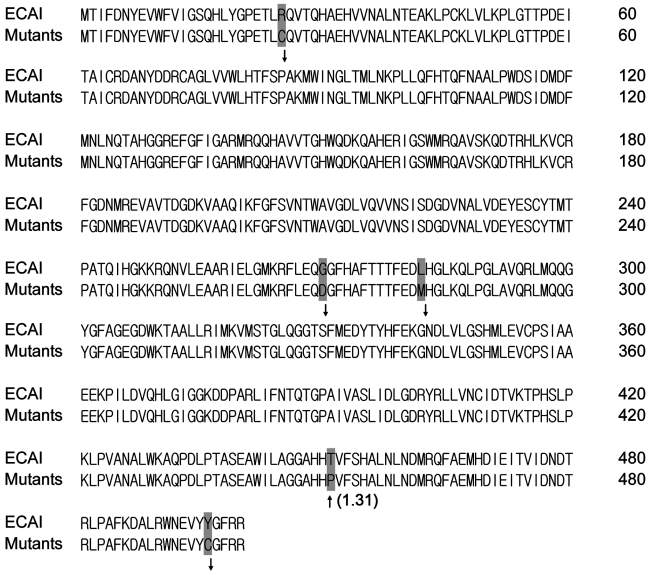

Fig. 3. Locations of mutations in ECAI showing more than a 20% increase or decrease in activity.
Shaded residues indicate locations of mutations. The upward-pointing arrow represents an increase in activity and the downward-pointing arrows represent a decrease in activity. Numbers in parentheses indicate the ratio of the specific activity of the mutant to that of the control.

increase in activity, whereas 41.4% showed a decrease, and 16 mutants showed a significant increase in activity and 1 showed a significant decrease. Therefore, a greater chance of increasing the isomerization of a non-natural substrate was obtained by random mutation of the non-metallic SATI rather than the metallic ECAI.

Mutant ECAI and SATI clones showing a significant change in activity were sequenced and the specific increase in activity was determined (Fig. 3 and 4). The greatest increase in activity among the 515 mutated ECAIs was 131% of the control (T451P), whereas that among the mutated SATIs was 190% (F160S). The rate of increase [% of increase/number of mutants, expressed in arbitrary units (au)] in activity of the ECAIs achieved by directed evolution was 0.1 au (30% increase/245 mutants), whereas that of the SATIs was 0.3 au (90% increase/302 mutants), which is 3 times higher.

The average mutation rate was 0.2±0.02 mutations/kb in the cases of ECAI and SATI. Mutation of Thr451 to Pro enabled ECAI to increase its activity by 31%, whereas mutation of Arg25, Leu282, Gly270, and Tyr496 to Cys, Met, Asp, and Cys, respectively, caused a decrease in activity of more than 20%. No mutations were found that significantly decreased the activity of SATI, but mutation of Val54, Glu132, Asp158, and Phe160 to Ala, Asp, Gly, and Ser, respectively, enhanced the activity of the enzyme by more than 20%. In particular, Asp158Gly and Phe160Ser mutations occurred twice and three times, respectively, in SATI mutants showing a significant increase in activity. The locations of mutations in ECAI showing a significant increase or decrease in activity were relatively scattered throughout the gene, whereas those of SATI mutants were relatively clustered at the C-terminus. The level of protein expression as observed by SDS–PAGE was not significantly changed (data not shown).

Non-metallic SATI showed better characteristics as a target for directed evolution than did metallic ECAI in terms of the isomerization of non-natural substrate. When

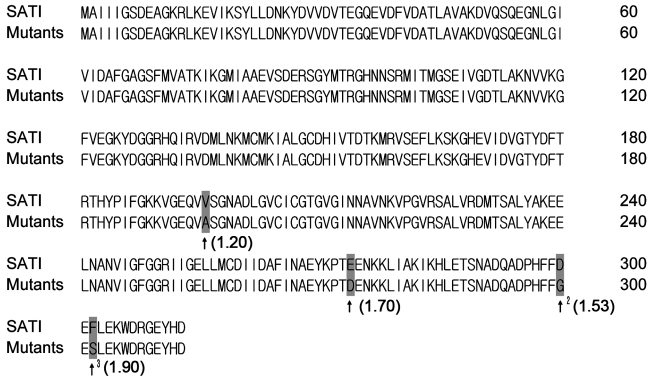

Fig. 4. Locations of mutations in SATI showing more than a 20% increase in activity.
Shaded residues indicate locations of mutations. The upward-pointing arrow represents an increase in activity and the downward-pointing arrows represent a decrease in activity. Numbers in superscript indicate the number of mutants in which the mutation was found. Numbers in parentheses indicate the ratio of the specific activity of the mutant to that of the control.

random mutations were introduced, the probability that enzyme activity toward the non-natural substrate would increase was higher for SATI (58.6% of mutations) than for ECAI (47.5%). The degree of increase in activity was also greater for SATI (0.3 au) than for ECAI (0.1 au) (Fig. 2). Therefore, SATI seems to be a better template than ECAI for directed evolution aimed at modification of substrate specificity in isomerization.

The absence of a metallic cofactor in SATI may explain its superiority as a template for directed evolution over ECAI, which requires a divalent cation cofactor. The exact location of the metal cofactor is essential in isomerases using a hydride transfer mechanism (Fig. 1A); a slight conformational change induced by mutation would disrupt the positioning of the metal ion, thus leading to a decrease in activity. The number of mutable positions would be limited in the directed evolution of such an enzyme since an altered amino acid should not be involved in the positioning of the metal ion and should contribute to an increase in activity at the same time. In ECAI, mutations of Arg25→Cys, Leu282→Met, Tyr496→Cys and Gly270 →Asp had a negative effect on enzyme activity (Fig. 3); the above hypothesis is strengthened by the fact that the mutated positions were conserved among members of the arabinose isomerase family in the alignment test (data not shown). The Thr451→Pro mutation of ECAI enhanced activity (Fig. 3), a result that also supports the above hypothesis. The catalytic residues of ECAI are reported to be His450, Glu333, His350, and Glu306 [9, 12], and a proline mutation has contributed to the stability of metal ions in the structures of other metallic isomerases [7, 15, 16]. Therefore, the mutation to proline of Thr451 in ECAI, which is immediately adjacent to the catalytic residue His450, would have also contributed to the increase in activity. On the other hand, non-metallic isomerases using the enediol mechanism do not require a metal ion (Fig. 1B). Therefore, such enzymes would have more nonessential positions available for modification of substrate specificity during directed evolution.

In conclusion, activity of a non-metallic isomerase toward a non-natural substrate tended to increase, whereas that of a metallic isomerase tended to decrease, during directed evolution. The three-dimensional structure of SATI, not yet available, would enable a better understanding of the effect of specific mutations on the modification of activity.

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