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Archives of Biochemistry and Biophysics 413 (2003) 1–8

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Purification and preliminary characterization of brain aspartoacylase

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Received 30 December 2002, and in revised form 23 January 2003

Abstract

Aspartoacylase catalyzes the deacetylation of *N*-acetylaspartic acid (NAA) in the brain to produce acetate and L-aspartate. An aspartoacylase deficiency, with concomitant accumulation of NAA, is responsible for Canavan disease, a lethal autosomal recessive disorder. To examine the mechanism of this enzyme the genes encoding murine and human aspartoacylase were cloned and expressed in *Escherichia coli*. A significant portion of the enzyme is expressed as soluble protein, with the remainder found as inclusion bodies. A convenient enzyme-coupled continuous spectrophotometric assay has been developed for measuring aspartoacylase activity. Kinetic parameters were determined with the human enzyme for NAA and for selected *N*-acyl analogs that demonstrate relaxed substrate specificity with regard to the nature of the acyl group. The clinically relevant E285A mutant reveals an altered enzyme with poor stability and barely detectable activity, while a more conservative E285D substitution leads to only fivefold lower activity than native aspartoacylase.

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Keywords: Aspartoacylase; Activity assay; Enzyme purification; Substrate specificity

Aspartoacylase (EC 3.5.1.15) catalyzes the deacetylation of *N*-acetyl-L-aspartic acid (NAA)¹ to produce acetate and L-aspartate and appears to be the only enzyme in brain that can effectively metabolize NAA. Although the exact role of this enzymatic reaction in the brain has not been completely elucidated, NAA appears to be necessary for the formation of myelin lipids [1]. NAA is synthesized in neuronal mitochondria from aspartate and acetyl-CoA, catalyzed by the enzyme L-aspartate-*N*-acetyltransferase [2]. Following transport to the cytoplasm, NAA is hydrolyzed by the enzyme aspartoacylase to aspartate and acetate [3]. A decrease in neuronal NAA concentration has been observed in many neurodegenerative diseases, including epilepsy [4], multiple sclerosis [5], myotrophic lateral sclerosis [6], and Alzheimer's disease [7]. In contrast, an increase in NAA levels in neurons has been hypothesized to be the

causative agent for Canavan disease, a fatal neurodegenerative disorder occurring with highest frequency among Ashkenazi Jews of Eastern European extraction [8]. The disease is characterized by atonia of the neck muscles, hyperextension of legs and flexion of arms, blindness, severe mental defect, megaloccephaly, and then death at an early age [9]. Excretion of the excess NAA in these patients results in urinary NAA concentrations that are elevated by more than 60-fold above normal levels [10]. The demyelination that is observed in Canavan disease is proposed to be a consequence of the osmotic pressure generated by the accumulation of NAA to extremely high levels in oligodendrocytes [11]. A deficiency in brain aspartoacylase activity has been correlated with abnormally high NAA levels, thus establishing aspartoacylase as the biochemical defect in Canavan disease [8].

Aspartoacylase has been partially purified from rat brain [12] and was subsequently purified to homogeneity with a final yield of 70 µg from bovine brain [13]. Immunostaining techniques have suggested that aspartoacylase may be a membrane-associated enzyme [13], although soluble preparations have been obtained in the presence of low levels of various detergents. Based upon

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¹ Abbreviations used: DTT, dithiothreitol; ESI, electrospray ionization; ICP, ion-coupled plasma; IPTG, isopropylthiogalactoside; NAA, *N*-acetyl-L-aspartic acid; Caps, 3-(cyclohexylamino)-1-propane-sulfonic acid.

its inactivation by diisopropylfluorophosphate, a classic inactivator of serine proteases, aspartoacylase was suggested to be a member of this enzyme family, and the presence of a catalytic serine, histidine, glutamate triad was postulated [14]. However, alignment studies show few similarities with this protease family [15], while local sequence similarities with the zinc-carboxypeptidase family led to the suggestion that aspartoacylase is a zinc-dependent peptidase [16]. Despite having as low as 10% overall sequence identity between these enzyme families, the essential zinc-binding functional groups that are present in carboxypeptidases are conserved in the aspartoacylases.

The divalent cations Zn^{2+} , Mn^{2+} , Mg^{2+} , and Ca^{2+} have each been reported to activate the enzyme. However, addition of these cations to the purified enzyme leads to only slight increases in catalytic activity, while assays conducted in the presence of metal ion chelators showed no loss in activity [13]. It has also been suggested that aspartoacylase activity can be regulated both by glycosylation and by phosphorylation/dephosphorylation. A putative *N*-glycosylation site (Gln117 in the human enzyme), consisting of a Gln-X-Thr recognition sequence, and several potential phosphorylation sites have been proposed [14]. Human aspartoacylase is encoded by six exons, and a number of defects, including deletions, missense mutations, and terminations, have been identified throughout the entire *ACY2* gene [17]. Analysis of DNA isolated from Canavan disease patients has identified two mutation sites that are responsible for greater than 95% of the enzyme defects among patients of Jewish origin. The most prevalent mutant, glutamic acid-285 to alanine, is found in nearly 85% of the patients and results in an aspartoacylase with very low catalytic activity [18]. A nonsense mutation at tyrosine-231, seen in about 10% of these patients, leads to a truncated, inactive enzyme.

With the aim of addressing the paucity of mechanistic information regarding aspartoacylase, we have cloned, expressed, and purified samples of both the murine and the human enzymes from *Escherichia coli*. A simple, continuous spectrophotometric assay for measuring aspartoacylase activity has been developed, and several alternative substrates have been identified by this assay.

Materials and methods

N-Acetyl-L-aspartate, *N*-chloroacetyl-L-aspartate, *N*-formyl-L-aspartate, and *N*-trifluoroacetyl-DL-aspartate were purchased from Aldrich. Dichloroacetyl chloride and trichloroacetyl chloride were purchased from Aldrich and used as received. Restriction enzymes, Vent polymerase, and ligase were purchased from New England Biolabs. The pET vectors and Nova Blue, Rosetta, and BL21(DE3) cell lines were from Novagen.

Plasmid miniprep kits and gel extraction kits were purchased from Qiagen. Tryptone, yeast, and agar were obtained from Difco Laboratories. Chromatography resins used for protein purification were from Amersham Biosciences. BugBuster detergent cell lysis mixture was purchased from Novagen. Site-directed mutagenesis was performed with the Stratagene QuikChange kit according to the manufacturer's protocol.

Enzyme assays

Assays were performed on a Perkin-Elmer Lambda-1 spectrophotometer equipped with a thermostated cell holder connected to a circulating water bath held at 30 °C. In this new coupled assay, the rate of aspartate produced was measured by deamination and then monitoring of the increasing fumarate concentration at 240 nm ($\epsilon = 2.53 \text{ mM}^{-1} \text{ cm}^{-1}$). A typical activity assay consisted of 50 mM Hepes, pH 7.0, $\sim 5 \mu\text{g}$ of aspartoacylase, 20 μg of aspartase, and 5 mM *N*-acetyl-L-aspartate in a total volume of 1.0 ml. Kinetic parameters for the alternative substrates were determined by varying each of the *N*-acylaspartate concentrations and fitting the initial rate data to an Enzyme Kinetics software package adapted from the programs of Cleland [19]. Compounds that were determined not to be substrates were tested as potential inhibitors by adding them in increasing amounts to the standard assay in the presence of NAA.

Cloning, expression, and purification of aspartoacylase

The murine and human genes encoding aspartoacylase (*mACY2*, *hACY2*)² were each amplified by PCR with an *NdeI* site at the 5' end and an *EcoRI* site at the 3' end. To facilitate the subsequent cloning each gene was subjected to site-directed mutagenesis to change a tyrosine codon from TAT to TAC, resulting in a silent mutation that removes an internal *NdeI* restriction site. Mutagenic colonies were identified based upon the absence of the internal *NdeI* recognition sequence (CA-TATG). Ligations were performed with the appropriate amplified gene and pET41 vector, each of which had been digested with *NdeI* and *EcoRI*. Plasmid DNA was sequenced and then transformed into Rosetta Codon-Plus *E. coli* cells for expression. In a typical purification a starter culture (50 ml) of LB medium containing kanamycin (30 $\mu\text{g/ml}$) was inoculated with *hACY2*-containing cells and grown to midlog phase. Four liters of LB medium was inoculated with 0.1% culture volume of the starter culture and grown until $A_{600} \cong 0.7$, then induced with 1 mM IPTG, and further shaken at 32 °C for

² Since the gene name *ASPA* is used to describe both aspartoacylase (EC 3.5.1.15) and L-aspartate (EC 4.3.1.1), to avoid confusion the alternative gene name *ACY2* is used here to identify the aspartoacylase coding sequence.

3.5 h. Cell paste was isolated by centrifugation at 12,000g for 20 min. A portion of the cell paste was treated with BugBuster detergent containing benzonase at room temperature for 20 min with stirring and then subjected to centrifugation. While some soluble aspartoacylase activity was observed, a significant portion of the enzyme was collected in the form of insoluble inclusion bodies that were separated from the soluble fraction. Inclusion bodies were washed twice with 20 mM Tris, pH 7.5, 10 mM EDTA, 1% Triton X-100 and then resolubilized at room temperature in 50 mM Caps, pH 11.0, with 0.3% *N*-laurylsarcosine. The remaining insoluble debris was separated by centrifugation. The supernatant was exhaustively dialyzed against 50 mM Tris, pH 7.5, 1 mM Mg(OAc)₂, 1 mM DTT, and 0.05% Triton X-100. A portion of this solution was passed through a Q-Sepharose XL anion-exchange column and then immediately eluted with a potassium chloride gradient up to 250 mM KCl to obtain homogeneous enzyme. Protein concentrations were determined according to the method of Bradford [20].

Synthesis of *N*-dichloroacetyl- and *N*-trichloroacetyl-*L*-aspartate

N-Dichloroacetyl-*L*-aspartate. Those *N*-acylated *L*-aspartate derivatives not commercially available were prepared by minor modifications to a published method [21]. Briefly, a stirred suspension of *L*-aspartic acid (2.01 g, 15.1 mmol), carbon tetrachloride (20 ml), granular zinc (~0.9 g), and dichloroacetyl chloride (4.6 ml, 47.8 mmol) in anhydrous ethyl acetate (120 ml) was heated under reflux for 5 h and then concentrated. Treatment of the viscous oil with dichloromethane (80 ml) prompted the formation of a white precipitate (*N*-dichloroacetyl-*L*-aspartic anhydride) that was isolated by filtration and dried in vacuo. A stirred suspension of the anhydride (1.02 g, 4.5 mmol) in water (10 ml) slowly dissolved upon formation of product. After removal of water by rotary evaporation the crude *N*-dichloroacetyl-*L*-aspartic acid was triturated with chloroform and further dried: yield, 0.711 g (46%); ¹H NMR (DMSO-*d*₆, 400 MHz) δ 12.78 (bs, 2H, COOH), 8.89 (d, 1H, *J* = 8 Hz, NH), 6.57 (s, 1H, CHCl₃), 4.53 (m, 1H, *J* = 8 Hz), 2.72 (m, 2H, AB₂ system); ¹³C NMR (DMSO-*d*₆) δ 171.45 (COOH), 171.34 (COOH), 163.37 (CO), 66.36 (CHCl₂), 49.21 (CH), 35.49 (CH₂). Elemental anal. Calcd for C₆H₇Cl₂NO₅: requires C, 29.53; H, 2.89. Found: C, 29.17; H, 2.96.

N-trichloroacetyl-*L*-aspartate. The intermediate *N*-trichloroacetyl anhydride (1.00 g, 4.0 mmol), prepared as described above, was stirred as a suspension in water (10 ml) that dissolved upon addition of sodium hydroxide. The *N*-trichloroacetyl-*L*-aspartic acid was separated as a white powder by filtration after treatment with 1 M HCl: yield, 0.711 g (37%); ¹H NMR (DMSO-

*d*₆, 400 MHz) δ 12.78 (bs, 2H, COOH), 9.19 (d, 1H, *J* = 8 Hz, NH), 4.59 (m, 1H, *J* = 8 Hz), 2.80 (m, 2H); ¹³C NMR (DMSO-*d*₆) δ 171.54 (COOH), 171.14 (COOH), 161.22 (CO), 92.41 (CCl₃), 50.53 (CH), 34.94 (CH₂). Elemental anal. Calcd for C₆H₆Cl₃NO₅: requires C, 25.88; H, 2.17. Found: C, 26.16; H, 2.22.

Purification of the coupling enzyme

Aspartase was expressed in *E. coli* by ligation of a 1.8-kb *NdeI/HindIII* fragment containing the *ASPA*² gene into a pET41a vector. A positive clone was transformed into *E. coli* BL21(DE3) cells for expression. Four liters of LB medium containing kanamycin (30 μg/ml) were inoculated with 0.1% culture volume and grown until *A*₆₀₀ ≈ 0.6, then induced with 1 mM IPTG, and further shaken at 32 °C for 3.5 h. Aspartase was then purified by an optimization of our published method [22]. Cell paste (3.5 g) was isolated by centrifugation and disrupted by sonic oscillation in 35 ml of buffer (50 mM potassium phosphate, pH 7.0, 1 mM EDTA, 1 mM DTT). The clarified extract was loaded onto a Q-Sepharose XL anion-exchange column (1.6 × 40 cm) and eluted with a linear KCl gradient (0 to 1 M). Active fractions were pooled and dialyzed against 30 mM Hepes (3 × 2 L), pH 7.0, with 5 mM Mg(OAc)₂ and 1 mM DTT. Yield of purified enzyme: 240 mg, ca. 95% homogeneous by SDS-PAGE, with specific activity of 50 μmol/min/mg. The activity of aspartase was monitored during purification by measuring the formation of fumarate at 240 nm (*E*₂₄₀ = 2.53 mM⁻¹ cm⁻¹) in 30 mM Hepes, pH 7.0, 1 mM Mg(OAc)₂, and 15 mM *L*-aspartic acid.

Electrophoresis and sequencing

Precast Tris-glycine gels (4% stacking; 10% resolving or 4–20% linear gradient gels) purchased from Bio-Rad were used for SDS-polyacrylamide gel electrophoresis. Coomassie brilliant blue R-250 staining for 2–4 h and overnight destaining with a 5% methanol/7% acetic acid solution were used for visualization. To quantitate the relative intensities of the stained bands, gel images were scanned into a personal computer and examined with digitizing software (UN-SCAN; Silk Scientific). Gene inserts were characterized by restriction mapping and sequencing of the plasmid DNA by the fluorescent dideoxyterminator method (ACTG, Inc.).

Spectrometry

Mass spectra were obtained from The Ohio State University CCIC-Mass Spectrometry Facility on a Micromass Q-ToF spectrometer with an orthogonal nanospray source operated in positive-ion mode. The enzyme was prepared in a solution containing 50%

acetonitrile/50% water, 0.1% formic acid at ca. 1 pmol/ μ l. ESI data were deconvoluted using MaxEnt I (Micromass).

The metal ion content of the purified aspartoacylase samples was measured by ion-coupled plasma (ICP) spectroscopy on a Perkin–Elmer Plasma II emission spectrometer. After zinc was identified as the major metal ion present, zinc standards were run to establish the linear response region and the limits of detectability in the buffer matrix.

Results

Aspartoacylase purification

The *ACY2* genes encoding human and murine brain aspartoacylase were amplified by PCR from plasmid DNA and expressed with a pET expression system in Rosetta Codon-Plus *E. coli* cells. The enzyme was expressed primarily as inclusion bodies; however, a moderate portion of aspartoacylase was obtained as soluble extract (Fig. 1), with a specific activity of 0.05 μ mol/min/mg. Variation of the postinduction temperature from 25 to 37 $^{\circ}$ C showed no significant change in the fraction of soluble enzyme obtained. The soluble extract can be kept at 4 $^{\circ}$ C for 2–3 days without significant loss of activity. The human and murine enzymes displayed similar activity, and no significant differences were observed in either the solubility or the stability of these enzymes. Several approaches were explored to purify the soluble fraction of unmodified aspartoacylase. *E. coli*-expressed

aspartoacylase can be partially captured by anion-exchange chromatography. However, precipitation of aspartoacylase on the column significantly diminishes the yield of purified enzyme, resulting in less than 10% recovery of total units. The same phenomenon was observed upon passage of a hexahistidine-tagged extract through a Ni–NTA column under nondenaturing conditions. Although the enzyme displayed no affinity for cation-exchange resin (HiTrap SP; Amersham) in preliminary trials, some impurities were removed in this manner by virtue of their affinity for this resin. Hydrophobic interaction chromatography was generally not applicable due to the tendency of the enzyme to form a nonrecoverable precipitate on this family of resins.

Growth of transformed *E. coli* cells in 4 L of LB medium yields ca. 9 g of cell paste and 500 mg of washed inclusion bodies with a specific activity of 0.2 U/mg. Samples of up to 200 mg and with 90–95% purity were obtained from refolded inclusion bodies carried out in a Tris or Hepes buffer near neutral pH in the presence of 1 mM DTT, \sim 0.1% Triton X-100 or NP-40, 0.5 mM Zn(OAc) $_2$, and 1 mM Mg(OAc) $_2$. However, removal of the detergent from the buffer, either by sequential dialysis or with β -cyclodextrin, results in a dramatic loss of aspartoacylase solubility.

Enzyme activity assay

A new aspartoacylase assay has been developed in which the production of aspartate is coupled to the formation of fumarate catalyzed by aspartase (Scheme 1). Initial attempts with this assay were successful in observing the catalytic activity of aspartoacylase, although some refinement was required to optimize the assay conditions. Adjusting the levels of the aspartase coupling enzyme eliminated an initial lag that was observed in the course of the reaction. The high levels of detergent and DTT used in the original end-point assay were found to produce an unacceptably high background absorbance at the wavelength at which fumarate is detected (240 nm), so these components were either reduced or eliminated from the assay protocol. The optimized assay gives a linear response across a wide range of substrate concentrations and functions throughout the pH range in which aspartoacylase is relatively stable and active.

Substrate specificity

Several *N*-acylated aspartate analogs were examined as potential alternative substrates for aspartoacylase,

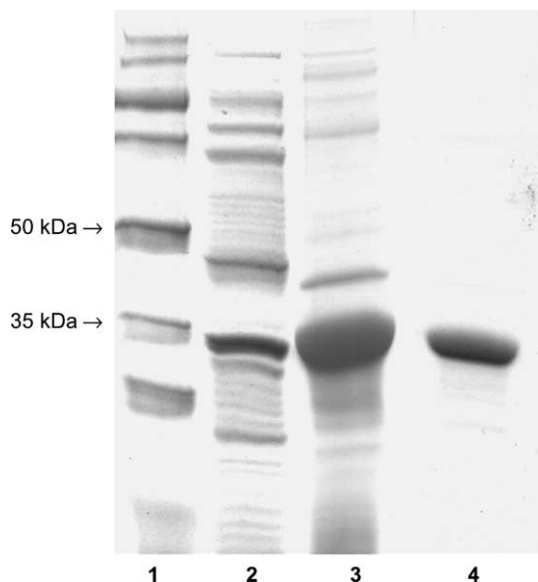
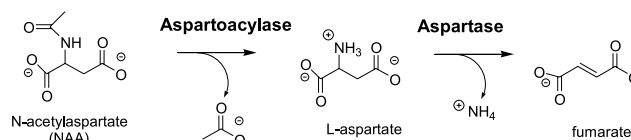


Fig. 1. Lane 1—MW markers. Lane 2—soluble extract of *mACY2* expressed in *E. coli* Rosetta cells. Lane 3—insoluble portion of cell paste. Lane 4—refolded inclusion bodies purified by anion-exchange chromatography.



Scheme 1. Aspartase-coupled aspartoacylase assay.

Table 1
Kinetics of alternative substrates for aspartoacylase

Substrate	k_{cat} (s^{-1})	K_{m} (mM)	$k_{\text{cat}}/K_{\text{m}}$ ($\text{M}^{-1} \text{s}^{-1}$)	Relative $k_{\text{cat}}/K_{\text{m}}$
<i>N</i> -Acetyl-L-aspartate	$0.083 \pm .006$	$0.36 \pm .06$	2.3×10^2	1.0
<i>N</i> -Formyl-L-aspartate	$0.25 \pm .03$	$0.95 \pm .23$	2.6×10^2	1.1
<i>N</i> -Chloroacetyl-L-aspartate	$0.62 \pm .04$	$0.59 \pm .09$	1.0×10^3	4.3
<i>N</i> -Dichloroacetyl-L-aspartate	$0.77 \pm .03$	$0.23 \pm .03$	3.4×10^3	15
<i>N</i> -Trifluoroacetyl-DL-aspartate ^a	$1.2 \pm .09$	$0.21 \pm .04$	5.8×10^3	25

^a Kinetic parameters were corrected for the presence of the D-isomer, which is presumed to be inert.

with the concentrations varied from 0.1 to 5 mM. Since the stability of refolded enzyme was considerably lowered when the detergent was decreased to acceptably low levels for the assay, fresh extract was used for the substrate specificity determinations. The results show that *N*-formylaspartate is a better substrate for aspartoacylase, with a 3-fold higher k_{cat} than the physiological substrate NAA (Table 1). The *N*-chloroacetyl analog shows a further 2-fold improvement in k_{cat} , with an overall 4-fold increase in $k_{\text{cat}}/K_{\text{m}}$, and the *N*-dichloroacetyl derivative has a 15-fold higher $k_{\text{cat}}/K_{\text{m}}$. This trend is reversed by the *N*-trichloroacetyl analog, which is an extremely poor substrate with a barely detectable rate. Those derivatives containing the more sterically hindered *N*-*t*-butylacetyl, *t*-butoxyacetyl, or guanidino groups were also found to be very poor substrates. Likewise, aspartate analogs in which the β -carboxyl group is derivatized (*N*-acetyl-L-asparagine) or longer homologs (*N*-acetyl-L-glutamate) do not show appreciable affinity for aspartoacylase. However, the *N*-trifluoroacetyl derivative is the best substrate that was examined, with a $k_{\text{cat}}/K_{\text{m}}$ that is 25-fold higher than that of NAA.

While the enzyme does have some flexibility in the acyl group structure, it is highly specific for aspartate as the amino acid. Neutral amino acids such as *N*-acetylvaline, -leucine, or -alanine are neither substrates nor inhibitors, nor are amides such as *N*-acetyl-asparagine or *N*-acetylglutamine. Even the homologous amino acid *N*-acetylglutamate is neither a substrate nor an inhibitor of aspartoacylase at the levels (10 mM) that were examined.

Effect of metal ions

To examine the proposed role of metal ions in the activity of aspartoacylase several divalent metal ions were introduced. The insoluble inclusion bodies of aspartoacylase were refolded by dialysis as before, but now in the presence of either 0.5 mM ZnCl_2 or 0.5 mM CoCl_2 . These enzyme samples were further dialyzed against several changes of buffer containing no divalent metal ions in order to remove any unbound metals. A control sample was refolded as above, but without added metal ions, and then exhaustively dialyzed against Tris buffer containing 2 mM EDTA to remove any trace metal ions. The samples treated with EDTA displayed

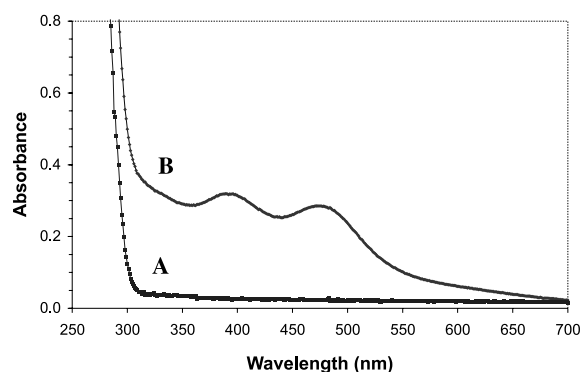


Fig. 2. UV-visible spectrum of aspartoacylase refolded from inclusion bodies after treatment with (A) 0.5 mM EDTA or (B) 0.5 mM CoCl_2 . Unbound CoCl_2 was removed by dialysis prior to analysis.

three- to fourfold lower V_{max} values relative to those values reported in Table 1 for the untreated native enzyme. The UV-visible spectrum of the enzyme in the presence of Co^{2+} (Fig. 2) shows broad absorption peaks with maxima at 390 and 480 nm that are indicative of cobalt binding to aspartoacylase. The Zn^{2+} -treated sample of aspartoacylase was analyzed by ICP emission spectroscopy to determine the presence of bound zinc. This aspartoacylase sample was found to contain approximately two atoms of zinc per enzyme subunit (data not shown). However, the zinc- and cobalt-treated enzymes have approximately the same activity as the native enzyme that was refolded without added metal ions.

Posttranslational modification

A purified sample of refolded murine aspartoacylase was examined by ESI mass spectrometry to screen for any deviation from the predicted molecular mass of 35,304 Da that would indicate that the enzyme had been covalently modified after translation. A fit to the family of m/z peaks from this spectrum gave a mass of $35,171 \pm 1$ (Fig. 3). This result is consistent with the mass that would be expected upon removal of the *N*-terminal methionine after expression, a phenomenon frequently observed for proteins expressed in *E. coli* [23]. No other peaks were detected that would suggest covalent modifications to the primary amino acid sequence of aspartoacylase. However, two sets of m/z peaks were observed at a ratio of approximately 2 to 1. The major

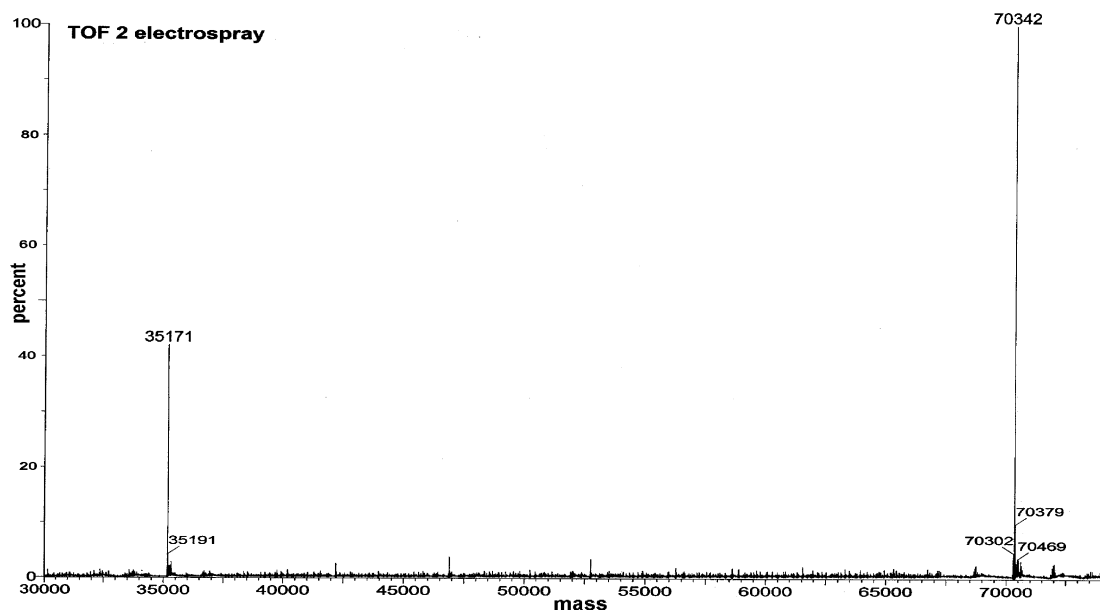


Fig. 3. Electrospray time of flight mass spectrum of murine aspartoacylase. The peak at 35,171 is the aspartoacylase monomer, and the peak at 70,342 is the enzyme dimer. The theoretical MW of aspartoacylase after cleavage of the *N*-terminal methionine is 35,172.

peak is consistent with the mass predicted for an aspartoacylase dimer, and the minor peak matches the mass of an intact monomer.

Site-directed mutagenesis of glutamic acid-285

A large number of mutations have been identified throughout the *ACY2* genes that have been isolated from Canavan patients, with mutations at glutamate-285 being the most common [18]. Two mutations, E285A and the more conservative E285D, were constructed at this position in the *hACY2* gene to determine whether these mutations have a direct effect on the catalytic properties of human aspartoacylase. The E285A mutant has approximately 1% catalytic activity relative to native enzyme. This variant is also quite unstable, with no residual catalytic activity detected after several hours. In contrast the E285D mutant has a k_{cat} value that is approximately 20% that of the native enzyme and is relatively stable, with at least 90% residual activity after several hours.

Discussion

The published assay for aspartoacylase requires incubation of the enzyme with NAA for a fixed (30 min to 2 h) time, followed by an end-point quantitation of the L-aspartic acid produced using an aspartate aminotransferase/malate dehydrogenase-coupled enzyme system [13]. If the substrate specificity and the role of different amino acid functional groups are to be systematically examined, then a sensitive continuous activity assay is highly desirable. Such a protocol was

devised by coupling the aspartoacylase-catalyzed deacetylation of NAA to the deamination of aspartic acid catalyzed by aspartase. Initial rate data are typically obtained with this assay in several minutes, although the reaction remains linear for at least several hours. Aspartase has been shown to be highly specific for aspartic acid, so the activity measurements should not suffer from interference by reaction with NAA. Indeed, it has previously been shown that NAA is not a substrate of aspartase, but only a weak inhibitor at high concentrations [24]. Control experiments conducted with aspartase and each *N*-acylated aspartic acid showed no fumarate production when aspartoacylase was omitted from the assay mixture.

Cell extracts were typically isolated in BugBuster, a proprietary blend of detergents designed not to absorb in the UV region. Purified enzyme obtained from refolded inclusion bodies is somewhat unstable without Triton X-100 or other nonionic detergents. However, these detergents generally absorb in the region of the UV spectrum in which the assay is conducted, thus preventing their inclusion as stabilizing additives. Detergent removal by dialysis, or by stripping with β -cyclodextrin, results in precipitated enzyme. Refolding in the presence of very low levels of Triton X-100 ($\sim 0.01\%$) gave insoluble enzyme. Therefore, substrate specificity kinetics were carried out with the soluble cell extract containing the overexpressed human enzyme. The soluble enzyme in a typical extract represented approximately 10–15% of the total protein and retained aspartoacylase activity for several days at 4°C. These levels of aspartoacylase in cell extracts extrapolate to a specific activity of about 0.5 units/mg for the purified

enzyme. This is well below the reported value of approximately 20 units/mg determined with the fixed-point assay for the enzyme isolated from bovine brain [13] and suggests the need for additional processing to achieve a fully active enzyme. Control experiments with extracts from uninduced cells showed no interference from competing activities with our newly developed assay, allowing us to measure the kinetic parameters of aspartoacylase in cell extracts. The measured K_m value for NAA (Table 1) is within a factor of 2 of the reported value for the bovine enzyme.

Aspartoacylase has been shown to accommodate a range of derivatized *N*-acyl groups on *L*-aspartate as alternative substrates. Increasing the electron withdrawing capability of the acyl group leads to an accelerated rate of enzyme-catalyzed hydrolysis, with up to a 25-fold increase for a substrate with an *N*-trifluoroacetyl group. This observation suggests that the mechanism of hydrolysis of NAA involves an initial nucleophilic attack at the carbonyl group. Such a mechanism, consistent with that proposed for the metalloproteases, would be accelerated by derivatives that make the carbonyl carbon more electron deficient. However, there are steric limitations to the nature of the derivatized groups that can be accepted at the active site of aspartoacylase. For example, when the *N*-acetyl group is replaced by either a *t*-butylacyl or a guanidino group there is only a very slow (overnight) yet measurable rate. It had been reported that *N*-acetyl-*L*-asparagine is an alternative substrate that yields aspartic acid upon treatment with aspartoacylase [25], suggesting that the enzyme could catalyze a second amide hydrolysis of this substrate. However, we were unable to measure any production of aspartic acid using our coupled activity assay. This compound also did not inhibit the rate with NAA as the substrate, indicating a lack of interaction at the active site of aspartoacylase, and thus shows the importance of a free β -carboxyl group for substrate binding.

A purified and dialyzed sample of native human aspartoacylase was analyzed by ICP emission spectroscopy to test for the presence of metal ions. Zinc was the only metal ion identified at significant levels in the enzyme sample. The effect of metal ions on aspartoacylase activity was examined by refolding the inclusion bodies obtained during cell growth using the protocol described above. The refolding experiments were conducted either in the presence of zinc or with added cobalt, which has been shown to substitute for zinc in some metalloenzymes and can be detected by its UV-visible spectrum. The spectrum of the Co^{2+} -enzyme shows broad absorption peaks at 390 and 480 nm and a molar absorptivity that, based on model compounds, is characteristic of cobalt in an octahedral coordination environment. The aspartoacylase samples that were refolded in the presence of either Zn^{2+} or Co^{2+} showed a three- to fourfold increase in catalytic activity compared with the

same enzyme preparation refolding in the presence of EDTA, but no additional enhancement over the native activity. These kinetic and spectroscopic results support the presence of a metal ion binding site in aspartoacylase; however, a critical functional role for this metal ion has not been definitively established.

The observed mass values suggest the absence of metal ion cofactors in the enzyme, but any bound metal ions could have dissociated under the mass spectral experimental conditions. Interestingly, the major component of the mass spectrum was not the monomer, but a dimer. There have been no reports suggesting that aspartoacylase exists as a dimer, and it is unusual for such noncovalent intersubunit interactions to persist under these experimental conditions. The identification of a dimer as the major species suggests a high affinity for subunit association that is consistent with the tendency of the purified enzyme to aggregate and precipitate.

The most prevalent clinical mutation of aspartoacylase is a single A-to-C base change that leads to the replacement of glutamate-285 with an alanine [14]. To probe the importance of this amino acid, an E285A and a more conservative E285D mutant were constructed. The E285A variant is quite unstable, displaying only a trace of catalytic activity that is lost after several hours. In contrast the E285D mutant is more stable and has a k_{cat} value that is nearly 20% that of the native enzyme. The instability observed upon removal of the carboxylate at position 285 is consistent with the notion that many of the nonconservative clinical mutants exert their deleterious effect because of a loss of structural integrity, rather than causing a direct disruption of the catalytic events taking place at the active site.

The instability and low catalytic activity of purified aspartoacylase, and the proposed presence of phosphorylation and glycosylation sites, suggests a requirement for additional processing to achieve the mature enzyme. The human aspartoacylase has been overexpressed and purified from inclusion bodies and from soluble extract using an *E. coli* expression system. This expression system is quite efficient, but is unable to support any posttranslational modifications that may be necessary for full in vivo activity or stability of aspartoacylase. Expression in a suitable eukaryotic system may be necessary to produce a fully functional, mature enzyme.

Acknowledgments

The authors thank Dr. Aryan Namboodiri for his generous gift of the *mACY2* and *hACY2* genes. Kari B. Greenchurch at The Ohio State University CCIC–Mass Spectrometry Facility is thanked for helpful discussions on the interpretation of the mass spectral results. We acknowledge Dr. Pannee Burckel and David Coe at the

University of Toledo for help with the ICP metal analyses.

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