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## Characterization of the *N*-acetylaspartate biosynthetic enzyme from rat brain

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### Abstract

Aspartate *N*-acetyltransferase (Asp-NAT; EC 2.3.1.17) activity was found in highly purified intact mitochondria prepared by Percoll gradient centrifugation as well as in the three sub-fractions obtained after the sucrose density gradient centrifugation of Percoll purified mitochondria; citrate synthase was used as a marker enzyme for mitochondria. The proportion of recoverable activities of Asp-NAT and citrate synthase were comparable in mitochondrial and synaptosomal fractions but not in the fraction containing myelin. Asp-NAT was solubilized from the pellet of the rat brain homogenate (26 000 *g* for 1 h) for the recovery of maximum activity and partially purified using three protein separation methods: DEAE anion exchange chromatography, continuous elution native gel electrophoresis and size-exclusion high performance liquid chromatography. Asp-NAT activity and the optical density pattern of the eluted protein from size-exclusion column indicated a single large protein (~670 kDa), which on sodium

dodecyl sulfate–polyacrylamide gel electrophoresis showed at least 10 bands indicative of an enzyme complex. This seemingly multi-subunit complex Asp-NAT was stable towards ionic perturbations but vulnerable to hydrophobic perturbation; almost 95% of activity was lost after 10 mM 3-[(3-cholamidopropyl)dimethylammonia]-1-propanesulfonate (CHAPS) treatment followed by size-exclusion chromatography. Asp-NAT showed an order of magnitude difference in  $K_m$  between L-aspartate (L-Asp, ~0.5 mM) and acetyl CoA (~0.05 mM). Asp-NAT showed high specificity towards L-Asp with 3% or less activity towards L-Glu, L-Asn, L-Gln and Asp-Glu. A model on the integral involvement of NAA synthesis in the energetics of neuronal mitochondria is proposed.

**Keywords:** enzyme kinetics, high performance liquid chromatography, mitochondrial energetics, native gel electrophoresis, phosphor imaging, thin-layer chromatography. *J. Neurochem.* (2003) **86**, 824–835

*N*-Acetylaspartate (NAA) is an abundant (5–10 mM) amino acid derivative of the nervous system (Tallan *et al.* 1956) and is used clinically as a non-invasive marker for the functional integrity of neurons using magnetic resonance spectroscopy (for reviews see Birken and Oldendorf 1989; Tsai and Coyle 1995). Aspartate-*N*-acetyltransferase (Asp-NAT; EC 2.3.1.17) catalyses acetylation of L-aspartate (Asp) by acetyl CoA to form NAA (Goldstein 1959; Knizley 1967; Truckenmiller *et al.* 1985). Earlier studies have shown that NAA is localized primarily in neurons (Moffett *et al.* 1991; Simmons *et al.* 1991; Moffett and Namboodiri 1995) and also demonstrated in cultured oligodendrocytes (Bhakoo and Pearce 2000). Subsequent studies have shown decreases in NAA in specific neuronal systems in a number of neurological disorders (cf. Tsai and Coyle 1995; Clark 1998). The importance of NAA in neuronal functions has been further emphasized by recent reports that show a strong association between NAA and cognitive ability and intelligence in

humans (Jung *et al.* 1999a,b). Also, decreased degradation of NAA resulting from a congenital defect in the degradative enzyme aspartoacylase (EC 3.5.1.15) causes Canavan disease, an autosomal-recessive neurodegenerative disorder that develops after birth and results in death before 10 years of age (cf. Matalon *et al.* 1995).

NAA synthesis was reported to be mitochondrial (Patel and Clark 1979) and hence decrease in the level of NAA is thought to indicate mitochondrial dysfunction (cf. Clark

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**Abbreviations used:** Asp-NAT, aspartate *N*-acetyltransferase; CHAPS, 3-[(3-cholamidopropyl)dimethylammonia]-1-propanesulfonate; L-Asp, L-aspartate; NAA, *N*-acetylaspartate; NAAG, *N*-acetylaspartylglutamate; NAG, *N*-acetylglutamate; TLC, thin-layer chromatography.

1998; Signoretti *et al.* 2001). Though NAA has been implicated in a number of neurological disorders, very little is known about the enzyme that synthesizes NAA. This lack of information poses a major hurdle in understanding the precise role of NAA in neurological disorders, especially in attributing whether it is a cause or effect, or merely a marker reflecting the overall physiology of mitochondria. In the present study, we show that Asp-NAT activity is present in Percoll gradient purified brain mitochondria and in the subfractions of the Percoll purified mitochondria after sucrose density gradient purification, and that the partially purified enzyme from rat brain might be an enzyme complex having a molecular weight of about 670 kDa. Also, we have characterized the enzyme in terms of substrate specificity, enzyme kinetics and product inhibition. Furthermore, we have proposed a model involving mitochondrial energetics to further study the functional roles of NAA.

## Materials and methods

### Materials

Solvents, Percoll and unlabelled L-Asp were bought from Sigma (St. Louis, MO, USA). Tris, glycine, 30% acrylamide solution, ammonium persulfate, N,N,N,N'-Tetra-methyl-ethylenediamine (TEMED) and DEAE cellulose were from Bio-Rad (Hercules, CA, USA) and 3-[(cholamidopropyl)dimethylammonia]propane-sulfonate (CHAPS) was from Calbiochem (La Jolla, CA, USA). Ultra-pure glycerol, 10× phosphate-buffered saline solution and pre-cast 10% Tris-glycine polyacrylamide gel electrophoresis gels were bought from Invitrogen (Carlsbad, CA, USA). NaCl solution (5 M) was from Digene (Beltsville, MD, USA). L-[<sup>14</sup>C]Asp and L-[<sup>14</sup>C]Glu (specific activity of 207 mCi/mmol) were purchased from Amersham Pharmacia Biotech (Piscataway, NJ, USA). [<sup>14</sup>C]Acetyl CoA was obtained from Moravsek Biochemicals (Brea, CA, USA). Aluminum supported flexible thin-layer chromatography (TLC) plates were purchased from Whatman Ltd (Maidstone, Kent, England). Phosphor image exposure cassettes were bought from Molecular Dynamics (Sunnyvale, CA, USA). Size-exclusion columns for HPLC were bought from Thomson Instruments (Clear Brook, VA, USA). All other chemicals were of analytical grade and used without further purification. Deionized and filtered Super Q water (Millipore, Bedford, MA, USA) was used for all purposes.

### Animals

Rat brains for Asp-NAT purifications were obtained from Sprague-Dawley male (12 weeks old) and female rats (6 weeks old), which were maintained according to the guidelines of National Institutes of Health (NIH). After dissection, the brains were immediately processed for mitochondrial localization studies of Asp-NAT and for Asp-NAT purification purposes, they were frozen on dry ice and stored under -80°C until further use.

### Mitochondrial purification from rat brain

Non-synaptic adult rat brain mitochondria were isolated on a Percoll gradient as described previously (Sims 1990) with minor modifications. Male Sprague-Dawley rats (300–350 g) were used for this

study. All animal procedures were carried out according to NIH and the University of Maryland, Baltimore animal care use committee guidelines. Rats were killed by decapitation, forebrains were rapidly removed, chopped and homogenized in ice-cold isolation buffer containing (in mM): mannitol 225, sucrose 75, HEPES 5, EGTA 1, pH 7.4 (adjusted with KOH). Homogenate was centrifuged at 1250 g for 3 min; pellet was discarded and the supernatant (fraction, F1) was subjected to 20 000 g for 10 min. The supernatant (fraction, PM) was discarded and the pellet (fraction, F2) was resuspended in 15% Percoll (Sigma, St Louis, MO, USA) and layered on pre-formed Percoll gradients (40 and 23%). The Percoll gradients were centrifuged at 30 000 g for 6 min. Three distinguishable fractions were identified: the band at the interface of 23% and 40% Percoll (fraction, F5) corresponding to pure intact mitochondria, a synaptosomal band (fraction, F4) above fraction F5, and the top fraction above synaptosomal band containing myelin (fraction, F3). The mitochondrial fraction located at the interface of the lower two layers was removed, diluted with isolation buffer and centrifuged at 16 600 g for 10 min. Supernatant was discarded, the loose pellet was washed further with isolation buffer (6700 g for 10 min) and the pellet was suspended in 100 µL of isolation medium without EGTA. All fractions were assayed for Asp-NAT and citrate synthase, which was used as the marker enzyme for mitochondria.

For Asp-NAT assay, aliquots from all the fractions were incubated for 1 h on ice with 10 mM CHAPS in a medium consisting of 10 mM sodium phosphate, 150 mM NaCl, 1 mM dithiothreitol, 10% glycerol, pH adjusted to 7.2. Following incubation, the samples were sonicated for 3 s (4710 Series, Ultrasonic Homogenizer, Cole-Parmer Instruments Co., Chicago, IL, USA) with the microtip probe limit set to 4 and duty cycle set to 20%. The sonicated suspensions were extensively dialysed (against a dilution of 1 : 20 000) for 1 h in a cold room. The dialysis medium consisted of 1 mM CHAPS, 1 mM dithiothreitol, 10% glycerol, 150 mM NaCl, 10 mM sodium phosphate, pH adjusted to 7.2 and the dialysed samples were used for Asp-NAT assay.

### Subfractionation of purified mitochondria

The Percoll gradient purified mitochondrial fraction (F5) was subjected to sucrose density gradient centrifugation subsequent to an osmotic swelling/shrinking and sonication as previously described (Sandri *et al.* 1988), to remove sources of potential contamination from plasma membrane, endoplasmic reticulum and cytosolic proteins. The mitochondrial suspension after osmotic swelling was adjusted to 1.37 M sucrose (density of 1.75 w/v, 0°C), sonicated and applied (1.2 mL) to the bottom of the centrifuge tubes. On the top, a discontinuous sucrose gradient was manually layered, i.e. 1.7 mL of 1.21 M (density of 1.15) followed by 2.0 mL of 1.025 M (density of 1.13). The sucrose gradient was centrifuged at 270 000 g for 2 h at 4°C. There were three distinct bands formed: a top band in between sucrose density of 1.13 and 1.15, containing the outer mitochondrial membrane; an intermediate band in between sucrose density of 1.15 and 1.17, containing the contact sites of outer and inner mitochondrial membranes; a pellet containing the inner mitochondrial membrane + matrix of the mitochondria. These three subfractions of mitochondria were verified according to the location of the bands on the sucrose gradient as described by Sandri *et al.* (1988) as well as by their protein profile: succinate dehydrogenase, pyruvate dehydrogenase complex and cytochrome oxidase as inner mitochondrial

membrane + matrix; porin as outer mitochondrial membrane and contact site marker by western blots. All these fractions were free from contamination by plasma membrane proteins as verified for the absence of marker proteins of Na/K ATPase subunits alpha 1 and alpha 3; free from contamination by endoplasmic reticulum proteins as verified for marker protein calnexin; and virtually free from any cytosolic proteins. The high sensitivity radiometric assay (L-[<sup>14</sup>C]Asp; 216 mCi/mmol) for Asp-NAT was performed on all these samples in triplicate as described below.

#### Enzyme assay

The media used in various steps of isolation and purification of Asp-NAT invariably consisted of NaCl (up to 150 mM). NaCl was maintained in the media, for the following reasons: (i) presence of salt generally increases protein stability and reduces problems of protein aggregation (cf. Timasheff 1993); (ii) concentration of NaCl up to 150 mM did not have adverse effect on Asp-NAT activity; (iii) presence of some salt in the medium decreases binding of weakly charged proteins to the resins in ion-exchange chromatography. Similarly, presence of glycerol in the medium preserved the enzyme activity during various processes of enzyme purification and handling, including freezing and thawing.

Citrate synthase activity was determined spectrophotometrically as previously described (Srere 1969). Asp-NAT activity was determined radiometrically using L-[<sup>14</sup>C]Asp and acetyl CoA as substrates in a final volume of 50  $\mu$ L. Asp-NAT activity in various fractions of mitochondrial purification was determined with final substrate concentrations of 1 mM L-Asp (with L-[<sup>14</sup>C]Asp specific activity of 10.4 mCi/mmol) and 0.5 mM acetyl CoA. Otherwise, in most cases the final concentrations of the ingredients in the assay medium were 0.5 mM L-Asp (specific activity = 10–200 mCi/mmol), 0.5 mM acetyl CoA, 40 mM sodium phosphate buffer, pH 7.1, 60–90 mM NaCl, 4–6% (w/v) glycerol, 1 mM CHAPS (which was used for solubilization of the membrane-bound enzyme proteins), 1 mM dithiothreitol and the enzyme protein. Glycerol and NaCl in the assay medium were from the enzyme buffer, which invariably consisted of phosphate-buffered saline, pH 7.1, 10% (w/v) glycerol, 1 mM CHAPS and 1 mM dithiothreitol. Assay was started by addition of the enzyme and incubation (37°C) was continued for 1 h. Reaction was stopped by adding 50  $\mu$ L of ice-cold ethanol containing 1 mM NAA (the unlabeled product) and vortex mixing the contents. Precipitates were removed by centrifuging at 30 000 g for 10 min. About 10  $\mu$ L of the supernatant was chromatographed on silica-gel TLC flexible plate. Substrates and the product were separated in a solvent system consisting of formic acid, methanol and chloroform (5 : 25 : 70). Excellent separation was achieved between L-[<sup>14</sup>C]Asp ( $R_F$ , 0.2–0.3) and the product L-[<sup>14</sup>C]NAA ( $R_F$ , 0.7–0.8). The substrate and product were identified by phosphor imaging, by exposing the plastic sheet wrapped TLC plate on to a phosphor-imaging screen overnight (about 14–16 h). Quantitation was done by determining the intensity/cpm, obtained by regression, of the L-[<sup>14</sup>C]Asp of known radioactivity spotted on the TLC plate.

#### Enzyme kinetics for Asp-NAT

The steady-state kinetic parameters  $K_m$  and  $V_{max}$  for L-Asp and acetyl CoA were determined using the DEAE ion exchange enriched fraction of the enzyme preparation (*vide infra*) by the radiometric assay described as above. In the case of L-Asp, a fixed amount of

L-[<sup>14</sup>C]Asp and varying unlabeled L-Asp (0.05–2 mM, final concentration) were used at a fixed acetyl CoA concentration (2 mM) in each assay. In the case of acetyl CoA, L-Asp was used at a constant concentration (2 mM), and acetyl CoA was varied (1  $\mu$ M–1 mM). The activity was determined by quantitating the intensity/cpm of the product [<sup>14</sup>C]NAA. Respective controls (without enzyme protein) were performed and subtracted to account for non-enzymatic acetylation of L-Asp. The activity (pmol NAA formed/h) was plotted against the respective substrate concentration and the values of the steady-state kinetic parameters were determined by non-linear iterative regression using Sigma-Plot software for Windows.

#### Inhibition of the Asp-NAT activity by products

Inhibition of the enzyme activity by NAA and CoA were determined using the DEAE ion exchange enriched fraction of the enzyme preparation (*vide infra*) by the radiometric assay described above. The assay medium consisted of 0.5 mM L-Asp (specific activity of 20 mCi/mmol), 0.5 mM acetyl CoA, 40 mM sodium phosphate buffer, pH 7.1, 60 mM NaCl, 4% (w/v) glycerol, 1 mM CHAPS, 1 mM dithiothreitol and the enzyme protein. Concentration of the product was varied from 0 mM to 10 mM. The enzyme activity was normalized to percentage activity with respect to the activity in the absence of either CoA or NAA, respectively, taken as 100%. The concentration at which enzyme activity was inhibited to 50% ( $IC_{50}$ ) by NAA and CoA was determined by fitting the data to a two constant hyperbolic decay using the Sigma Plot software for Windows.

#### Substrate specificity of Asp-NAT

Enzyme activity was determined against L-Asp, L-Glu, L-Asn, L-Gln and dipeptide L-Asp-L-Glu by using [<sup>14</sup>C]acetyl CoA as the second substrate. Assay medium consisted of 0.4 mM amino acid/dipeptide, 0.4 mM acetyl CoA (specific activity of 2 mCi/mmol), 45 mM sodium phosphate buffer, pH 7.2, 1 mM CHAPS, 60 mM NaCl, 6% glycerol, 1 mM dithiothreitol and the enzyme protein. Except in case of Asp-Glu, TLC was employed to determine the activity as described above. In the case of Asp-Glu, product *N*-acetylaspartyl-glutamate (NAAG) was separated using isocratic elution (flow rate = 1 mL/min) in a HPLC anion exchange column with a buffer of 200 mM  $KH_2PO_4$  buffer, pH 5.0. In this system, NAAG retention time was found to be ~40 min [<sup>14</sup>C]acetyl CoA was retained on the column for a longer time and was eluted with 0.5 M  $KH_2PO_4$  buffer, pH 2.0 after every third run. NAAG was detected by optical density at 231 nm and the fractions corresponding to NAAG were counted for radioactivity in a scintillation counter.

#### Asp-NAT purification

About 200 g of rat brain tissue was homogenized in a domestic blender in a medium consisting of phosphate-buffered saline, pH 7.2, 1 mM dithiothreitol and protease inhibitor cocktail (obtained either from Boehringer Mannheim or Sigma). The homogenate was centrifuged at low speed (3000 g) for 10 min to remove the debris and the resulting supernatant was again centrifuged (26 000 g for 1 h). The pellet was collected and solubilized overnight with 10 mM CHAPS solution containing 10% glycerol, 0.15 M NaCl, 10 mM sodium phosphate buffer pH 7.1 and 1 mM dithiothreitol and the protease inhibitor cocktail. Partial purification of the enzyme was achieved by following a three-step purification scheme involving

ion-exchange chromatography and native gel electro-elution (Bio-Rad) followed by size-exclusion HPLC. All equilibration and elution solutions contained 10 mM sodium phosphate buffer pH 7.1, 1 mM CHAPS, 1 mM dithiothreitol and 10% glycerol for optimal stability of the enzyme.

#### Anion-exchange chromatography

Bio-Rad macro-prep DEAE cellulose resin was used as an anion exchange matrix to separate the Asp-NAT from the bulk of the proteins. The solubilized preparation of Asp-NAT (250 mL) in a medium containing 10 mM sodium phosphate buffer pH 7.1, 0.15 M NaCl, 10 mM CHAPS, 1 mM dithiothreitol and 10% glycerol was gently mixed for 1 h in a cold room with the washed and equilibrated (0.15 M NaCl containing solution) DEAE cellulose resin (100 mL bed volume). The preparation was then poured into a glass column and the unbound fraction was collected. The column was washed sequentially: (i) with 10 column volumes of equilibrating buffer (0.15 M NaCl, 10 mM sodium phosphate buffer pH 7.1, 1 mM CHAPS, 1 mM dithiothreitol and 10% glycerol) and (ii) with five column volumes of equilibrating buffer containing 0.25 M NaCl. The enzyme activity was eluted with the equilibration buffer containing 0.5 M NaCl, collecting six fractions of 45 mL each. The fractions 2 and 3, which contained the bulk of the eluted enzyme activity were concentrated and used for further purification by native gel electro-elution.

#### Native gel electro-elution

Bio-Rad Model 491 Prep Cell was used to purify Asp-NAT after DEAE ion exchange chromatography, by continuous flow electrophoresis under native conditions. Continuous buffer of Tris-Glycine-HCl of pH 8.0 was used in both the upper and lower chamber. Concentrations of Tris and Glycine were 25 mM and 192 mM, respectively. Asp-NAT activity could not be detected in the eluates even after 48 h of electro-elution when pH 7.1 was used. Asp-NAT activity was consistently detected in eluates after 21 h at pH 8.0 and higher pH was not attempted due to enzyme stability considerations. In order to retain the enzyme activity this buffer also consisted of 6% glycerol, 1 mM CHAPS and 1 mM dithiothreitol. The elution buffer composition was phosphate-buffered saline, 10% glycerol, 1 mM CHAPS, 1 mM dithiothreitol, pH adjusted to 7.2. Resolving gel (6%) of 3 cm height and stacking gel (3%) of 3 cm height were cast overnight according to the manufacturer's instructions using 30% acrylamide/Bis, 37.5 : 1 (2.6% C) stock solutions. The gel solution also consisted of 10% glycerol, 1 mM CHAPS and Tris-Glycine-HCl buffer of pH 8.0 in order to protect the enzyme activity. Entire electro-elution was carried out in a cold room (2–4°C) at a constant power of 5 W. About 2 h of pre-electrophoresis was carried out before loading the protein sample, to remove the unreacted catalysts from the polyacrylamide gel. Sample protein (~6–8 mL) was dialysed against 2 L of a medium containing 10 mM sodium phosphate pH 7.2, 10% glycerol, 1 mM CHAPS and 1 mM dithiothreitol before loading on to the native gel column, which retained the activity of the enzyme at the same level as opposed to dialysing in the electrophoresis buffer (Tris-HCl-Glycine, pH 8.0), which resulted in a loss of activity by ~20–25%. At the elution rate of 0.3 mL/min, about 250 fractions of 5 mL were collected. Every fourth fraction was assayed after concentrating 2 mL into ~0.25 mL (Centricon YM 10 concentrator), using

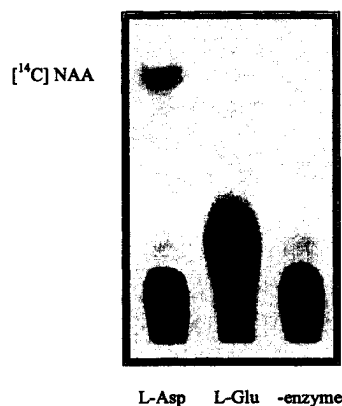
high specific activity (~100 mCi/mmol) L-[<sup>14</sup>C]Asp as substrate as described above.

#### Size-exclusion HPLC

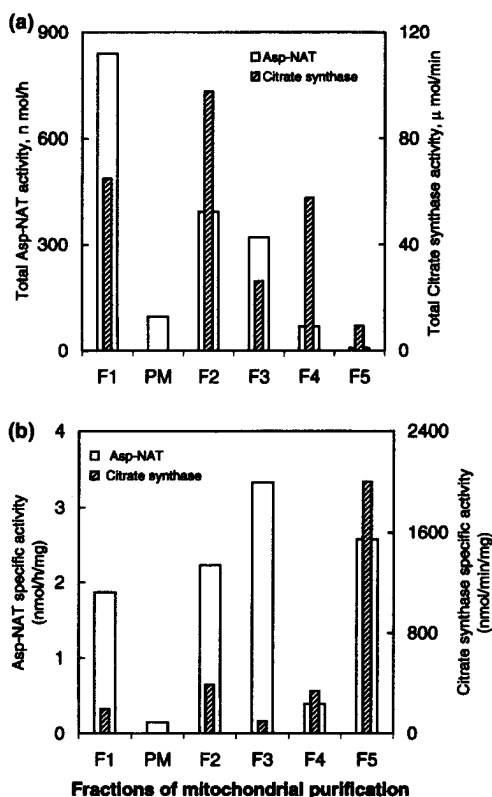
The fractions showing peak Asp-NAT activity from the native gel electro elution step were concentrated and 0.2 mL aliquot was passed over an analytical size-exclusion HPLC column (Shodex PROTEIN KW-804; ~850 kDa cut-off) equilibrated with 10 mM sodium phosphate, pH = 7.2 containing 0.15 M NaCl, 1 mM CHAPS, 1 mM dithiothreitol and 10% glycerol (flow rate = 0.5 mL/min). About 30 fractions (0.5 mL) were collected and assayed for the enzyme activity. The fractions showing the peak activity were pooled, concentrated and re-chromatographed in the same column to reduce the contaminating proteins from either side of the peak activity. The re-chromatographed fraction showing peak activity was analyzed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Water based staining of Commassie blue by the Pierce Gel Code stain with a sensitivity of ~5 ng protein/band was used for staining the gel. Protein standards (thyroglobulin, apoferritin, alcohol dehydrogenase and BSA) were also chromatographed under similar conditions to ascertain the approximate size of Asp-NAT.

## Results

A TLC based assay for Asp-NAT is shown in Fig. 1. This is a modification of an earlier assay we developed for aspartoacylase that cleaves NAA to form acetate and aspartate (Madhavarao *et al.* 2002). It is clear that the solvent system used provides excellent separation between [<sup>14</sup>C]Asp ( $R_F$  0.3) and [<sup>14</sup>C]NAA ( $R_F$  0.8) making the assay highly reliable. The excellent separation by TLC also enables this assay to be improved, if required, by using: (i) [<sup>14</sup>C]Asp with higher specific activity, (ii) reduced total volume of the assay



**Fig. 1** Phosphor image showing the thin-layer chromatography (TLC) separation of the radiolabel substrates L-[<sup>14</sup>C]Asp ( $R_F$  0.3), L-[<sup>14</sup>C]Glu ( $R_F$  0.4) and the product [<sup>14</sup>C]NAA ( $R_F$  0.8). Assay was performed as described in the Methods section. No detectable spot was found corresponding to *N*-acetylglutamate ( $R_F$  = 0.8) when substrate was L-[<sup>14</sup>C]Glu and in control, corresponding to NAA spot.



**Fig. 2** Asp-NAT and citrate synthase activity distribution (a) and their specific activity (b) in various fractions of mitochondrial purification. The various fractions are: F1, supernatant after low speed centrifugation (1250 g) of the tissue homogenate; F2, pellet obtained after subjecting F1 to high speed centrifugation (20 000 g), which was subjected to Percoll gradient centrifugation; F3, the topmost fraction containing myelin in the Percoll gradient after centrifugation; F4, the fraction consisting of synaptosomes; F5, the interface band between 23% and 40% Percoll, corresponding to purified intact mitochondria, that was twice washed; PM, the supernatant obtained after subjecting F1 to high speed centrifugation (20 000 g). PM was almost clear, in that citrate synthase activity could not be detected by spectrophotometric assay, whereas the more sensitive radiometric assay detected Asp-NAT activity. The ratio of activities of Asp-NAT to citrate synthase was comparable in mitochondria (F5) and synaptosomes (F4) but not in fraction F3, which consists primarily of myelin. Repetition of this experiment gave similar results.

components to achieve higher concentrations and (iii) stopping the reaction by heat-denaturing the sample without adding ethanol. We have used this TLC assay for the subsequent purification of the enzyme described below.

Figure 2 shows the quantitative distribution of recoverable Asp-NAT and citrate synthase activity (Fig. 2a) and their specific activity (Fig. 2b) in various fractions during mitochondrial purification. Most of the Asp-NAT activity was recovered in fraction F2, the crude mitochondrial pellet; only about 10% of the Asp-NAT activity in F1 was detected in the supernatant (PM). Subjecting fraction F2 to Percoll gradient

centrifugation yielded highly pure and intact mitochondria as reflected by the fivefold increase of citrate synthase specific activity [from  $\sim$ 400 nmol/(min mg) in F2 to 2000 nmol/(min mg protein) in F5, the purified mitochondria]. In all the fractions of the Percoll gradient purification, both Asp-NAT activity and citrate synthase activity were easily detectable. Asp-NAT specific activity was similar in the highly purified mitochondrial fraction (F5) and that containing myelin (F3). Maximum recoverable Asp-NAT activity was in the fraction F3 consisting of myelin, whereas for citrate synthase it was in the synaptosomal fraction (F4).

Further subfractionation of Percoll purified mitochondria (F5) on sucrose gradient centrifugation resulted in even higher level of purification for the mitochondrial membrane systems, namely, inner mitochondrial membrane + matrix, outer mitochondrial membrane, and contact sites. These three subfractions were free of potential sources of contaminating proteins from plasma membrane (Na/K ATPase,  $\alpha$ 1 and  $\alpha$ 3 subunits), endoplasmic reticulum (calnexin) and the cytosolic proteins as determined by western blots (data not shown). Asp-NAT activities in the three subfractions were as follows: inner mitochondrial membrane + matrix = 17 pmol/(h mg protein); outer mitochondrial membrane = 31 pmol/(h mg protein); contact sites = 35 pmol/(h mg protein). Cross-contamination between the three fractions does not permit any conclusion on the exact location of the Asp-NAT within the mitochondria (cf. Sandri *et al.* 1988). The relatively low level of Asp-NAT activity reflects possible loss in the recovery of enzyme activity during this additional step of sucrose gradient centrifugation following the osmotic swelling/shrinking and sonication.

Our purification plan for Asp-NAT involved solubilization of Asp-NAT activity from the crude mitochondrial pellet, followed by purification by: (i) anion exchange chromatography, (ii) native gel electrophoresis/continuous elution (Model 491 Prep Cell, Bio-Rad), (iii) HPLC size-exclusion chromatography and (iv) HPLC size-exclusion rechromatography. We have successfully tested all the methods separately using crude solubilized enzyme. Subsequently, we have combined them in the order given above.

Solubilization of Asp-NAT activity increased with increase in concentration of CHAPS up to 10 mM. Further increase in CHAPS concentration decreased the enzyme activity in an irreversible manner. In subsequent studies, we found that increasing the solubilization time increased the recovery of Asp-NAT activity to some extent. Therefore, we routinely solubilized the enzyme overnight in all the subsequent purification studies. About 50% of the total activity is recovered in the solubilized fraction.

DEAE cellulose chromatography was used as the first chromatographic step for purification of the enzyme. As high as 80% of the enzyme activity bound to the column under the present chromatographic condition. About 90% of the bound enzyme was eluted from the column by 0.5 M NaCl medium.

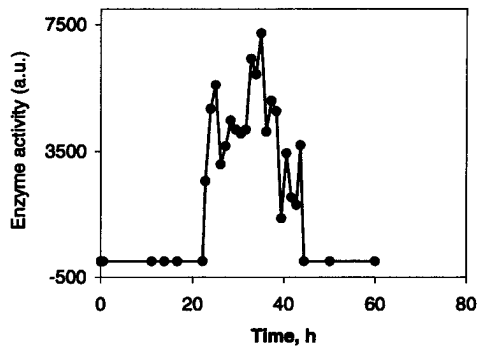


Fig. 3 Asp-NAT activity profile from the native gel electro-elution. Asp-NAT activity was determined after concentrating 2 mL into ~0.25 mL and using high sensitive radiometric assay described in the Methods section.

This method was highly reproducible and about four- to fivefold enrichment of Asp-NAT specific activity was routinely achieved by this chromatographic step [from 5–6 nmol/(h mg protein) to 24–26 nmol/(h mg protein)]. The fractions 2 and 3 containing most of the enzyme activity were pooled and concentrated before being used to further purify the enzyme by native gel electrophoresis.

Figure 3 shows the Asp-NAT activity profile from the eluted fractions of the continuous buffer native-gel electrophoresis. Asp-NAT was eluted late, reflecting high molecular weight nature of the enzyme complex. Also, the activity was eluted in a rather large volume, perhaps reflecting the presence of multiple species of enzyme complex. Although the enzyme activity was quite detectable, the overall recovery of the enzyme activity was in the 5–10% range. This necessitated the use of high specific radioactivity (~100–200 mCi/mmol) in subsequent steps, resulting in reduced substrate (L-Asp) concentration, which did not reflect the maximal activity of Asp-NAT. However, this lack of true level of Asp-NAT activity was not a hindrance in the purification of the enzyme. The fractions showing Asp-NAT activity were pooled, concentrated and subjected to size-exclusion chromatography in an analytical HPLC column (~850 kDa cut-off) in order to ascertain the size of the partially purified enzyme preparation and also to further purify it for generation of monoclonal antibodies. The fractions showing the peak Asp-NAT activity were collected, pooled, concentrated and rechromatographed.

Elution profile from the rechromatography step is shown in Fig. 4. A single protein peak was detected, which coincided with the enzyme activity peak. The protein peak corresponded to that of the molecular weight standard thyroglobulin (~669 kDa). However, on sodium dodecyl sulfate–polyacrylamide gel electrophoresis analysis, the protein showed multiple (at least 10 distinct) bands (inset, Fig. 4). Although all these protein bands may not be part of

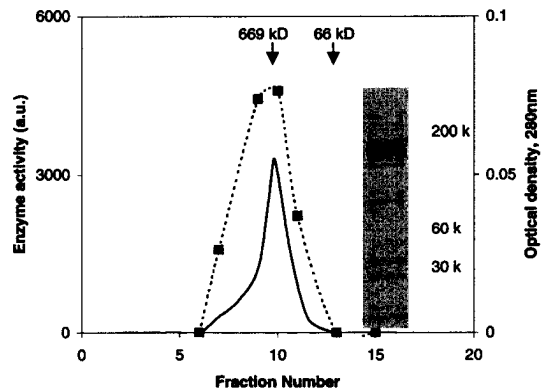
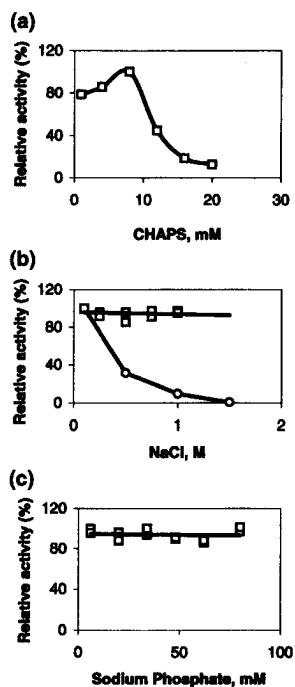


Fig. 4 Profile of Asp-NAT activity and optical density from the HPLC size-exclusion column rechromatography. Size-exclusion chromatography was done in a HPLC column (Shodex PROTEIN KW804) using concentrated enzyme preparation from the native gel electroelution step. Asp-NAT activity (broken line) was determined as described in Methods section by radiometry. The phosphor image intensity corresponding to the NAA spot (arbitrary units) is given in the y-axis. Optical density (solid line) of the elution profile was recorded at sensitivity set to 0.1 OD full scale. Elution was carried out in a Gilson HPLC system at the flow rate of 0.5 mL/min. Two-minute fractions were collected, concentrated and assayed for Asp-NAT activity. The inset shows the sodium dodecyl sulfate–polyacrylamide gel electrophoresis (10% Tris-glycine gel) image of the sample showing the peak activity.

Asp-NAT, the indication was that of an enzyme complex containing multiple enzymes or subunits, which is a characteristic of a variety of mitochondrial enzymes.

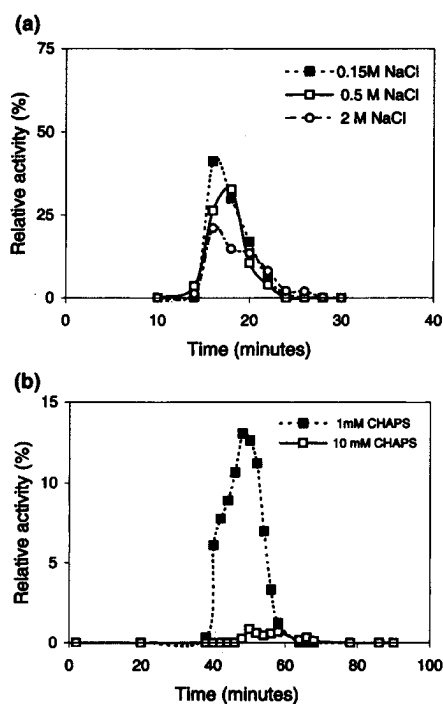
This unusually high molecular weight and seemingly multimeric nature of Asp-NAT prompted us to study its stability characteristics as a function of CHAPS and salt concentrations. Enriched Asp-NAT preparation from DEAE column step was used in these studies in view of its higher enzyme activity. The results are given in Fig. 5. It is clear CHAPS has a biphasic effect on the Asp-NAT stability revealed in terms of its activity (Fig. 5a). In this experiment, Asp-NAT preparation was pre-incubated (0–4°C, 1 h) in the presence of varying concentrations of CHAPS and the enzyme activity was determined using the TLC assay; CHAPS concentration decreased to about 80% due to dilution caused by the addition of substrates during the assay. Asp-NAT activity decreased to 40% of control at 12 mM CHAPS and 10% of the control at 20 mM CHAPS. After pre-incubating in media containing > 12 mM CHAPS, dialysis of the pre-incubated preparation to remove CHAPS did not increase the enzyme activity, indicating that there was an irreversible modification of the enzyme complex. Presence of NaCl during Asp-NAT assay at concentrations greater than 0.15 M decreased the activity progressively with increase in NaCl concentration. However, reducing the NaCl concentration to ~0.15 mM by dialysis of



**Fig. 5** Asp-NAT activity in the presence of varying concentrations of CHAPS (a), NaCl (b) and sodium phosphate (c). Relative activity with respect to 1 mM CHAPS in (a), 0.15 M NaCl in (b) and 40 mM sodium phosphate in (c) is represented in the y-axis. Enzyme activity was determined at constant protein concentrations using the radiometric assay described in the Methods section. Enzyme activity was more or less fully restored by dialysis (squares) after treating in high concentrations of NaCl (b).

the preparation after incubating with higher NaCl, reversed this effect up to 1 M NaCl (Fig. 5b). Surprisingly, increase in sodium phosphate did not have any significant effect up to 80 mM (Fig. 5c).

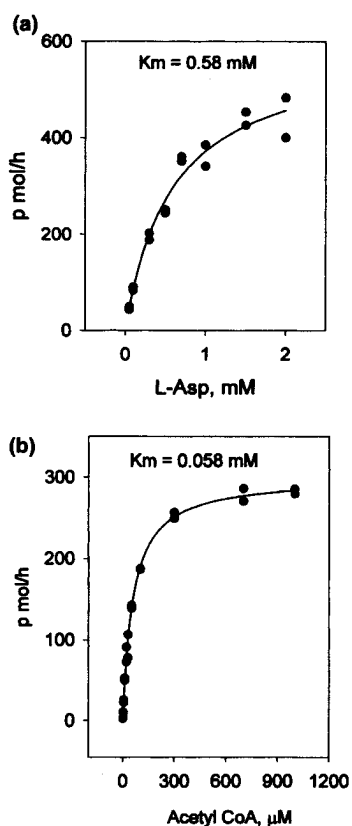
We further studied the stability of the enzyme using HPLC size-exclusion chromatography. Pre-treatment for 1 h in 0.15 M, 0.5 M and 2.0 M NaCl media and separating on a size-exclusion column (~850 kDa cut-off) by HPLC resulted in unimodal distribution of Asp-NAT activity in the eluted fractions (Fig. 6a). Somewhat decreased enzyme activity was observed at 0.5 and 2.0 M NaCl, probably due to incomplete dialysis because extensive dialysis restored the activity to the level of 0.15 M NaCl (cf. Figure 5b). However, when the enzyme preparation was pre-treated with 10 mM CHAPS (as opposed to 1 mM CHAPS normally employed throughout after solubilizing with 10 mM CHAPS) and separated on a size-exclusion column, the enzyme suffered significant loss of activity, which was irreversible even after dialysis to reduce the concentration of CHAPS to the level of 1 mM. Also, the distribution pattern was different, showing a right shift (Fig. 6b) and increased flatness, as compared to that of 1 mM CHAPS pre-treated Asp-NAT. These results are



**Fig. 6** Structural perturbation of Asp-NAT by NaCl (a) and CHAPS (b) and its impact on enzyme complex size and activity. The sample from the DEAE cellulose chromatography step was further purified by two sequential HPLC size-exclusion chromatography on a semi-preparative column (Shodex PROTEIN 804KW) and the resulting preparation was used. Samples were pre-treated with the given concentration of NaCl (a) and CHAPS (b) in the media for 1 h before injecting into the columns. The columns were pre-equilibrated and eluted with the respective media. (a) Shows the activity profile from the column of the analytical size (14 mL), wherein 2-min fractions were collected with the flow rate of 0.5 mL/min. Asp-NAT activity was determined after dialyzing the sample against 0.15 M NaCl medium. The recovery of activity in (a) was 95%, 80% and 64% for 0.15 M, 0.5 M and 2.0 M NaCl media, respectively. The decrease in activity at 0.5 M and 2.0 M NaCl reflects interference of NaCl with activity due to incomplete dialysis. (b) Shows the activity profile from the column of the size 100 mL from which 2-min fractions were collected with the flow rate of 1.0 mL/min. Asp-NAT activity was determined after dialyzing the sample against 1 mM CHAPS-containing medium. About 85% of the activity was recovered in 1 mM CHAPS-treated sample and only 5% of the activity was recovered in 10 mM CHAPS-treated sample. The y-axis represents the activity recovered, relative to the input activity normalized to 100.

consistent with the possibility that some subunits that contribute to the enzyme activity or regulate the activity are separated from the complex during the chromatography, which results in the loss of enzyme activity. That these enzyme components are held primarily by hydrophobic interactions is indicated by the sensitivity to CHAPS but not to high ionic strength.

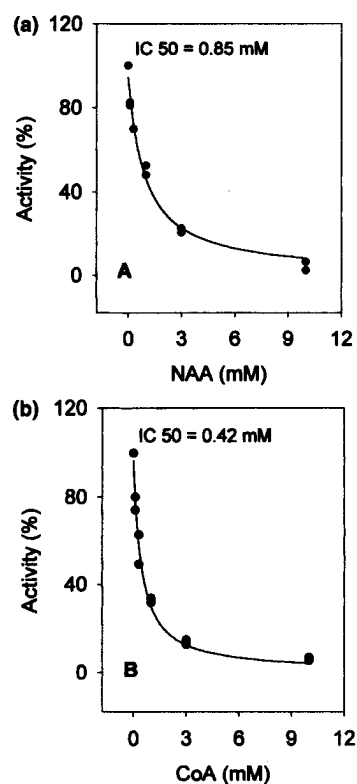
It was of interest to determine the steady-state kinetic parameters for the substrates L-Asp and acetyl CoA for this



**Fig. 7** Enzyme activity as a function of varying substrate concentration: L-Asp (a) and acetyl CoA (b). Enzyme activity was determined using the radiometric assay described in the Methods section using [ $^{14}$ C]L-Asp. Steady-state kinetic parameters were determined by non-linear regression using Sigma Plot software for Windows. The  $K_m$  values obtained for L-Asp and acetyl CoA were 0.58 mM and 0.058 mM, respectively. The  $V_{max}$  values obtained for L-Asp and acetyl CoA were 27 nmol/(h mg protein) and 23 nmol/(h mg protein), respectively. In the assays of (a) and (b), 22  $\mu$ g and 13  $\mu$ g of enzyme protein were used, respectively.

enzyme. Figure 7(a) shows the typical velocity versus substrate concentration curve for L-Asp, which revealed an apparent  $K_m$  to be  $\sim$ 0.5 mM. Surprisingly, for acetyl CoA, Asp-NAT showed a  $K_m$  of  $\sim$ 0.05 mM, an order of magnitude lower in concentration compared to L-Asp (Fig. 7b). Earlier, Goldstein (1959) had reported an order of magnitude higher  $K_m$  values for both L-Asp ( $\sim$ 5 mM) and acetyl CoA ( $\sim$ 0.5 mM) in a crude preparation of the enzyme. We also studied the effect of the products on the Asp-NAT activity. Both NAA and CoA inhibited Asp-NAT activity (Figs 8a and b) with  $IC_{50}$  (inhibitory concentration corresponding to 50% loss of activity compared to control) values for NAA at  $\sim$ 0.9 mM and for CoA  $\sim$ 0.4 mM. These results are summarized in Table 1.

Substrate specificity was determined using amino acid/peptide analogs. About 3% of the activity obtained with



**Fig. 8** Inhibition of Asp-NAT activity in the presence of varying concentrations of NAA (a) and CoA (b). Enzyme activity was determined as described in the Methods section using L-[ $^{14}$ C]Asp as the radiolabel, with enzyme protein of 22  $\mu$ g per assay. The data were analyzed using Sigma Plot software by subjecting to a two constant hyperbolic decay to extract the  $IC_{50}$  concentrations. The  $IC_{50}$  values for NAA and CoA were 0.85 mM and 0.42 mM, respectively.

**Table 1** Summary of steady-state kinetic parameters (a) and inhibitory concentrations of NAA and CoA (b) for Asp-NAT

(a)		
Substrate	$K_m$ ( $\mu$ M)	$V_{max}$ [nmol/(h mg protein)]
L-Asp	580	27
Acetyl CoA	58	23
(b)		
Product	$IC_{50}$ ( $\mu$ M)	$I_{max}$ (%) <sup>a</sup>
NAA	850	95
CoA	420	96

All assays were done using partially purified Asp-NAT, i.e. the 0.5 M NaCl medium eluted DEAE fractions, which was put through size-exclusion chromatography. Assays were performed in duplicates; all data points (16 for L-Asp and 20 for acetyl CoA, for enzyme kinetics; 12 points for inhibitory studies) were included for analysis.

<sup>a</sup> $I_{max}$  is the percentage maximum inhibition exerted by the compound on Asp-NAT activity.

**Table 2** Asp-NAT specificity with respect to amino acid and peptide substrates<sup>a</sup>

Substrate	Relative activity (%)
L-Asp	100.0
L-Glu	2.1
L-Asn	2.7
L-Gln	2.3
L-Asp-L-Glu	Undetectable

<sup>a</sup>Asp-NAT activity was determined using L-[<sup>14</sup>C]Asp or [<sup>14</sup>C]acetyl CoA by TLC assay except for Asp-Glu, in which case HPLC anion exchange chromatography was employed.

L-Asp was found with L-Glu, L-Asn and L-Gln. However, enzyme activity was undetectable against Asp-Glu (Table 2). This plausibly rules out acetylation of Asp-Glu as a viable pathway for the biosynthesis of NAAG.

## Discussion

The bioinformatics approach based on the availability of the Human Genome Data Base towards cloning Asp-NAT was without success. Although glutamate *N*-acetyl transferase has been recently cloned and expressed from mouse and human (Caldovic *et al.* 2002a,b), no assigned gene/sequence to be aspartate *N*-acetyltransferase was identifiable in the entire data base. Therefore, purification of Asp-NAT was pursued in order to get at the gene coding Asp-NAT.

Several observations indicate Asp-NAT to be a neuronal enzyme (Moffett *et al.* 1991; Simmons *et al.* 1991; Moffett and Namboodiri 1995) and reported to be mitochondrial in location (Patel and Clark 1979; Clark 1998; Signoretti *et al.* 2001). As expected, most of the Asp-NAT activity was detected in the crude mitochondrial fraction (F2). Also, Percoll gradient purification with differential centrifugation technique showed Asp-NAT activity in the highly purified brain mitochondria. As the mitochondria became purified, as indicated by the increase in citrate synthase specific activity by fivefold, Asp-NAT specific activity remained at the comparable level amongst F2, F3 and F5 (Fig. 2b), ruling out Asp-NAT as a contamination. Furthermore, the three sub-fractions obtained from F5 after sucrose density gradient centrifugation, which were free from potential contaminant proteins of plasma membrane, endoplasmic reticulum and cytosol proteins, demonstrated Asp-NAT activity. This established Asp-NAT localization to mitochondria. However, the highest recoverable activity was found in the fraction containing myelin (fraction F3), unlike the case with citrate synthase, the mitochondrial marker enzyme. Two factors could contribute to such a difference: (i) higher proportion of non-neuronal mitochondria in the fraction F5 and (ii) possible association of Asp-NAT with the myelin. The observations that NAA provides acetyl group for myelin

synthesis in brain (D'Adamo and Yatsu 1966; D'Adamo *et al.* 1968; Burri *et al.* 1991; Chakraborty *et al.* 2001) and that aspartoacylase (EC 3.5.1.15), which generates acetate from NAA, is localized in oligodendrocytes and myelin (Chakraborty *et al.* 2001; Kirmani *et al.* 2002, 2003) are consistent with possible association of Asp-NAT with myelin.

The results indicate that solubilized Asp-NAT is a high molecular weight protein, plausibly a complex containing multiple subunits. In this regard, two important observations made over several purification cycles need to be mentioned. First, as the bulk of the protein was removed with different separation techniques, specific activity of Asp-NAT did not increase by orders of magnitude as one would normally expect. Second, the peak of the activity shifted slightly to the right (towards lower molecular weight) in the size-exclusion column. In this context, structural integrity and activity relationship of Asp-NAT was probed. The structure and function of the enzyme do not appear to be affected by changes in ionic strength perturbation (cf. Figs 5 and 6a). On the other hand, the quaternary structure seems to be vulnerable to hydrophobic perturbations, as demonstrated by 10 mM CHAPS treatment followed by size-exclusion chromatography (Fig. 6b). A surprising aspect of this effect of CHAPS on the enzyme complex is that mere treatment of 10 mM CHAPS does not result in loss of enzyme activity; enzyme was solubilized by treatment with 10 mM CHAPS. It appears that mere loss of hydrophobic interactions among the subunits does not destroy the enzyme activity. Instead, separation of subunits by size-exclusion chromatography seems to be required for the loss of enzyme activity (cf. Fig 5a and Fig. 6b). Presence of Asp-NAT in mitochondria raises the issue of whether it is a membrane-bound enzyme like many other mitochondrial enzyme complexes involved in degradation of carbon source during respiration. The requirement of 10 mM CHAPS for optimal extraction of the enzyme suggests such a possibility.

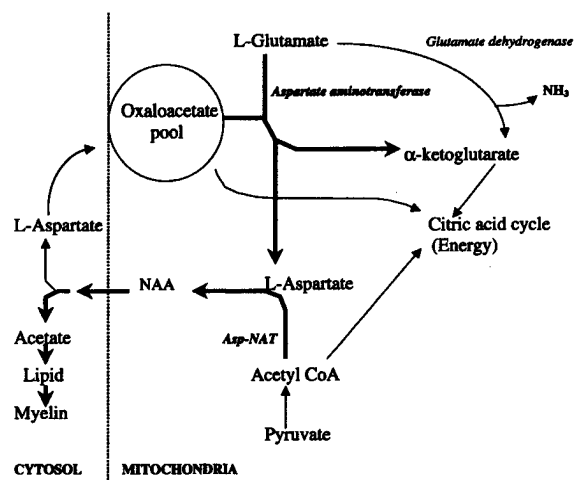
Another interesting point that merits some discussion is the high specificity of this enzyme toward aspartate. Aspartoacylase, the enzyme that degrades NAA, also exhibits such a high degree of specificity toward NAA. Whereas NAA appears to be localized primarily in neurons (Moffett *et al.* 1991; Simmons *et al.* 1991; Moffett and Namboodiri 1995) and is also found in cultured oligodendrocytes (Bhakoo and Pearce 2000), aspartoacylase is not detectable in neurons (Baslow *et al.* 1999; Kirmani *et al.* 2002). These observations suggest that NAA serves some important but complex functional roles in the brain. Of these, perhaps the most understood is the role as an acetyl source for acetyl CoA required for lipid synthesis during myelination; Canavan disease appears to be caused by a deficiency of this role (Mehta and Namboodiri 1995; Kirmani *et al.* 2002, 2003). Another role of NAA is as a precursor for *N*-acetylaspartyl-glutamate, the most abundant peptide neurotransmitter in the

mammalian nervous system (Cangro *et al.* 1987). NAA also has been proposed to be involved in other functions, such as osmoregulation (Taylor *et al.* 1995).

A somewhat parallel biochemical system exists in the liver with an established functional role. This system involves a less complex mitochondrial enzyme, *N*-acetyl glutamate synthetase (EC 2.3.1.1) that acetylates glutamate with high specificity to form *N*-acetylglutamate (NAG) (cf. Sonoda and Tatibana 1983; Caldovic *et al.* 2002a). NAG is degraded by a less specific enzyme, amidohydrolase 1 (EC 3.5.1.14). NAG is known to play an important role in the control of the urea cycle by allosterically activating carbamoyl phosphate synthetase I (EC 6.3.4.16). Mutations that cause deficiency in this acetylating enzyme result in hyperammonemia, leading to death in early childhood. Given that neuronal mitochondria do not have an active urea cycle system, the NAA system in brain seems to perform some other roles.

The present studies provide three main evidences for the mitochondrial localization of Asp-NAT: (i) co-localization of Asp-NAT and citrate synthase activities in the crude mitochondrial pellet as well as Percoll gradient fractions; (ii) comparable Asp-NAT specific activity in pure and myelin-containing impure fractions of the mitochondria after Percoll gradient centrifugation; (iii) the highly purified three subfractions obtained from the Percoll purified mitochondria (F5) on sucrose density gradient centrifugation, demonstrated Asp-NAT activity. Taken together with all other related data in the literature, these new data suggest a mitochondrial localization of Asp-NAT.

Presence of Asp-NAT in mitochondria and the seemingly complex nature of the enzyme prompted us to propose a working model to stimulate further research on the functional roles of NAA. This model is represented schematically in Fig. 9. We propose that NAA is an integral part of the neuronal mitochondrial energy metabolism that is required to support the extraordinarily high energy needs of neurons. The extra demand for ATP in neurons appears to be met to a large extent by oxidation of glutamate via aspartate aminotransferase (EC 2.6.1.1) pathway. The importance of this pathway in the neuronal mitochondria has been recognized by earlier investigators, and the name 'mini citric acid cycle' has been coined to emphasize its role in neuronal energetics (cf. Erecinska *et al.* 1988; Yudkoff *et al.* 1994). It appears that neuronal mitochondria use this reaction instead of glutamate dehydrogenase reaction to generate  $\alpha$ -ketoglutarate that is required for oxidation because this avoids the problem of ammonia toxicity caused by the lack of an effective urea cycle system in neurons. We think NAA synthesis is intimately associated with the proper functioning of the mini citric acid cycle via aspartate aminotransferase reaction in the neuronal mitochondria. NAA synthesis serves two important functions. First, by converting the product aspartate into NAA, NAA synthesis helps to steer the aspartate aminotransferase reaction toward  $\alpha$ -ketoglutarate



**Fig. 9** A model on the integral involvement of NAA synthesis in the energetics of neuronal mitochondria. The pathways in which NAA is involved or might play a role are indicated in thicker lines. Glutamate oxidation in mitochondria takes place via aspartate aminotransferase to generate  $\alpha$ -ketoglutarate that is used for oxidation via 'mini citric acid cycle'. NAA synthesis by Asp-NAT reduces L-Asp build up, facilitating the aspartate aminotransferase reaction toward  $\alpha$ -ketoglutarate. NAA that is transported into the site of aspartoacylase provides acetate for lipid/myelin synthesis.

facilitating energy production. Second, it substitutes for citrate as the acetate carrier to the cytoplasm and thus compensates for the lack of citrate production during the 'mini citric acid cycle'. Aspartoacylase (EC 3.5.1.15) responsible for NAA degradation into acetate and L-Asp seems to be localized primarily in the cytosol of oligodendrocytes (cf. Kaul *et al.* 1991; Baslow *et al.* 1999; Kirmani *et al.* 2002) and it is likely that there is *trans*-axonal transport of NAA into the cytosol of oligodendrocytes for supplying acetate for lipid synthesis (Chakraborty *et al.* 2001).

There are several findings in the literature that are consistent with or supportive of this model. First, inhibition of aspartate aminotransferase activity by  $\beta$ -methyleneaspartate decreased the oxygen consumption in state 3 respiration in the presence of glutamate and malate as substrates (Cheeseman and Clark 1988). Second, efflux of NAA increased and that of L-Asp decreased with increasing concentration of pyruvate in the presence of glutamate and malate as substrates in neuronal mitochondria (Patel and Clark 1979). Third, ATP synthesis and NAA synthesis were directly correlated with oxygen consumption in studies involving respiratory chain inhibitors (Bates *et al.* 1996). Fourth, concentrations of NAA and ATP were directly correlated in a study involving diffuse traumatic brain injury in rats (Signoretti *et al.* 2001). Fifth, there is substantial evidence presented in the literature that NAA supplies acetyl groups for the lipids synthesized to form myelin (D'Adamo and Yatsu 1966; D'Adamo *et al.* 1968; Burri *et al.* 1991;

Chakraborty *et al.* 2001). Canavan disease appears to be caused by a defect in the supply of acetyl group from NAA for myelin synthesis (Mehta and Namboodiri 1995; Kirmani *et al.* 2002; Kirmani *et al.* 2003). Studies are in progress to develop tools, such as inhibitors and antibodies of the NAA biosynthetic enzyme, to understand its role in the mitochondrial energetics of neurons.

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