Modulation of Substrate Specificities of D-Sialic Acid Aldolase through Single Mutations of Val-251

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In a recent directed-evolution study, Escherichia coli D-sialic acid aldolase was converted by introducing eight point mutations into a new enzyme with relaxed specificity, denoted RS-aldolase (also known formerly as L-3-deoxy-manno-2-octulosonic acid (L-KDO) aldolase), which showed a preferred selectivity toward L-KDO. To investigate the underlying molecular basis, we determined the crystal structures of D-sialic acid aldolase and RS-aldolase. All mutations are away from the catalytic center, except for V251I, which is near the opening of the [(α/β)8]-barrel and proximal to the Schiff base-forming Lys-165. The change of specificity from D-sialic acid to RS-aldolase can be attributed mainly to the V251I substitution, which creates a narrower sugar-binding pocket, but without altering the chirality in the reaction center. The crystal structures of D-sialic acid aldolase-L-arabinose and RS-aldolase-hydroxypyruvate complexes and five mutants (V251I, V251L, V251R, V251W, and V251I/V265I) of the D-sialic acid aldolase were also determined, revealing the location of substrate molecules and how the contour of the active site pocket was shaped. Interestingly, by mutating Val251 alone, the enzyme can accept substrates of varying size in the D-aldolase reactions and still retain stereoselectivity. The engineered D-sialic acid aldolase may find applications in synthesizing unnatural sugars of C6 to C10 for the design of antagonists and inhibitors of glycoenzymes.

Carbohydrates on cell surfaces play a pivotal role in the molecular recognition processes in various cellular interactions. Interfering with the recognition processes between pathogens and hosts may present therapeutic strategies for infectious diseases (1, 2). D-Sialic acids are widely found in nature, from bacteria to plants and animal tissues. Cell-surface glycoproteins and glycolipids frequently contain D-sialic acids at the termini of the oligosaccharide chains. The invading bacteria and viruses also target the cell-surface sialic acids of their hosts, which constitutes an important step for infectivity (3). On the other hand, the monosaccharide D-3-deoxy-manno-2-octulosonic acid (D-KDO) has never been identified in vertebrates, but it is an important component in Gram-negative bacteria (4–6). Because D-KDO is vital to the structural stability of bacterial outer membrane, targeting D-KDO and the related biosynthetic enzymes may be a new strategy for future antibacterial agent discovery.

Currently, unnatural enantiomeric compounds become increasingly important in pharmaceutical implementations. Enantiomers (D-form) of natural peptides have been used to target cell-surface D-sugars. These D-peptide cell-surface sugar binders might serve as new drug candidates for infectious disease (7) and as tools for studying the interactions between proteins and carbohydrate. Owing to the resistance to hydrolases and high binding affinity to sugars, the unnatural D-peptides can become potential therapeutic agents. Through mirror-image phage display, natural L-peptides can be identified for L-sugars, and enantiomers (D-form) of the natural peptides are then synthesized by chemical methods (8). Hence, efficient production of the enantiomeric L-sugars of the natural D-carbohydrates is the prerequisite for future applications. However, L-sugars are usually difficult to synthesize in large scale by chemical methods. It is well known that enzymes can be engineered to cover a wide range of biological activities and that directed evolution represents an efficient way to improve the desirable activity and specificity of an enzyme. For pharmaceutical implementations, enzymes are able to offer good tools for the synthesis of enantiomeric L-sugars.

In a recent directed evolution study (Fig. 1), the enzyme D-sialic acid aldolase (EC 4.1.3.3) from Escherichia coli (9) was converted into a new enzyme with relaxed specificity, denoted RS-aldolase and also known formerly as L-KDO aldolase, by introducing eight point mutations (10). D-Sialic acid aldolase catalyzes the reversible conversion of N-acetyl-D-mannosamine (D-ManNAc) and pyruvate to D-sialic acid (NeuAc), and it is important for sialic acid catabolism (11). The enzyme has a wide application in the synthesis of D-sialic acid and its derivatives (12–16). The crystal structures of D-sialic acid aldolases from both E. coli (9) and Haemophilus influenzae (17) have been reported, which showed a tetramer with each subunit...
unit comprising an eight-stranded α/β barrel. Based on the structures of *E. coli* (18) and *H. influenzae* D-sialic acid aldolase (17), a strictly conserved lysine residue (Lys-165 in the *E. coli* enzyme) has been proposed to form a Schiff base intermediate with the substrate (9, 18).

As mentioned above, the *E. coli* D-sialic acid aldolase was converted into an efficient RS-aldolase by directed evolution (10). Because L-KDO is structurally similar to D-sialic acid, it is a good starting substrate for screening. After five rounds of error-prone PCR, a new RS-aldolase was created, which showed an ~1,000-fold (*k_\text{cat}/K_m (L-KDO))/(*k_\text{cat}/K_m (D-sialic acid)) improvement compared with the original D-sialic acid aldolase toward accepting L-KDO as its substrate. These studies indicated that the new enzyme is applicable for the synthesis of L-KDO and its derivatives and that the substrate specificity of an enzyme can be rapidly altered by the directed evolution methods.

According to the crystal structure of *E. coli* D-sialic acid aldolase (9), none of the mutations that created the RS-aldolase (10) occurred in the catalytic center. How these mutations make the subtle changes of enzyme conformation and substrate specificity still remains to be answered. To elucidate the underlying molecular basis, the crystal structures of *E. coli* D-sialic acid aldolase and RS-aldolase and their complexes with substrate were solved. The structures clearly show that all mutated residues are away from the catalytic center, except for V251I, which is near the opening of the active site cavity (19, 20) and critical for altering the substrate specificity of the enzyme. To clarify the role of Val-251 in substrate binding and enzyme catalysis, many mutants were studied, including V251I, V251L, V251R, V251W, V265I, V251I/V265I, V251L/V265I, V251I/G252R, V251I/W265I, and V251I/G252R/W265I. V251I was also implicated in the specificity change (10). The substrate specificity and kinetic parameters of these enzymes were characterized. In conjunction with the observed structural information, the result provides useful insight to produce desirable sugar products by fine-tuning the enzyme specificity.

**EXPERIMENTAL PROCEDURES**

**Protein Expression, Purification, and Crystallization**—The gene coding for D-sialic acid aldolase (nanA gene) was amplified from the genomic DNA of *E. coli* by PCR and inserted into the vector pET-16b (Novagen) via the NdeI and XhoI sites, with a His\text{10} tag sequence at its 5′-end. The vector was transformed into *E. coli* BL21(DE3) cells (Novagen), which were screened using ampicillin, propagated at 37 °C, and subsequently induced by isopropyl 1-thio-β-D-galactopyranoside for protein production at 16 °C. The cells were harvested and lysed in 150 mM NaCl, 20 mM imidazole, and 50 mM Tris-HCl, pH 7.8, and the protein was purified on a nickel nitrolotriacetic acid-agarose column using an imidazole gradient in the same buffer. The eluate was desalted in a 50 mM Tris-HCl, pH 7.8, buffer and concentrated to 12–15 mg/ml.

The eight mutations for the RS-aldolase (10) were reintroduced into the D-sialic acid aldolase vector in seven steps, employing a QuickChange Site-directed Mutagenesis kit and seven DNA primers (supplemental Table S4). The recombinant RS-aldolase was expressed and purified as described above. The other mutants were prepared by using the QuickChange kit (supplemental Table S4), and expressed and purified likewise. All enzymes had a purity of >95% as judged by SDS-PAGE.

The wild-type and all mutants of D-sialic acid aldolase and RS-aldolase were crystallized at room temperature by the hanging drop vapor diffusion method by using a reservoir containing 2.0 M ammonium sulfate and 0.1 M Bis-Tris, pH 6.5. The ligand-bound D-sialic acid aldolase and RS-aldolase crystals were obtained by soaking, respectively, with 1 mM L-arabinose and 1 mM hydroxypyruvate in the crystallization buffer. For the cryoprotectant, 20–25% glycerol (v/v) was used.

**Data Collection, Structure Refinement, and Model Building**—The X-ray diffraction data were collected at National Synchrotron Radiation Research Center (Hsinchu, Taiwan), SPring-8 (Hyogo, Japan), and Photon Factory (Tsukuba, Japan) and processed using the HKL2000 package (see supplemental Tables S1 and S2) (21). The structure was solved by molecular replacement. All crystals belonged to the trigonal space group *P3_21*, with four molecules in an asymmetric unit. Model building was done with the programs O (22) and XtalView (23). The computational refinements were carried out using CNS (Crystallography and NMR System) (24). The R\text{free} values were calculated by using 5% randomly selected reflections. Stereochemical quality was assessed by PROCHECK (25). All structural figures were generated by using PyMOL (DeLano Scientific LLC) and UCSF chimera (University of California, San Francisco). The atomic coordinates and structure factors have been deposited in the Protein Data Bank (D-sialic acid aldolase, 3LB; D-sialic acid aldolase-L-arabinose complex, 3LB; D-sialic acid aldolase-L-arabinose complex, 3LC; RS-aldolase, 3LCX; RS-aldolase-hydroxypyruvate complex, 3LCW; D-sialic acid aldolase mutants, V251I, 3LCH; V251L, 3LCG; V251R, 3LCL; V251W, 3LCI; and V251I/V265I, 3LCL).

Based on the structure similarity of the *E. coli* D-sialic acid aldolase and its *H. influenzae* homologue, the crystal structure of *E. coli* sialic acid aldolase and RS-aldolase were superimposed on that of *H. influenzae* enzyme (Protein Data Bank code 1F7B) (17) with a bound 4-oxo-α-acetyl neuraminic acid (an analogue of D-sialic acid) attached to it (supplemental Fig. S1). Minor adjustments of the model with reference to the RS-aldolase-hydroxypyruvate complex allowed rational docking of the substrates L-KDO and D-sialic acid into the active site. The models were further energy-minimized by CNS to eliminate unreasonable contacts.

**Enzyme Activity Measurement**—L-KDO was enzymatically synthesized from pyruvate and L-arabinose using the RS-aldolase at 37 °C for 2 days (26). Subsequently L-KDO was purified by Dowex 1 × 8 (HCO_3^-) anion-exchange chromatography (Sig-
sugars were purchased from Sigma-Aldrich.

Aliquot was withdrawn and quenched with 5 ml of 150 l. Reactions were initiated by addition of the aldolase. The fluorescence of NADH was monitored at an emission at 340 nm after the intensity remained constant and fluorescence of NADH was recorded with excitation at 340 nm and an emission of 450 nm using a Fluorolog3 spectrofluorimeter (Horiba Jobin Yvon, Edison, NJ). The fluorescence of NADH was recorded with excitation at 340 nm and an emission of 450 nm using a Fluorolog3 spectrofluorimeter (Horiba Jobin Yvon, Edison, NJ). The fluorescence of NADH was recorded with excitation at 340 nm and an emission of 450 nm using a Fluorolog3 spectrofluorimeter (Horiba Jobin Yvon, Edison, NJ).

RESULTS AND DISCUSSION

Crystal Structures of D-Sialic Acid Aldolase and Its Mutants—Previously, the crystal structure of D-sialic acid aldolase from E. coli has been solved at a 2.2-Å resolution (9). We have reconstructed an expression vector, crystallized the protein, and improved the structure to a 1.48-Å resolution (supplemental Table S1). Each subunit of the tetrameric enzyme consists of an (α/β)n barrel with eight β-strands (βα–βh), eight α-helices (αA–αH), and three additional C-terminal α-helices denoted αl, αj, and αk (supplemental Fig. S2) and makes contacts with only two of the other subunits in the tetramer. A sulfate ion was identified in each active site, bound by Ser-47 Oγ, Thr-48 Oγ1 and N, Tyr-137 Oη, and Lys-165 NZ.

The enzyme was converted to RS-aldolase by introducing the eight point mutations (E60A/Y98H/F115L/D150G/N153Y/V251I/V265I/Y281C) according to the directed evolution study (10). The crystal structure of RS-aldolase was refined to 1.98-Å resolution (Fig. 2). Its comparison with D-sialic acid aldolase showed no significant conformational change. The structure clearly indicates that Ile-251 is the only mutated residue pointing toward the catalytic center that may interact with the substrate upon binding, whereas all other mutated residues are away from the active site.

Most of the eight mutated residues are on the protein surface, and none occur at the inter-subunit interface. The larger side chain of Ile-265 is directed toward the protein interior surrounded by several hydrophobic amino acids. A sulfate ion is also found adjacent to the side chain of Lys-165 in the active site of RS-aldolase (Fig. 2), as in D-sialic acid aldolase. In addition, another sulfate ion is found on the surface of RS-aldolase, forming hydrogen bonds with Ser-94 Oγ, Arg-97 Nη1 and Nη2, and His-98 Nε. It seems to replace the original acidic side chain of Glu-60, which has been mutated into Ala-60 in RS-aldolase. The sulfate-binding His-98 is also a mutation from Tyr-98. Under physiological conditions, this site may be occupied by a phosphate ion. The subtle structural changes on the surface may have some effects on the enzyme, albeit not as significant as does the mutation of V251I.
Substrate Specificities of d-Sialic Acid Aldolase

The structure of the d-sialic acid aldolase-L-arabinose complex revealed that an L-arabinose was bound to each monomer (supplemental Fig. S3). The bound L-arabinose lies in a cavity neighboring the substrate-binding site, not in the catalytic center but near the subunit interface, via a hydrogen bond to Glu-192 and some hydrophobic interaction (supplemental Fig. S4). In addition to the sulfate ion bound to the active site as in the native enzyme, a second sulfate was found near the subunit interface, in a site adjacent to the L-arabinose molecule. These additional ligands may interfere with the proper binding of substrate to the active site (see below). The observation that L-arabinose was bound to the secondary site instead of the active center also suggests that it is not a good substrate.

Various attempts were made to obtain other enzyme-substrate complexes. One of these turned out to be the RS-aldolase in complex with hydroxypyruvate, an analogue of the natural substrate pyruvate (supplemental Fig. S5). The hydroxypyruvate molecule was covalently attached to the side chain of Lys-165 in the active site, forming a Schiff base. Its C2 formed a single bond to Lys-165 NZ and its carboxylate moiety was positioned so as to make hydrogen bonds with Ser-47 N and Thr-48 Oγ1 and Tyr-137 Oγ (Fig. 3A). The side chain conformations of the residues surrounding the active site show no significant deviation from those observed in the substrate-free structure, with the exception of Lys-165 (supplemental Fig. S6). In particular, the ordered water molecules in the active site are essentially identical. The locations of active-site waters in the hydroxypyruvate complex may mimic those of substrate O7 and O8, and allow rational docking of the substrate L-KDO into the active site (supplemental Fig. S6). The sulfate ion was expelled from the active site upon the binding of hydroxypyruvate. The other sulfate bound to the surface near Ala-60 and His-98 was consistently observed as in the previous structure. Although the other substrate L-arabinose was included in the crystallization solution, it was not visible in the complex crystal structure. Presumably, the additional hydroxyl group of the first substrate hindered the entry of the second substrate into the active site.

The E. coli d-sialic acid aldolase shares 37% sequence identity with its H. influenzae homologue. Structural comparison of these two enzymes along with the bound 4-oxo-N-acetyl neuraminic acid (an analogue of d-sialic acid) allowed analysis of the active site of E. coli sialic acid aldolase, which was then docked with d-sialic acid. And the structure of the RS-aldolase-hydroxypyruvate complex allowed rational docking of the substrate L-KDO into the active site. The models of d-sialic acid aldolase with bound d-sialic acid and RS-aldolase with bound L-KDO are shown in Fig. 3, B and C, respectively. Each substrate is covalently bonded to the NZ atom of Lys-165. The carboxylate group of L-KDO interacts with Ser-47 N, Thr-48 Oγ1, and Tyr-137 Oγ of RS-aldolase in a fashion identical to that observed in the model of d-sialic acid aldolase with bound d-sialic acid. The O5 hydroxyl is oriented towards the active site and forms stable hydrogen bonds with Thr-48 Oγ1 and Ser-208 N. The O6 hydroxyl makes hydrogen bond with Gly-189 O or Asp-191 Oδ1, and the O8 hydroxyl is capable of forming a hydrogen bond with Glu-192 Oε2. Comparison of the d-sialic acid aldolase/d-sialic acid and RS-aldolase/L-KDO models shows that the C4 hydroxyl group in the substrates d-sialic acid and L-KDO is oriented outward from the active site and possibly forms a hydrogen bond with the active-site Tyr-137. As described above, the catalytically essential residues and the water molecules in the catalytic center are highly conserved between the two enzymes, suggesting that their catalytic mechanism should be very similar.

The structures of the double mutant V251I/V265I and four of the single mutants (V251I, V251L, V251R, and V251W) of d-sialic acid aldolase (supplemental Table S2) are nearly identical to the wild-type enzyme. As will be shown below, the

![FIGURE 3. Substrate docking into the active site of d-sialic acid aldolase and RS-aldolase. A, the molecular surface of the RS-aldolase-hydroxypyruvate complex is colored according to the electrostatic potential from red (negative) to blue (positive). The catalytically important residues and the mutated residue Ile-251 are depicted with stick models in green and magenta, respectively. The bound hydroxypyruvate is drawn as a ball-and-stick model in cyan and found to form a Schiff base with Lys-165. Hydrogen bonds are shown as black dashed lines. The docked sugars in d-sialic acid aldolase with bound d-sialic acid (B) and RS-aldolase with bound L-KDO (C) are depicted as stick-and-ball models in cyan and yellow, respectively. The C4 hydroxyl groups of both substrates is oriented outward from the active site and possibly forms a hydrogen bond with the active site residue Tyr-137. Note that the stereogenic configuration at the C4 carbon is identical in both cases.](image-url)
mutations at Val-251 changed the active site contour of the enzyme. On the other hand, the mutation of V265I had little structural effects, with the additional methylene group very well accommodated in a nonpolar pocket.

**Active Site Environment and Effects of Mutations at Val-251**—The active site of d-sialic acid aldolase has been identified as a pocket located near the C-terminal end of the (α/β)8 barrel (9). The deep pocket is lined primarily by amino acids Ser-47, Thr-48, Tyr-137, Lys-165, Gly-189, Asp-191, Glu-192, and Ser-208 (supplemental Fig. S7). Lys-165 at the C terminus of strand f plays an important role in the catalysis process by forming the Schiff base intermediate with the substrate (d-sialic acid or L-KDO regarding to the retro-aldol reaction). Tyr-137 at the C terminus of strand e is also a catalytically important residue, whose role was proposed as to accept a proton from the C4 hydroxyl group of d-sialic acid and to help stabilize the Schiff-base intermediate during catalysis (supplemental Fig. S8). Ser-47 and Thr-48 are situated in the loop between strand b and helix B, and they are associated with binding the carboxylate group of the common α-keto acid moiety of the substrate. Gly-189, Asp-191, and Glu-192 are situated in the loop between strand g and helix G, and Ser-208 is situated in the loop between strand h and helix H. These residues are important for substrate binding but are not involved in catalysis itself. For Asp-191 and Glu-192, both the size and the charge of these residues seem to be absolutely necessary for substrate binding.

When the catalytically important residues in the four subunits of the tetrameric d-sialic acid aldolase are superimposed (supplemental Fig. S7A), the side chain conformation of all pivotal residues and the ordered waters in the catalytic centers are essentially identical among the four subunits. Similarly, the catalytic centers of the four subunits of RS-aldolase are observed alike (supplemental Fig. S7B). Furthermore, overlay of the residues in subunit A of d-sialic acid aldolase and RS-aldolase reveals that the largest difference between their Ca coordinates was 0.45 Å (supplemental Fig. S7C), again showing identical disposition of the residues. The sulfate ions and the highly conserved waters in the catalytic centers also showed no difference between the two enzymes. Water 1 was proposed to mediate the proton transfer during catalysis process (supplemental Fig. S8). These observations suggest that the underlying catalytic mechanisms of d-sialic acid aldolase and RS-aldolase are similar, but with different substrate specificity, due to the size of the remaining part of the substrate molecule (see below and also supplemental Fig. S9).

Ring-opening of substrates is the probable first step in the aldol reaction, with the enzyme active site environment presumably distorting the equilibrium of the compound away from the closed ring form toward the linear form (17). By addition of the active site lysine to the carbonyl group, the carbinolamine cation is formed, followed by proton transfers that lead to dehydration of an iminium intermediate and formation of a Schiff base between the lysine residue of the enzyme and C2 of the substrate. The proton abstraction from the hydroxyl at the C4 position leads to the cleavage of the C3–C4 bond and thus to the release of the first product (d-ManNAc when d-sialic acid is the substrate) and the formation of enamine. A hydrogen bond is observed between the hydroxyl group at C4 of the substrate and the Tyr-137 hydroxyl as well as between the latter hydroxyl group and the carboxylate group of the substrate, hence suggesting that the tyrosine mediates the proton abstraction by the carboxylate in a substrate-assisted reaction. Next, protonation of the methylene carbon converts the enamine to imine. After the active-site water (Wat1) attaches itself to the imine, the oxonium intermediate is formed and a proton is transferred from the oxygen to nitrogen atom. Consequently, the pyruvate is produced and released from the active site.

Given the virtually unchanged tetramer structures of d-sialic acid aldolase and RS-aldolase, the modified substrate specificity from d-sialic acid to L-KDO cannot be explained by the overall protein conformation. Whereas all other mutations occurred away from the active site, the important residue Val-251 appeared to play a key role. Hence, we focused on the V251I substitution to clarify the change of the substrate specificity. The sugar-binding pockets in the structures of d-sialic acid aldolase and RS-aldolase are shown in Fig. 4. The pocket in d-sialic acid aldolase (10.2 and 9.3 Å, see Fig. 4) is wider than that in RS-aldolase (8.4 and 7.7 Å). The replacement of Val-251 with Ile caused the sugar-binding pocket of RS-aldolase to become narrower, thus creating significant steric restraints for binding with large-sized sugars. In d-sialic acid aldolase, the distance between Val-251 C and Tyr-190 Ce1 is 9.3 Å. However, the distance between Ile-251 C and Tyr-190 Ce1 in RS-aldolase is 7.7 Å. Sialic acid is a nine-carbon sugar that includes an N-acetyl group at the C5 position, whereas KDO is an eight-carbon sugar having the C5-hydroxyl group. These structural differences could rationalize the change of substrate specificity.
Single point mutations on Val-251 alone are sufficient to change the size of the sugar-binding pocket, the reported turnover rate and binding affinity of this enzyme toward D-sialic acid are essentially high (10). Upon L-KDO binding to the active site of D-sialic acid aldolase, the sugar-binding pocket is relatively large; thus, the backbone of bound L-KDO can rotate freely. Only when the hydrogen bond between the C4 hydroxyl group of L-KDO and the active-site Tyr-137 is formed can the L-KDO cleavage proceed. Hence, the untrammeled rotation of the substrate in the sugar-binding pocket leads to a decrease in the cleavage activity.

On the other hand, D-sialic acid may fit more properly to the broad sugar-binding pocket of D-sialic acid aldolase. Namely, the bound D-sialic acid could not rotate freely and the orientation of the C4 hydroxyl group can easily form hydrogen bond with Tyr-137. In addition, the wide pocket of D-sialic acid aldolase may allow the product L-arabinose to slip into a cavity neighboring the active site during catalysis for the L-KDO cleavage, as observed in the complex crystal structure (supplemental Fig. S10). The slow product release likely results in a decreased cleavage activity. The bound L-arabinose molecule may also interfere with further binding of the substrate L-KDO to the active site. Therefore, D-sialic acid aldolase exhibits a higher cleavage rate for D-sialic acid than for L-KDO. In RS-aldolase, the larger side chain of Ile-251 can prevent L-arabinose from slipping into this pocket (supplemental Fig. S10), and neither substrate binding nor product release should thus cause any problem.

Analysis of the D-sialic acid cleavage activity shows that a single point mutation is sufficient to hinder the entry of substrates. Compared with D-sialic acid aldolase, mutant V251I and mutant V251L have a 60% decrease in D-sialic acid cleavage activity (supplemental Table S3). Remarkably, mutation of Val-251 to either arginine or tryptophan led to a further drastic decrease in the enzyme activity because of the expected significant steric hindrance. Moreover, the double mutant V251I/V265I has an 80% decrease in D-sialic acid cleavage activity. It is thus reasonable to assume that the long-range V265I substitution may cause a subtle change of enzyme conformation as well. However, the precise effect of Ile-265 on the active site environment still remains to be elucidated.

Conversely, analysis of the L-KDO cleavage activity shows that one point mutation is enough to improve the catalytic activity toward L-KDO. The L-KDO cleavage activity of mutant V251L is identical to that of RS-aldolase and other related mutants have similar activities for L-KDO. Interestingly, that mutant V251W exhibits the highest ratio of cleavage activity for L-KDO and D-sialic acid (supplemental Table S3), revealing that the significant steric hindrance created by the V251W substitution effectively blocks the D-sialic acid binding, but does not completely hinder the entry of L-KDO. Presumably, all of the other mutations that created RS-aldolase make subtle changes of the enzyme activity when compared with V251I. The V251I substitution chiefly mediates the change of enzyme conformation and substrate specificity, and the remaining seven mutations at remote sites may influence the enzyme activity in a synergistic manner. These enzymatic analyses indicate that mutations on Val-251 alone are sufficient to change the size of sugar-binding pocket, in good agreement with the structural observations described above.
The aldolase-catalyzed reactions are in fact reversible. Because the specificity of sialic acid aldolase can be modified by the mutations at Val-251, by which the size of the active site pocket is altered, we further measured the activity of the condensation reaction. With pyruvate as the first substrate, a number of aldose sugars with carbon numbers ranging from 3 to 7 were used as the second substrates, including D-glucoheptose, D-ManNAc, L-arabinose, D-erythrose, D-glyceraldehyde, and D-glucoheptose. The enzymes tested for activity include the wild-type D-sialic acid aldolase, the RS-aldolase, and four single mutants V251I, V251R, V251W, and V251G. The results are shown in Table 1. The remaining two mutants V251L and V251V/V265I are expected to display similar activity as that of RS-aldolase, judged by the previous results in Fig. 5.

For the aldol condensation reaction, D-sialic acid aldolase is most active and highly specific for D-ManNAc (its natural substrate), as compared with L-arabinose and D-glucoheptose. Specificity is modified once there are multiple mutations (RS-aldolase) or a single mutation at Val-251 (the other mutants), which is particularly supported by the $K_m$ values. Both mutants V251R and V251W are relatively inactive for D-ManNAc (V251R and V251W have $K_m$ values of 963 mM and 901 mM, respectively) and L-arabinose (V251R and V251W have $K_m$ values of 642 and 784 mM, respectively) but show increasing activity as the size of substrates decreased to D-erythrose (V251R and V251W have $K_m$ values of 263 and 305 mM, respectively) and D-glyceraldehyde (V251R and V251W have $K_m$ values of 516 and 307 mM, respectively). The smaller active-site pockets in these two mutant enzymes can account for their preference for smaller substrates. Interestingly, when the side chain was absent in the mutant V251G, the enzyme showed increased activity with increased size of the substrate. D-Glucoheptose has the $K_m$ value of 53.5 mM, ~6- and 4-fold lower than those of D-ManNAc and L-arabinose, dependent solely on the size of the substrate. In fact, these results suggest that the aldol condensation activity shows an overall correlation between the size of substrate and its binding pocket, without alteration of the stereoselectivity. The condensation carbon center still retains the same chirality.

In fact, our results of aldol condensation reactions already showed that some unnatural sugars can be manufactured in this way. Although the C10 sugar has no natural equivalent reported so far, its structural similarity to sialic acid can be exploited to block the wild-type D-sialic acid aldolase. We carried out the enzymatic synthesis of the C10, C8, and C7 sugar products by the aldol reactions of pyruvate with D-glucoheptose, L-arabinose, and D-erythrose, respectively. Supplemental Fig. S12 shows the desired molecular weights of the products and the stereoselectivity by the corresponding mass and $^1$H NMR spectra, respectively. The C6 sugar can find use in the inhibitor development for many important enzymes, including those that produce glucose, and its further modifications can lead to the discovery of useful new drugs. The C7 product is actually 3-deoxy-D-heptulosonate (DAH), which is related to DAH 7-phosphate (DAH7P). DAH7P is synthesized in nature by DAH7P synthase using phosphoenolpyruvate and erythrose 4-phosphate, and it is important in the shikimate pathway that eventually leads to production of the essential aromatic amino acids tyrosine, phenylalanine, and tryptophan in bacteria and plants (27, 28) but not in mammals. The DAH produced by the D-sialic acid aldolase mutant V251W can serve as a starting material for derivatives, which can be developed into herbicidal and antimicrobial agents. Similarly, D-KDO is synthesized in nature by reacting phosphoenolpyruvate and arabinose 5-phosphate. By engineering of the active-site pocket, the RS-aldolase can also be modulated to produce D-KDO. Furthermore, phosphoenolpyruvate can also be used to produce L-sialic acid by reacting it with D-ManNAc. The corresponding enzyme can be developed by engineering of pseudoaminic acid synthase, or Psel, which is involved in the pseudoaminic acid pathway for O-linked flagellin glycosylation in *Helicobacter pylori* (29, 30). Conversely, because of its structural similarity to pseudoaminic acid, the

### Table 1: Kinetic parameters of the reverse reactions catalyzed by d-sialic acid aldolase, RS-aldolase, and four other mutants

| Enzyme* | Substrate      | $K_m$ (mM) | $k_{cat}$ (s$^{-1}$) | $k_{cat}/K_m$ (mM$^{-1}$ s$^{-1}$) |
|---------|----------------|------------|----------------------|----------------------------------|
| d-sialic acid aldolase (WT) | D-Glucoheptose | 194 ± 21 | 0.14 ± 0.01 | 7.2 × 10$^{-4}$ |
|         | D-ManNAc       | 64.4 ± 12.5 | 10.2 ± 0.4 | 1.6 × 10$^{-1}$ |
|         | L-Arabinose    | 220 ± 64 | 0.05 ± 0.05 | 2.0 × 10$^{-3}$ |
| RS-aldolase | D-ManNAc       | 181 ± 48 | 7.30 ± 0.66 | 4.0 × 10$^{-2}$ |
|         | L-Arabinose    | 199 ± 24 | 1.10 ± 0.05 | 5.5 × 10$^{-3}$ |
| V251I   | D-ManNAc       | 173 ± 36 | 10.0 ± 0.7 | 5.8 × 10$^{-2}$ |
|         | L-Arabinose    | 153 ± 25 | 1.06 ± 0.05 | 6.9 × 10$^{-3}$ |
| V251R   | D-Glucoheptose | N.D.$^b$ | N.D.$^b$ | – |
|         | D-ManNAc       | 963 ± 441 | 3.23 ± 1.02 | 3.3 × 10$^{-3}$ |
|         | L-Arabinose    | 901 ± 356 | 0.34 ± 0.10 | 3.8 × 10$^{-4}$ |
|         | D-Erythrose    | 263 ± 84 | 1.44 ± 0.20 | 5.4 × 10$^{-3}$ |
|         | D-Glyceraldehyde | 305 ± 75 | 1.44 ± 0.17 | 4.7 × 10$^{-3}$ |
| V251W   | D-Glucoheptose | N.D.$^b$ | N.D.$^b$ | – |
|         | D-ManNAc       | 642 ± 102 | 0.41 ± 0.04 | 6.4 × 10$^{-4}$ |
|         | L-Arabinose    | 784 ± 287 | 2.33 ± 0.55 | 3.0 × 10$^{-3}$ |
|         | D-Erythrose    | 516 ± 180 | 2.38 ± 0.46 | 4.6 × 10$^{-3}$ |
|         | D-Glyceraldehyde | 307 ± 70 | 1.87 ± 0.19 | 6.1 × 10$^{-3}$ |
| V251G   | D-Glucoheptose | 53.5 ± 16.3 | 0.94 ± 0.05 | 1.8 × 10$^{-2}$ |
|         | D-ManNAc       | 335 ± 82 | 12.5 ± 1.4 | 3.7 × 10$^{-2}$ |
|         | L-Arabinose    | 203 ± 91 | 1.25 ± 0.20 | 6.2 × 10$^{-3}$ |

* All reactions were carried out with 10 mM pyruvate and various sugar substrates at 37 °C.

$^b$ Enzyme activity was not detectable when the concentration of D-glucoheptose was $\leq$500 mM.


**Substrate Specificities of d-Sialic Acid Aldolase**

D-sialic acid produced by the wild-type D-sialic acid aldolase can serve as a perspective lead in the future design of useful drugs against the pathogen.

**Concluding Remarks and Future Perspectives**—For the efficient production of the enantiomeric l-sugars of the natural D-sugars (2, 7), *E. coli* D-sialic acid aldolase was converted into RS-aldolase for synthesizing l-KDO (10). In the current study, structural and biochemical analyses have elucidated that among the eight mutations, the most important one is V251L. Based on the results presented here, just a single mutation is sufficient to improve the catalytic activity toward l-KDO. Mutation of V251L endows the engineered enzyme with most of the RS-aldolase activity, attributed to the resulting narrow sugar-binding pocket. The relatively long Ile-251 in RS-aldolase may hinder the entry of larger substrates and thus l-KDO can be better accommodated in the narrow binding pocket.

The previously observed difference in kinetic parameters of D-sialic acid aldolase and RS-aldolase for D/L-sialic acid and D/L-KDO (10) can also be explained in terms of the active site structures (supplemental Table S5). It is expected that l-KDO can thus be efficiently synthesized by the mutant V251L (30 mg/liter culture) and efficient production of l-KDO, the mutant V251L can be used as an ideal candidate for further engineering to generate efficient biocatalysts for the synthesis of biologically important sugars.

**Acknowledgments**—We thank the National Synchrotron Radiation Research Center of Taiwan, Photon Factory and SPring-8 of Japan for beam time allocation.

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