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Mechanistic model for the synthesis of *N*-acetylneuraminic acid using *N*-acetylneuraminate lyase from *Escherichia coli* K12

Anna Groher, Kathrin Hoelsch*

Institute of Biochemical Engineering, Technische Universität München, Boltzmannstr. 15, 85748 Garching, Germany

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ABSTRACT

N-Acetylneuraminate lyase (NAL) from *Escherichia coli* K12 is an important enzyme for the production of *N*-acetylneuraminic acid (Neu5Ac), catalyzing the reversible aldol condensation between *N*-acetyl-D-mannosamine (ManNAc) and pyruvate. Despite the industrial importance of this enzyme, its kinetic mechanism has never been elucidated before. The initial rate patterns were consistent with a rapid-equilibrium ordered bi uni mechanism with pyruvate binding first. Based on progress curve analysis, a mechanistic model was developed to predict the reaction course of Neu5Ac synthesis. The model accurately reproduced the experimental data in a wide range of initial conditions. The correct assignment of the kinetic mechanism is a critical element in optimizing enzymatic syntheses by means of mathematical models, which have become indispensable tools for the design of cost-effective biocatalytic processes.

1. Introduction

Sialic acids are a remarkably diverse family of α-keto acids with a nine-carbon backbone [1,2]. They are typically found at the terminal position of glycan chains present on many eukaryotic surface-exposed glycoproteins and glycolipids and play an important role in a variety of biological processes such as cellular recognition and adhesion, tumor metastasis, and bacterial and viral infections [1,3–5]. There are more than fifty naturally occurring forms of sialic acid, with the most abundant one being *N*-acetylneuraminic acid (Neu5Ac) [2]. Due to its involvement in many (patho)physiological events, Neu5Ac is an important starting material for the synthesis of pharmacologically active molecules. A well known example is the Neu5Ac derivative zanamivir (tradename Relenza, GlaxoSmithKline) that is used to treat influenza virus A and B infections [5].

The most efficient method for Neu5Ac production is the enzymatic synthesis from *N*-acetyl-D-mannosamine (ManNAc) and pyruvate with *N*-acetylneuraminate lyase (NAL, EC 4.1.3.3) as catalyst [6] (Fig. 1). ManNAc is an expensive compound that can be generated from the cheaper *N*-acetyl-D-glucosamine (GlcNAc) by epimerization under alkaline conditions [7]. As an alternative, the epimerization can be catalyzed by *N*-acylglucosamine-2 epimerase (AGE, EC 5.1.3.8) [8,9]. The synthesis of Neu5Ac suffers from several disadvantages, including an unfavorable reaction equilibrium and difficult product separation due to the presence of pyruvate, which has a similar pK_a to Neu5Ac [8]. Thus, optimization of the production process has been the subject of many studies [8,10–13]. Despite this considerable effort, the synthesis of Neu5Ac is still very expensive and further research is needed to optimize the current production methods [14].

The identification of optimal operating conditions for an enzymatic synthesis is greatly facilitated by using a mechanistic kinetic model [15–17]. The development of such a model requires a detailed knowledge of the kinetic mechanisms and accurate estimates of the kinetic parameters of all involved enzymes. For the industrial production of Neu5Ac, the NAL from *Escherichia coli* is employed [18]. Nevertheless, to date, the kinetic mechanism of this enzyme has not been investigated. For the NAL from *Clostridium perfringens* an ordered reaction mechanism has been described in which pyruvate forms a Schiff base with a lysine residue in the active site before ManNAc binds [19,20]. This is why developed mathematical models of Neu5Ac synthesis also assumed a steady-state ordered bi uni mechanism for the NAL from *E. coli* [21,22], which shares only 38% amino acid identity with the *Clostridium* enzyme.

In this article, the first investigation of the kinetic mechanism of Neu5Ac synthesis catalyzed by the NAL from *E. coli* K12 is presented. A novel mechanistic model was developed and its suitability for the prediction of reaction rates in a wide range of conditions was demonstrated.

Abbreviations: GlcNAc, *N*-acetyl-D-glucosamine; ManNAc, *N*-acetyl-D-mannosamine; NAL, *N*-acetylneuraminate lyase; Neu5Ac, *N*-acetylneuraminic acid.

* Corresponding author. Tel.: +49 089 28915729; fax: +49 089 28915714.
E-mail address: k.hoelsch@lrz.tum.de (K. Hoelsch).

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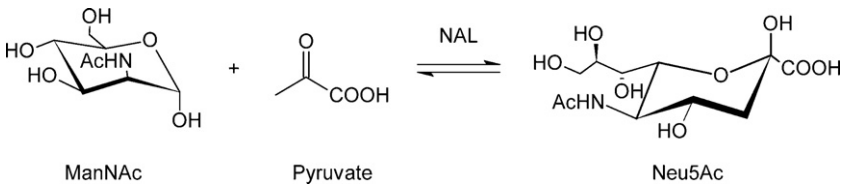

Fig. 1. Enzymatic synthesis of *N*-acetylneuraminic acid (Neu5Ac) from *N*-acetyl-D-mannosamine (ManNAc) and pyruvate using *N*-acetylneuraminate lyase (NAL).

2. Material and methods

2.1. Chemicals

ManNAc monohydrate (99%) and Neu5Ac (97%) were purchased from Alfa Aesar (Karlsruhe, Germany). Sodium pyruvate (≥99%) was obtained from Carl Roth (Karlsruhe, Germany). All enzymes used for DNA manipulation were purchased from New England Biolabs (Frankfurt, Germany). Primers were synthesized by Eurofins MWG Operon (Ebersberg, Germany). All other chemicals were of analytical grade from various suppliers.

2.2. Bacterial strains and plasmids

E. coli K12 DSM 498 was provided by the *German Collection of Microorganisms and Cell Cultures* (DSMZ, Braunschweig, Germany). *E. coli* strains DH5α (Invitrogen, Carlsbad, USA) and BL21(DE3) (Novagen, Madison, USA) were used for cloning and overexpression experiments, respectively. The vector pET28a(+) was purchased from Novagen (Madison, USA).

2.3. Cloning

The NAL gene was amplified by standard PCR from isolated genomic DNA from *E. coli* K12 DSM 498. The primers were designed on the basis of the available sequence of *E. coli* K12 substr. MG1655 (GenBank accession number U00096; region from 3370705 to 3371598): forward primer, 5′-AGA GAT CAT ATG GCA ACG AAT TTA CGT GG-3′; reverse primer, 5′-AGA GAT GGA TCC TCA CCC GCG CTC TTG CAT C-3′). NdeI and BamHI restriction sites are underlined. The purified and digested PCR product was ligated into the linearized pET28a(+) plasmid, in frame with an N-terminal hexahistidine tag (His_6-tag). The resulting vector was transformed into *E. coli* DH5α. After isolation from DH5α cells and DNA sequencing (Eurofins MWG Operon, Ebersberg, Germany), the plasmid was transformed into *E. coli* BL21(DE3) cells.

2.4. Protein expression and purification

A 4-mL preculture was inoculated with a single colony, grown overnight (15-mL test tubes, 37 °C, 150 rpm, 16 mm excentricity) and subcultured (1:200 [v/v]) into 4× 200 mL of LB-medium supplemented with 30 mg L^{-1} kanamycin (1 L shaking flask without baffles, 37 °C, 250 rpm, 3.5 cm excentricity). When the cells had reached an optical density at 600 nm (OD_{600}) of 0.8, protein production was induced by adding isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 1 mM. Thereafter, the cells were incubated at 20 °C and 250 rpm for 16 h and collected by centrifugation (3260 × *g*, 4 °C, 10 min). The cell pellets were resuspended in ice-cold immobilized metal affinity chromatography (IMAC) binding buffer (50 mM sodium phosphate, pH 7.4, 500 mM NaCl, 20 mM imidazole) at a ratio of 1 g wet weight to 5 mL IMAC-binding buffer. After addition of 1 mM PMSF, the cells were disrupted using 50% (v/v) glass beads (0.25–0.5 mm; Carl Roth, Karlsruhe, Germany) in a mixer mill (Retsch, Haan, Germany) for 3 min at 30 Hz. The homogenate was subsequently centrifuged at 50,377 × *g*, 4 °C, for 30 min. The supernatant fractions were applied to a 1-mL HisTrap FF crude column (GE Healthcare, Uppsala, Sweden). Bound proteins were then eluted with a linear gradient from 0% to 100% elution buffer (50 mM sodium phosphate, pH 7.4, 500 mM NaCl, 500 mM imidazole) over 20 column volumes (CV) and a final 10 CV wash was performed with 100% elution buffer. Subsequently, the buffer of the eluted protein was exchanged to IMAC-binding buffer by dialysis (molecular weight cut off 10–12 kDa). In preliminary experiments, it was shown that the His_6-tag influenced the catalytic properties of the enzyme. Hence, it was cleaved at 37 °C within 3 h by adding 50 μL thrombin solution (20 U mL^{-1}) per mg protein. After cleavage, the His_6-tag and the protease were removed by passing the solution through a 1-mL HisTrap FF crude column connected in series to a HiTrap-Benzamidine column (GE Healthcare, Uppsala, Sweden). After this second purification step, the buffer was changed to 0.1 M sodium phosphate (pH 7.5). The purified protein appeared homogeneous in a 12.5% Tris-Glycine SDS–PAGE stained with Coomassie. Protein concentrations were determined with the bicinchoninic acid (BCA) assay (Pierce, Rockford, USA).

2.5. Enzymatic synthesis of Neu5Ac

All reactions were carried out at 30 °C unless stated otherwise. The reaction buffer was 0.1 M sodium phosphate (pH 7.5). In preliminary tests, this buffer was found to be optimal for NAL activity. One Unit (U) corresponds to the amount of enzyme converting 1 μmol substrate per min under these conditions. Initial rate measurements were performed in 1.5 mL safe lock tubes using a thermomixer (Eppendorf, Hamburg, Germany), whereas progress curves were recorded using a milliliter-scale bioreactor system [23,24]. In this system, 48 parallel single-use stirred-tank reactors on a 10 mL-scale are arranged in a 'bioreaction block'. The impeller speed was set to 650 min^{-1} and a liquid-handling system (Freedom EVO, Tecan, Crailsheim, Germany) was used for sampling. An optical sensor spot (Presens, Regensburg, Germany) immobilized onto the bottom of each reactor was applied for online pH measurements. Adjustments of the pH were not necessary. Evaporation was minimized by headspace cooling at 4 °C. Substrate concentrations ranged from 2 to 298 mM for pyruvate and from 10 to 210 mM for ManNAc. Enzyme concentrations varied between 25 and 130 mg L^{-1}. Under these conditions, the enzyme activity was proportional to the NAL concentration. At various time intervals, aliquots were withdrawn and the reactions were stopped by 1:10 (v/v) dilution in 0.1 mol L^{-1} sulfuric acid. Samples were analyzed by high-performance liquid chromatography (HPLC).

2.6. High-performance liquid chromatography

The concentrations of pyruvate, ManNAc and Neu5Ac were determined by HPLC (Agilent 1100 Series, Santa Clara, USA) equipped with an Aminex HPX-87H column (Biorad, Munich, Germany) and with 5 mM sulfuric acid as the mobile phase. The column was operated at 60 °C and the flow rate was set to 0.5 mL min^{-1}. The elution was monitored using a UV detector (S 3300, Knauer, Berlin, Germany) at 210 nm. The mean relative standard deviation of HPLC measurements was 4%.