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Protein engineering of a bacterial *N*-acyl-D-glucosamine 2-epimerase for improved stability under process conditions

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ABSTRACT

Enzymatic cascade reactions, i.e. the combination of several enzyme reactions in one pot without isolation of intermediates, have great potential for the establishment of sustainable chemical processes. However, many cascade reactions suffer from cross-inhibitions and enzyme inactivation by components of the reaction system. This study focuses on the two-step enzymatic synthesis of *N*-acetylneuraminic acid (Neu5Ac) using an *N*-acyl-D-glucosamine 2-epimerase from *Anabaena variabilis* ATCC 29413 (AvaAGE) in combination with an *N*-acetylneuraminate lyase (NAL) from *Escherichia coli*. AvaAGE epimerizes *N*-acetyl-D-glucosamine (GlcNAc) to *N*-acetyl-D-mannosamine (ManNAc), which then reacts with pyruvate in a NAL-catalyzed aldol condensation to form Neu5Ac. However, AvaAGE is inactivated by high pyruvate concentrations, which are used to push the NAL reaction toward the product side. A biphasic inactivation was observed in the presence of 50–800 mM pyruvate resulting in activity losses of the AvaAGE of up to 60% within the first hour. Site-directed mutagenesis revealed that pyruvate modifies one of the four lysine residues in the ATP-binding site of AvaAGE. Because ATP is an allosteric activator of the epimerase and the binding of the nucleotide is crucial for its catalytic properties, saturation mutagenesis at position K160 was performed to identify the most compatible amino acid exchanges. The best variants, K160I, K160N and K160L, showed no inactivation by pyruvate, but significantly impaired kinetic parameters. For example, depending on the mutant, the turnover number k_{cat} was reduced by 51–68% compared with the wild-type enzyme. A mechanistic model of the Neu5Ac synthesis was established, which can be used to select the AvaAGE variant that is most favorable for a given process condition. The results show that mechanistic models can greatly facilitate the choice of the right enzyme for an enzymatic cascade reaction with multiple cross-inhibitions and inactivation phenomena.

1. Introduction

N-Acetylneuraminic acid (Neu5Ac) is a nine-carbon amino sugar with a variety of (patho-)physiological functions in the human body [1–3]. It is therefore an important starting material for the production and development of pharmaceutical agents [4,5]. Because Neu5Ac also occurs in very high levels in the human brain and in breast milk, its use as dietary supplement in infant formula is also currently under investigation [6].

The growing interest in Neu5Ac synthesis is reflected by intensified scientific effort in this field and many different production strategies have been pursued during the last decade. In addition to the biocatalytic synthesis employing isolated enzymes [7–9], which is also focused on in this study, a chemoenzymatic route with base-catalyzed epimerization and a spore-displayed NAL [10], various whole-cell biotransformations [11–15], and a fermentative Neu5Ac production starting from glucose have been investigated [16]. The diversity of the approaches illustrates that there is no clearly preferable production method at the moment. For example, Neu5Ac production by fermentation using inexpensive glucose as carbon source is very attractive, but suffers from a quite low space-time yield of 1.9 g L^{-1} d^{-1} [16]. In contrast, enzymatic two-step synthesis of Neu5Ac from *N*-acetyl-D-glucosamine (GlcNAc) and pyruvate in an enzyme membrane reactor (EMR) reaches a very high space time yield of 109 g L^{-1} d^{-1} [17], but employs isolated enzymes which are costly. This can be compensated by using highly stable biocatalysts in continuous processes.

Abbreviations: AGE, *N*-acyl-D-glucosamine 2-epimerase; AvaAGE, *N*-acyl-D-glucosamine 2-epimerase from *Anabaena variabilis* ATCC 29413; GlcNAc, *N*-acetyl-D-glucosamine; ManNAc, *N*-acetyl-D-mannosamine; NAL, *N*-acetylneuraminate lyase; Neu5Ac, *N*-acetylneuraminic acid; EMR, enzyme membrane reactor; CSTR, continuous stirred tank reactor.

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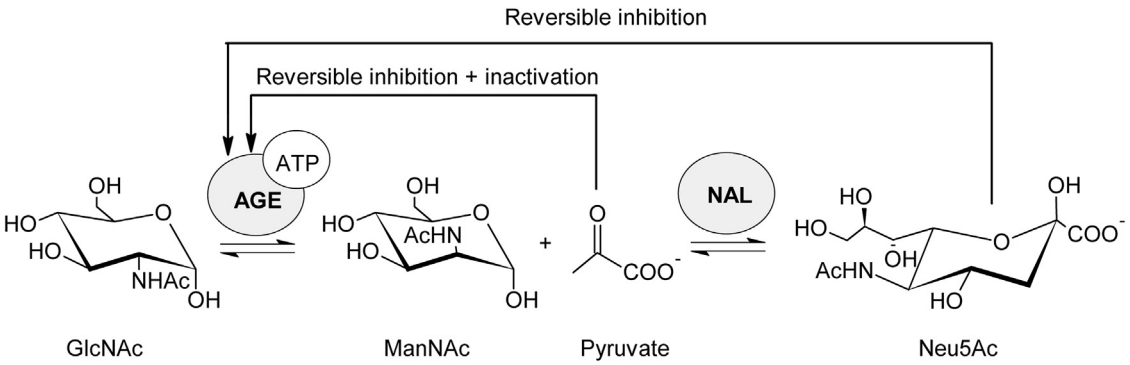

Fig. 1. Two-step enzymatic synthesis of *N*-acetylneuraminic acid (Neu5Ac). The *N*-acyl-D-glucosamine 2-epimerase (AGE) epimerizes *N*-acetyl-D-glucosamine (GlcNAc) into *N*-acetyl-D-mannosamine (ManNAc), which then undergoes an *N*-acetylneuraminate lyase (NAL)-catalyzed aldol condensation with pyruvate to yield Neu5Ac. All currently known AGEs are allosterically activated by ATP. The inhibiting and inactivating effects of reaction components on the AGE from *Anabaena variabilis* are indicated.

In contrast to chemical reactions, biocatalytic transformations are generally regarded as easy to combine since most enzymes operate under similar "mild" conditions, i.e. in aqueous medium, at moderate temperatures, neutral pH and ambient pressure. However, cross-inhibitions as well as enzyme inactivation by components of the reaction system are common problems encountered in cascade reactions [18]. The phenomena of cross-inhibitions and enzyme inactivation have to be considered when combining an *N*-acyl-D-glucosamine 2-epimerase (AGE, EC 5.1.3.8) with an *N*-acetylneuraminate lyase (NAL, EC 4.1.3.3) for the synthesis of Neu5Ac. In this reaction system, the AGE epimerizes GlcNAc to *N*-acetyl-D-mannosamine (ManNAc), the latter being more expensive and not readily available in large amounts. Then, ManNAc undergoes a NAL-catalyzed aldol condensation with pyruvate to form Neu5Ac (Fig. 1). Because the enzymatic synthesis of Neu5Ac suffers from an unfavorable position of the thermodynamic equilibrium [19], an excess of pyruvate is used to push the reaction toward the product side. However, high Neu5Ac and pyruvate concentrations strongly inhibit most of the currently known AGEs [20]. The undesired reversible inhibition by pyruvate can be circumvented by using the AGE from the cyanobacterium *Anabaena variabilis* ATCC 29413 (AvaAGE), which has an up to 10-fold higher inhibition constant for pyruvate than other AGEs indicating a much weaker inhibitory effect [20]. AvaAGE also displays the highest catalytic efficiency ever reported for this kind of epimerase with GlcNAc as substrate.

In this study, the long-term stability of AvaAGE in the two-enzyme system was investigated and it was discovered that the stability of this enzyme is strongly reduced at high pyruvate concentrations. The mechanism of enzyme inactivation was elucidated and site-directed saturation mutagenesis was performed to improve the stability of AvaAGE toward pyruvate.

2. Material and methods

2.1. Chemicals

GlcNAc monohydrate (99%) and ManNAc monohydrate (99%) were purchased from Alfa Aesar (Karlsruhe, Germany). Adenosine triphosphate (ATP) (≥98%) and sodium pyruvate (≥99%) were purchased from Carl Roth (Karlsruhe, Germany). NAL from *Escherichia coli* (*E. coli*) K12 was obtained from Sigma-Aldrich (Schnelldorf, Germany). Primers were synthesized by Eurofins MWG Operon (Ebersberg, Germany). All other chemicals were of analytical grade from various suppliers.

2.2. Site-directed mutagenesis

Site-directed mutations were planned using the HotSpot Wizard webserver [21]. As input served a homology model of AvaAGE that was constructed using the SWISS-MODEL automated comparative protein modelling web server (http://swissmodel.expasy.org/) [22,23]. Site-directed amino acid substitutions were made by using the QuikChange® Lightning Site-Directed Mutagenesis Kit (Stratagene, Agilent Technologies Company, La Jolla, CA, USA) according to the manufacturer's instructions. For saturation mutagenesis, the QuikChange® Multi Site-Directed Mutagenesis Kit (Stratagene, Agilent Technologies Company, La Jolla, CA, USA) was used and the generated plasmids were transformed into *E. coli* NovaBlue (DE3) (Novagen, Darmstadt, Germany). The sequences of the mutagenic primers are given in Table 1. As template DNA served a pET-28a(+)-vector with the gene encoding AvaAGE in frame with a N-terminal His_6-tag, which had been generated previously [20]. Confirmatory DNA sequencing was performed by Eurofins MWG Operon (Ebersberg, Germany).

2.3. Protein expression and purification of mutants

Protein expression and purification were performed as described previously [24]. After purification, the buffer was changed to 0.1 M sodium phosphate buffer (pH 7.5) by dialysis. Protein purity was judged by SDS-PAGE stained with Coomassie. Protein concentrations were determined with the bicinchoninic acid assay (Pierce, Rockford, USA) using bovine serum albumin as standard.

2.4. Mutant library screening

For cultivation of the clones of the saturation mutagenesis library, deep well plates made from polystyrene with 96 × 2 mL U-bottom cavities (nerbe plus GmbH, Winsen/Luhe, Germany) were used. Each well was filled with 800 μL LB-medium supplemented with 30 μg mL^{-1} kanamycin and inoculated with a single colony from an agar plate. The cultures were placed into an orbital shaker at 200 rpm (WiseCube, Witeg, Wertheim, Germany) for 8 h at 37 °C. Protein expression was initiated by adding 1 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG) and the temperature was lowered to 30 °C. After overnight expression, 200 μL of each sample were mixed with 2 μL phenylmethylsulfonyl fluoride (100 mM stock in isopropanol) and 20 μL PopCulture® reagent (Novagen, Darmstadt, Germany) to lyse the cells. The mixtures were incubated for 15 min at room temperature.

The epimerase activity was measured using an *N*-acylmannosamine 1-dehydrogenase (ManDH, E.C. 1.1.1.233) from