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Glutaminase catalyzes reaction of Glutamate to GABA

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ABSTRACT

Here, for the first time, we report an NMR spectroscopy study of L-Glutamine (Gln) conversion by Glutaminase (Glnase), which shows that the reaction involves two distinct steps. In the first step, Glnase rapidly hydrolyzes Gln to Glutamate (Glu) (~16.87 μmol of Gln/min/mg of Glnase) and in the second step, Glu generated in the first step is decarboxylated into gamma-amino butyric acid (GABA) with a much slower rate (~0.185 μmol/min/mg). When Glnase was added to the sample containing L-Glu alone, it was also converted to GABA, at a similar rate as in the second step mentioned above. The rate of Glu decarboxylation into GABA by Glnase is about an order of magnitude lower than that by commonly known enzyme, Glutamate decarboxylase. Potential impact of these findings, on the mechanistic aspects of Gln–Glu shuttle in neuroscience and glutaminolysis in tumors, is discussed.

1. Introduction

Glnase-mediated Gln metabolism plays a key role in both tumor glutaminolysis and in neurons. It is well known that increased glucose utilization in tumor cells is due, in part to PI3K/Akt/mTOR-mediated up-regulation of glucose transporters [1,2]. Even in the presence of sufficient oxygen, tumor cells derive their energy from glycolysis (Warburg effect), thereby causing over-production of lactic acid [3–6]. Consequently, the amount of glucose-derived metabolites entering into the tri-carboxylic acid (TCA) cycle decreases significantly. As a result, cancer cells rely on alternate metabolites to replenish TCA cycle intermediates. Glutamine (Gln), an amino acid, has been identified as a key contributor [7] to important metabolic processes that augment the proliferation of the cancer cells. In addition to being an important source of nitrogen for amino acid synthesis and participation in bioenergetics, Gln plays a key role against oxidative stress and complements glucose metabolism [8,9] (Fig. 1). '*Myc*' is the only oncogene known to influence Gln uptake, and it induces increased Gln uptake by certain cancers. '*Myc*' can directly regulate the levels of Glutamate transporter SLC1A5, and indirectly regulate Gln expression via miR-23a/b, thus rendering tumor cells dependent on Gln uptake for their viability [10]. Gln is converted into Glu by Glnase, a key enzyme, the expression of which has been shown to be important for tumor growth [5,10]. It has been suggested that tumors have the ability to optimize Glnase activity, thereby enabling increased Glutamine uptake [11]. Indeed, blocking of Glnase activity is being explored as a means to arrest various types of tumor growth [3,12].

Glnase also plays a vital role in the Gln–Glu cycling that occurs during neurotransmission. Upon the release of Glu from the presynaptic neuron into the synaptic cleft, some of it will be processed by the Glu receptors on the post synaptic neuron. Excess Glu from the synaptic cleft is transported by Glu transporters into astroglia, where it is converted into Gln by Glutamine synthetase (GS). Gln then shuttles into neurons where it gets converted to Glu by Glnase and the cycle continues.

It is commonly known that Glnase hydrolyzes Gln into Glu and ammonia. In this study, using NMR spectroscopy, an additional step, Glnase conversion of Glu to GABA, is demonstrated. The formation of GABA in the second step is further confirmed by mass spectrometry. Also, shown is that the rate of conversion of Glu to GABA by Glnase is around 1/10th than that reported for the Glutamate decarboxylase (GAD).

2. Materials and methods

35.7 ml of 0.1 M acetic acid was added to 64.3 ml of 0.1 M sodium acetate to give 100 ml of 0.1 M acetate buffer with a pH of 4.96 as measured with a pH meter (UB-10, Denver Instruments). Samples of 10 mM L-Gln, L-Glu, AS, GABA, L-Asn and L-Asp (all from

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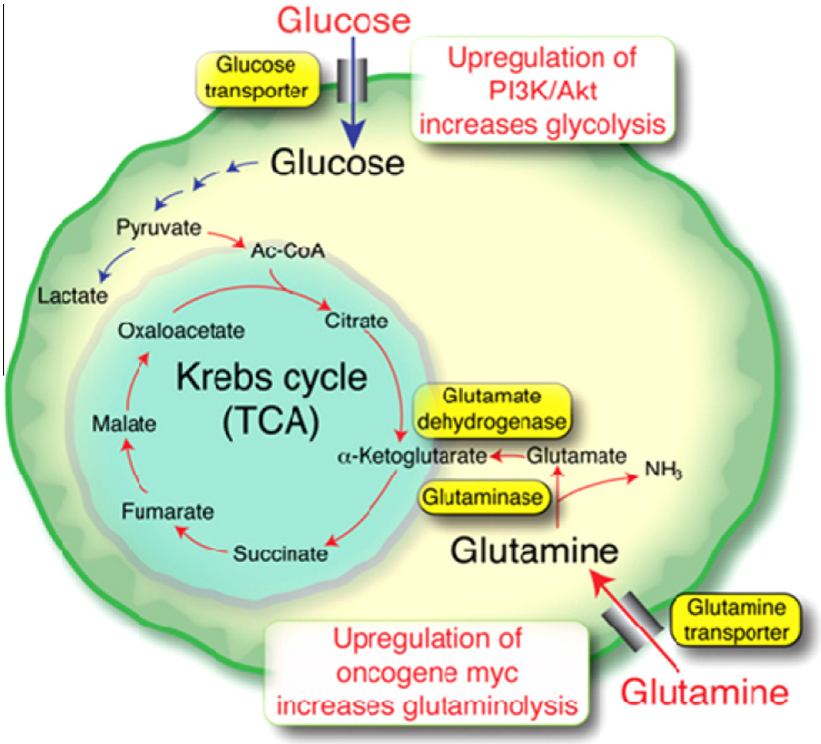

Fig. 1. Schematic drawing of intracellular metabolism of glucose and Glutamine and potential metabolic changes in tumor cells using glycolysis or glutaminolysis are shown. This figure was adapted from the research originally published in JNM [2]. © by the Society of Nuclear Medicine and Molecular Imaging, Inc.

Sigma–Aldrich, St. Louis, MO, USA) were prepared in 0.1 M acetate buffer and the final pH of all the samples were within 4.96 ± 0.04 units.

2.1. Preparation of Glutaminase

To the vial containing 2.4 mg of Glnase powder (Source: *Escherichia coli*; Lot# 113M4054V; Sigma–Aldrich, St. Louis, MO, USA), 1 ml of 0.1 M acetate buffer at pH 4.96 was added to prepare the stock solution. From the Sigma–Aldrich data sheet provided, the optimal condition for the Glnase activity was 0.1 M acetate buffer at pH 4.9 and at 37 °C. For the NMR experiments, 60 µl of this stock solution that corresponds to 3 U of Glnase was added to 540 µl of 10 mM Glutamine solution (5.4 µmol Gln) in a 5 mm Norell NMR tube just before the NMR acquisition and the reaction was monitored for 355 min at 37 °C. Since the conversion of Gln to Glu is very rapid (<6 min), we have diluted the enzyme by addition of 10 µl of stock solution to 490 µl of acetate buffer. From this diluted solution, 60 µl that corresponds to 0.05 U was used to follow the kinetics of Gln to Glu for over a period of 156 min. Similarly, separate samples of L-Glu, L-Asn and L-Asp were incubated with 3 U of Glnase at 37 °C for 4–6 h before NMR acquisition.

2.2. Data acquisition and processing

All the high resolution one-dimensional proton (1D-^{1}H) NMR experiments were performed at 310 K (by spinning the sample) using Bruker Avance DMX 400 MHz spectrometer equipped with a 5 mm PABBI proton probe. For all the NMR samples, a sealed capillary containing a mixture of D_2O (for lock) and 10 mM tetramethylsilane (TMS) (for reference) was inserted into the tubes. The spectral parameters used were 2 dummy acquisitions followed by 24 acquisitions (pulse sequence: 'zgpr45.sw'), 65536 TD (real+imaginary), 6775 Hz sweep width, and a relaxation delay of 4 s. All of the 1D-^{1}H NMR spectra were processed using Spin Works (version 4.0.0, Copyright ©2013, Kirk Marat, University of Manitoba). All the spectra were referenced to TMS. We have calculated the first order rate constant by plotting the peak integral against time in minutes. This data was fitted to following exponential equation: $Y(t) = Y_0 * (1 - \exp(-k * t))$, where '$k$' is the exponential recovery rate constant and 'Y' is peak integral at time 't'. For the first and second steps, peak integrals of Glu at 2.39 ppm and GABA at 3.03 ppm were measured, respectively.

2.3. Mass spectrometry (MS)

All the four samples (Gln+Glnase, Gln, Glu and GABA) were submitted to Metabolomic core at The Children's Hospital of Philadelphia for the small molecule MS using Agilent 6410 triple quad (LC/MS) mass spectrometer.

3. Results

Fig. 2 shows the high-resolution one dimensional (1D)-^{1}H NMR spectra of ammonium sulfate (AS), Glu, Gln and Gln at 6 min after addition of 3 U of Glnase. The spectrum obtained at 6 min after the addition of Glnase to Gln solution is clearly identical to that of Glu spectrum indicating that Glnase catalyzed the conversion of Gln to Glu. Truncated resonance at 1.98 ppm is from acetate buffer with its spinning side bands at 1.82 and 2.14 ppm. The peak at around 2.5 ppm corresponds to succinate from the enzyme solution.

Fig. 3a shows 1D-^{1}H NMR spectra from GABA, Glu, Gln and Gln+Glnase at different time points after the addition of 3 U of Glnase. Formation of Glu from the solution containing Glnase can be seen in (D) (data gathered within 6 min of adding Glnase to Gln) and the subsequent spectra shows the formation of GABA from Glu and the final spectrum shows majority (>85%) of Glu conver-

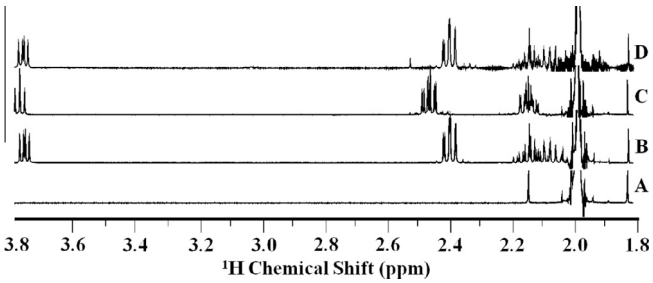

Fig. 2. High resolution NMR spectra from AS (A), Glu (B), Gln (C) and Gln+3 U of Glnase (D). No proton signal is seen from the AS while Glnase mediated conversion of Gln to Glu can be seen in (D). The sharp resonances at 1.82 and 2.14 ppm are the spinning side bands of acetate peak from buffer. Peak at ~2.5 ppm corresponds to the succinate from enzyme solution.

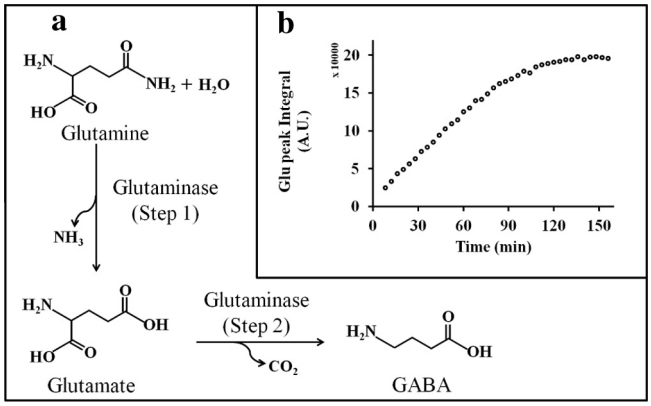

Fig. 4. (a) Glnase catalyzed Gln conversion to Glu and GABA. Step 1 shows Gln hydrolysis by Glnase to produce Glu and ammonia and in step 2 to decarboxylation of Glu by Glnase to produce GABA and CO_2 is shown. (b) Plot of Glu peak (2.39 ppm) integral (open circles) at different time points obtained by addition of 0.05 U of Glnase to Gln and shows the complete conversion to Glu at ~148 min. Exponential recovery rate constant (k) is 0.012 min^{-1} meaning that it takes 84 min to convert 63% of Gln to Glu.

sion to GABA by about 360 min, with a rate of ~16.87 μmol/min/mg of Glnase for the conversion of Glu to GABA (Fig. 3b).

Fig. 4a outlines the two-step conversion process of Gln to GABA by Glnase. The result from an additional experiment performed with 0.05 U of enzyme concentration reveals complete conversion of Gln to Glu in about 148 min (Fig. 4b) with a rate of ~0.185 μmol/min/mg of Glnase. From these data, the first step seems to be ~91 times faster than the second step.

Additionally, the formation of GABA was confirmed by liquid chromatography–mass spectrometry (LC–MS) of the sample containing the product of Glnase reaction with Gln at the end of 6 h, as well as the samples of Gln, Glu and GABA alone (Fig. S1). In addition, when Glnase was added to solution containing L-Glu alone, it catalyzed the conversion of Glu to GABA with the same rate (Fig. S2). In order to check the possibility of autonomous decarboxylation of Glutamate to GABA, we have acquired NMR spectra of L-Glu alone in the absence of Glnase every 1 h for 6 h and an additional spectra at the end of ~17 h (Fig. S3), which clearly shows that L-Glu is quite stable within as well as beyond our time frame of experiments.

4. Discussion

High-resolution NMR spectra clearly demonstrate that Glnase converted Gln first to Glu and later to GABA or catalyzed Glu solution to GABA in the same time frame. These results were further confirmed by mass spectrometry of the samples. This is a rather atypical observation, as the enzyme that is reportedly specific to Gln is also able to convert Glu to GABA. These observations suggest that the Glnase has two active sites, one of which hydrolyzes Gln to Glu and a second site that decarboxylates the resulting Glu to GABA. In order to determine the specificity of these active sites, we further performed separate experiments of Glnase reaction with L-Asparagine and L-Aspartic acid. In both these cases, the enzyme did not have any effect on these amino acids (Fig. S4) indicating that Glnase is specific to Gln and Glu only.

Glnase used in this study has been isolated from *E. coli*. Titrating active sites with the specific analog inhibitor, C-14C-diazo-oxo-L-norleucine, two active sites were revealed [13]. It was further shown that the enzyme will bind only substances having an unsubstituted L-glutamyl acyl portion and a substituent in the Y position no larger than 2-hydroxyethylamino or 2,2,2,-trifluroethoxy, and will hydrolyze only those in which the substituent is no larger than methylamino or ethoxy [13]. As described above, while there is

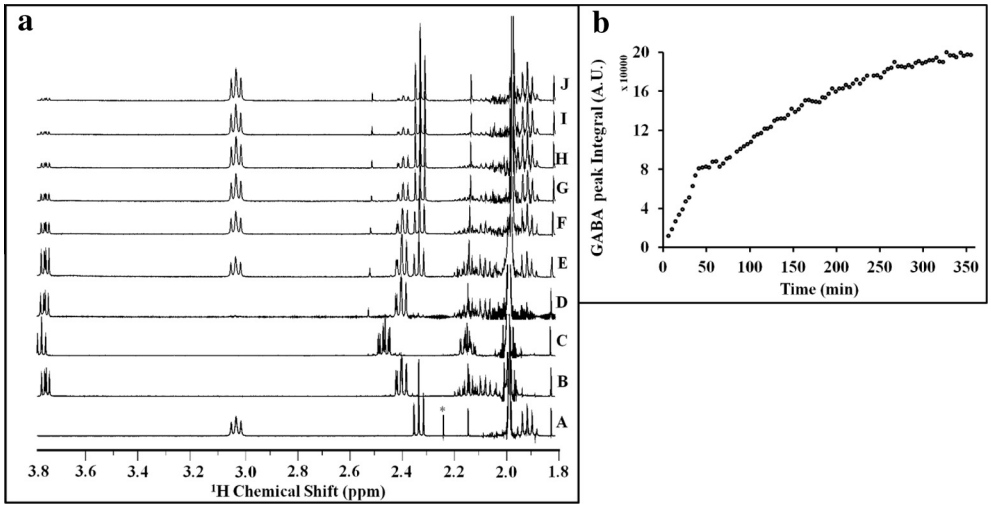

Fig. 3. (a) High-resolution NMR spectra from GABA (A), Glu (B), Gln (C), Gln+3 U of Glnase (D–J; ~6, 61, 120, 180, 243, 303 and 355 min after addition of Glnase to Gln), are shown. Formation of Glu from the solution containing enzyme can be seen (data gathered within 6 min of adding enzyme to Gln) (D) and conversion of Gln to GABA can be seen in (E–J). Peak at ~2.5 ppm corresponds to the succinate from enzyme solution. Peak marked with * in GABA spectra could either be artifact or impurity from the sample. (b) Plot of GABA peak (3.03 ppm) integral (open circles) at different time points obtained by addition of 3 U of Glnase to Gln. Exponential recovery rate constant (k) is 0.008 min^{-1} meaning that it takes 128 min to convert 63% of Glu to GABA.

some information in the literature regarding the possibility of Glnase having two active sites, there are no studies demonstrating either the activity or specificity of the second active site on this enzyme to Glu or any other amino acid.

Since, in *in vivo* conditions, Glu concentration is 10–15 mM where as GABA is 1–2 mM, the observed reaction kinetics suggests that Glnase might potentially play a role in the synthesis of GABA. Recent studies on GAD report the specific activity of this enzyme on L-Glu conversion to GABA in the range of 2–3 μmol of CO_2 formed/min/mg [14,15] whereas in our study the rate for Glnase mediated GABA formation (~0.185 μmol/min/mg) is 1/10th to 1/16th lower. In a published enzyme activity study [16], performed on the frontal lobe brain tissues obtained post mortem from normal subjects the activity for Glnase was reported to be ~10-fold higher than that of GAD (0.287 vs. 0.026 μmol product formed/hour/mg of protein cytosolic enzyme) while the activity for Glnase was ~30-fold higher in schizophrenic subjects (1.246 vs. 0.040 μmol product formed/hour/mg of protein cytosolic enzyme). If these results hold true for the *in vivo* conditions then this study will open up the discussion on the role of Glnase in the synthesis of GABA under *in vivo* situations and potentially have significant implications in studies involving underlying mechanisms of Glu–Gln cycle in the brain as well as in glutaminolysis in tumors.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.04.059.

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