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Analysis of whole body ammonia metabolism in *Aedes aegypti* using [^{15}N]-labeled compounds and mass spectrometry

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Abstract

We have established a protocol to study the kinetics of incorporation of ^{15}N into glutamine (Gln), glutamic acid (Glu), alanine (Ala) and proline (Pro) in *Aedes aegypti* females. Mosquitoes were fed 3% sucrose solutions containing either 80 mM $^{15}NH_4Cl$ or 80 mM glutamine labeled with ^{15}N in either the amide nitrogen or in both amide and amine nitrogens. In some experiments, specific inhibitors of glutamine synthetase or glutamate synthase were added to the feeding solutions. At different times post feeding, which varied between 0 and 96 h, the mosquitoes were immersed in liquid nitrogen and then processed. These samples plus deuterium labeled internal standards were derivatized as dimethylformamidine isobutyl esters or isobutyl esters. The quantification of ^{15}N-labeled and unlabeled amino acids was performed by using mass spectrometry techniques. The results indicated that the rate of incorporation of ^{15}N into amino acids was rapid and that the label first appeared in the amide side chain of Gln and then in the amino group of Gln, Glu, Ala and Pro. The addition of inhibitors of key enzymes related to the ammonia metabolism confirmed that mosquitoes efficiently metabolize ammonia through a metabolic route that mainly involves glutamine synthetase (GS) and glutamate synthase (GltS). Moreover, a complete deduced amino acid sequence for GltS of *Ae. aegypti* was determined. The sequence analysis revealed that mosquito glutamate synthase belongs to the category of NADH-dependent GltS.

Keywords: Labeled amino acids; Glutamine synthetase; Glutamate synthase; Metabolic pathways; Sequence analysis

1. Introduction

During the process of taking blood, female mosquitoes can transmit disease-causing pathogens such as malaria protozoans, and dengue, West Nile, and yellow fever viruses, which altogether affect millions of people annually worldwide. Attempts to control mosquito populations using biorational approaches depend on a thorough understanding of mosquito biology and metabolism.

Aedes aegypti females use only about 3% of the amino acids derived from the digestion of blood meal proteins for egg protein production. Some of the remaining amino acids are used to synthesize egg lipids, while others are used to build energy reserves of the female. But, more than 60% of the amino acids are oxidized to CO_2 to provide the energy needed for egg production (Briegel, 1985; Zhou et al., 2004). An important by-product of amino acid oxidation is ammonia, which is highly toxic to animal tissues. However, *Ae. aegypti* females are able to survive the massive deamination that takes place during the catabolism of the amino acids derived from blood meal proteins, suggesting that mosquitoes have efficient physiological mechanisms to detoxify ammonia. In this paper we will use ammonia to refer to both NH_3 and NH_4^+ or a combination of the two (Campbell, 1997).

We previously proposed that mosquitoes utilize proline as a temporary nitrogen sink to store ammonia arising from deamination of blood meal protein amino acids (Goldstrohm et al., 2003) and that proline can also be used

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as fuel during flight (Scaraffia and Wells, 2003). However, when proline serves as a source of energy, alanine and glutamine seem to be involved in the shuttling of the amino group between the flight muscle and the fat body to avoid ammonia's toxic effects (Scaraffia and Wells, 2003). In correlation with these observations, when *Ae. aegypti* females were given access to solutions containing ammonium chloride, hemolymph glutamine and proline concentrations increased markedly, suggesting that mosquitoes are able to detoxify ammonia mainly through the synthesis of these two amino acids. A mechanism for ammonia assimilation in mosquitoes, involving glutamine synthetase and glutamate synthase, was proposed based on inhibitor studies, and the fat body was implicated as the main tissue involved in the ammonia detoxification in *Ae. aegypti* females (Scaraffia et al., 2005). Glutamate synthase is also known as GOGAT (from the initial acronym assigned to glutamate synthase), but in this paper, we will use the currently accepted acronym, GltS.

To better understand how mosquitoes metabolize ammonia, we have begun to take a systems biology approach (Hellerstein, 2003) based on the use of $^{15}NH_4Cl$ and mass spectrometry in order to study the kinetics of whole body ammonia metabolism. As a prelude to this work, we have recently investigated the fragmentation mechanism of derivatized glutamine and developed a method to identify and quantify both ^{15}N-labeled and unlabeled glutamine (labeled glutamine includes ^{15}N-amide labeled, ^{15}N-amine labeled and ^{15}N-labeled at both amide and amine positions) and glutamic acid at a series of different neutral losses by performing multiple-reaction monitoring scans in a triple-quadrupole mass spectrometer (Zhang et al., 2005). The results presented in this paper confirm and extend the role of the glutamine synthetase (GS)–glutamate synthase (GltS) reactions as the main metabolic pathway involved in ammonia metabolism in mosquitoes. In addition, we report the entire amino acid sequence of *Ae. aegypti* GltS (see Appendix A). The molecular signatures involved in electron donors and the previous biochemical studies (Scaraffia et al., 2005) confirm that *Ae. aegypti* GltS is a NADH-dependent enzyme.

2. Materials and methods

2.1. Insects

Ae. aegypti (NIH-Rockefeller strain) were reared under standard conditions (Scaraffia and Wells, 2003). Adults were allowed to feed on 3% sucrose for the first 3–4 days and starved for 24 h before experimental feeding.

2.2. Feeding procedure

Females were placed individually in 10-ml plastic scintillation vials covered with nylon mesh for feeding. Mosquitoes were allowed to feed on one of several solutions containing either Ammonium-^{15}N Chloride ($^{15}NH_4Cl$), L-Glutamine-amide-^{15}N ([5-^{15}N]-Gln) or L-Glutamine-$^{15}N_2$ ([2,5-$^{15}N_2$]-Gln) in 3% sucrose for 15 min. Solutions were directly applied to the mesh in a volume of 5 μl. In some experiments, specific inhibitors of GS or GltS were added to the feeding solutions. Controls were performed by feeding mosquitoes on 3% sucrose. Only mosquitoes that fed to repletion were used.

2.3. Sample preparation and derivatization methods

At different times post-feeding, which varied between 0 and 96 h (as is indicated in figure legends), the mosquitoes were immersed in liquid nitrogen. Whole bodies of 10 insects were homogenized in a Potter–Elvehjem tissue grinder in 150 μl of water. The suspension was boiled for 1 min, centrifuged at 13,000 rpm for 5 min, and the supernatant collected.

For the quantification of unlabeled and labeled amino acids, 25 μl of supernatant was mixed with 25 μl of 1 mM deuterium labeled amino acids as internal standards. Then, the solution was dried using a vacuum centrifuge and derivatized as dimethylformamidine isobutyl esters or as isobutyl esters as described previously (Zhang et al., 2005). Briefly, samples mixed with deuterium labeled amino acid standards ([$^2H_5$]-Gln, [$^2H_5$]-Glu and [$^2H_4$]-Ala) were treated with 240 μl of dimethylacetal dimethylformamide (DMF–DMA)-acetonitrile-methanol (2:5:5 by volume) for 10 min at room temperature. Excess reagents were removed under a gentle stream of nitrogen. The residue was treated with isobutanol/3 M hydrogen chloride at room temperature for 50 min and then the solvent was removed under a stream of nitrogen. For proline analysis, samples with [$^2H_7$]-Pro were only treated with isobutanol/3 M hydrogen chloride at room temperature for 50 min and the excess reagent was removed under a stream of nitrogen. The derivatization products were dissolved in a solution containing methanol-water-acetic acid (50:50:1 by volume) before the mass spectrometry (MS) analysis.

2.4. Nanospray ionization tandem mass spectrometry

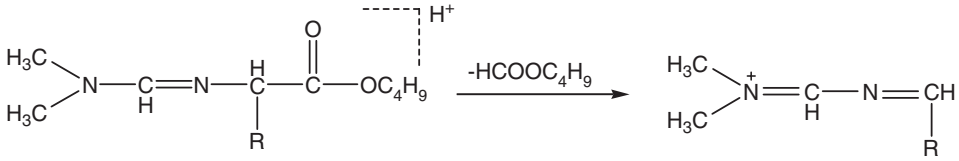
ESI/MS/MS analyses were performed on a Finnigan MAT TSQ-700 triple quadrupole mass spectrometer (San Jose, CA) equipped with a nanospray ion source operating in the positive ion mode.

The samples were loaded into the nanospray capillary with a tip I.D. of 5–10 μm. The needle voltage used was 1.2–1.6 kV and the skimmer was at ground potential. The capillary temperature was maintained at 200 °C. Collision induced dissociation was performed with argon (3 mTorr) using a collision energy of 20 eV. The instrument was tuned to unit mass resolution and the mass spectra were acquired in profile mode. The identification of unlabeled and labeled amino acids was performed by

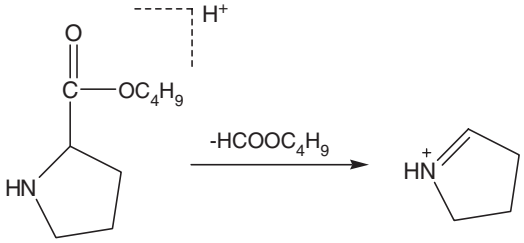
Table 1
m/z of precursor ions and neutral losses for unlabeled and labeled dimethylformamidine isobutylester of amino acids* or isobutylester of amino acids**

Amino acids	Precursor ions after derivatization	Neutral losses
[^{14}N]-Ala, [^{15}N]-Ala, [$^{2}H_4$]-Ala	*201, *202, *205	102
[^{14}N]-Glu, [^{15}N]-Glu, [$^{2}H_5$]-Glu	*315, *316, *320	102
[^{14}N]-Pro, [^{15}N]-Pro, [$^{2}H_7$]-Pro	**172, **173, **179	102
[2,5-$^{14}N_2$]-Gln	*258	73
[5-^{15}N]-Gln	*259	74
[2-^{15}N]-Gln	*259	73
[2,5-$^{15}N_2$]-Gln	*260	74
[$^{2}H_5$]-Gln	*263	73

Multi-reaction monitoring (MRM) scan is a mass spectrometry technique which can detect the compound of interest from a mixture with high sensitivity and selectivity (Vogg et al., 1999; Nagy et al., 2003). In the present study, MRM scan was performed in a triple quadrupole mass spectrometer with two mass analyzers and a collision cell. The precursor ion is fragmented in the collision cell. The two analyzers are synchronized to transmit the precursor and a fragment ion for only the compound of interest, i.e. only the compound with certain neutral loss can be transmitted and detected. For the detection of alanine, glutamic acid and proline, a neutral loss of 102 Da ($HCOOC_4H_9$) occurs to the amino acid derivatives, as shown below.Neutral loss of 102 Da ($HCOOC_4H_9$) from dimethylformamidine isobutyl esters of Ala and Glu (Johnson, 2001).



Neutral loss of 102 Da from proline isobutyl ester.



For the detection of unlabeled glutamine, a neutral loss of 73 Da from dimethylformamidine glutamine isobutyl ester is used. The neutral loss corresponds to a two-step process: the loss of NH_3 (17 Da) from the glutamine side chain and the loss of isobutene (56 Da) from the isobutyl ester group. For glutamine with label(s) at different positions, different neutral losses (73 or 74 Da) are used depending on that whether the ^{15}N label(s) is involved in the neutral loss or not (Zhang et al., 2005).

multiple-reaction monitoring scans (Table 1). Data were exported out as a text file and processed with the Xcalibur program (Xcalibur software version 1.4; Thermo Electron Corporation) using the peak area for the quantification of each amino acid. Data were expressed as nmol amino acid/animal. The natural abundance of isotopes for each amino acid and isotope effect corrections for glutamine were considered in the calculation (Zhang et al., 2005).

2.5. Statistical analyses

Data are presented as mean ± standard error of three to six independent samples. Student's t-test and one-way analysis of variance (ANOVA) followed by Dunnett's Multiple Comparison Test were used. A p-value less than 0.05 was considered significant. All the statistical analyses were carried out using GraphPad Prism version 4.0 (GraphPad Software, San Diego, CA, USA).

2.6. Reagents

Acetyl chloride, azaserine, isobutanol, sucrose, DL-methionine-DL-sulfoximine were purchased from Sigma-Aldrich (St. Louis, MO, USA). Ammonium-^{15}N Chloride and L-Glutamine-amide-^{15}N were purchased from Isotec (Miamisburg, OH, USA). L-Glutamine-$^{15}N_2$ was purchased from Sigma-Aldrich (Milwaukee, WI, USA). The deuterium labeled amino acid standards were purchased from Cambridge Isotope Laboratories (Andover, MA, USA). Methanol and acetonitrile were obtained from EMD Chemicals Inc. (Gibbstown, NJ, USA). *N*, *N*-Dimethylformamide

dimethyl acetal was purchased from Pierce (Rockford, IL, USA). Isobutanol/3 M HCl was prepared according to Johnson (2001).

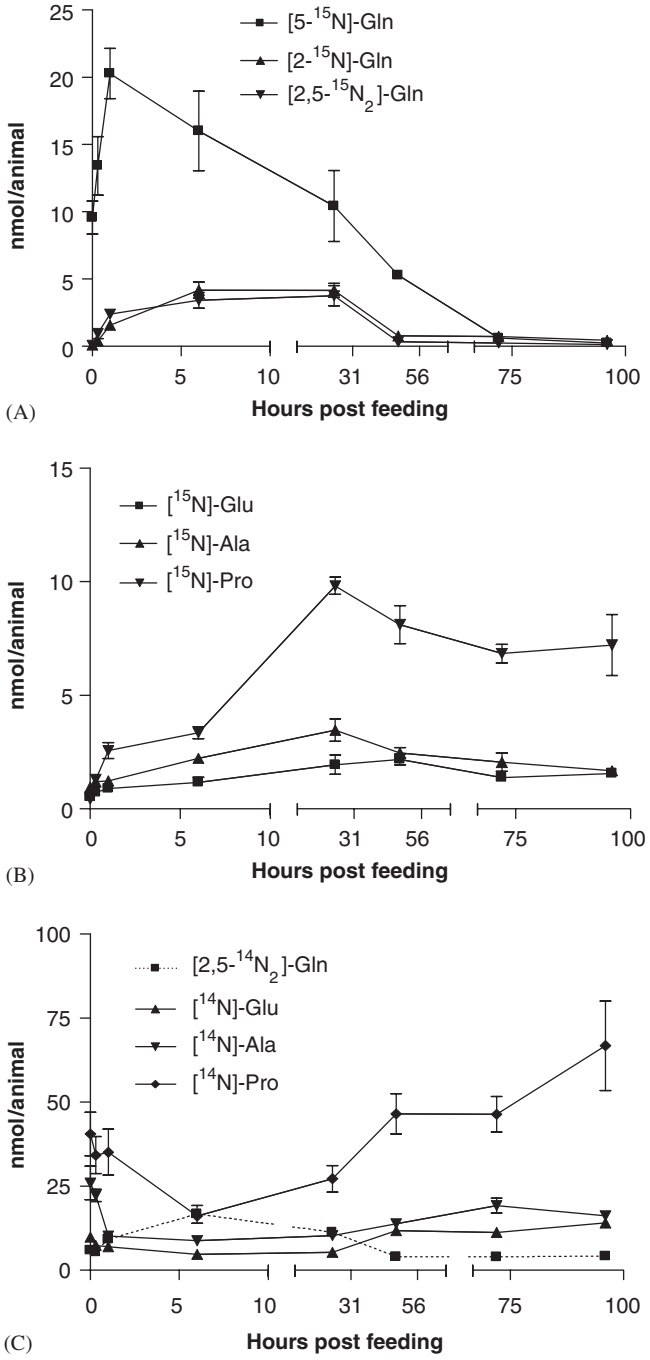


Fig. 1. Effect of 80 mM $^{15}NH_4Cl$ on whole body amino acid concentrations from *Ae. aegypti*. (A) Time course of [5-^{15}N]-Gln, [2-^{15}N]-Gln and [2,5-$^{15}N_2$]-Gln, (B) time course of [^{15}N]-Glu, [^{15}N]-Ala and [^{15}N]-Pro, (C) time course of [2,5-$^{14}N_2$]-Gln, [^{14}N]-Glu, [^{14}N]-Ala and [^{14}N]-Pro. Mosquitoes were fed on labeled ammonia solution for 15 min, after that they were immersed in liquid nitrogen at 0, 0.25, 1, 6, 24, 48, 72 and 96 h post-feeding (see Section 2 for details). Data are presented as mean ± standard error of three to six independent samples.

3. Results

3.1. Kinetics of the incorporation of ^{15}N from $^{15}NH_4Cl$ into amino acids

The time course for the incorporation of ^{15}N from $^{15}NH_4Cl$ into whole body amino acids from *Ae. aegypti* is shown in Fig. 1 (note that these are whole body amino acid concentrations, which are about 10-fold higher than the hemolymph amino acid concentrations reported previously; Goldstrohm et al., 2003; Scaraffia et al., 2005). Immediately after feeding mosquitoes for 15 min with 80 mM $^{15}NH_4Cl$, labeled amino acids were detected, indicating a rapid incorporation of ^{15}N into amino acids (Fig. 1A and B). The highest labeled amino acid concentrations were [5-^{15}N]-Gln (labeled amide nitrogen) followed by [^{15}N]-Pro. The concentration of [5-^{15}N]-Gln reached a maximum at 1 h post-feeding, started to decline by 6 h post-feeding, and was close to zero by the end of the time course (Fig. 1A). The highest [^{15}N]-Pro level was observed at 24 h post-feeding, but even at 96 h post-feeding, the [^{15}N]-Pro concentration was still high (Fig. 1B)

During the time course, the changes in the concentrations of the other labeled amino acids were less pronounced than those observed for [5-^{15}N]-Gln and [^{15}N]-Pro. [2-^{15}N]-Gln and [2,5-$^{15}N_2$]-Gln concentrations reached a peak at 6 and 24 h post-feeding, and then decreased significantly (Fig. 1A). The kinetics of incorporation of ^{15}N into glutamate and alanine showed a similar pattern to that of [^{15}N]-Pro, although the levels of [^{15}N]-Glu and [^{15}N]-Ala were lower than that of [^{15}N]-Pro throughout the time course (Fig. 1B).

One advantage of using mass spectrometry for these analyses is that information about the concentration of unlabeled amino acids is also obtained (Fig. 1C). The concentration of [2, 5-$^{14}N_2$]-Gln reached a peak at 6 h post-feeding and started to decrease by 24 h post-feeding. At 48, 72 and 96 h post-feeding, the [2,5-$^{14}N_2$]-Gln concentration remained constant with values close to those observed at the beginning of the time course. In contrast, the concentration of [^{14}N]-Glu, [^{14}N]-Ala and [^{14}N]-Pro reached a minimum at 6 hours post-feeding, and then increased and remained almost constant until the end of the time course (Fig. 1C). Sucrose feeding, per se, did not significantly affect the whole body amino acid concentration (in nmol/animal [2, 5-$^{14}N_2$]-Gln = 6.4 ± 1.2; [^{14}N]-Glu = 11.1 ± 2.6; [^{14}N]-Ala = 23.1 ± 5.2; and [^{14}N]-Pro = 39.6 ± 5.1) over the same time course as shown in Fig. 1C.

3.2. Effect of inhibitors on kinetics of incorporation of ^{15}N from $^{15}NH_4Cl$ into labeled amino acids

When 20 mM MS, an inhibitor of GS (Eisenberg et al., 2000), was included in the meal, the concentration of [5-^{15}N]-Gln, [2-^{15}N]-Gln and [2,5-$^{15}N_2$]-Gln decreased significantly compared to controls, whereas the concentration

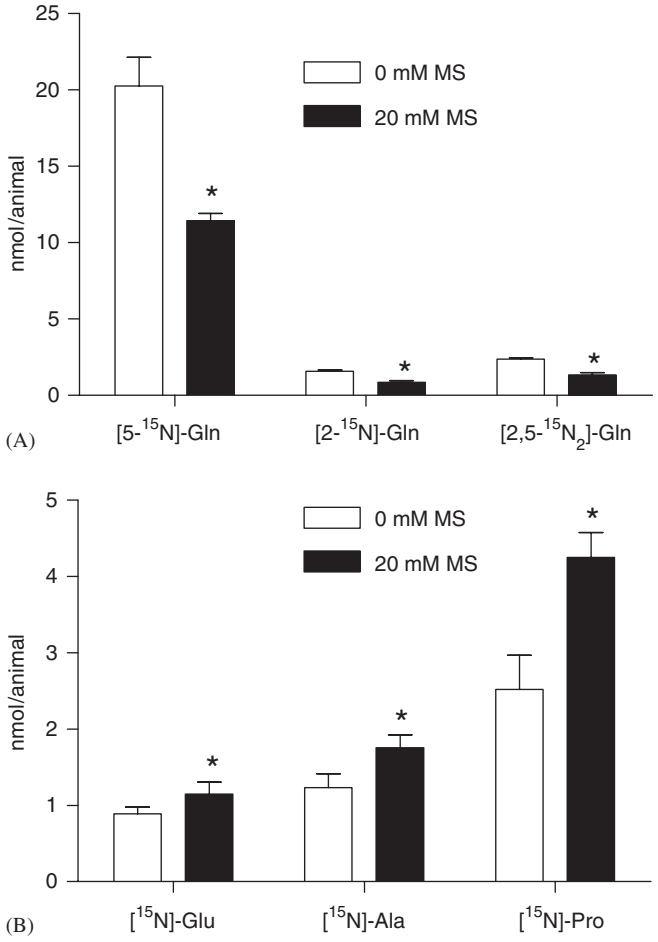


Fig. 2. Effect of $^{15}NH_4Cl$ and D,L-methionine D,L-sulfoximine on whole body labeled amino acid concentrations from *Ae. aegypti*. Labeled amino acid concentrations were measured 1 h after feeding mosquitoes on a sucrose solution containing 80 mM $^{15}NH_4Cl$ with or without 20 mM D,L-methionine D,L-sulfoximine (MS). (A) Labeled glutamine, (B) labeled glutamic acid, alanine and proline. Data are presented as mean ± standard error of three to six independent samples. $^*p < 0.05$ when compared to 0 by Student's *t* test.

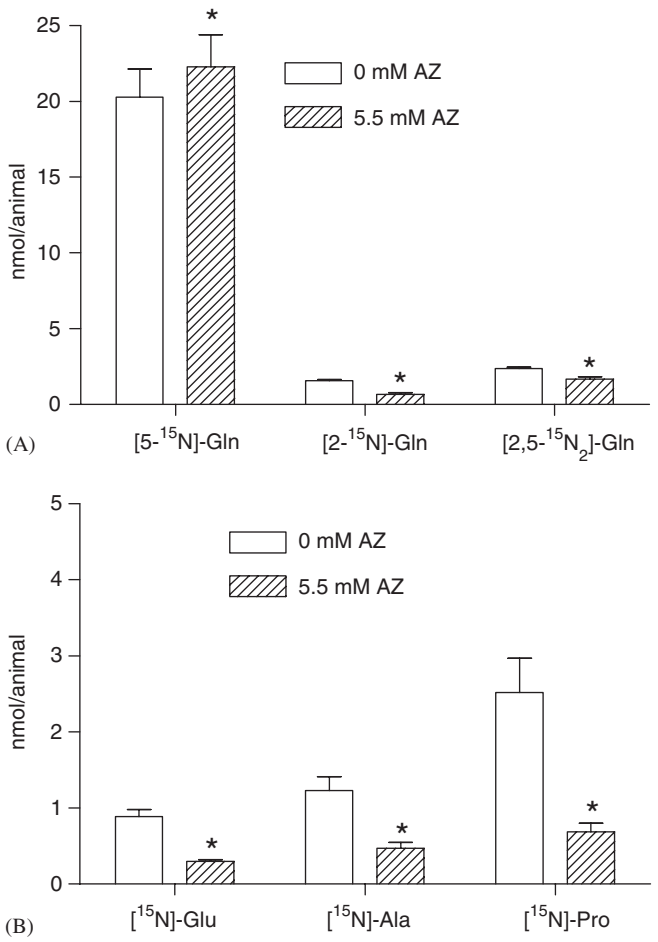


Fig. 3. Effect of $^{15}NH_4Cl$ and azaserine on whole body labeled amino acid concentrations from *Ae. aegypti*. Labeled amino acid concentrations were measured 1 h after feeding mosquitoes on sucrose solution containing 80 mM $^{15}NH_4Cl$ with or without 5.5 mM azaserine (AZ). (A) Labeled glutamine, (B) labeled glutamic acid, alanine and proline. Data are presented as mean ± standard error of three to six independent samples. $^*p < 0.05$ when compared to 0 by Student's *t* test.

of [^{15}N]-Glu, [^{15}N]-Ala and [^{15}N]-Pro increased significantly (Fig. 2A and B).

However, in the presence of 5.5 mM azaserine (AZ), an inhibitor of GltS (Miflin and Lea, 1977), the concentration of [5-^{15}N]-Gln increased while the concentration of [2-^{15}N]-Gln, [2,5-$^{15}N_2$]-Gln, [^{15}N]-Glu, [^{15}N]-Ala and [^{15}N]-Pro decreased significantly (Fig. 3A and B).

3.3. Kinetics of [^{15}N]-labeled-glutamine metabolism

Feeding mosquitoes with either 80 mM [5-^{15}N]-Gln or 80 mM [2,5-$^{15}N_2$]-Gln increased the whole body concentration of these amino acids rapidly, but their concentrations subsequently decreased abruptly after 6 h and they were essentially undetectable by 24 h (Fig. 4A). For each of the two labeled glutamine molecules, the [^{15}N]-Pro and [^{15}N]-Glu levels reached a peak at 6 h post-feeding and tended to remain constant until 24 h post-feeding (Fig. 4B and 4C), whereas the [^{15}N]-Ala concentration reached peaks at 1 and 6 h post feeding with [2,5-$^{15}N_2$]-Gln and [5-^{15}N]-Gln, respectively, and then remained constant (Fig. 4D). These data show that the label in [2,5-$^{15}N_2$]-Gln and [5-^{15}N]-Gln can equilibrate with these other amino acids. The label in these other amino acids accounts for about 50% of the whole body concentration of the supplied isotope. The fate of the remainder is unknown at present, but it may be excreted. In the case of post-feeding with [2,5-$^{15}N_2$]-Gln, the concentration of labeled Pro, Ala and Glu was about twice that produced when [5-^{15}N]-Gln was used. This is consistent with the fact that there is twice as much ^{15}N in [2,5-$^{15}N_2$]-Gln compared to [5-^{15}N]-Gln and the fact that [^{15}N]-Glu, [^{15}N]-Ala and [^{15}N]-Pro must be produced by a pathway in which both labeled nitrogens from [2,5-$^{15}N_2$]-Gln are conserved, i.e., the GltS pathway. The levels of [2-^{15}N]-Gln and [5-^{15}N]-Gln post-feeding [2,5-$^{15}N_2$]-Gln and the levels of [2-^{15}N]-Gln and [2,5-$^{15}N_2$]-Gln post-feeding [5-^{15}N]-Gln were negligible during the time course (data not shown).

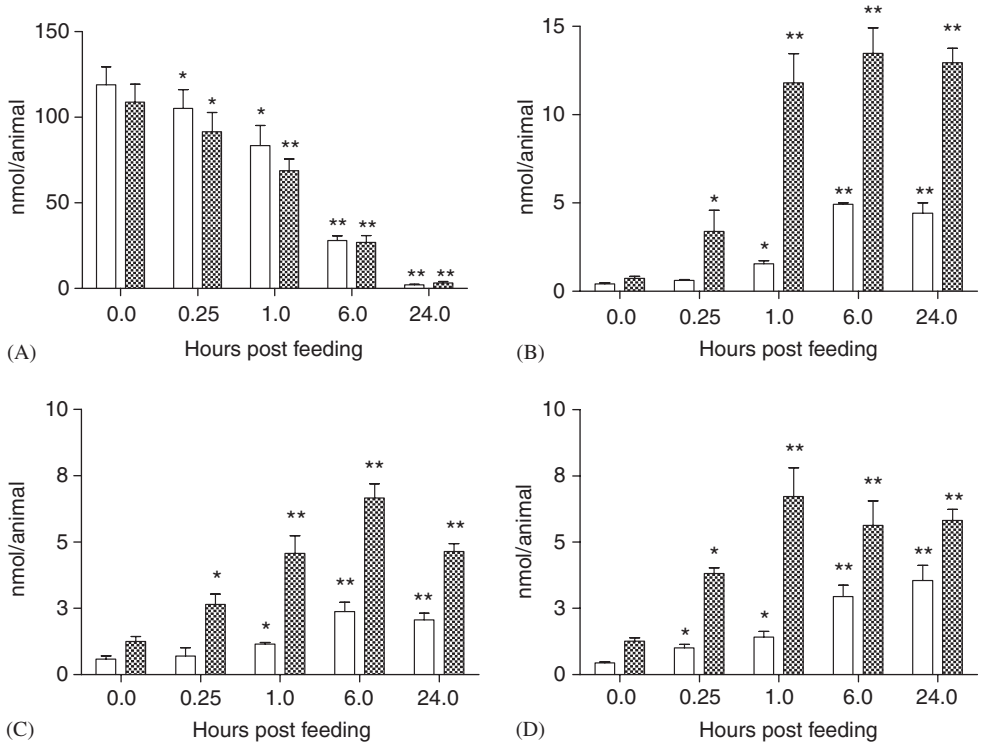


Fig. 4. Time course of whole body labeled amino acids from *Ae. aegypti* after feeding on 80 mM [5-^{15}N]-Gln (white bars) or [2,5-$^{15}N_2$]-Gln (dark bars) in 3% sucrose. (A) [5-^{15}N]-Gln (white bars) and [2,5-$^{15}N_2$]-Gln (dark bars), (B) [^{15}N]-Pro, (C) [^{15}N]-Glu, (D) [^{15}N]-Ala. Data are presented as mean ± standard error of three to four independent samples. $^{*}p<0.05$ and $^{**}p<0.01$ when compared to 0 post-feeding with 80 mM [5-^{15}N]-Gln or [2,5-$^{15}N_2$]-Gln by ANOVA.

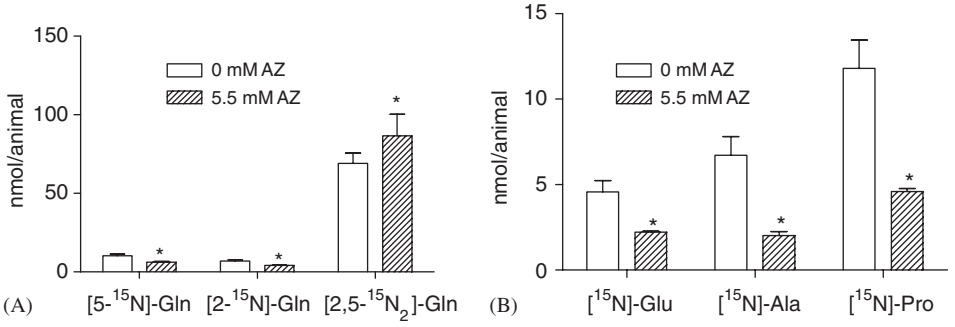


Fig. 5. Effect of [2,5-$^{15}N_2$]-Gln and azaserine on whole body labeled amino acid concentrations from *Ae. aegypti*. Labeled amino acid concentrations were measured 1 h after feeding on sucrose solution containing 80 mM [2,5-$^{15}N_2$]-Gln with or without 5.5 mM azaserine (AZ). (A) Labeled glutamine, (B) labeled glutamic acid, alanine and proline. Data are presented as mean ± standard error of three to four independent samples. $^{*}p<0.05$ when compared to 0 by Student's *t* test.

When mosquitoes were fed with 5.5 mM AZ in the presence of 80 mM [2, 5-$^{15}N_2$]-Gln, the levels of [5-^{15}N]-Gln, [2-^{15}N]-Gln, [^{15}N]-Glu, [^{15}N]-Ala and [^{15}N]-Pro were reduced significantly, whereas the [2,5-$^{15}N_2$]-Gln concentration increased significantly (Fig. 5A and B), indicating diminished metabolism. The effects observed in the presence of 80 mM [5-^{15}N]-Gln and AZ (data not shown) were very similar to those observed in Fig. 5 with [2,5-$^{15}N_2$]-Gln.

4. Discussion

In previous reports we outlined how mosquitoes are able to survive the large amount of ammonia produced during amino acid metabolism following a blood meal (Goldstrohm et al., 2003; Scaraffia et al., 2005). In those papers we relied on the chemical determination of amino acids, the use of enzyme inhibitors, and cloning of putative enzymes in new pathways. In the present research we studied the

kinetics of ammonia metabolism in *Ae. aegypti* in order to follow the rate of incorporation of ammonia into various amino acids. The use of $^{15}NH_4Cl$ and mass spectrometry enables one to measure the concentration of the [^{15}N]-amino acids over time and also to follow the kinetics of the changes in concentration of [^{14}N]-amino acids, which provides new information about the metabolism of preexisting amino acids in the mosquitoes during an ammonia challenge. The data obtained in this study confirm that *Ae. aegypti* females have an extraordinary capacity to deal with ammonia mainly through metabolic pathways involving glutamine and proline synthesis.

The kinetics of incorporation of ^{15}N from labeled ammonium chloride into amino acids occurs in two consecutive phases: one of fixation and another of assimilation (Fig. 6). The fixation phase starts immediately after mosquitoes ingest $^{15}NH_4Cl$ and continues for about 1 h. During this phase, the $^{15}NH_3$ is rapidly incorporated into glutamine by GS. This reaction fixes labeled ammonia into unlabeled glutamate to yield [5-^{15}N]-Gln, which accumulates in large amounts (Fig. 1). The assimilation phase turns on after the fixation phase starts and remains active through the entire time course. During the assimilation phase part of the ^{15}N from [5-^{15}N]-Gln is metabolized by GltS to produce [^{15}N]-Glu, which is mainly converted to [^{15}N]-Pro and to a lesser extent to [^{15}N]-Ala. The [^{15}N]-Glu produced by GltS can also be used by GS to fix another molecule of labeled ammonia to produce [2,5-$^{15}N_2$]-Gln, which can also be converted into [^{15}N]-Glu through GltS (Fig. 6). The [5-^{15}N]-Gln that is not metabolized by GltS, must have other metabolic fates such as uric acid synthesis. We have recently reported that in addition to ammonia, mosquitoes excrete uric acid after feeding a load of NH_4Cl (Scaraffia et al., 2005; see also von Dungern and Briegel, 2001). *Ae. aegypti* females could utilize the nitrogen of the amide group of two glutamine molecules to synthesize one uric acid molecule, if they follow the same metabolic pathway that vertebrates use for synthesizing uric acid (Sonne et al., 1956; Levenberg et al., 1956).

The changes observed in the unlabeled amino acid concentrations post-feeding with $^{15}NH_4Cl$ are also consistent with the role that GS/GltS pathway plays in the fixation and assimilation of ammonia in mosquitoes.

The fact that the concentrations of [^{15}N]-Glu, [2-^{15}N]-Gln and [2,5-$^{15}N_2$]-Gln are low compared to that of [5-^{15}N]-Gln during the initial fixation period indicates that glutamate dehydrogenase (GDH), which would fix labeled ammonia into α-ketoglutarate to synthesize [^{15}N]-Glu, is of only minor significance. Further support for the importance of GS/GltS pathway comes from the fact that in the presence of AZ, an inhibitor of GltS, the synthesis of [^{15}N]-Glu, [^{15}N]-Ala and [^{15}N]-Pro were significantly reduced. The amounts of [2-^{15}N]-Gln and [2,5-$^{15}N_2$]-Gln were almost negligible in the presence of AZ suggesting that [^{15}N]-Glu used to synthesize [2-^{15}N]-Gln and [2,5-$^{15}N_2$]-Gln is produced mainly by GltS and further confirming the secondary role played by GDH. In agreement with these observations, it was reported that GDH plays a minor role in ammonia fixation in the silkworm *Bombyx mori* (Hirayama et al., 1997, 1998; Hirayama and Nakamura, 2002).

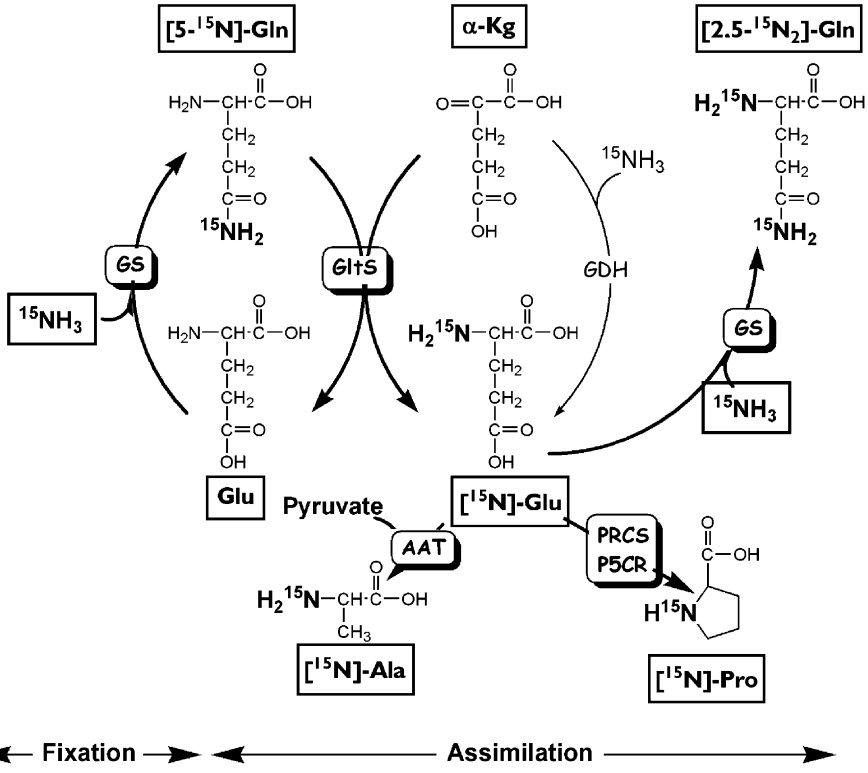


Fig. 6. Pathways for ammonia metabolism in mosquitoes.

However, the results obtained in the presence of $^{15}NH_4Cl$ and MS are consistent with the suggestion that when GS was inhibited with MS, GDH (Fig. 6) was involved in the detoxification of ammonia in *Ae. aegypti* (Scaraffia et al., 2005). Thus, when GS activity is reduced, the $^{15}NH_3$ is fixed to α-ketoglutarate by GDH to produce [^{15}N]-Glu which is mainly converted to [^{15}N]-Pro by pyrrolidine carboxylase synthase (P5CS) and pyrrolidine carboxylase reductase (P5CR). The presence of [^{15}N]-Ala indicates that [^{15}N]-Glu can also be used in transamination reactions (Fig. 6). Alanine aminotransferase (AAT), which catalyzes the transfer of the amino group of glutamate to pyruvate to produce alanine, is active in mosquitoes (Scaraffia et al., 2005).

The time course experiments performed using [2,5-$^{15}N_2$]-Gln or [5-^{15}N]-Gln clearly demonstrate that a significant portion of the glutamine is utilized for proline synthesis through GltS. In the presence of [2,5-$^{15}N_2$]-Gln, the concentration of [^{15}N]-Pro increased almost twice as much as in the presence of [5-^{15}N]-Gln. This happens because one molecule [2,5-$^{15}N_2$]-Gln can produce two molecules of [^{15}N]-Glu via GltS. Further, the participation of GltS in the metabolism of glutamine and its role in proline synthesis was demonstrated by the fact that in the presence of AZ the [^{15}N]-Pro concentration was reduced significantly.

In the literature, the GltS enzyme has been classified in three categories on the basis of the amino acid sequence and the nature of the electron donor: Ferredoxin-dependent GltS, NADPH-dependent and NADH-dependent (van den Heuvel et al., 2004; Suzuki and Knaff, 2005; Vanoni and Curti, 1999, 2005; Vanoni et al., 2005). Several lines of evidence suggest that mosquito GltS is a NADH-dependent GltS. First, according to the amino acid sequence, *Ae. aegypti* GltS is synthesized as a monomeric polypeptide and shares a high degree of identity with alfalfa (*Medicago sativa*) NADH-dependent GltS sequence (see Appendix A Fig. 1). The amino acid sequence encoding the *Ae. aegypti* NADH-dependent GltS constitutes the first complete amino acid sequence of GltS obtained from animals. Second, the NADH binding site, GXGXXG, in C-terminal of *Ae. aegypti* GltS, is conserved between mosquito and *M. sativa* GltS. Third, a previous study indicates that NADH is required as an electron donor for GltS activity (Scaraffia et al., 2005). Taken together, these data support our conclusion that mosquito GltS belongs to the NADH-dependent GltS class.

The importance of the GS/GltS pathway in the glutamic acid synthesis during the assimilation of ammonia in bacteria and plants has been reported in numerous papers (for reviews see Miflin and Lea, 1977; Lea and Miflin, 2003; Reitzer, 2003; Muro-Pastor et al., 2005; Suzuki and Knaff, 2005; Vanoni and Curti, 1999, 2005; Vanoni et al., 2005). However, in animals this pathway has been not investigated extensively. It has been reported that the GS/GltS pathway produces glutamic acid which is used for silk synthesis in *B. mori* larvae (Hirayama et al., 1997), whereas in *Samia cynthia ricini* larvae (Osanai et al., 2000) and in *Spodoptera frugiperda* insect cells (Drews et al., 2000; Doverskog et al., 2000), it was suggested that GS/GltS pathway synthesizes glutamic acid which is transaminated to alanine. In *Ae. aegypti* females, the GS/GltS pathway participates not only in the fixation and assimilation of ammonia but also produces the precursor for proline synthesis, the main amino acid in mosquito hemolymph.

The protocol developed here opens new ways to better understand mosquito whole body metabolism and to find new targets for mosquito control.

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Appendix A. Supplementary materials

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ibmb.2006.05.003.

References

Briegel, H., 1985. Mosquito reproduction: incomplete utilization of the blood meal protein for oogenesis. J. Insect Physiol. 31, 15–21.

Campbell, J.W., 1997. Mitochondrial ammonia metabolism and the proton-neutral theory of hepatic ammonia detoxification. J. Exp. Zool. 278, 308–321.

Doverskog, M., Jacobsson, U., Chapman, B.E., Kuchel, P.W., Häggström, L., 2000. Determination of NADH-dependent glutamate synthase (GOGAT) in *Spodoptera frugiperda* (Sf9) insect cells by a selective $^{1}H/^{15}N$ NMR in vitro assay. J. Biotechnol. 79, 87–97.

Drews, M., Doverskog, M., Öhman, L., Chapman, B.E., Jacobsson, U., Kuchel, P.W., Häggström, L., 2000. Pathways of glutamine metabolism in *Spodoptera frugiperda* (Sf9) insect cells: evidence for the presence of the nitrogen assimilation system, and a metabolic switch by $^{1}H/^{15}N$ NMR. J. Biotechnol. 78, 23–37.

Eisenberg, D., Gill, H.S., Pfluegl, G.M.U., Rotstein, S.H., 2000. Structure–function relationships of glutamine synthetases. Biochim. Biophys. Acta 1477, 122–145.

Goldstrohm, D.A., Pennington, J.E., Wells, M.A., 2003. The role of hemolymph proline as a nitrogen sink during blood meal digestion by the mosquito *Aedes aegypti*. J. Insect Physiol. 49, 115–121.

Hellerstein, M.K., 2003. In vivo measurement of fluxes through metabolic pathways: the missing link in functional genomics and pharmaceutical research. Annu. Rev. Nutr. 23, 379–402.

Hirayama, C., Nakamura, M., 2002. Regulation of glutamine metabolism during the development of *Bombyx mori* larvae. Biochim. Biophys. Acta 1571, 131–137.

Hirayama, C., Konno, K., Shinbo, H., 1997. The pathway of ammonia assimilation in the silkworm, *Bombyx mori*. J. Insect Physiol. 43, 959–964.

Hirayama, C., Saito, H., Konno, K., Shinbo, H., 1998. Purification and characterization of NADH-dependent glutamate synthase from the silkworm fat body (*Bombyx mori*). Insect Biochem. Mol. Biol. 28, 473–482.

Johnson, D.W., 2001. Analysis of amino acids as formamidene butyl esters by electrospray ionization tanden mass spectrometry. Rapid Commun. Mass Spectrom. 15, 2198–2205.

Lea, P.J., Miflin, B.J., 2003. Glutamate synthase and the synthesis of glutamate in plants. Plant Physiol. Biochem. 41, 555–556.

Levenberg, B., Hartman, S.C., Buchanan, J.M., 1956. Biosynthesis of the purines. X. Further studies *in vitro* on the metabolic origin of nitrogen atoms 1 and 3 of the purine ring. J. Biol. Chem. 220, 379–390.

Miflin, B.J., Lea, P.J., 1977. Amino acid metabolism. Annu. Rev. Plant Physiol. Plant Mol. Biol. 28, 299–329.

Muro-Pastor, M.I., Reyes, J.C., Florencio, F.J., 2005. Ammonium assimilation in cianobacteria. Photosynth. Res. 83, 135–150.

Nagy, K., Takats, Z., Pollreisz, F., Szabo, T., Vekey, K., 2003. Direct tandem mass spectrometric analysis of amino acids in dried blood spots without chemical derivatization for neonatal screening. Rapid Commun. Mass Spectrom. 17, 983–990.

Osanai, M., Okudaira, M., Naito, J., Demura, M., Asakura, T., 2000. Biosynthesis of L-alanine, a major amino acid of fibroin in *Samia cynthia ricini*. Insect Biochem. Mol. Biol. 30, 225–232.

Reitzer, L., 2003. Nitrogen assimilation and global regulation in *Escherichia coli*. Ann. Rev. Microbiol. 57, 155–176.

Scaraffia, P.Y., Wells, M.A., 2003. Proline can be utilized as an energy substrate during flight of *Aedes aegypti* females. J. Insect Physiol. 49, 591–601.

Scaraffia, P.Y., Isoe, J., Murillo, A., Wells, M.A., 2005. Ammonia metabolism in *Aedes aegypti*. Insect Biochem. Mol. Biol. 35, 491–503.

Sonne, J.C., Lin, I., Buchanan, J.M., 1956. Biosynthesis of the purines. IX. Precursors of the nitrogen atoms of the purine ring. J. Biol. Chem. 220, 369–378.

Suzuki, A., Knaff, D.B., 2005. Glutamte synthase: structural, mechanistic and regulatory properties, and role in the amino acid metabolism. Photosynth. Res. 83, 191–217.

van den Heuvel, R.H.H., Curti, B., Vanoni, M.A., Mattevi, A., 2004. Glutamate synthase: a fascinating pathway from L-glutamine to L-glutamate. Cell. Mol. Life Sci. 61, 669–681.

Vanoni, M.A., Curti, B., 1999. Glutamate synthase: a complex iron–sulfur flavoprotein. Cell Mol. Life Sci. 55, 617–638.

Vanoni, M.A., Curti, B., 2005. Structure–function studies on the iron–sulfur flavoenzyme glutamate synthase: an unexpectedly complex self-regulated enzyme. Arch. Biochem. Biophys. 433, 193–211.

Vanoni, M.A., Dossena, L., van den Heuvel, R.H., Curti, B., 2005. Structure–function studies on the iron–sulfur flavoenzyme glutamate synthase: the key enzyme of ammonia assimilation. Photosynth. Res. 83, 219–238.

Vogg, G., Achatz, S., Kettrup, A., Sandermann Jr., H., 1999. Fast, sensitive and selective liquid chromatographic-tandem mass spectrometric determination of tumor-promoting diterpene esters. J. Chromatogr. 855, 563–573.

von Dungern, P., Briegel, H., 2001. Enzymatic analysis of uricotelic protein catabolism in the mosquito *Aedes aegypti*. J. Insect Physiol. 47, 73–82.

Zhang, Q., Wysocki, V.H., Scaraffia, P.Y., Wells, M.A., 2005. Fragmentation pathway for glutamine identification: loss of 73 Da from dimethylformamidine isobutyl glutamine. J. Am. Soc. Mass Spectrom. 16, 1192–1203.

Zhou, G., Flowers, M., Friedrich, K., Horton, J., Pennington, J.E., Wells, M.A., 2004. Metabolic fate of [^{14}C]-labeled meal protein amino acids in *Aedes aegypti* mosquitoes. J. Insect Physiol. 50, 337–349.