Structure-guided engineering of N-acetylneuraminic acid lyase

Gavin Williams, Thomas Woodall, Adam Nelson and Alan Berry

Introduction
Analogues of N-acetylneuraminic acid (1, sialic acid, NANA), particularly 6-dipropylcarboxamides (such as (2)), have been found to be selective and potent inhibitors of influenza sialidase. Sialic acid analogues are, however, difficult to synthesise by traditional chemical methods and the enzyme N-acetylneuraminic acid lyase (NAL) has been used for the synthesis of a number of analogues. Although a number of hexoses and pentoses and their analogues are substrates for NAL, condensations involving shorter aldehydes are less promising: L- and D-erythrose and threose react at between 0.3% and 5% of the rate of N-acetyl mannosamine, and two- and three-carbon aldehydes are not substrates. The activity of this enzyme towards 6-dipropylcarboxamides is also low. To overcome this problem, we have used structure-guided saturation mutagenesis to produce variants of NAL with improved activity and specificity towards 6-dipropylcarboxamides.

Results
Using the known crystal structure of the E.coli and H. influenzae NAL (Fig.1), we identified three residues which contact the 6-glycerol moiety of sialic acid and reasoned that mutagenesis of these residues would produce variants with increased specificity towards analogues of sialic acid which had hydrophobic groups in place of the polar glycerol component. Residues Asp-191, Glu-192 and Ser-208 were mutated to all the other 19 amino acids by saturation mutagenesis to create three libraries, D191X, E192X and S208X.

Members of each library were tested for their ability to cleave the 6-dipropylamide (2). Only substitution at position 192 produced significant improvements in activity towards the dipropylamide, and a number of substitutions at this position resulted in significant switches in substrate specificity towards compound 2 (Fig 2A). One variant, E192N, was purified and characterized and showed a 49-fold improvement in catalytic efficiency towards the target analogue (2) and a 690-fold shift in specificity from sialic acid towards the analogue. The breadth of substrate specificity of the E192N variant was assessed by incubating the enzyme with pyruvate and various aldehydes and the reactions were followed synthetically. The results in Table 1 show that the E192N variant is a general purpose aldol catalyst for the production of a wide range of tertiary amide sialic acid analogues.

Another important property of any useful biocatalyst is the stereochemical purity with which the reaction is carried out. Naturally occurring NAL exhibits only poor facial selectivity during carbon-carbon formation, and as such, its scope as a general biocatalyst is limited, since products which are neither kinetic nor thermodynamically preferred are difficult, if not impossible to isolate. Engineering the stereochemical course of the NAL catalysed would
remove this limitation. We have used directed evolution to create a pair of stereochemically complementary variant lyases for the synthesis of sialic acid mimetics.

<table>
<thead>
<tr>
<th>R-</th>
<th>Yield</th>
<th>R-</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>37%</td>
<td>[ ]</td>
<td>48%</td>
</tr>
<tr>
<td></td>
<td>42%</td>
<td>[ ]</td>
<td>55%</td>
</tr>
<tr>
<td></td>
<td>42%</td>
<td>[ ]</td>
<td>47%</td>
</tr>
<tr>
<td></td>
<td>66%</td>
<td>[ ]</td>
<td>35%</td>
</tr>
</tbody>
</table>

Initially, error-prone PCR identified functional residues that were located in the active site of NAL, and subsequently this inspired an intense program of structure-guided saturation mutagenesis (Fig. 2). Finally, two variants were obtained which were 48-fold and 52-fold stereoselective towards products with R- and S-configuration at C4 of the product, respectively. Wild-type NAL cannot be used for the synthesis of a 6-dipropylamide mimetic of sialic acid with R configuration at C4 because the product is not kinetically favoured nor thermodynamically more stable than the S product. However, the evolved R selective variant was used to synthesise this R configuration product allowing isolation of the rare diastereoisomer. The conversion of an essentially non-stereoselective aldolase into a pair of complementary biocatalysts will be of enormous interest to synthetic chemists, and these novel biocatalysts will be used for the synthesis of a range of clinically relevant sialic acid analogues.

Publications

Funding
This work was funded by the BBSRC and The Wellcome Trust.