



## A novel UPLC-MS/MS based method to determine the activity of *N*-acetylglutamate synthase in liver tissue



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### ABSTRACT

**Background:** *N*-acetylglutamate synthase (NAGS) plays a key role in the removal of ammonia via the urea cycle by catalyzing the synthesis of *N*-acetylglutamate (NAG), the obligatory cofactor in the carbamyl phosphate synthetase 1 reaction. Enzymatic analysis of NAGS in liver homogenates has remained insensitive and inaccurate, which prompted the development of a novel method.

**Methods:** UPLC-MS/MS was used in conjunction with stable isotope (*N*-acetylglutamic-2,3,3,4,4- $d_5$  acid) dilution for the quantitative detection of NAG produced by the NAGS enzyme. The assay conditions were optimized using purified human NAGS and the optimized enzyme conditions were used to measure the activity in mouse liver homogenates.

**Results:** A low signal-to-noise ratio in liver tissue samples was observed due to non-enzymatic formation of *N*-acetylglutamate and low specific activity, which interfered with quantitative analysis. Quenching of acetyl-CoA immediately after the incubation circumvented this analytical difficulty and allowed accurate and sensitive determination of mammalian NAGS activity. The specificity of the assay was validated by demonstrating a complete deficiency of NAGS in liver homogenates from *Nags*  $-/-$  mice.

**Conclusion:** The novel NAGS enzyme assay reported herein can be used for the diagnosis of inherited NAGS deficiency and may also be of value in the study of secondary hyperammonemia present in various inborn errors of metabolism as well as drug treatment.

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### 1. Introduction

*N*-acetylglutamate synthase (NAGS, E.C:2.3.1.1) utilizes acetyl-CoA and L-glutamate to catalyze the formation of *N*-acetylglutamate (NAG), which is an obligatory allosteric activator of carbamyl phosphate synthetase 1 (CPS1) and is therefore indispensable for urea formation [1]. Inherited NAGS deficiency (OMIM: 237310) is an autosomal recessive disorder which is primarily associated with hyperammonemia [1]. Secondary hyperammonemia may occur in several organic acidemias and upon intake of valproate, which both result in inhibition of NAGS, reduced NAG level and subsequent dysfunction of CPS1 [2,3]. Primary (inherited) as well as acquired deficiency of NAGS is associated with various symptoms including lethargy, vomiting, hypotonia, seizures, and even coma or death [4]. Primary NAGS deficiency is currently confirmed by sequencing of the NAGS gene but confirmatory studies at the enzyme level have thus far been problematic due to the

requirement of a relatively large amount of liver tissue as well as insufficient sensitivity of the measurement of the reaction product, i.e. NAG [5]. Patients with NAGS deficiency, as confirmed by pathogenic mutations in the NAGS gene, do not always exhibit deficient NAGS activity in enzyme analyses of liver biopsies [5,6]. Consequently, this disorder may remain underdiagnosed due to the lack of specific biomarkers other than ammonia and limited availability of centers able to measure NAGS and CPS1 activity.

Various assays for hepatic and purified NAGS have been developed, which include the use of radioactive substrates, and either HPLC, GC-MS, or LC-MS/MS for substrate and product analysis [2,7–10]. Initial methods used acetyl-CoA and [ $^{14}$ C]-L-glutamate as substrate for NAGS, forming *N*-acetyl-[ $^{14}$ C]-L-glutamate ([ $^{14}$ C]-NAG) [2,7]. Lund and Wiggins [8] as well as Alonso and Rubio [9] suggested the determination of NAGS via hydrolysis of hepatic NAG followed by measurement of released L-glutamate by HPLC. All reported methods proved to be time-consuming and non-specific. A GC-MS stable isotope application was later developed to directly determine enzymatic production of NAG, but column separation and absolute quantification (due to multiple peak formation) were problematic rendering it unsuitable for large-scale kinetic experiments [10,11].

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The most promising methodology appears to use HPLC- or UPLC-MS/MS, in which direct measurement of NAGS and specific quantification is possible with NAG stable isotope used as an internal standard [12]. Specific reaction conditions have been investigated by several research groups which have shown that optimal substrate concentrations, pH, and the addition of L-arginine (which stimulates the activity of NAGS) are needed to accurately determine NAGS activity [2,7–10]. Although the MS-based assays have proven to be successful, they suffer from high backgrounds in samples with low enzyme activity due to the non-enzymatic (chemical) formation of NAG [2,7–10].

The aim of this study was to develop an optimized assay for the determination of NAGS activity in liver homogenates. To this end, we first determined the optimal reaction conditions and kinetic constants using purified recombinant human NAGS. The optimized method was subsequently used to measure the activity of NAGS in mouse liver homogenates, but we observed undesirable low signal-to-noise ratios. To circumvent this problem, we have now devised a novel method described herein.

## 2. Materials and methods

All materials were supplied by Sigma-Aldrich (St. Louis, MO) unless otherwise specified.

### 2.1. Purified NAGS enzyme assay

Human NAGS was expressed and purified as described before [13]. The activity of NAGS, as expressed and purified from *E. coli*, was measured essentially as described by Caldovic et al. [12] and Aires et al. [14] with some modifications. These modifications included an optimized sample preparation protocol and product determination by UPLC-MS/MS. Several conditions of the assay, including time of incubation, pH, and type of buffer (sodium phosphate, triethanolamine, Tris, and ammonium acetate) were evaluated. Subsequently, the  $K_m$  values for acetyl-CoA and L-glutamate were determined with GraphPad Prism version 5 (GraphPad Software Inc., La Jolla, CA, USA), which led to an optimized reaction mixture containing 50 mM ammonium acetate buffer (final pH = 9.0), 10 mM L-glutamate, 2.5 mM acetyl-CoA and 1 mM arginine in a total volume of 75  $\mu$ l. The reaction was started by adding 10  $\mu$ l homogenate containing 0.05  $\mu$ g purified enzyme (prepared in 0.1 mg/ml BSA/PBS solution). After a 10 min incubation period at 30 °C, reactions were stopped with 175  $\mu$ l acetonitrile and 10  $\mu$ l of a solution containing N-acetyl-L-glutamic-2,3,3,4,4-d<sub>5</sub> acid (CDN isotopes: Germany) at 1 mmol/l. All steps, except the incubation, were performed on ice to minimize non-enzymatic formation of NAG. The precipitated protein was removed by centrifugation (20,000 g, 5 min, 4 °C) and the supernatant fluid was dried under nitrogen at 40 °C. The reaction product was resuspended in 110  $\mu$ l of 0.4% heptafluorobutyric acid (HFBA) (pH = 4). Appropriate blank samples, containing all substrates with pre-quenched enzyme were used to establish the baseline for detection.

The amount of NAG formed was quantified using a Quattro Premier XE tandem mass spectrometer (MS/MS) from Waters (Milford, MA, USA) with an Acquity sample manager and an Acquity binary solvent manager. NAG and NAG-[2,3,3,4,4-d<sub>5</sub>] were analysed on a Waters C18-BEH column (100 mm-length  $\times$  2.1 mm-diameter, 1.7  $\mu$ m particle size), using a linear gradient from 100% solvent A (0.1% HFBA) to 50% solvent B (acetonitrile/water, 4/1, v/v) in 5 min. The flow rate was 500  $\mu$ l/min with a total run time of 9 min. Both compounds were detected and quantified by MRM acquisition electron positive ionization mode (ESI+), using the transitions  $m/z$  190 > 84 for NAG, and 195 > 88 for NAG-[2,3,3,4,4-d<sub>5</sub>]. The MS conditions furthermore consisted of a capillary voltage of 3.00 kV, cone voltage of 30 V, desolvation gas flow of 900 L/h as well as a source temperature and desolvation temperature of 120 °C and 300 °C, respectively. The optimized collision energy was achieved at 25 eV.

### 2.2. NAGS assay in mouse liver homogenate

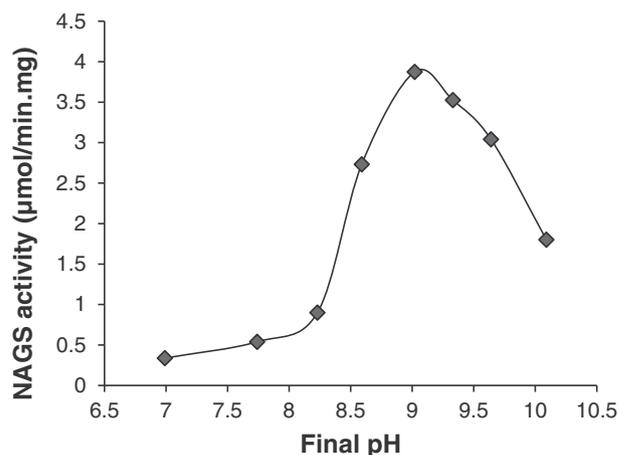
Wild type and NAGS-devoided mouse liver samples, obtained under a research protocol approved by the Children's Research Institute IACUC (with Institutional Animal Care and Use Committee approval), were used [15]. Liver samples, with a wet weight of approximately 20 mg, were homogenized in phosphate-buffered saline (PBS) and sonicated twice (40 J) on ice with intervals of approximately 30 s between bursts. The protein content of samples was determined with the BCA assay [16]. The activity of NAGS was measured using the same optimized conditions as described for purified human NAGS, with a final protein concentration of 2 mg/ml and one modification. At the end of the incubation (75  $\mu$ l final volume), excess acetyl-CoA was removed by adding 10.2  $\mu$ l solution of carnitine, N-ethylmaleimide (NEM) and carnitine acetyltransferase (CRAT) (Roche Holding AG, Basel: Switzerland), with a final concentration of 5.9 mM, 5.9 mM and 0.9 mU/ml, respectively. This was stopped after 10 min incubation at 30 °C with acetonitrile containing N-acetyl-L-glutamic-2,3,3,4,4-d<sub>5</sub> acid as internal standard, as mentioned above. This procedure which is based on earlier work by McGarry and Forster (1979), allowed for rapid conversion of unused acetyl-CoA into acetylcarnitine thereby minimizing further non-enzymatic formation of NAG [17].

## 3. Results and discussion

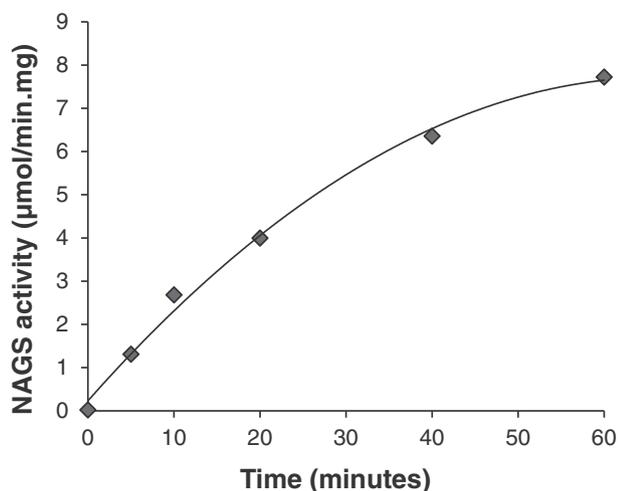
### 3.1. Optimization of the NAGS assay using purified NAGS

In order to determine the optimal assay conditions of purified human NAGS, we first expressed human NAGS in a His-tagged form in *E. coli* [13]. We subsequently incubated the enzyme for 15 min at different pH values in the presence of 2.5 mM acetyl-CoA and 10 mM L-glutamate in the presence of L-arginine (1 mM). The results depicted in Fig. 1 show that the enzyme activity is highest at a pH of 9.0. We then performed a time dependency study as illustrated in Fig. 2. The activity increased with time although the rate of NAG-formation steadily decreased with time. We therefore selected an optimal incubation time of 10 min. Under such conditions approximately 10% of the limiting substrate (acetyl-CoA) was consumed.

A relatively high rate of non-enzymatic production of NAG was observed. The mechanism has been previously attributed to the addition of acid (when terminating the reaction with trichloroacetic acid) [10.11]. In order to circumvent this problem, we changed the procedure to quench the NAGS reaction by replacing acid with acetonitrile. Unfortunately, also under these conditions formation of NAG was still



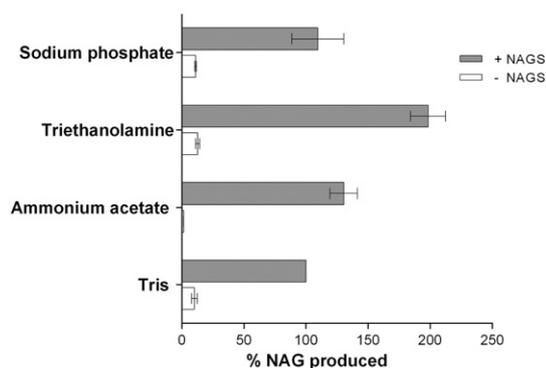
**Fig. 1.** The effect of the pH on the activity of NAGS. The pH of the standard reaction mixture was adjusted with ammonium hydroxide accordingly. Purified NAGS was incubated for 10 min and the production of NAG was quantified as described in the methods section. NAGS activity was measured in duplicate, with each point representing the mean.



**Fig. 2.** The effect of the incubation time on production of NAG. The assay was performed as described in the methods section using purified NAGS and the reaction was allowed to proceed for 5, 10, 20, 40 and 60 min after which the reactions were terminated with 70% acetonitrile. Values are the mean of duplicate incubations.

appreciable. Since it has been proposed that the free amine group of Tris may potentially react with enzymes and/or substrate, which would alter the kinetic properties of the enzyme assay [18] we first studied the contribution of the type of buffer used to the non-enzymatic production of NAG. The results depicted in Fig. 3 show that non-enzymatic NAG-formation was lowest with ammonium acetate used as buffer and highest with triethanolamine. On the other hand, the rate of enzyme-driven NAG-formation was highest with triethanolamine followed by ammonium acetate, sodium phosphate, and Tris. We observed the best signal-to-noise ratio using ammonium acetate as buffer, and used this system in all subsequent experiments.

Finally, we optimized the deproteinization step which must be effective in termination of the reaction as well as be compatible with a UPLC-MS/MS analysis. The use of perchloric acid/potassium carbonate (PCA/K<sub>2</sub>CO<sub>3</sub>) and trichloroacetic acid/potassium hydroxide (TCA/KOH) resulted in severe ion suppression as well as inconsistent peak formation. Also, inappropriate peak sharpening was observed with the use of PCA/K<sub>2</sub>CO<sub>3</sub> and peak broadening was seen with the use of TCA/KOH, which complicated quantitation. Acetonitrile or heat inactivation appeared to be better choices for the deproteinization of samples. Acetonitrile, which is easily removed by evaporation, was selected for

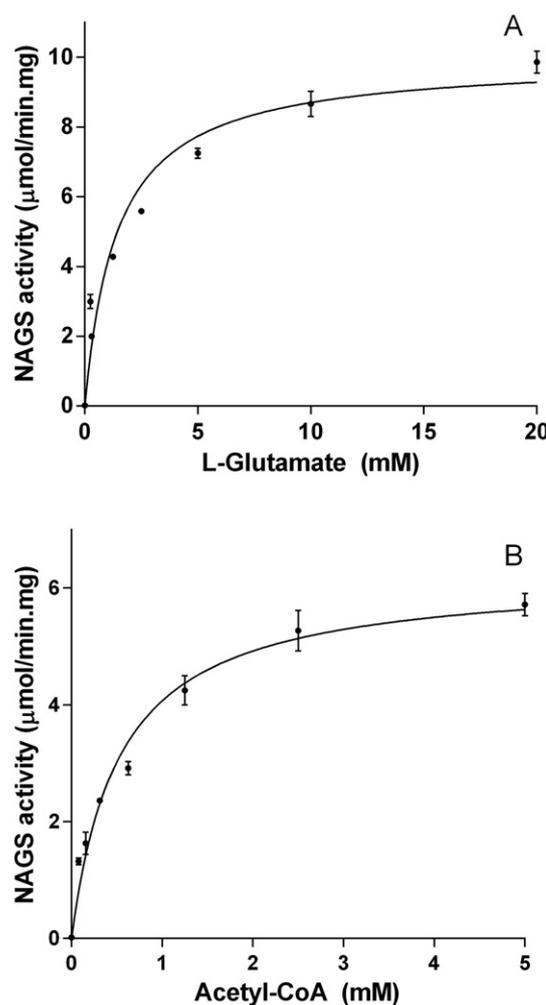


**Fig. 3.** Influence of the type of buffer on the enzymatic and non-enzymatic production of NAG. The formation of NAG from acetyl-CoA and L-glutamate was studied in a standard reaction medium containing each of the different buffers at a final concentration of 50 mM and a final pH of 9.0 in the absence (black bars) or presence (grey bars) of purified NAGS. The production of NAG was quantified as described in the material and methods section. The activity obtained with the original buffer conditions (Tris) as described by Aires et al. [13], was set to 100%. The experiment was performed in duplicate and error bars indicate the range.

all subsequent experiments. Furthermore, the resuspension of the sample, after evaporation under nitrogen (40 °C), in 0.4% HFBA was essential to avoid peak “splitting” [19]. Supplementary Fig. 1 depict the chromatography observed after optimising the analytical conditions and the mass spectra for unlabelled and labelled N-acetylglutamate.

### 3.2. Kinetic parameters of purified NAGS

Using the optimal assay conditions, as described for purified human NAGS, we varied the concentrations of the two substrates in the incubation mixture and determined the respective activity of purified human NAGS. Fig. 4 depicts Michaelis-Menten plots for the two substrates which were used for the calculation of the kinetic parameters by non-linear regression analysis. The apparent  $K_m$  values for L-glutamate and acetyl-CoA were  $1.43 \pm 0.28$  mM and  $0.53 \pm 0.04$  mM, respectively. The values concur with previous results of Caldovic et al. for purified human NAGS [20]. L-Glutamate and acetyl-CoA had apparent  $V_{max}$  values of  $9.95 \pm 0.5$  µmol/min/mg and  $6.2 \pm 0.3$  µmol/min/mg, respectively.



**Fig. 4.** L-Glutamate and acetyl-CoA response curve NAGS. The activity was measured using purified NAGS in a standard reaction mixture containing (A) different concentrations of L-glutamate with fixed acetyl-CoA concentration (2.5 mM) (B) different concentrations of acetyl-CoA with fixed L-glutamate concentration (10 mM). Michaelis-Menten plots were derived from non-linear regression analyses. The data points represent the mean values of three experiments.

### 3.3. Wild type and knock-out NAGS activity in mouse liver

We tested the new optimized assay for the determination of the activity of NAGS in mouse liver homogenates. The signal-to-noise ratio which is the ratio between the rates of formation of NAG in the presence and absence of homogenate, turned out to be very low compared to the formation of NAG with purified enzyme. This was partly due to the low activity of NAGS in total liver homogenates but more importantly due to the non-enzymatic formation of NAG. We reasoned that it would be best if we only had acetyl-CoA and L-glutamate present during the incubation time and to quench acetyl-CoA and/or L-glutamate immediately after the reaction period. To this end acetyl-CoA was rapidly depleted from the mixture by adding a solution of CRAT, carnitine plus NEM at the end of the incubation period. The latter compound was added, since it reacts with SH-group thereby trapping coenzyme A (CoASH) produced from acetyl-CoA and shifting the equilibrium towards acetylcarnitine. The initial signal-to-noise ratio of approximately 2 was improved to 5.8 after the application of the quenching step in liver samples with normal NAGS activity. The limit of detection (LOD) and quantitation (LOQ) were determined as 0.06 nmol/min·mg and 0.19 nmol/min·mg, respectively.

In order to provide final validation of the assay, we determined the activity of NAGS in murine liver homogenates in which the gene coding for NAGS had been deleted [15]. The NAGS activity in the liver homogenate from the *Nags*<sup>-/-</sup> mice (n = 4) was below the LOD and LOQ values. Liver samples of wild type mice (n = 2) showed NAGS activity of 1.55 and 1.71 nmol/min/mg, respectively which is almost 10 times the LOQ value. An additional advantage of our method was the use of low protein concentrations (2 mg/ml), compared to earlier studies which used 10–20 mg/ml of cell lysate, which mostly consisted of enriched mitochondrial fractions [14,21].

## 4. Conclusions

We have developed an improved assay for the enzymatic determination of NAGS based on the use of UPLC-MS/MS for the detection of NAG. This study illustrates the benefits of the use of UPLC-MS/MS in the rapid reliable monitoring of NAGS activity. An important advantage of the current assay is the optimization of conditions for mass spectrometric analyses, which included the use of an optimal buffer and the termination of the enzyme assay by adding acetonitrile. Furthermore, non-enzymatic formation of NAG was minimized by rapidly converting acetyl-CoA to acetylcarnitine at the end of the incubation. The current method can be of value for the enzymatic confirmation of NAGS-deficiency in patients with unexplained hyperammonemia [5,10,11]. In addition, this assay allows us to explore drug-induced inhibition of NAGS and other forms of secondary hyperammonemia.

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.ymgme.2016.10.004>.

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