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Directed Evolution of *N*-Acetylneuraminic Acid Aldolase to Catalyze Enantiomeric Aldol Reactions

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Abstract—Expanding the scope of substrate specificity and stereoselectivity is of current interest in enzyme catalysis. Using error-prone PCR for in vitro directed evolution, the Neu5Ac aldolase from *Escherichia coli* has been altered to improve its catalytic activity toward enantiomeric substrates including *N*-acetyl-L-mannosamine and L-arabinose to produce L-sialic acid and L-KDO, the mirror-image sugars of the corresponding naturally occurring D-sugars. The first generation variant containing two mutations (Tyr98His and Phe115Leu) outside the $(\alpha,\beta)_8$-barrel active site exhibits an inversion of enantioselectivity toward KDO and the second generation variant contains an additional amino acid change Val251Ile outside the α,β-barrel active site that improves the enantiomeric formation of L-sialic acid and L-KDO. The X-ray structure of the triple mutant epNanA.2.5 at 2.3 Å resolution showed no significant difference between the wild-type and the mutant enzymes. We probed the potential structural 'hot spot' of enantioselectivity with saturation mutagenesis at Val251, the mutated residue most proximal to the Schiff base forming Lys165. The selected variant had an increase in k_{cat} via replacement with another hydrophobic residue, leucine. Further sampling of a larger sequence space with error-prone PCR selected a third generation variant with significant improvement in L-KDO catalysis and a complete reversal of enantioselectivity.

Introduction

N-Acetylneuraminic acid aldolase (Neu5Ac aldolase, EC 4.1.3.3, also called *N*-acetylneuraminate lyase), which catalyses the reversible aldol reaction of *N*-acetyl-D-mannosamine and pyruvate to give *N*-acetyl-D-neuraminic acid (D-sialic acid), has been extensively used in the synthesis of sialic acids and its analogues.[1–4] This enzyme has been shown to be quite specific for pyruvate as the donor, but flexible to a variety of D- and, to some extent, L-hexoses and pentoses as acceptor substrates.[5,6]

N-Acetyl-D-neuraminic acid (D-sialic acid) is an interesting high-carbon sugar with important pharmaceutical implications. A potent neuraminidase inhibitor for the treatment of flu is prepared from D-sialic acid.[7] In addition, D-sialic acid is found as an essential sugar residue in many complex carbohydrates associated with inflammatory diseases and cancer metastasis.[8] The unnatural L-sialic acid, which is stable against enzymatic degradation in biological systems, has been used in the mirror-image phage display for identification of D-peptides to target cell-surface D-sialic acid.[9] However, the aldol condensation reaction of *N*-acetyl-L-mannosamine and pyruvate with wild-type Neu5Ac aldolase is too weak (<1% of the D-substrate) to be suitable for preparative synthesis of L-sialic acid. Thus, alteration of the Neu5Ac aldolase with increased activity toward the L-enantiomeric substrate is needed. Since the active site of Neu5Ac aldolase is composed of the $(\alpha/\beta)_8$-barrel[10] (a fold found in most structurally known aldolases[11]), it represents an ideal structural motif for directed evolution.[12–14] Our interest is to engineer the enantioselectivity of this enzyme to develop an effective catalyst for the synthesis of both D- and L-sugars. Our ultimate goal is to evolve an aldolase with a complete inversion of enantioselectivity (Figs 1 and 2).

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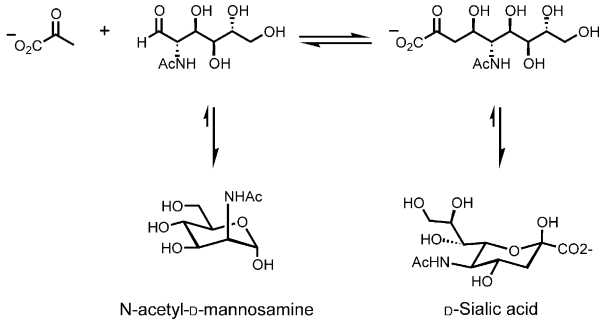
In general, it is very difficult to alter the stereospecificity of an enzyme, especially to invert the enantioselectivity. For example, there is no significant sequence homology between D-lactate dehydrogenase and L-lactate dehydrogenase. However, the wild-type Neu5Ac aldolase exhibits a very low level of activity toward mirror image substrates, such as 3-deoxy-L-manno-oct-2-ulosonic acid (KDO).[6] As demonstrated in our previous work on the evolution of KDPG aldolase[15] to new variants capable of accepting L-glyceraldehyde as an acceptor substrate, we intend to use the same strategy to alter the enantioselectivity of Neu5Ac aldolase. However, the activity of wild-type Neu5Ac aldolase toward L-sialic acid is too low to be useful as a good starting point for directed evolution. On the other hand, the wild-type Neu5Ac aldolase (Fig. 2) accepts both D- and L-KDO with 2.3 and 3.7% relative activity toward D- and L-KDO, respectively, compared to D-sialic acid. Thus, D- and L-KDO were chosen as substrates for screening, with hopes that the new enzymes evolved to accept L-KDO will also accept L-sialic acid as a substrate.

Results and Discussion

Directed evolution of Neu5Ac aldolase to cleave D- and L-KDO

A screening strategy similar to our previous approach to KDPG aldolase was employed for this study (Fig. 3). Coupling of the aldol cleavage with the reduction of pyruvate to lactate with a concurrent oxidation of NADH to NAD^+ in the presence of lactic dehydrogenase formed the basis of our screening methodology. Assay samples containing D- or L-KDO, the evolved crude enzyme, and the coupled system were prepared on 96-well plates. The fluorescence of NADH in each well was monitored (excitation at 340 nm and emission at 450 nm).

Screening of enzymes with low initial activities could be facilitated by the presence of a sufficient amount of enzyme in each sample, such that a good signal to noise ratio could be obtained over a reasonable period of time. The N-terminal 6x histidine fusion expression vector, pTrcHisB (Invitrogen) was used for our studies of Neu5Ac aldolase as it provides a high expression system and the availability of a rapid single-step affinity purification of the expressed protein for further analysis (Fig. 4). Subsequent libraries of mutant Neu5Ac aldolase genes were cloned into this vector.



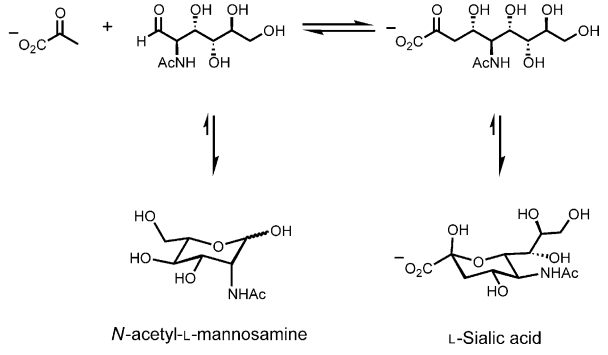


Figure 1. Neu5Ac aldolase catalyzed synthesis of enantiomeric sialic acid.

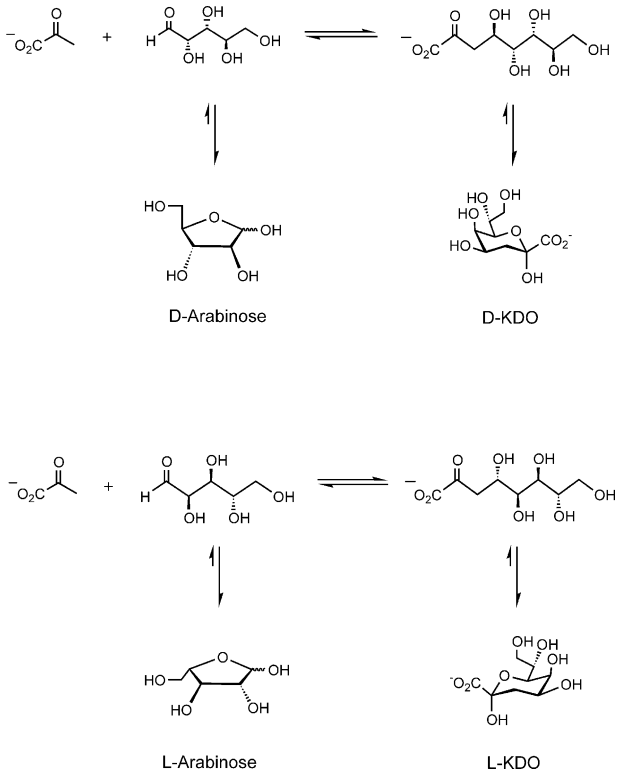


Figure 2. Neu5Ac aldolase catalyzed synthesis of D- and L-KDO.

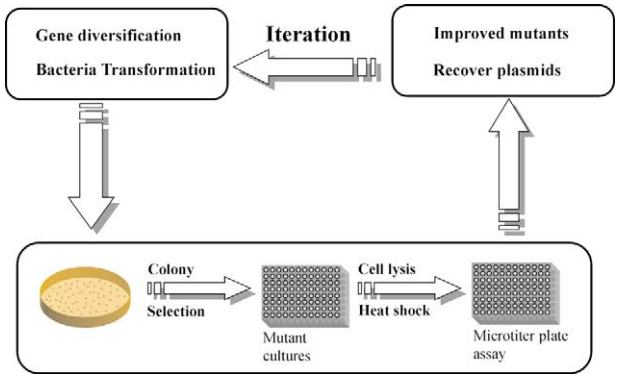


Figure 3. Schematic diagram showing the approach adopted for the directed evolution of Neu5Ac aldolase.

The wild-type aldolase gene was amplified under standard mutagenic PCR condition with a controlled mutation rate of 1–5 bases per gene to generate the first generation plasmid library. The protein library was prepared by lysis of re-suspended cell pellets harvested from 2-mL cultures of individually picked colonies obtained by transformation with the plasmid library. The lysed suspensions were then heat treated to reduce background interference due to other contaminants present in the cell lysate, and the supernatants were transferred to 96-well plates for assaying at 25 °C. About 25% of the first generation mutants still retained certain activity after the heat treatment. Hence, we adopted this strategy to reduce the background signal and for a more stringent evolution condition that restrains the thermal stability of selected mutants. One per cent of the 1600 members in the first generation were found more active toward D-KDO than the wild-type enzyme. Four mutants (0.25% screened population) amongst the first generation showed a significant enhancement in the rate of D-KDO cleavage. Unfortunately, no mutant with higher activity toward L-KDO was found. Compared with other studies, it is interesting that a relatively high fraction of population with beneficial mutations was observed in the first generation library, indicating that the enzyme may be poorly optimized toward the KDO cleavage reaction but is a good starting template for the evolution of this property. The genes from four of the selected first generation mutants (epNanA.1.1, epNanA.1.2, epNanA.1.3 and epNanA.1.4) that showed improvement in the cleavage of D-KDO were sequenced. It was found that epNanA.1.1, epNanA.1.2 and epNanA.1.3 had the same mutations. DNA shuffling was performed to produce the second generation library using epNanA.1.1 and epNanA.1.4. Eight hundred variants of this library were screened for both D- and L-KDO cleavage; however, no mutant with improved activity toward L-KDO was identified. Thus, the best first generation mutant (epNanA.1.1) was selected as a template for another round of mutagenic PCR and screening. 1600 variants of the error-prone PCR products were screened and one mutant (designated as epNanA.2.5) with cleavage activity toward L-KDO (0.06% screened population) was identified for further characterization. The number of colonies screened to fully explore the possible mutant combinations of these three spots is over 30,000 (32^3), after failing to select a more active mutant toward L-KDO cleavage with screening of 3000 clones, we decided to concentrate on Val251Ile, which is the closest mutation to the Schiff base forming lysine-165, located at strand β6 of the $(\alpha/\beta)_8$ TIM. Being the closest mutated residue at a distance of 13.9 Å to the active Lys165 residue, Val251Ile may play a more active role in the enantioselectivity of the aldolase. Thus, a small number of clones were screened with saturation mutagenesis at Val251. Without significant improvements with saturation mutagenesis, we proceeded to explore more sequence space by screening with another round of error-prone PCR using epNanA.2.5 as a template. After screening 1600 mutants, we selected a variant epNanA.3.B7 with a complete reversal of enantioselectivity.

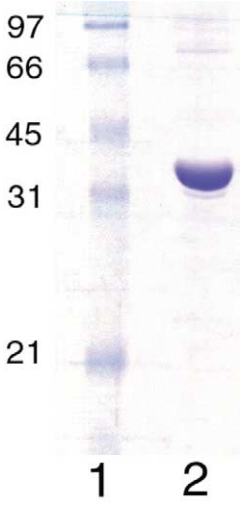


Figure 4. SDS-PAGE analysis of the expression and purification of 6xHis-tagged *N*-acetylneuraminate lyase. Lane 1, molecular weight standard. Lane 2, Ni^{2+} column purified *N*-acetylneuraminate lyase.

Characterization of the catalytic properties of the evolved Neu5Ac aldolases

The selected mutants were purified and analyzed for their ability to cleave D- and L-KDO (Table 1). While the first generation mutant epNanA1.1 and the second generation mutant epNanA.2.5 have higher k_{cat} and lower K_m toward D-KDO than the wild-type, the third generation mutant had a higher k_{cat} and K_m. Their specificity constant (k_{cat}/K_m) toward D-KDO showed a 3.4- and 4.0-fold increase from the wild-type for epNanA.1.1 and epNanA.2.5 but a 0.5-fold decrease for epNanA.3.B7. Progressive improvement of both k_{cat} and K_m was observed in the evolution of the aldolase toward L-KDO cleavage. Compared to the wild-type, the second generation mutant epNanA.2.5 has a 1.7-fold increase in k_{cat} and a 1.4-fold reduction in K_m for L-KDO and hence a 2.3-fold improvement in k_{cat}/K_m.

Table 1. Kinetics parameters of wild-type and evolved sialic acid aldolase

Enzyme	Substrate	K_m (mM)	k_{cat} (min^{-1})	k_{cat}/K_m (mM^{-1} min^{-1})
Wild-type	D-Sialic acid	2.60	0.81	0.311 (100%)
	L-Sialic acid	4.43	0.01	0.002 (1.0%)
	D-KDO	24.5	0.18	0.007 (2.3%)
	L-KDO	13.3	0.15	0.012 (3.7%)
epNanA.1.1	D-Sialic acid	2.07	0.81	0.389 (125%)
	L-Sialic acid	N.D.[a]	N.D.[a]	N.D.[a]
	D-KDO	12.3	0.29	0.024 (7.6%)
	L-KDO	14.1	0.16	0.011 (3.7%)
epNanA.2.5	D-Sialic acid	3.90	0.75	0.195 (61.6%)
	L-Sialic acid	369.4	0.27	0.001 (0.2%)
	D-KDO	14.9	0.39	0.026 (8.4%)
	L-KDO	9.5	0.26	0.028 (8.9%)
epNanA.3.B7	D-Sialic acid	2.6	0.28	0.109 (35.1%)
	L-Sialic acid	N.D.[a]	N.D.[a]	N.D.[a]
	D-KDO	116.1	0.38	0.003 (1.1%)
	L-KDO	5.0	0.45	0.089 (28.5%)

[a]No detectable activity was observed.

Interestingly, epNanA.2.5 has a 27-fold increase in k_{cat} toward L-sialic acid compared to the wild-type, while the k_{cat} toward D-sialic acid remains basically the same. The K_m value is much higher for L-sialic acid, resulting in a relatively low k_{cat}/K_m value. For synthetic applications, however, the substrate concentration is often relatively high so that the enzyme operates at the maximum velocity. Similarly, epNanA.1.1 and epNanA.2.5 exhibit higher k_{cat} toward D- and L-KDO compared to the wild-type. With regard to the specificity constant measured by k_{cat}/K_m, epNanA.2.5 showed slightly better L-enantioselectivity toward L-KDO (0.028 for L vs 0.026 for D), while no inversion of enantioselectivity was observed for KDO or sialic acid (Table 1). It is interesting to note that residue 251 seems to play a hydrophobic role in L-KDO cleavage, since saturation mutagenesis at that residue resulted in the change of Val (wild-type) to Ile (epNanA.2.5) and to Leu (sm251.2.4). Though the most active mutant (sm251.4), showed a 1-fold improvement in k_{cat} by replacement of Ile with Leu, a concomitant 6-fold increase in K_m was also observed. Unlike the previous generations, a much improved L-enantioselectivity toward L-KDO and a complete reversal of enantioselectivity was observed with epNanA.3.B7 as it favors L-KDO in k_{cat} (0.45 for L vs 0.38 for D) and the specificity constant, k_{cat}/K_m (0.089 for L vs 0.003 for D). The specificity constant of epNanA.3.B7 for L-KDO was 7.7-fold that of the wild-type enzyme. Thus, while the wild-type enzyme has remarkable high substrate specificity for D-sialic acid over L-KDO, the evolved enzymes are significantly less specific for these substrates and an improved L-enantioselectivity was observed with KDO.

Condensation reaction of evolved aldolases

The wild-type Neu5Ac aldolase catalyzes reversible aldol reactions between pyruvate and sugar substrates. The enzyme is selective for sugar substrates with D-configuration; however it accepts several L-sugars at low reaction rate. We examined the effect of mutations on the addition reactions between pyruvate and D-, L-sugars respectively. Compared with wild-type, epNanA.2.5 has a 3.3-fold improvement in the rate for addition of *N*-acetyl-L-mannosamine to pyruvate. *N*-Acetyl-L-mannosamine has been reported as a very weak substrate for the wild-type *E. coli* Neu5Ac aldolase,[1] but no detectable activity under the current assay condition. Though epNanA.2.5 exhibits a relatively high k_{cat} for L-sialic acid, a relatively high concentration of substrate is required; however, as the affinity for the sugar substrate is relatively high. Further improvement in this regard is to lower the K_m of epNanA.2.5 for D-sialic acid and for *N*-acetyl-L-mannosamine. The activities of epNanA.2.5 toward L- and D-arabinose are relatively high compared with the wild-type and have been used in the preparative synthesis of D- and L-KDO using the condition described previously.[6]

Structural study and remodeling of mutations observed in the evolved aldolase

The first generation mutants possess two common mutations, Tyr98His and Phe115Leu. A favorable cumulative mutation Val251Ile for L-KDO was introduced in the second round of mutagenesis and screening. According to the three-dimensional structure (Fig. 5) of this Schiff base forming class I aldolase,[10] Phe115 is at the end of the loop between strand d and helix D. Tyr98 is located towards the C-terminal end of helix C. Val 251 is located between helix I and helix J. None of these residues is in the active site. How do the changes of these amino acids affect the enzyme structure and enantioselectivity remains an interesting subject for investigation. To address this question, we have determined the X-ray crystal structure of epNanA.2.5 at 2.3 Å and found that there is no significant difference between the wild-type and the mutant enzymes (Fig. 6). The largest deviation is observed for residue LysA71 with an rmsd of 1.4 Å. In our structure the Nζ of LysA71 forms hydrogen bonds to the side chain of GlnC19 of a symmetry related molecule, whereas in 1NAL the side chain of LysA71 adopts a different conformation. The overall quaternary structure of the tetramer is also very similar for both enzymes. Based on the previous alignment the rms deviation for the second molecule of the tetramer is 1.13 Å, and 1.23 Å and 1.43 Å for the third and fourth molecule, respectively. Work is in progress to obtain a high-resolution structure and to elucidate the molecular mechanism of the enantiomeric aldol reactions, especially the origin of enantioselectivity in the enzymatic catalysis.

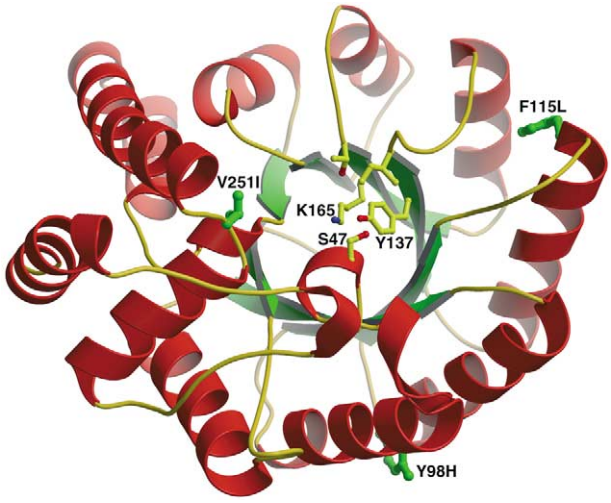


Figure 5. X-ray structure of *E. coli N*-acetylneuraminate lyase (PDB code 1NAL).[10] The enzyme forms an $(\alpha/\beta)_8$ TIM barrel structure with the reactive lysine, Lys165 located at strand β6 of the barrel. The evolved aldolase contains three mutated residues Tyr98His, Phe115-Leu and Val251Ile, which are displayed in green. None of these residues are located in close proximity to the Schiff base forming lysine. The Tyr98His mutation is located towards the C-terminal end of helix C. The second mutation, Phe115Leu, is at the end of the loop between strand d and helix D. The final mutation of Val251Ile is located between helix I and helix J. This is the only residue pointing toward the center of the molecule and may interact with the substrate upon binding. However, the distance between Lys165-Nζ and Val251-Cα is 13.9 Å. Structural alignment and sequence comparison of different NAL members is adapted from Barbosa et al.[27] The structural figures were made with Bobscript[29] and Raster3-D.[30]

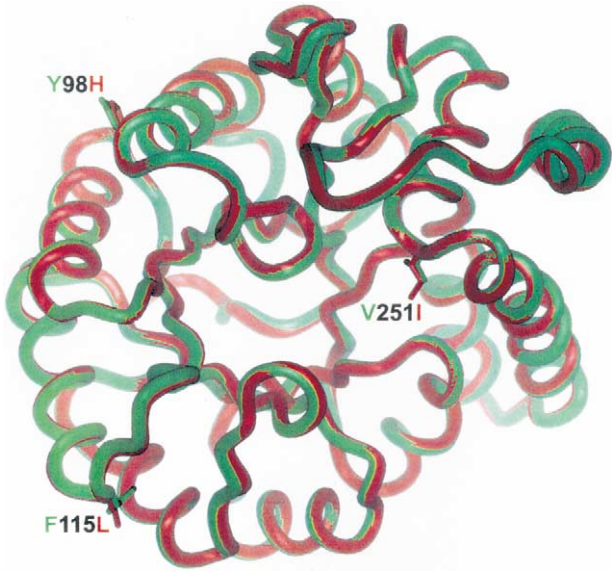


Figure 6. Superposition of *E. coli* *N*-acetylneuraminate lyase (PDB code 1NAL)[10] colored in green and the mutant structure colored in red. Mutated residues are displayed and labeled for both enzymes. There are no large structural changes observed between both structures. The rms deviation is 0.33 Å between molecule A of 1NAL and the mutant structure. This figure was made using Molecular Simulations Insight2000.

Conclusion

This research has demonstrated the effectiveness of using in vitro evolution to alter the catalytic properties of Neu5Ac aldolase for use in synthesis of both D- and L-sugars. An evolved aldolase exhibits a significant activity for condensation of pyruvate and *N*-acetyl-L-mannoamine to form L-sialic acid, while retaining its natural activity for the synthesis of D-sialic acid. Interestingly, this evolved enzyme is more effective toward both enantiomeric D- and L-KDO and is thus useful for the synthesis of D- and L-KDO. The aldolase evolved contains mutation outside the active site according to the X-ray structure of the mutant, and the work demonstrates that a small number of mutations outside the active site of an enzyme could significantly affect the specificity and enantioselectivity of the enzyme catalysis. Further work on the evolution of Neu5Ac aldolase and structural investigation is in progress to understand the origin of enantioselectivity.

Experimental

General procedures

Nucleic acid manipulations were done according to standard procedure.[16] Restriction endonucleases and T4 DNA ligase were purchased from New England Biolabs. Taq polymerases were purchased from Stratagene. UV kinetic assays were performed on a Cary 3 Bio UV–vis spectrophotometer. DNA sequencing was performed on an ABI 377A automated sequencer. The reactions were performed using thermal cycle sequencing conditions with fluorescent labeled terminators. Curve fitting were done by non-linear least squares method using KaleidaGraph (Abelbeck Software). 96-well plate samples were analyzed with a Packard-fluorescence spectrophotometer (Fusion-Universal Microplate Analyzer) for microtiter plate assays.

Chemical synthesis of *N*-acetyl-L-mannosamine. *N*-Acetyl-L-mannosamine was prepared as reported previously.[9] Starting from 5 g of L-glucose, 800 mg of *N*-acetyl-L-mannosamine was obtained (overall-yield 13%).

Synthesis of 3-deoxy-D-manno-2-octulosonic acid (D-KDO) and 3-deoxy-L-manno-2-octulosonic acid (L-KDO). D-KDO was synthesized by chemical condensation of D-arabinose and oxalacetate as described previously.[1,9] L-KDO was made by enzymatic synthesis[4,9] using the wild-type Neu5Ac aldolase expressed in *E. coli*. Both enantiomers of KDO were purified by AG-1 X-8 (HCO_3^-) anion-exchange chromotography resin (Bio-rad) and re-crystallization from ethanol–water to remove pyruvate contamination.

Plasmid construction

The 0.9-kb *E. coli* Neu5Ac aldolase gene was amplified by standard PCR from a genomic preparation of *E. coli* JM109, with primers N-NanA (5′-ATC GCG GAT CCG ATG GCA ACG AAT TTA CGT G) and C-NanA (5′-ATC CGG AAT TCT CAC CCG CGC TCT TGC ATC) flanking the gene with restriction sites BamH I and EcoR I. The resulting PCR product was subcloned into vector pTrcHisB (Invitrogen). The ligation product was transformed into *E. coli* XL1Blue-MRF' by electroporation.[17] Plasmids recovered from transformants were screened by PCR for the presence of the aldolase insert. A positive plasmid clone NanA-pTrcHisB was sequenced, used for protein expression and as mutation template for the construction of the first generation library.

Library construction

Mutagenic PCR was carried out under standard error-prone conditions.[18] Primers N-NanA and C-NanA were used to amplify and mutate the template gene. 40 pM each of the primers were used and the reaction conditions were: 10 ng template/10 mM Tris–Cl, pH8.3/50 mM KCl/1.5 mM MgCl2/0.5 μL DMSO/0.2 mM MnCl2/0.2 mM each of dATP, dTTP, dCTP, dGTP/2.5 units Taq polymerase (Stratagene), in a total volume of 50 μL. The mixture was thermocycled for 30 rounds of 94 °C, 1 min; 55 °C, 1 min; 72 °C 1 min, and then 1 round of 72 °C for 2 min. The library fragment was purified on agarose gel and cloned into pTrcHisB as described above and the resulting library construct was transformed into XL1Blue-MRF′, amplified and purified as plasmid miniprep (Qiagen). The presence of gene insert in the library constructs was confirmed by gel-electrophoresis with the parent vector pTrcHisB as reference. Heterogeneity of the first generation library construct was examined by transforming the plasmid into JM109 following by random picking of three

colonies, plasmid extraction, and DNA sequencing. Each of the selected mutants contained one to five mutations.

DNA shuffling was done according to the method of Stemmer.[19] Fragments of 50–100 bp were isolated and used for the reassembly. The substrates for shuffling were prepared by standard PCR of the two selected plasmids, epNanA.1.1 and epNanA.1.4, from the first generation library. A thermal cycling program of 95 °C for 120 s; 94 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s (25 cycles) was used for reassemble PCR and the amplification of the reassembled product.

Saturation mutagenesis at three sites, Tyr98, Phe115 and Val251 were carried out with overlap extension PCR.[20] The following oligonucleotide primers were used in constructing the libraries: (randomized 251) for 251, 5′-TTCCGCGGCCTGAAAACTGTCC-3′ and rev251ran, 5′-GGCCGCGGAANN(G/C)GCCCGTTTTG-3′, (randomized 115) for 115, 5′-GAAGAACACTGCGATCACTATCG-3′ and rev115ran, 5′-CGCAGTGTTCTTCNN(G/C)GCTGAAA-3′, (randomized 98) for 98, 5′-GGCTTCGATGCCGTCTCCGCCGTC-3′ and rev98ran, 5′-CGAAGCCNN(G/C)ACGTTTAGCCGAT-3′. Plasmid epNanA.2.5 was used as a template. Primers N-NanA and rev98ran were used to generate fragment 1ran98. Primers for98 and rev115ran were used to generate fragment 2ran115. Primers for115 and rev251ran were used to generate fragment 3ran251. Primers for251 and C-NanA were used to generate fragment 4rev251. The fragments were then purified by agarose gel and a QIAEX II agarose gel extraction kit (Qiagen). Primerless PCR amplification was carried out using fragments 1ran98, 2ran115, 3ran251 and 4rev251 under the following conditions: 94 °C, 45 s; 58 °C, 45 s; and 72 °C, 80 s (10 cycles). PCR amplification was carried out for the primerless PCR products using primers N-NanA and C-NanA under the following conditions: 94 °C for 4 min, followed by 30 cycles of 94 °C, 30 s; 60 °C, 1 min 30 s; and 74 °C, 1 min 30 s and then 74 °C for 7 min. Saturation mutagenesis at Val251were carried out with the same method as described above.

Library screening for KDO cleavage

Constructs harboring the mutant library were transformed into JM109 by electroporation. The transformed culture was spread on Luria-Bertani (LB) agar plates containing 100 μg/mL of ampicillin and incubated at 37 °C for 16 h. Individual colonies were picked, replicated on a LB agar-ampicillin plate, and dispensed into 96-well plates that contained 1 mL of 2X YT/50 μg/mL ampicillin/0.2 mM IPTG (the broth was shaken vigorously before dispensing into the plates). The plates were sealed with gas permeable membranes (Breathe Easy, Diversified Biotech) and shaken at 37 °C/200 rpm in an incubator for 18 h, centrifuged at 10,000 *g*/4 °C for 1 h, and the supernatant was carefully decanted. Each cell pellet was re-suspended in 0.5 mL of 50 mM potassium phosphate buffer, pH 7.5 containing 0.5 mg/mL of lysozyme. The plates were rapidly frozen in liquid nitrogen followed by thawing at room temperature, and then incubated at 65 °C for 20 min. Cell debris was collected by centrifugation at 10,000 *g*, 4 °C for 1 h. Supernatant (100 μL) from each well was transferred to a 96-well plate. Upon incubation at 25 °C for 10 min, 40 μL of an assay solution containing 50 mM potassium phosphate, NADH and L-lactic dehydrogenase at pH 7.5 was dispensed into each well with an 8-channel-repeating pipette. In each well, the starting concentration of NADH was 360 μM, and 0.1 U of lactic dehydrogenase was present. Baseline drift was monitored for 2 min. All samples had leveled or insignificant baseline-drift. 10 μL of D- or L-KDO (13 mM for the first two generation of mutants, 8 mM for the subsequent generations) in 50 mM potassium phosphate, pH 7.5 was added to each well and the fluorescence at exitation of 340 nm and absorbance of 450 nm was monitored at 1-min intervals continuously for 25 min with mild shaking. The activity of each mutant was reflected by the rate of decrease in fluorescence. Mutants that had the highest activity were selected. The selected mutant colonies were picked from the replicated LB agar-ampicillin plate, grown overnight in LB-ampicillin medium, and their plasmids extracted with the DNA minipreps (Qiagen) and sequenced.

Enzyme expression and purification

Selected plasmids were transformed into JM109. To express the protein, culture was prepared by picking individual colonies and inoculated into 5 mL of LB-ampicillin medium, at 37 °C, 220 rpm overnight. Culture was added to 500 mL of LB-ampicillin medium, and was incubated at 37 °C, 220 rpm. Protein expression was induced with 0.2 mM IPTG when OD600 is at 0.4. Cells were harvested 6 h after the induction, by centrifugation at 4 °C, 1200 *g* for 10 min and were stored at −78 °C. Cell pellet from 500 mL culture was re-suspended in 20 mL of 50 mM potassium phosphate pH 7.5/5 mM β-mercaptoethanol/300 mM NaCl, chilled on ice, and was lysed by passing through a French Press (SLM instruments, Urbana, IL) compressed to 1500 psi and then released to ambient pressure. The process was repeated three times. Cell debris was pelleted by centrifugation at 12,000 *g*, 4 °C for 1 h. The supernatant was filtered through a 0.2 μm cellular acetate membrane filter (Corning), and loaded onto a Ni^{2+}-NTA-agarose column with a bed volume of 2.5 mL pre-equilibrated with the cell re-suspension buffer. The column was washed with 20 mL of buffer containing 50 mM potassium phosphate pH 7.5/5 mM β-mercaptoethanol/300 mM NaCl/5% glycerol/10 mM imidazole, and then 20 mL of buffer containing 50 mM potassium phosphate pH 7.5/5 mM β-mercaptoethanol/10 mM imidazole. Bound enzyme was eluted with 50 mM potassium phosphate pH 7.5/5 mM β-mercaptoethanol/250 mM imidazole, and was dialyzed extensively against 50 mM potassium phosphate pH 7.5/5 mM β-mercaptoethanol at 4 °C. Eluted enzymes were analyzed with SDS–PAGE and were found to be >90% pure in all cases (Fig. 4). Enzyme solution was frozen in liquid nitrogen and was stored at −78 °C prior to use. No activity lost was observed upon freezing and thawing of the enzymes. Enzyme concentrations were determined by the Bradford procedure (Bio-Rad) using BSA as the calibration standard.

KDO cleavage assay

The activity was determined by the standard coupled assay with L-lactic dehydrogenase (EC 1.1.1.27, Type II from rabbit muscle) and NADH. A typical assay was initiated by addition of an appropriate amount of D-KDO or L-KDO in 50 mM potassium phosphate, pH 7.5/5 mM β-mercaptoethanol, to a mixture of L-lactic dehydrogenase (0.8 U)/NADH (0.43 mM)/ aldolase (10–100 μg) in 50 mM potassium phosphate, pH 7.5/5 mM β-mercaptoethanol. The total reaction volume was 600 μL. Prior to the addition of the substrate, the mixture was pre-incubated at 25 °C for 5 min. UV absorbance at 340 nm was recorded continuously for 2 min and the slope of the absorbance curve during the first 30 s was used for rate estimation.

Assay for addition reaction

The aldol condensation activity was determined by the rate of depletion of pyruvate. Pyruvate concentration was determined by a method similar to that previously reported.[6] Reactions were initiated by the addition of the aldolase to a mixture of pyruvate and sugars pre-incubated at 37 °C. Reactions were performed in 1 mL of 50 mM phosphate buffer, pH 7.5. Starting concentrations of pyruvate, sugars and enzyme were 10 mM, 250 mM and 30–300 μg/mL, respectively. Aliquots (100 μL) were withdrawn from the reaction mixture at different time points and quenched with 30 μL of 7% perchloric acid. Samples were neutralized with 20 μL of 1 M NaOH. 150 μL of each neutralized sample was diluted to 1150 μL and assayed for pyruvate.

Preliminary structural determination of mutant *N*-acetylneuraminate lyase

The mutant enzyme (22.5 mg/mL) crystallizes at 22.5 °C from 15% PEG 600, 0.2 M imidazole malate pH 5.5 in space group $P2_1$ and four molecules in the asymmetric unit. Data were collected on a Siemens generator equipped with a MAR345 detector and an Oxford cryosystem low-temperature device at −150 °C using 20% glycerol as a cryoprotectant. Data were processed and scaled with HKL2000.[21] The unit cell dimensions are $a = 84.3$ Å, $b = 95.3$ Å, $c = 93.7$ Å, $\beta = 116.3°$. The crystal lattice exhibits pseudo-symmetry in space group $C222_1$, but this is not reflected by the symmetry equivalent reflections as shown by XPREP.[22] The data set is 97.6% complete with $R_{sym} = 6.9\%$ for a resolution range 25–2.3 Å. The structure was determined by molecular replacement with Amore[23] using one molecule of *N*-acetylneuraminate lyase (PDB code 1NAL)[10] as a search model. Four molecules were found and after a fitting step the correlation coefficient was 79.2 with $R = 29.8\%$. After rigid-body refinement in CNS,[24] the structure is currently being refined in CNS using positional and slow-cooling refinement protocols. CNS restraints relating the four molecules in the asymmetric unit are applied during refinement. Intermittent cycles of model building are done with the program O.[25] Current refinement statistics are $R_{cryst} = 23.7\%$ and $R_{free} = 26.4\%$ for all data to 2.3 Å resolution. Comparison of the mutant sequence with the published structure sequence[10] revealed in addition to the Tyr98His, Phe115Leu and Val251Ile mutations three additional mutations: Gly70Ala, Thr84Ser and Gln282Leu. The *E. coli* sequence of NAL used in the sequence alignment and comparison of related *N*-acetylneuraminate lyase enzymes[26] contains Gly70, Thr84 and Gln282 in agreement with the 1NAL structure. However, in a similar sequence alignment,[27] the sequence is in agreement with our mutant sequence and contains Ala70, Ser84 and Gln282. Structure superposition of 1NAL with our structure using ProFit[28] resulted in an rms difference of 0.33 Å based on Cα atoms for residues A6 to A292 used in the alignment.

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References and Notes

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