Protein Expression and Purification 119 (2016) 11–18

Contents lists available at ScienceDirect

Protein Expression and Purification

journal homepage: www.elsevier.com/locate/yprep

Purification and characterization of aspartate *N*-acetyltransferase: A critical enzyme in brain metabolism

Qinzhe Wang [1], Mojun Zhao [1], Gwenn G. Parungao, Ronald E. Viola*

Department of Chemistry and Biochemistry, University of Toledo, Toledo, OH 43606, USA

ARTICLE INFO

Article history:
Received 24 September 2015
Received in revised form
2 November 2015
Accepted 3 November 2015
Available online 10 November 2015

Keywords:
Canavan disease
aspartate N-acetyltransferase
membrane protein
enzyme kinetics
enzyme purification

ABSTRACT

Canavan disease (CD) is a neurological disorder caused by an interruption in the metabolism of N-acetylaspartate (NAA). Numerous mutations have been found in the enzyme that hydrolyzes NAA, and the catalytic activity of aspartoacylase is significantly impaired in CD patients. Recent studies have also supported an important role in CD for the enzyme that catalyzes the synthesis of NAA in the brain. However, previous attempts to study this enzyme had not succeeded in obtaining a soluble, stable and active form of this membrane-associated protein. We have now utilized fusion constructs with solubilizing protein partners to obtain an active and soluble form of aspartate *N*-acetyltransferase. Characterization of the properties of this enzyme has set the stage for the development of selective inhibitors that can lower the elevated levels of NAA that are observed in CD patients and potentially serve as a new treatment therapy.

Canavan disease (CD) is a fatal, neurological disease that is caused by an interruption in the metabolism of a critical amino acid, *N*-acetyl-L-aspartic acid (NAA) [1]. Defects at multiple locations in the *aspA* gene that codes for aspartoacylase [2] lead to mutant forms of this enzyme that are either not expressed or rapidly degraded [3], or have significantly impaired catalytic activity [4]. As a consequence the substrate NAA accumulates to unusually elevated levels and the products of this reaction, aspartic acid and acetate, are not made in oligodendrocytes. Various hypotheses of the molecular basis for CD have been proposed and tested, including toxic accumulation of NAA [5], increased NAAG neurotransmitter production [6], osmotic effects of NAA accumulation [7], and defects in fatty acid biosynthesis as a consequence of acetate deficiency [8,9]. Animal models in which the *aspA* gene had been knocked out were found to reproduce many of the disease symptoms [10]. Unexpectedly, a recent study in which a second gene knock-out was introduced in the *Nat8l* gene which codes for the enzyme that synthesizes NAA appears to reverse these adverse effects, leading to normal myelination and a decrease in CD symptoms [11].

These new results place an increased importance on the characterization of aspartate *N*-acetyltransferase (ANAT), the brain enzyme that catalyzes the synthesis of NAA. Unfortunately, attempts to express and purify this enzyme had resulted in only limited progress to date. This enzyme has been shown to be membrane-associated [12], with a structural model suggesting the presence of a membrane anchor region [13]. Attempts to extract a functional enzyme from membrane preparations have not succeeded, thwarted by the apparent sensitivity of this enzyme to the presence of detergents [12] and the suggestion of additional protein components that may be required to maintain a catalytically active enzyme [14].

A survey of a wide range of detergents with a variety of properties has led to improved conditions for the extraction of this enzyme from membranes. Coupling this approach with the production of a fusion construct with a solubilizing protein partner has now resulted in the successful extraction and purification of active ANAT. The catalytic properties of this purified enzyme have been examined, along with its substrate specificity and optimized stabilization conditions.

1. Experimental procedures

1.1. Materials

5,5′-Dithiobis(2-nitrobenzoic acid) (DTNB) was purchased from Sigma. Detergents were obtained from Anatrace, and the protease

* Corresponding author.
E-mail address: ron.viola@utoledo.edu (R.E. Viola).
[1] These authors contributed equally to this work.

http://dx.doi.org/10.1016/j.pep.2015.11.001
1046-5928/

inhibitor cocktail (P8340) was from Sigma–Aldrich. *NiCo*21 (DE3) competent *E. coli* cells were from New England Biolabs. A modified pET28a plasmid with the incorporation of a human rhinovirus 3C protease cleaving site was a gift from Dr. Don Ronning (University of Toledo). NuPAGE Bis-Tris 4–12% gradient gels were purchased from Life Technologies.

1.2. Expression and solubilization of native ANAT

The human *nat8l* gene was cloned into the pETDEST42 plasmid using the Gateway cloning technology (Life Technologies). This plasmid was used to transform *E. coli* BL21(DE3) cells for protein production. The cells were grown at 37 °C to an A_{600} of 0.6–0.8 in Luria–Bertani (LB) medium containing 100 μg/mL ampicillin, and gene expression was then induced with 1 mM isopropyl α-D-thio-galactopyranoside (IPTG) at 28 °C for 5 h. The resulting *E. coli* cells were collected and resuspended in buffer A [50 mM HEPES, 500 mM NaCl and 10% glycerol (pH 7.4)] and lysed by sonication on ice for 8 min with a 30 s pulse on and 2 min pulse off cycle. The cell lysate was centrifuged at low speed (15,560 × g) for 30 min to pellet cell debris and unlysed cells. The membrane fraction was collected by high-speed centrifugation of the supernatant (193,000 × g) for 1 h. Solubilization trials of ANAT from the membrane fraction were conducted with a variety of different detergents that include ionic, non-ionic and zwitterionic detergents. Twenty microliters aliquots of the resuspended membrane fraction were mixed with different amounts of stock detergent solutions each prepared at 10 times their critical micelle concentration (CMC), leading to a final concentration of 1.5× CMC and 8.7× CMC for each detergent. These solutions were incubated either at room temperature for 1 h or at 4 °C overnight on a rocking platform. The supernatant was separated from the pellet by centrifugation and the proteins that were extracted into the supernatant were analyzed by SDS-PAGE and by Western blotting with an anti-his-tag antibody. ANAT extraction levels were calculated relative to the buffer control run in the absence of added detergents.

1.3. Expression and purification of ANAT fusion enzymes

The *E. coli* codon optimized gene (Supplemental Fig. S1) was designed, gene synthesized (Genescript, Piscataway, NJ) flanked by EcoRI and XhoI restriction sites, and then inserted into the modified pET28 plasmid. Different versions of this fusion expression vector have been used to subclone the genes for thioredoxin (TRX), glutathione S-transferase (GST) or maltose binding protein (MBP), followed by a linker region (21–68-amino acids) containing various cleavage site sequences, and then connected to the *N*-terminal of the *nat8l* gene (Fig. 1). These fusion constructs also contain polyhistidine tags located at both the *N*- and C-termini of each fusion protein. The ANAT fusion enzymes were expressed in *E. coli* BL21(DE3) cells grown in LB media. Each culture was grown at 37 °C until reaching an A_{600} of between 0.6 and 0.8, followed by induction with IPTG at 30 °C for 5 h. The recombinant fusion enzymes were purified by using Ni-immobilized metal affinity chromatography (IMAC).

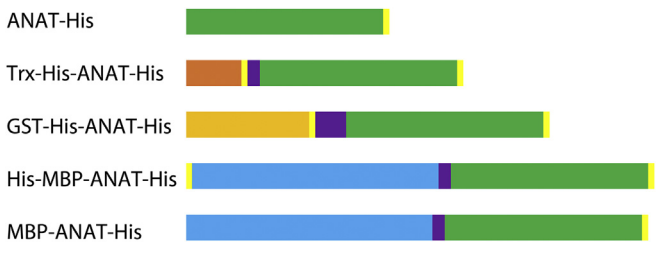

Fig. 1. Different fusion constructs of human aspartate *N*-acetyltransferase (ANAT). The DNA lengths are drawn roughly to scale, with the *nat8l* gene shown in green, the his-tag in yellow, and the linker region in purple. The thioredoxin (TRX) fusion partner is shown in red, the glutathione *S*-transferase (GST) in orange and the maltose binding protein (MBP) is in blue.

1.4. Expression and dual affinity purification of MBP-ANAT fusion enzyme

The MBP-ANAT-his construct (Fig. 1) was produced from the his-MBP-ANAT-his construct by site-directed mutagenesis using the forward and reverse primers: AAAACTGAAGAAGGTAAACTGG and GCCCATGGTATATCTCCTTC, respectively. *NiCo*21(DE3) competent *E. coli* cells containing the MBP-ANAT-his construct were selected on LB plates with 30 μg/ml kanamycin at 37 °C for 16 h. Colonies from these plates were used to inoculate starter cultures containing 10 ml of LB media. After 16 h growth at 37 °C, each starter culture was diluted 100-fold into 1 L of LB media, and cell growth was continued for about 2 h until A_{600} reached 0.6. IPTG was then added to a final concentration of 0.5 mM and protein expression was induced at 16 °C for 20 h. The dual affinity tagged human ANAT was initially purified by metal-affinity chromatography (IMAC). The column was washed with Buffer A, containing 20 mM potassium phosphate, pH 7.4, 300 mM sodium chloride, 10% glycerol and 20 mM imidazole and then eluted with a linear gradient of Buffer B (buffer A containing 400 mM imidazole). The active fractions were pooled and loaded onto an amylose column and highly purified ANAT fusion was then obtained by elution with a 0–10 mM linear maltose gradient (Fig. 2). Protein concentration was determined by NanoDrop 2000 UV–Vis Spectrophotometer (Thermo Scientific) and enzyme activity was measured as described below.

1.5. Enzyme activity assay

The ANAT activity was measured by an established DTNB-based assay [15] in which the coenzyme A product participates in a thiol exchange reaction with DTNB. The resulting TNB^{2-} ion was monitored at 412 nm ($\varepsilon = 14.15\ mM^{-1}\ cm^{-1}$) using a SpectraMax 190 spectrophotometer plate reader (Molecular Devices, CA). A typical activity assay contains 8 mM potassium phosphate, 4 mM Tris–HCl, 120 mM NaCl, 4% glycerol, 40 μM DTNB, 40 μM acetyl-CoA, 2 mM L-aspartate at pH 7.4 in a total volume of 200 μl. The results of the DTNB-based assay were validated by an HPLC end point assay that directly measures the coenzyme A product at 260 nm.

1.6. Enzyme kinetic and stability studies

The DTNB-based assay was used to measure the kinetic parameters for the physiological substrates and for some alternative ANAT substrates. The reaction rates were measured with varying substrate concentrations of L-aspartate (0.01–2 mM) and acetyl-CoA (2.5–50 μM), and the data were fitted to the equation for a sequential enzyme mechanism (Eq. (1)) to determine the kinetic parameters.

$$\text{Velocity} = \frac{V_{max}[A][B]}{K_a[B] + K_b[A] + [A][B] + K_{ia}K_b} \quad (1)$$

where K_a and K_b are the Michaelis constants and K_{ia} is the binding constant for substrate A.

To examine the effects of pH on enzyme activity the rates were measured at pH values from 6.0 to 9.5 by varying the L-aspartate concentration at fixed, saturating levels of acetyl CoA. The V_{max} and