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Reexamination of aspartoacylase: Is this human enzyme really a glycoprotein?

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ABSTRACT

Aspartoacylase catalyzes the metabolism of an important amino acid in the brain, with the release acetate serving as the source for fatty acid biosynthesis. Defects in this enzyme lead to a loss of activity and the symptoms of a fatal neurological disorder called Canavan disease. Extensive evidence, including deglycosylation studies, differential activity upon eukaryotic host expression and site directed mutagenesis, have supported the presence of a glycan that plays an essential role in the stability and catalytic activity of mammalian aspartoacylase. However, the structure of this enzyme did not show the presence of any non-amino acid components at the putative glycosylation site. A more extensive study specifically designed to resolve this discrepancy has now shown that recombinantly-expressed human aspartoacylase is not glycosylated, but is still fully functional and stable even when produced from a bacterial expression system. Alternative interpretations of the prior experiments now present a consistent picture of the structural components of this essential brain enzyme.

Introduction

Aspartoacylase catalyzes the selective breakdown of *N*-acetyl-L-aspartate (NAA)[1] to release acetate in the brain [1]. This acetate provides the building blocks required for fatty acid biosynthesis. Defects in the ASPA gene that codes for aspartoacylase causes a dramatic elevation in NAA levels, depletion of brain acetate, and leads to demyelination in neuronal cells [2]. The brains of newborns with this enzymatic deficiency do not make sufficient numbers of neuronal connections, causing a range of developmental symptoms known as Canavan disease (CD) [3]. There is a clear connection between this genetic deficiency and the symptoms of CD [4], and a correlation has been established between the levels of aspartoacylase activity and the severity of the symptoms [5]. The mechanism of the aspartoacylase-catalyzed reaction has been elucidated [6], and the structure of this enzyme has been determined both as an apo-enzyme [7] and in the presence of a bound transition state analog [6].

The previous case for aspartoacylase functioning as a glycoprotein is quite compelling. The enzyme contains a consensus **NxT/S** glycosylation site [8] that is conserved throughout this acylase enzyme family. Expression of human aspartoacylase in *Escherichia coli* cells leads to an enzyme form with low catalytic activity and low stability [9], suggesting the need for additional post-translational processing to produce a fully functional enzyme. Switching to a eukaryotic host (*Pichia pastoris*) for enzyme expression that is capable of protein glycosylations yielded an enzyme with good stability and greater than 100-fold higher catalytic activity compared with the bacterial-expressed enzyme [10]. Treatment of the *Pichia*-expressed enzyme with a glycosidase (PNGase F) that is known to cleave *N*-glycosidic linkages caused aspartoacylase to revert to its low activity form. A similar loss of activity and stability was seen when this glycosylation site was mutated (N117Q) to prevent subsequent glycosylation [10]. Finally, cleavage and isolation of the glycan resulted in a peak observed by MALDI-mass spectrometry that corresponded to the mass expected for a typical *N*-glycosylation oligosaccharide [10]. Taken together this experimental evidence supported an essential role for an *N*-linked glycan in the stability and catalytic activity of this human brain enzyme.

The first experimental evidence that was inconsistent with this glycoprotein picture came when the structure of *Pichia*-expressed aspartoacylase was determined. No additional electron density was observed at the putative *N*-glycosylation site, and the N117 side chain that was proposed to be glycosylated was completely

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[1] *Abbreviations used:* NAA, *N*-acetyl-L-aspartate; CD, Canavan disease; PNGase F, Peptide:*N*-glycosidase F; Endo H, endoglycosidase H; YPDS, yeast extractpeptone-dextrose-sorbitol; IMAC, metal-affinity chromatography; DTT, dithiothreitol; IAA, iodoacetamide; TFA, trifluoroacetic acid; SPE, solid phase extraction; PMF, peptide mass fingerprinting; DHB, 2,5-dihydroxybenzoic acid; PAS, periodic acid–schiff; MALDI-MS, matrix-assisted laser desorption ionization mass spectrometry; SPEG, solid-phase extraction of a glycoprotein/glycopeptide.

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buried and not exposed to solvent [6]. This structural inconsistency has necessitated a more extensive examination of the presence and possible role of a glycan component in the structure and activity of aspartoacylase. These additional studies were unable to detect the presence of a glycan component in the recombinantly-expressed human enzyme. Alternative interpretations and explanations of the previous results are offered that lead to a consistent picture of the available experimental data from a variety of studies that have been conducted to definitively address this question. Based on these new studies we can now conclude that aspartoacylase is, in fact, not glycosylated when expressed in *P. pastoris* and that glycosylation is not required to produce a fully functional enzyme.

Experimental procedures

Chemicals

Yeast Nitrogen Base without amino acids and ammonium sulfate was obtained from Research Products International Corp, and the protease inhibitor cocktail (P8340) was from Sigma–Aldrich. NuPAGE® LDS Sample Buffer was from Life Technologies Corporation. Trypsin was from Promega Corp. Peptide:*N*-glycosidase F (PNGase F) and endoglycosidase H (Endo H) were obtained from New England Biolabs. Additional chemicals and reagents were obtained from either Sigma or Fischer Scientific. L-aspartase was purified as previously described [9].

Enzyme expression and purification

Native human aspartoacylase was expressed from *P. pastoris* as previously described [10] with only slight modifications. Briefly, the KM71H *P. pastoris* cell line containing the human ASPA gene, integrated using pPICZ A vector for intracellular expression, was selected on yeast extract-peptone-dextrose-sorbitol (YPDS) plates with 100 μg/ml zeocin at 30 °C for 2–3 days. Colonies from these plates were used to inoculate starter cultures containing 10 ml of minimal glycerol medium. After 22 h growth at 28 °C each starter culture was diluted 100-fold into 1 L minimal glycerol medium, and cell growth was continued for 36 h until A_{600} reached ~15. The cells were centrifuged and resuspended in minimal methanol medium to induce *aspA* expression, controlled by the alcohol oxidase regulator, with 1% methanol supplementation after 24 h to compensate for methanol consumption and evaporation. Cells were harvested 48 h after induction and either processed immediately for enzyme purification or stored at −80 °C until used.

The His-tagged human aspartoacylase was purified by metal-affinity chromatography (IMAC) with elution by a linear imidazole gradient from 20 to 400 mM [10]. The active fractions were pooled and dialyzed into 50 mM Hepes buffer, pH 7.5, with 1 mM dithiothreitol (DTT). Highly purified aspartoacylase was then obtained by anion exchange chromatography using a 10 ml Source 15Q column (GE Healthcare) with a linear gradient of NaCl from 0 mM to 500 mM.

Enzyme kinetic assay

The aspartoacylase activity was measured by a standard coupled-enzyme assay [9], in which the *N*-acetyl-L-aspartate substrate is hydrolyzed into L-aspartate and this product is then converted to fumarate by the L-aspartase coupling enzyme. The formation of fumarate was monitored at 240 nm ($\varepsilon = 2.53\ mM^{-1}\ cm^{-1}$) using a SpectraMax 190 spectrophotometer plate reader (Molecular Devices).

Tryptic digestion of aspartoacylase

500 μg of *Pichia*-expressed aspartoacylase was concentrated to 4 mg/ml and the buffer exchanged into 0.1 M ammonium bicarbonate buffer (pH 7.9) containing 8 M urea using an Amicon Ultra 10 K NMWL concentrator (Millipore). The protein was denatured in this buffer at 37 °C for 1 h, 5 mM DTT was added and the sample incubated at 37 °C for an additional hour. Protein thiols were then protected by adding 10 mM iodoacetamide (IAA) and the enzyme incubated at 37 °C for another hour. After quenching the excess IAA with DTT the sample was diluted 10-fold by using 0.1 M ammonium bicarbonate buffer (pH 7.9) to reduce the urea concentration to less than 1 M. Trypsin was added at an enzyme:protein ratio of 1:50 and aspartoacylase was digested for 23 h at 37 °C. Tryptic digestion was terminated by adding 1% trifluoroacetic acid (TFA). Trypsin, urea, DTT, IAA and ammonium bicarbonate were removed by using a Sep-Pak C18 Solid Phase Extraction (SPE) cartridge (Waters) according to the standard protocol. Peptides were loaded onto the cartridge and salts removed with 0.1% TFA. Tryptic peptides were eluted by 50% acetonitrile/water with 0.1% TFA and dried by Centrivap Concentrator (Labconco).

Peptide mass fingerprinting (PMF) analysis

Tryptic peptides obtained from aspartoacylase were dissolved in 33% acetonitrile/water with 0.067% TFA. 1 μl of sample was mixed with 1 μl of 10 mg/ml 2,5-dihydroxybenzoic acid (DHB) in 33% acetonitrile/water with 0.067% TFA and 1 μl was spotted onto the MALDI target. MALDI spectra were recorded by accumulating 4000–5000 laser shots per spectrum in positive reflection mode with 0–4 kDa/z as the detection range in a Bruker Ultraflextreme MALDI-TOF/TOF mass spectrometer. Peaks in the resulting spectra were searched against the SwissProt database with Homo sapiens as the taxonomy filter using the Mascot search algorithm (Matrix Science).

Nano-HPLC-MALDI-TOF/TOF

An UltiMate 3000 Nano LC System with an Acclaim PepMap100 C18 Protein and Peptide Column (75-μm i.d. × 15 cm) was used for sample preparation and analysis. Mobile phase A was HPLC grade water plus 0.05% TFA and mobile phase B was 90% HPLC grade acetonitrile/water plus 0.05% TFA. A 75 min linear gradient from 3% B to 60% B, followed by a linear gradient from 60% B to 100% B in 10 min, were used at a flow rate of 300 nl/min to separate the peptides. Fractions were collected, mixed with the DHB matrix and loaded onto a MALDI target (AnchorChip, Bruker) by a PROTEINEER fc II system (Bruker). The spotting interval was 15 s, each spot contained 75 nl of sample, and more than 300 spots were typically collected for each nano-HPLC separation. A quick MS scan of each spot was conducted using similar parameters as those for PMF analysis to identify peaks at around the 3300 m/z expected for the N117-containing peptide. The spots containing the targeted peaks were analyzed by MS/MS using the LIFT method in positive mode. Laser induced dissociation occurs for the selected peptides and the tandem mass spectra were recorded by Compass 3.0 (Bruker).

PNGase F and Endo H digestion of aspartoacylase

Peptide:*N*-glycosidase F (PNGase F) and endoglycosidase H (Endo H) are two endo-glycosidases that can either completely [11] or partially [12] remove *N*-glycans from glycoproteins, respectively. For deglycosylation of peptides 500 U of PNGase F was added to the tryptic peptides obtained from 500 μg of aspartoacylase in 25 mM ammonium bicarbonate buffer (pH 7.9) and digested at 37 °C overnight (>10 h). Peptides were desalted using a