Novel enzymes based on the *N*-acetyl neuraminic acid scaffold

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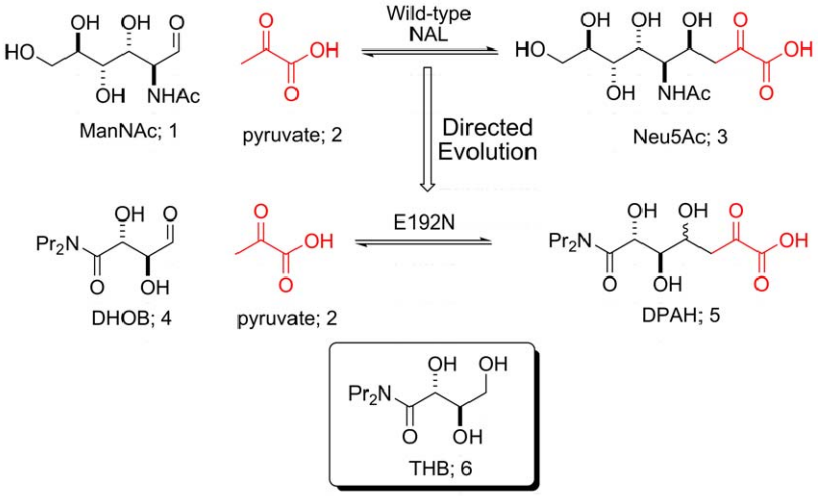
Introduction

N-acetylneuraminic acid lyase (NAL) catalyses the reversible aldol condensation of *N*-acetyl mannosamine (ManNAc; **1**) with pyruvate (**2**) to yield the sialic acid, *N*-acetyl neuraminic acid (Neu5Ac; **3**). As such this enzyme plays an important role in controlling the cellular levels of sialic acids as they are being incorporated into growing complex sugars. In our continuing studies of altering aldolases for a variety of uses in constructing complex biomolecules, we have used rational protein engineering and directed evolution to alter a number of properties of these enzymes.



In previous studies on NAL, we have successfully evolved novel enzymes with altered substrate specificity and stereochemistry. The substrate specificity of the *Escherichia coli* NAL was switched from the natural condensation, **1** + **2** → **3**, to an aldol condensation which generated *N*-alkylcarboxamide analogues of Neu5Ac (**5**). This was achieved by the single mutation of Glu-192 to Asn. In order to analyze the structural changes involved and to more fully understand the basis of this switch in specificity, we isolated all 20 variants of the enzyme at position 192 and determined the activities with a range of substrates. We also determined five high-resolution crystal structures.

Results

In order to determine the relationship between the amino acid at position 192 and the activity with various compounds, we isolated all 20 possible variants of NAL at position 192 and assessed their activity with either the natural substrate **3** or the new analogue **5**. These results (Figure 1) show that the activity towards Neu5Ac is highest for the wild-type enzyme (E192), and only the E192Q variant shows significant activity towards this substrate whereas the activity profile of the E192X library with DPAH, **5** differs dramatically from that with Neu5Ac **3**, as many more of the variants (for example E192F, E192H, E192M, E192N, E192Q, E192P and E192V) show significant activity with the new substrate. These results suggest that specific hydrogen bonding interactions are important for the wild-type enzyme and its natural substrate, but that a variety of mechanisms are in play to allow the variants to bind and utilize DPAH as a substrate.

In order to gain further insights into the mechanism of substrate specificity in the E192 variants we embarked on an X-ray crystallo-graphic analysis of the 'best' enzyme for DPAH

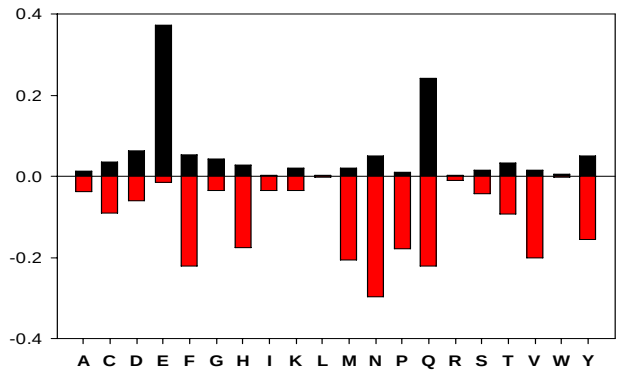


Figure 1: the activity profile of the E192X variants with Neu5Ac (black) and with DPAH (red).

cleavage, namely E192N, and structural modeling of the possible binding modes of interaction of the DPAH with other E192 variants.

Five high resolution crystal structures of NAL were solved; the structures of the wild-type *E. coli* NAL in the presence and absence of pyruvate, the E192N variant in the presence and absence of pyruvate and the E192N variant in the presence of pyruvate and a competitive inhibitor (2*R*, 3*R*)-2, 3, 4-trihydroxy-*N*, *N*-dipropylbutanamide (THB; **4**). This latter structure (Fig. 2) revealed that the inhibitor mimics the substrate and can bind in two orientations in the enzyme active site. It also reveals details of the mechanism of the specificity switch. The natural E192 would clash with the substrate explaining why the natural enzyme only poorly accepts DPAH, whereas the extensive hydrophobic surface generated in the active variants can all accept the new substrate.

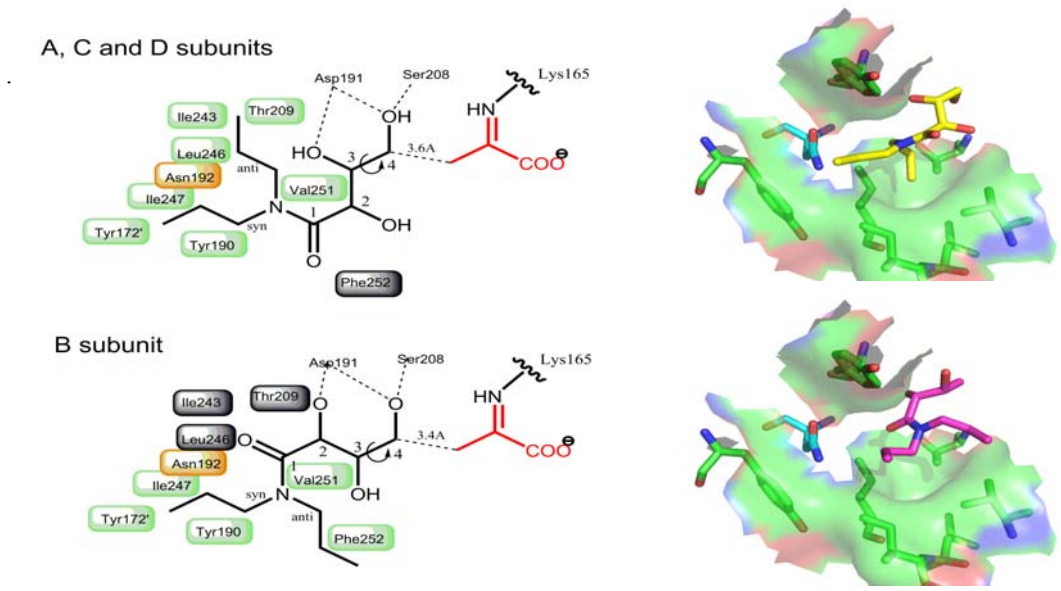


Figure 2: **right**; structures of *E.coli* NAL in complex with pyruvate and the competitive inhibitor DHOB (**6**) (yellow and purple) in subunits A, C and D (upper) and subunit B (lower). Active site residues forming the hydrophobic surface of the active site are shown in green and the introduced Asn-192 is shown in cyan. **Left**; schematic representations of the conformations of the substrates inferred from these structures showing the two major conformations of binding. Asn-192 lies at the bifurcation of the dipropylamide

This work demonstrates the subtleties of enzyme-substrate interactions and the importance of determining structures of enzymes produced by directed evolution, where the specificity determinants may change from one substrate to another.

Publications

Campeotto, I., Bolt, A., Harman, T., Dennis, C., Trinh, C., Phillips, S., Nelson, A., Pearson, A. & Berry, A. (2010) Structural insights into substrate specificity in variants of *N*-acetylneuraminic acid lyase produced by directed evolution. *J. Mol. Biol.* **404**:56-69.

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