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RESEARCH ARTICLE

Stable isotope studies reveal pathways for the incorporation of non-essential amino acids in *Acyrthosiphon pisum* (pea aphids)

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ABSTRACT

Plant roots incorporate inorganic nitrogen into the amino acids glutamine, glutamic acid, asparagine and aspartic acid, which together serve as the primary metabolites of nitrogen transport to other tissues. Given the preponderance of these four amino acids, phloem sap is a nutritionally unbalanced diet for phloem-feeding insects. Therefore, aphids and other phloem feeders typically rely on microbial symbionts for the synthesis of essential amino acids. To investigate the metabolism of the four main transport amino acids by the pea aphid (*Acyrthosiphon pisum*), and its *Buchnera aphidicola* endosymbionts, aphids were fed defined diets with stable isotope-labeled glutamine, glutamic acid, asparagine or aspartic acid (U-^{13}C, U-^{15}N; U-^{15}N; α-^{15}N; or γ-^{15}N). The metabolic fate of the dietary ^{15}N and ^{13}C was traced using gas chromatography–mass spectrometry (GC-MS). Nitrogen was the major contributor to the observed amino acid isotopomers with one additional unit mass (M+1). However, there was differential incorporation, with the amine nitrogen of asparagine being incorporated into other amino acids more efficiently than the amide nitrogen. Higher isotopomers (M+2, M+3 and M+4) indicated the incorporation of varying numbers of ^{13}C atoms into essential amino acids. GC-MS assays also showed that, even with an excess of dietary labeled glutamine, glutamic acid, asparagine or aspartic acid, the overall content of these amino acids in aphid bodies was mostly the product of catabolism of dietary amino acids and subsequent re-synthesis within the aphids. Thus, these predominant dietary amino acids are not passed directly to *Buchnera* endosymbionts for synthesis of essential amino acids, but are rather are produced *de novo*, most likely by endogenous aphid enzymes.

KEY WORDS: *Buchnera aphidicola*, Aphid, Endosymbiont, Glutamic acid, Glutamine, Aspartic acid, Asparagine, Biosynthesis, Unbalanced diet

INTRODUCTION

The majority of inorganic nitrogen assimilation in plants occurs in the roots via the glutamine synthase–glutamine oxoglutarate aminotransferase metabolic cycle (Coruzzi and Last, 2000; Oaks, 1992). Aspartic acid aminotransferase and asparagine synthase use glutamic acid and glutamine, respectively, as nitrogen donors to produce aspartic acid and asparagine. Assimilation of inorganic nitrogen sources varies somewhat among different plant species (Fischer et al., 1998; Okumoto and Pilot, 2011; Oliveira et al., 2013). Nevertheless, the four amino acids of primary nitrogen assimilation, and less commonly also serine and/or alanine, constitute more than half of all transported nitrogen in most plant species (Douglas, 1993; Febvay et al., 1988; Fukumorita and Chino, 1982; Girousse and Bournoville, 1994; Girousse et al., 1996; Hunt et al., 2006; Karley et al., 2002; Lohaus and Moellers, 2000; Ponder et al., 2000; Riens et al., 1991; Sandström and Pettersson, 1994; Sasaki et al., 1990; Urquhart and Joy, 1981; Valle et al., 1998; Wilkinson and Douglas, 2003). Whereas these four non-essential amino acids are relatively abundant, phloem plant sap typically is deficient in essential amino acids that cannot be synthesized by animals (Atkins et al., 2011; Girousse et al., 2005; Lohaus et al., 1994; Zhang et al., 2010).

Animals that feed on nutritionally unbalanced resources such as phloem sap, which typically has sub-optimal amounts of essential amino acids, have evolved mechanisms for acquiring or synthesizing these amino acids. In many cases, this involves symbiotic interactions with microorganisms that produce essential amino acids whose synthesis is not encoded in animal genomes (Crotti et al., 2010; Defossez et al., 2011; Nardi et al., 2002; Oh et al., 2010; Russell et al., 2009; Schloss et al., 2006; Spiteller et al., 2000). Some of the best-studied symbioses of this type are found in aphids, whiteflies and other phloem-feeding Hemiptera (Braendle et al., 2003; Douglas et al., 2006; Kikuchi et al., 2007, 2009; Wilkinson et al., 2001).

Phloem-feeding insects benefit from having evolved rapid and efficient pathways for the uptake and metabolic conversion of glutamine, glutamic acid, asparagine and aspartic acid into essential amino acids. Aphids accomplish this in part through close interactions with endosymbiont bacteria, *Buchnera aphidicola*, that are contained in specialized cells called bacteriocytes (Buchner, 1965). Although all *Buchnera* synthesize essential nutrients for their aphid hosts, there is variation among different aphid species and even lineages within a species in the metabolic functions of their bacterial endosymbionts and their utilization of host plant nutrients (MacDonald et al., 2011; Vogel and Moran, 2011). In addition to *Buchnera*, several aphid species have been shown to contain facultative endosymbionts that can contribute to survival and fitness (Leonardo and Muiru, 2003; Oliver et al., 2003), including through amino acid biosynthesis (Richards et al., 2010).

Sequencing of the pea aphid (*Acyrthosiphon pisum*) and *Buchnera* genomes, gene expression profiling, and proteomic studies have provided new insight into the predicted pathways of amino acid biosynthesis in the *A. pisum–Buchnera* symbiosis (Brinza et al., 2010; Hansen and Moran, 2011; Poliakov et al., 2011; Richards et al., 2010; Shigenobu et al., 2000). Notably, there are several examples of shared biosynthesis pathways for essential amino acids that are partly encoded by the endosymbiont bacteria and partly by the aphid host (Russell et al., 2013; Wilson et al., 2010). Genomic analyses indicate that aspartic acid and glutamic acid contribute to the biosynthesis of all amino acids in the aphid–*Buchnera* system by providing nitrogen and/or the carbon backbone (Hansen and Moran, 2011).

The *de novo* synthesis of essential amino acids from other phloem nitrogen sources has been explored in the symbiosis between

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A. pisum and its *Buchnera* endosymbiont. Analysis of *A. pisum* fed on *Vicia faba* plants and artificial diet containing varying amounts of non-essential and essential amino acids showed a similar overall amino acid composition, suggesting biosynthesis of essential amino acids within the insects (Liadouze et al., 1995). Compared with aphids containing *Buchnera*, aposymbiotic aphids that had been cleared of endosymbionts by antibiotic treatment accumulated greater amounts of free amino acids when feeding from plants (Liadouze et al., 1995). Whereas asparagine, aspartic acid and glutamine were more abundant, several essential amino acids were less abundant, suggesting that the endosymbiont bacteria contribute to the conversion of non-essential amino acids into essential amino acids.

Experiments with isolated bacteriocytes showed that they actively take up [U-^{14}C]glutamine and convert it into glutamic acid, which is efficiently taken up by *Buchnera* bacteria (Sasaki and Ishikawa, 1995). In whole-aphid experiments, *de novo* synthesis of essential amino acids in *A. pisum* was demonstrated by incorporation of carbon from [^{14}C]sucrose in the diet (Febvay et al., 1999). The relative incorporation of ^{14}C from labeled amino acids and sucrose was markedly different in *A. pisum* feeding on a diet containing excess amino acids compared with those on a diet that was designed to mimic host phloem sap components, indicating that aphids and/or their symbionts adjust amino acid synthesis based on their dietary needs (Febvay et al., 1999; Liadouze et al., 1995).

Although ^{14}C labeling experiments are very sensitive for detecting dietary carbon incorporation into essential amino acids, measurement of the radioactive signal provides only partial information about metabolic pathways. In particular, ^{14}C incorporation by itself cannot determine whether intact dietary amino acids are provided to the *Buchnera* endosymbionts for the synthesis of essential amino acids. In contrast, mass spectrometry (MS) and/or nuclear magnetic resonance (NMR) techniques, in conjunction with labeled non-radioactive stable isotopes such as ^{13}C and ^{15}N, permit the detection of specific labeled atoms in the reaction products (Bier, 1997; Crown and Antoniewicz, 2013; Kahana et al., 1988; Pond et al., 2006; Schatschneider et al., 2011; Tsvetanova et al., 2002; Venema et al., 2007). For instance, stable isotope labeling has been used to elucidate pathways in metabolic engineering studies (Alonso et al., 2007; Chen et al., 2011; Schwender et al., 2003; Suthers et al., 2007).

The MS fragmentation pattern and the elemental composition of the fragments can be used to trace the origin of the carbon and nitrogen incorporation from dietary non-essential amino acids into essential amino acids in the aphid–*Buchnera* system. Stable isotope labeling experiments with [^{15}N]glutamine provided insight into aphid nitrogen incorporation (Sasaki et al., 1990; Sasaki and Ishikawa, 1995). When [δ-^{15}N]glutamine was added to bacteriocytes, the ^{15}N was incorporated into glutamic acid, indicating likely *de novo* synthesis from α-ketoglutarate. Isolated *Buchnera* actively took up labeled [^{15}N] glutamic acid and incorporated the labeled nitrogen into other amino acids. Together, these experiments suggested that amide (δ) nitrogen and amine (α) nitrogen of glutamine are utilized differently in the aphid–*Buchnera* system.

Here, we describe experiments in which we used gas chromatography–mass spectrometry (GC-MS) to determine the metabolic fates of both carbon and nitrogen derived from the main phloem transport amino acids after they are taken up by whole aphids. Specifically, we set out to answer the following questions in the *A. pisum–Buchnera* metabolic system. (i) Does utilization of the four predominant nitrogen transport amino acids, aspartic acid, asparagine, glutamic acid and glutamine, follow the predicted metabolic pathways? (ii) Are the two nitrogen atoms in asparagine functionally equivalent? (iii) Is the carbon backbone of dietary amino acids maintained during the synthesis of essential amino acids?

MATERIALS AND METHODS

Aphid rearing

A clonal culture of the pea aphid (*Acyrthosiphon pisum* Harris) strain CWR09/18, derived from a single female collected in June 2009 from an alfalfa crop at Freeville Farms (Freeville, NY, USA) (Russell et al., 2014), was reared on faba beans (*Vicia faba*, var. Windsor; Johnny's Selected Seeds, Winslow, ME, USA), which were grown in Metromix 200 (Scotts, Marysville, OH, USA) in a growth chamber at 23°C and 100 µmol photons m^{-2} s^{-1} light intensity, with a 18 h:6 h light:dark cycle; 10 day old pre-flowering plants were used for aphid rearing.

Chemicals and reagents

All labeled amino acids were purchased from Cambridge Isotope Laboratories (Tewksbury, MA, USA). *N*-Methyl-trimethylsilyltrifluoroacetamide (MSTFA) was purchased from Sigma-Aldrich (St Louis, MO, USA). Reagent grade hydrochloric acid was purchased from VWR (Radnor, PA, USA).

Aphid diets

To study the specific uptake and incorporation of phloem transport amino acids, aphids were fed with artificial diet prepared as described by Prosser and Douglas (1992), with modifications (A. E. Douglas, personal communication) (Table S1). The diets for labeling experiments were modified so that they contained combinations of either aspartic acid and asparagine, or glutamic acid and glutamine, as the only nitrogen sources. To achieve the required pH 7 in the diet, aspartic acid and asparagine were added in an approximately 1:3.5 ratio (33.2 mmol l^{-1} aspartic acid and 115.1 mmol l^{-1} asparagine). Experimental diets were prepared with combinations of isotope-labeled and unlabeled aspartic acid and asparagine. Similarly, diets were prepared with isotope-labeled and unlabeled glutamic acid and glutamine in an approximately 1:2 ratio (50.4 mmol l^{-1} glutamic acid and 99.0 mmol l^{-1} glutamine). *Acyrthosiphon pisum* did not survive for the duration of the experiments when other buffers were used to adjust the pH to 7 in diets containing only aspartic acid or glutamic acid as the nitrogen source.

Baseline values for *m/z* of fragment ions for all detectable unlabeled amino acids in aphids were obtained using a diet of unlabeled aspartic acid+asparagine to obtain the natural isotopic abundance values. To test the incorporation of carbon and nitrogen into amino acids, experiments were conducted with one of the tested amino acids universally labeled with ^{13}C and ^{15}N. These diets consisted of: (i) [U-^{13}C, ^{15}N]aspartic acid+asparagine; (ii) aspartic acid+[U-^{13}C, U-^{15}N]asparagine; (iii) [U-^{13}C, ^{15}N]glutamic acid+glutamine; and (iv) glutamic acid+[U-^{13}C, U-^{15}N]glutamine. To determine nitrogen incorporation, two combinations of diets were used: (i) aspartic acid+[U-^{15}N]asparagine; and (ii) glutamic acid+[U-^{15}N] glutamine. To investigate differential nitrogen uptake of the α- and γ-nitrogen of asparagine, aphid feeding experiments were conducted with: (i) aspartic acid+[α-^{15}N]asparagine; (ii) aspartic acid+[γ-^{15}N]asparagine; and (iii) aspartic acid+[U-^{15}N]asparagine. The percentage of isotope-labeled nitrogen in each aphid diet is shown in Table 1.

Table 1. Percentage of isotope-labeled nitrogen in different aphid diets

Amino acids in the diet	% ^{15}N
50.4 mmol l^{-1} glutamic acid+99 mmol l^{-1} glutamine	0
50.4 mmol l^{-1} [U-^{13}C, ^{15}N]glutamic acid+99 mmol l^{-1} glutamine	20
50.4 mmol l^{-1} glutamic acid+99 mmol l^{-1} [U-^{13}C, U-^{15}N]glutamine	80
50.4 mmol l^{-1} glutamic acid+99 mmol l^{-1} [U-^{15}N]glutamine	80
33 mmol l^{-1} aspartic acid+115 mmol l^{-1} asparagine	0
33 mmol l^{-1} [U-^{13}C, ^{15}N]aspartic acid+115 mmol l^{-1} asparagine	13
33 mmol l^{-1} aspartic acid+115 mmol l^{-1} [U-^{13}C, U-^{15}N]asparagine	87
33 mmol l^{-1} aspartic acid+115 mmol l^{-1} [U-^{15}N]asparagine	87
33 mmol l^{-1} aspartic acid+115 mmol l^{-1} [α-^{15}N]asparagine	44
33 mmol l^{-1} aspartic acid+115 mmol l^{-1} [γ-^{15}N]asparagine	44

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Aphid bioassays

Experiments were conducted with 2 day old larvae that had been deposited by apterous adults onto *V. faba*. Twenty to 30 individual larvae were transferred to 3 cm wide, 10 ml plastic cups that were covered with fine polypropylene mesh at the bottom. Then, the cups were covered with thinly stretched Parafilm (Pechiney Plastic Packaging Company, Chicago, IL, USA). A 100 µl sample of the diets to be tested was pipetted onto the center of the Parafilm and covered with a second layer of stretched Parafilm. Cups were transferred to an airtight 60×30×30 cm Plexiglas box with a 250 ml beaker containing saturated salt solution to maintain humidity. The box was placed at 22–23°C, 25 µmol photons m^{-2} s^{-1} light intensity, with a 18 h:6 h light:dark cycle. Aphids were harvested for GC-MS analysis of metabolites after 5 days.

Acid hydrolysis for extraction of total amino acids

A weighed amount of aphids from each diet cage was transferred to a 2 ml glass vial. After addition of 400 µl of 6 mol l^{-1} HCl, the vials were flushed with nitrogen for 30 s, and immediately capped. Aphids were extracted for 36–40 h in a heating block maintained at 110°C. The reaction mixture was cooled and the samples were filtered using 42 µm, 500 µl microcentrifuge filters (Millipore, Billerica, MA, USA). The clear extracts were then evaporated to dryness in a 2 ml glass vial under a nitrogen stream at 60–65°C. The residue was re-dissolved in 10 µl of 20 mmol l^{-1} HCl per mg of aphids and used for amino acid analysis by GC-MS.

Amino acid analysis

GC-MS analyses were carried on a Varian CP-3800 GC coupled to a CombiPal autosampler (LEAP Technologies, Carrboro, NC, USA) and a Varian 1200L triple quadrupole MS (Varian, Carey, NC, USA). The aphid extract samples in the 2 ml glass GC vials were evaporated to dryness. To each sample, 50 µl of MSFTA derivatizing agent was added. The vials were closed tightly and heated to 110–115°C for 1.5 h. The samples were then directly injected into a VF-17ms FS, 30 m×0.25 µm FactorFour GC column (Varian) for chromatography. The flow rate was 1 ml min^{-1} of helium and the temperature gradient was 70°C for 2 min; to 100°C at 5°C min^{-1} and held for 5 min; to 150°C at 5°C min^{-1}, held for 0 min; and to 300°C at 12°C min^{-1} and held for 3 min. Electron ionization (EI)-MS spectra were collected at 70 eV. The mass of individual chromatographic peaks was compared with a spectral library (Palisade Corporation, Ithaca, NY, USA), as well as with the retention times and mass spectra of isotope-labeled and unlabeled amino acid standards. Chemical ionization (CI)-MS data, using methane as the ionizing gas, were collected with GC parameters at 70 eV, as described above for EI-MS, and the spectral data were used in assessing parent ions of components that were labeled with more than one atom. Table 2 shows the fragment ions that were monitored to identify and measure the ion abundance of the amino acids seen in the mass spectra.

Table 2. Ions monitored for different metabolite GC retention times

Metabolites measured	Retention time (min)	Base peak in EI mode	[Molecular ion +H]$^+$ in CI mode
Alanine	6.614	116	234 (2 TMS)
Glycine	7.074	102	220 (2 TMS)
Valine	8.922	144	262 (2 TMS)
Leucine	11.018	158	276 (2 TMS)
Isoleucine	11.839	158	276 (2 TMS)
Proline	14.967	142	260 (2 TMS)
Serine	15.126	218	322 (3 TMS)
Threonine	15.475	218	336 (3 TMS)
Aspartic acid	21.487	232	350 (3 TMS)
Glutamic acid	24.097	246	364 (3 TMS)
Pyroglutamic acid	24.317	156	274 (2 TMS)
Phenylalanine	24.917	192	310 (2 TMS)
Lysine	27.042	174	291 (2 TMS)
Tyrosine	28.718	179	398 (3 TMS)

EI, electron ionization; CI, chemical ionization; TMS, trimethylsilyl group.

Data analysis

The total ion abundances for selected ion peaks were calculated by integrating the area under each component and were converted to a percentage proportion of the peak area of the natural isotope mass species $[M]^+$ (carbon 12, hydrogen 1, sulfur 32, silicone 28 and oxygen 16). In addition to molecular species, less abundant natural isotopes produce $[M+1]^+$ and $[M+2]^+$, depending on the molecular composition of the intact compound. This could be observed best in CI-MS. In EI-MS mode, we could measure the fragment ion mass of the base peak [M-117, M-COOTMS], with loss of the trimethylsilyl (TMS)-derivatized carboxyl group, hereafter referred to as FM throughout the paper. If one or more ^{13}C- or ^{15}N-labeled atoms are incorporated into the molecule, the intensity of $[FM+n]^+$ would be much higher than the natural abundance. This is illustrated in Fig. 1 and Fig. S1.

Statistical analyses (one-way ANOVA) were conducted to identify significant differences between different experiments using JMP 9 and 11 (SAS Statistical Software, Cary, NC, USA) or Statistix 8 (Analytical Software, Tallahassee, FL, USA). Mean values and standard errors for graphs were calculated using one of the above software packages or Excel (Microsoft, Redmond, WA, USA).

RESULTS

Incorporation of nitrogen from phloem nitrogen transport amino acids

To investigate the incorporation of dietary glutamine, glutamic acid, asparagine and aspartic acid, aphids were raised for 5 days on diets containing these amino acids with ^{13}C and ^{15}N stable isotope labeling. During the course of these experiments, the mass of the aphids increased approximately threefold, indicating uptake and incorporation of nutrients from the artificial diet (Fig. S2).

Incorporation of both the carbon and the nitrogen atoms of dietary amino acids into other amino acids can be detected by GC-MS. As an example, Fig. 1 shows scenarios for the conversion of [U-^{13}C, U-^{15}N]asparagine into isoleucine in the *A. pisum–Buchnera* system. The predicted pathway for isoleucine biosynthesis from asparagine includes both aphid- and *Buchnera*-encoded enzymes (Hansen and Moran, 2011; Russell et al., 2013). If four carbons of [U-^{13}C, U-^{15}N]asparagine are incorporated into isoleucine, this would result in an M+4 isotopomer (Fig. 1A). The nitrogen atoms of [U-^{13}C, U-^{15}N]asparagine would be lost in the isoleucine biosynthesis pathway, but ^{15}N could be added back during the final transamination step, resulting in an M+5 isotopomer, or instead an M+1 isotopomer if the carbon backbone of asparagine is not conserved. For aphids on diets with [U-^{13}C, U-^{15}N]asparagine, both the M+5 and the M+1 isomers of isoleucine were detected. For aphids on diets with [U-^{15}N]asparagine, the M+1 isomer became more abundant than for those on diets with unlabeled asparagine (Fig. 1C–E), indicating that this M+1 mass increase primarily results from dietary ^{15}N, rather than incorporation of a single ^{13}C atom.

In further experiments, diets containing each of the four [U-^{13}C, U-^{15}N]-labeled amino acids (aspartate, asparagine, glutamate and glutamine) resulted in a mass unit increase in *m/z* [FM+1] in the [M-117, M-COOTMS] fragment ion (after loss of the TMS-derivatized carboxyl group) in most of detected amino acids (Fig. 2). This indicated that ^{15}N or ^{13}C from the labeled amino acids was incorporated into the newly formed amino acids. However, it is more likely that nitrogen is incorporated into the molecule, as the detected ion fragment retains the amino group from the corresponding amino acids (M-117, shown for isoleucine in Fig. 1E). In the case of lysine, a smaller amount of the FM+2 molecular ion was observed (data not shown), likely as a result of the less frequent incorporation of ^{15}N into both nitrogen atoms of this amino acid.

The incorporation of nitrogen from the [U-^{13}C, U-^{15}N]asparagine and [U-^{13}C, U-^{15}N]glutamine diets was similar for all of the

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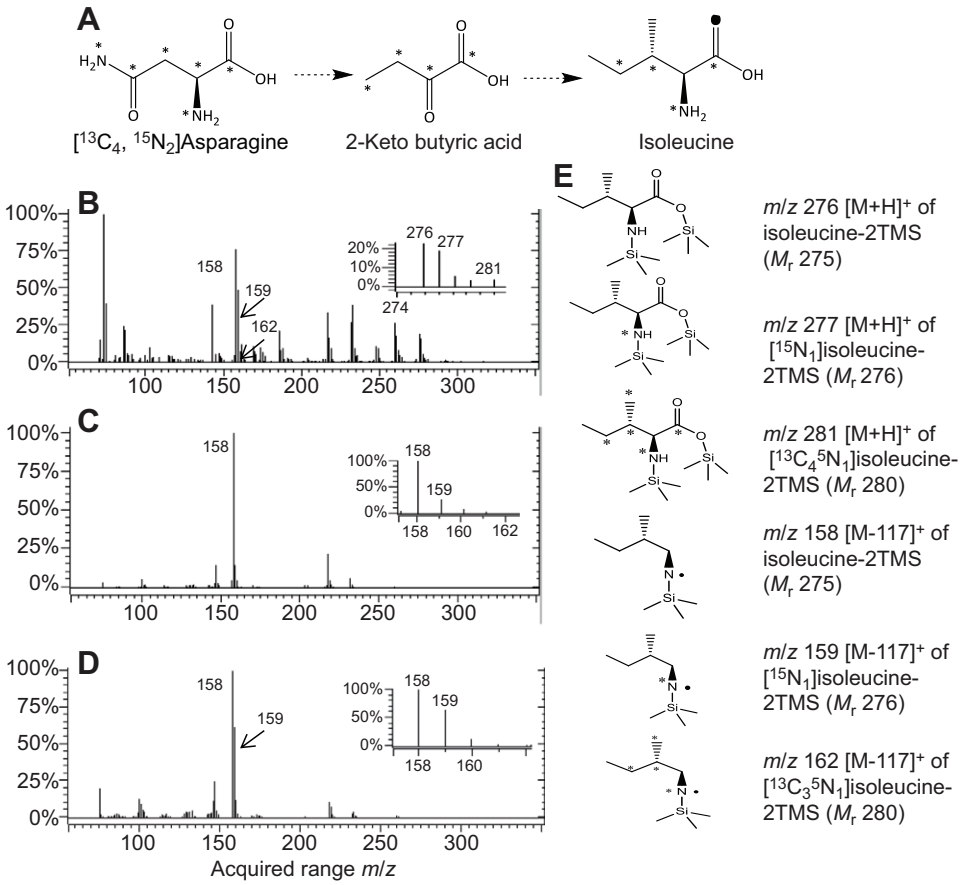

Fig. 1. Incorporation of ^{13}C and ^{15}N from asparagine into isoleucine in *Acyrthosiphon pisum*. Aphids were fed on a diet containing [U-^{13}C, U-^{15}N]asparagine, amino acids were extracted by acid hydrolysis, and isoleucine was detected by GC-MS. (A) Metabolic pathway for the biosynthesis of isoleucine from asparagine, with 2-oxobutanoic acid as an intermediate. M+1 atoms (^{13}C and ^{15}N) are marked with an asterisk. Isoleucine made from [U-^{13}C, U-^{15}N]asparagine has a predicted +4 mass increase, +5 if the nitrogen is also derived from the breakdown products of labeled dietary asparagine. (B–E) Mass spectra and representative structures that were analyzed. (B) Chemical ionization (CI) mode mass spectrum of isoleucine produced from [U-^{13}C, U-^{15}N]asparagine, showing incorporation of either four ^{13}C and one ^{15}N atom, or one ^{15}N atom (*m/z* 281 and 277, respectively). Inset shows an expansion of the area containing the molecular ion peaks of the labeled isomers. (C,D) Mass spectra of unlabeled and labeled isoleucine produced from [U-^{15}N]asparagine, respectively, showing incorporation of one ^{15}N atom in electron ionization (EI) mode. Insets show an expansion of the area containing the base peak and the labeled isomer. (E) Major peaks in the spectra and their sources.

detected amino acids (Fig. 2). After 5 days on the artificial diet, up to 50% of the individual aphid amino acids were labeled with a single ^{15}N or ^{13}C. Nitrogen or carbon incorporation into isoleucine was significantly higher with [U-^{13}C, U-^{15}N] asparagine relative to [U-^{13}C, U-^{15}N]glutamine in the diet. In contrast, labeled alanine, glycine, serine and valine were more abundant in aphids fed with [U-^{13}C, U-^{15}N]glutamine diet (Fig. 2).

In the experiments with labeled aspartic acid or glutamic acid, the observed incorporation of ^{15}N or ^{13}C into aphids was much lower than for asparagine and glutamine (Fig. 2). This is largely as a result of limitations of the experimental system. Whereas aspartic acid and glutamic acid each have one nitrogen that can be labeled, asparagine and glutamine have two. Moreover, to achieve the required pH 7 in the diet, it was necessary to add larger amounts of asparagine and glutamine than aspartic acid and glutamic acid. Thus, the [U-^{13}C,

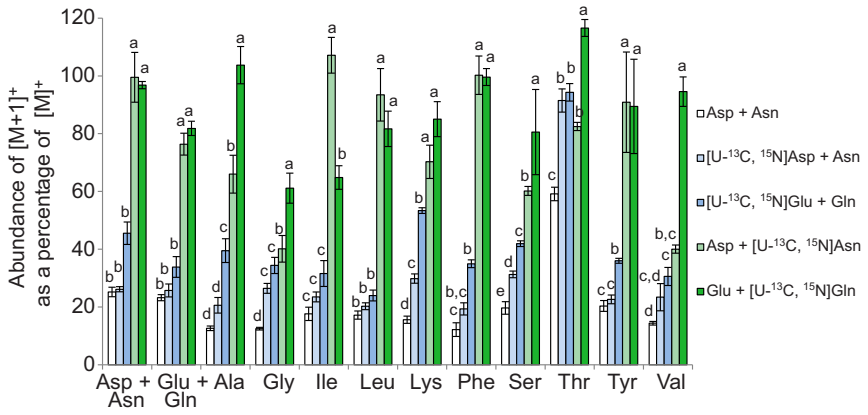

Fig. 2. Incorporation of nitrogen from phloem transport amino acids into other amino acids in *A. pisum*. Aphids were fed the indicated amino acid diets, balanced to pH 7. Abundance of the EI-MS fragment [FM+1] ion ([FM=M-117, loss of COOTMS]) resulting from ^{15}N or a single ^{13}C carbon atom incorporation is shown as a percentage of the abundance of the unlabeled molecule in the same samples, i.e. a value of 100 indicates that ions for [FM+1] and [FM] are equally abundant. The total amino acid component of the samples was prepared by hydrolysis with 6 mol l^{-1} HCl. Under this condition, asparagine and glutamine are hydrolyzed to the respective acids. Therefore, we cannot differentiate the aspartic acid and asparagine, or glutamic acid and glutamine, content of the samples. Methionine was detected only in some samples, hence it was not included in the analysis of the data, but it also showed an increase of 1 mass unit for the fragment ion [M-117]. Each bar represents the mean and standard error of 5–9 samples. For each amino acid, different letters indicate significant differences (P<0.05 ANOVA followed by Tukey's HSD test).

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U-^{15}N]asparagine diet contained sevenfold more ^{15}N than the [U-^{13}C, U-^{15}N]aspartic acid diet, and the [U-^{13}C, U-^{15}N]glutamine diet contained fourfold more ^{15}N than the [U-^{13}C, U-^{15}N]glutamic acid diet (Table 1). This could explain the lower incorporation of labeled nitrogen in the aphids fed on [^{15}N]aspartic acid and [^{15}N]glutamic acid diets compared with those on the [U-^{13}C, U-^{15}N]asparagine or [U-^{13}C, U-^{15}N]glutamine diets (Fig. 2).

To confirm that the mass increase [M+1-117] was due to the labeled nitrogen addition, we conducted experiments with diets that contained [U-^{15}N]asparagine or [U-^{15}N]glutamine, together with unlabeled aspartic acid and glutamic acid, respectively. Analysis of amino acids from aphids fed on these diets showed that, in all amino acids detected, there was incorporation of labeled nitrogen that caused [M+1] increases in the nitrogen-containing fragment [M-117, M-COOTMS] (Fig. 3). However, there were differences in the abundance of the [M+1] fragment ion in the two labeling experiments (Figs 2, 3). For instance, isoleucine was equally labeled by diets containing [U-^{15}N]asparagine or [U-^{15}N]glutamine (Fig. 2). However, it was more highly labeled with [U-^{13}C, U-^{15}N]asparagine than with [U-^{13}C, U-^{15}N]glutamine (Fig. 2). The opposite labeling effect was observed with alanine and valine. Some amino acids showed overall higher incorporation of +1 mass unit in the amine-containing fragments [M-117] with the [U-^{13}C, U-^{15}N]-labeled diets (Fig. 2) than with the [U-^{15}N]-labeled diets (Fig. 3), suggesting that, in addition to the nitrogen, a carbon from the labeled amino acids may be incorporated. Other labeling differences could be due to unexplained variation in the phenology of the aphids at the time of processing in the respective experiments.

Detection of fragments [M+1-117] of aspartic acid/asparagine, and glutamic acid/glutamine with a mass increase due to ^{15}N (Figs 2, 3) demonstrates that there is *de novo* synthesis of these amino acids in the aphids, even when they were abundant in the diet. One likely metabolic pathway is the rapid degradation of these amino acids after uptake from the diet and incorporation of the nitrogen into other amino acids in the aphid–*Buchnera* system. Thus, the newly synthesized amino acids come from the conversion of their respective keto-acids. Additionally, this is evidence that the dietary aspartic acid/asparagine, and glutamic acid/glutamine are utilized differently from those that are synthesized *de novo* in the aphids.

Differential incorporation of α- and γ-nitrogen from asparagine

To determine whether the two nitrogen atoms of asparagine are incorporated in the same manner by the *A. pisum–Buchnera* system, we conducted artificial diet experiments with specifically labeled [α-^{15}N]asparagine and [γ-^{15}N]asparagine. The nitrogen incorporation from the diet containing [U-^{15}N]asparagine was higher than that with either [α-^{15}N]asparagine or [γ-^{15}N]asparagine, indicating that both nitrogen atoms from [U-^{15}N]asparagine can be incorporated (Fig. 3). However, in most cases, there was significantly greater incorporation of the α-nitrogen than the γ-nitrogen of asparagine into other amino acids. Although the two nitrogen atoms of dietary asparagine are not functionally equivalent during *A. pisum* amino acid biosynthesis, they were incorporated similarly in the biosynthesis of the nucleic acids uracil and thymine (Fig. S3).

Fate and conversion of the carbon from transport amino acids into other amino acids

We conducted isotopomer analysis of all of the detected amino acids, choosing those fragments of the mass spectrum that were the base peaks and retained the nitrogen atom of the amine group. For example, for aspartic acid, we chose the fragment ion *m/z* 232 and, if all the carbons and nitrogen atoms were retained, then we would see an isotopomer for *m/z* 232+4. Similarly, for glutamine (pyroglutamic acid after acid hydrolysis), we chose fragment ion *m/z* 156 and, if all of the labeled carbon and nitrogen atoms were retained, we would observe an isotopomer at *m/z* 156+5. If only nitrogen were to be labeled, then we would see *m/z* 232+1 and 156+1 for aspartic acid and glutamic acid, respectively (Fig. 4). If we observed the isotopomers with masses between the fully labeled and nitrogen-labeled isotopomers, it would suggest that the labeled carbon atoms are also incorporated into these amino acid isotopomers. In that case, there would be various combinations of labeled atoms being incorporated into the various locations. Examples of possible combinations of labeled nitrogen and carbon for aspartic acid and pyroglutamic acid are shown in Fig. S1.

The EI-MS isotopomer analysis of the dietary amino acids in the aphids showed that a only a small percentage of aspartic acid/asparagine has an intact, isotope-labeled carbon backbone (FM+ 4 is about 4% of the total isotopomers or 13% of the

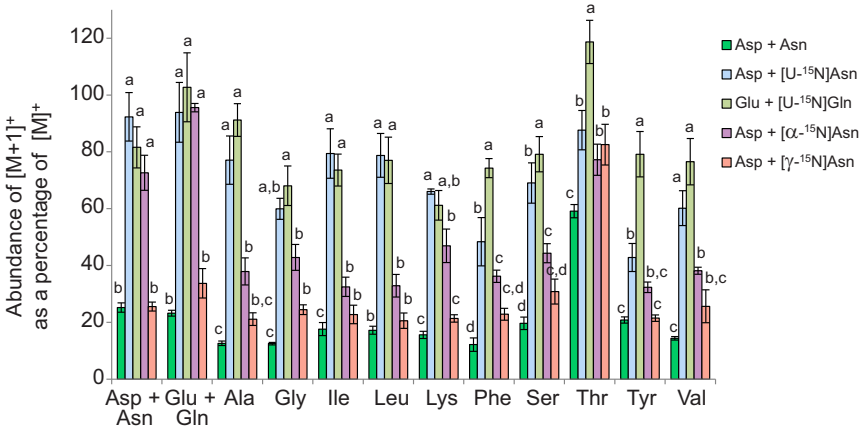

Fig. 3. Incorporation of nitrogen from ^{15}N-labeled amino acids into other amino acids in *A. pisum*. Aphids were fed the indicated amino acid diets, balanced to pH 7. Abundance of the EI-MS fragment [FM+1] ion ([FM=M-117]) resulting from ^{15}N incorporation is shown as a percentage of the abundance of the unlabeled fragment ion [FM] in the same samples, i.e. a value of 100 indicates that ions for [FM+1] and [FM] are equally abundant. The total amino acid component of the samples was prepared by hydrolysis with 6 mol l^{-1} HCl. Under this condition, asparagine and glutamine are hydrolyzed to the respective acids. Therefore, we cannot differentiate the aspartic acid and asparagine, or glutamic acid and glutamine, content of the samples. Each bar represents the mean and standard error of 6–12 samples. For each amino acid, different letters indicate significant differences (P<0.05 ANOVA followed by Tukey's HSD test).

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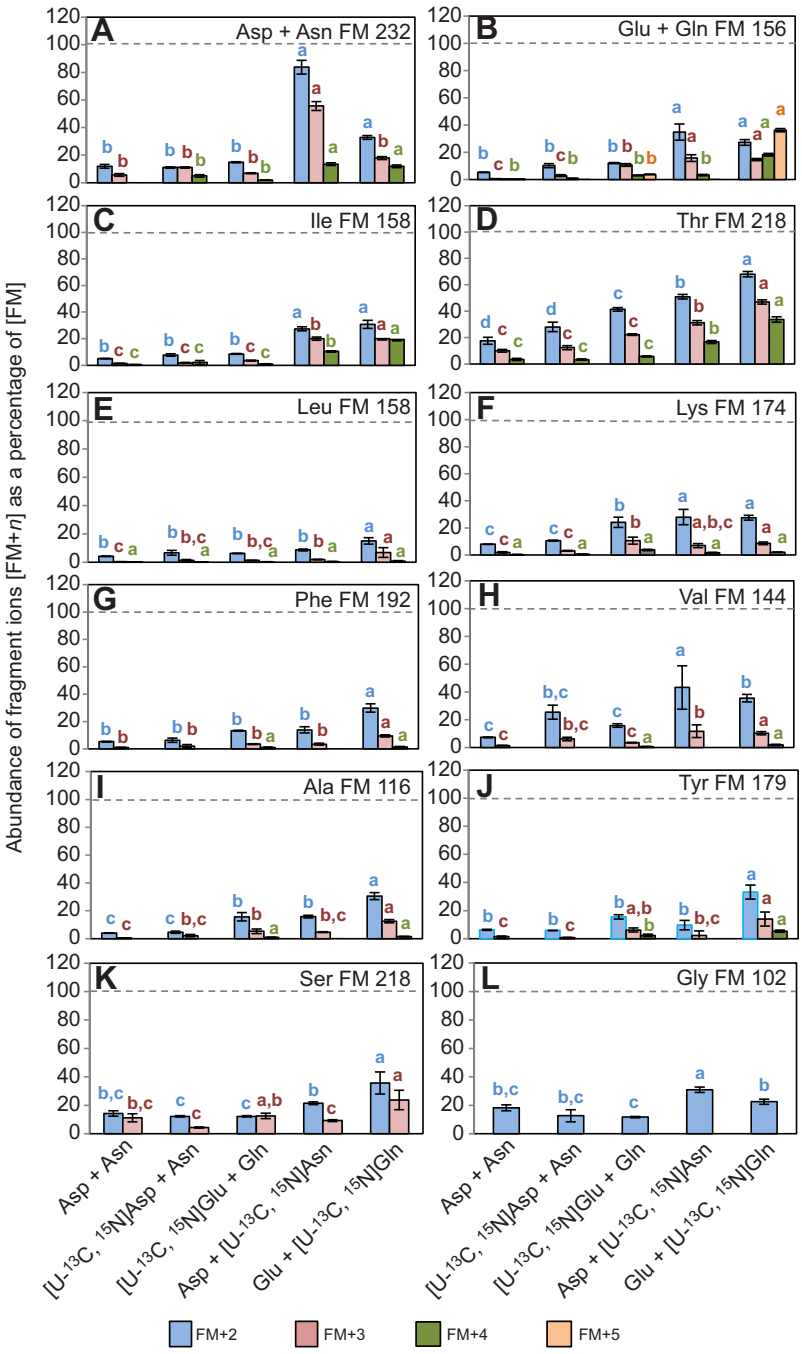

Fig. 4. Incorporation of more than one nitrogen and carbon from phloem transport amino acids into other amino acids in *A. pisum*. Aphids were fed the indicated amino acid diets, balanced to pH 7. Abundance of the fragment [FM+*n*] ion ([FM=M-117]; *n*=2, 3, 4 or 5) resulting from ^{15}N and/or ^{13}C carbon incorporation is shown as a percentage of the abundance of the unlabeled molecule [FM] for the same amino acid, i.e. a value of 100 indicates that ions for [FM+*n*] and [FM] are equally abundant. For each amino acid, bars with the same color indicate the same isotopomer. Different letters indicate significant differences for the isotopomers with the same color bars in different ion different treatments (P<0.05 ANOVA followed by Tukey's HSD test).

monoisotopomer) for diets containing [U-^{13}C, U-^{15}N]glutamine and [U-^{13}C, U-^{15}N]asparagine, respectively (Fig. 4A). In contrast, somewhat more glutamic acid and glutamine were observed as direct diet-derived amino acids (FM+5 was about 12% of the total isotopomers or 28% of the monoisotopomer) (Fig. 4B). There are some limitations to this assay: (i) we cannot determine whether these isotopomers came from unreacted glutamine and asparagine or whether they were resynthesized after degradation to keto-acids; (ii) as decarboxylated amino acid fragments are being measured in this assay, it is not possible to determine whether the carboxyl carbon atom is ^{13}C-labeled in the measured amino acids; (iii) as total amino acids were prepared by acid hydrolysis, asparagine and glutamine were converted to their respective acids, and we cannot determine whether glutamic acid and aspartic acid are synthesized directly from asparagine or glutamine, respectively, by the aphids; and (iv) at any given time some isotope-labeled dietary amino acids are still in the gut and have not yet been metabolized by the aphids.

To determine whether there is *de novo* synthesis of otherwise abundant dietary amino acids in the aphid–*Buchnera* system, we measured the abundance of other isotopomers. In addition to the higher amounts of FM+1 (^{15}N incorporation; Fig. 2) and FM+4 (all ^{15}N and ^{13}C retained; Fig. 4A), we observed significant amounts of the FM+2 and FM+3 isotopomers for aspartic acid/asparagine in aphids that were fed on the [U-^{13}C, U-^{15}N]asparagine and [U-^{13}C, U-^{15}N]glutamine diets (Fig. 4A). Similarly, relative to control samples, we measured increased amounts of FM+2, FM+3 and FM+4 for the [U-^{13}C, U-^{15}N]glutamic acid and [U-^{13}C, U-^{15}N]glutamine diets (Fig. 4B). The presence of variable numbers of ^{13}C-labeled carbon atoms suggests breakdown of the dietary amino acids and *de novo* synthesis that reincorporates some

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of the isotope-labeled atoms. In fact, the relatively high abundance of these isotopomers shows that most of the dietary amino acids are catabolized and re-synthesized.

Significant incorporation of the carbon backbone from the dietary amino acids was detected in isoleucine and theronine (Fig. 4C,D). In the respective experiments, the abundance of FM+4 labeled isoleucine, expressed as a percentage of native unlabeled isoleucine, was 15% (7.2% of total isotopomers) for [U-^{13}C, U-^{15}N]asparagine and 20% (7.2% of total isotopomers) for [U-^{13}C, U-^{15}N]glutamine. In the case of threonine, the results were similar, with incorporation efficiencies of approximately 11% (5% of total isotopomers) from [U-^{13}C, U-^{15}N]asparagine and 33% (12% of total isotopomers) for [U-^{13}C, U-^{15}N]glutamine of native unlabeled threonine. In contrast, aphids fed on [U-^{13}C, ^{15}N]aspartic acid or [U-^{13}C, ^{15}N]glutamic acid diet did not show any significant incorporation of the amino acid carbon skeleton relative to control aphids reared on unlabeled diet. The isotopomer analysis also showed that there was addition of other numbers of labeled carbon atoms (FM+2 or FM+3) into isoleucine and threonine (Fig. 4C,D), indicating that the backbone carbon chain of these amino acids can be synthesized with carbon atoms that were derived from degraded dietary asparagine or glutamine.

We also observed incorporation of isotope-labeled carbon atoms into leucine, lysine, phenylalanine, valine, alanine, tyrosine and serine (Fig. 4E–K). Almost all of these amino acids showed some incorporation of two carbon atoms or one carbon and one nitrogen, but the amounts varied for different dietary inputs. In most cases, the highest incorporation was from [U-^{13}C, U-^{15}N]asparagine and [U-^{13}C, U-^{15}N]glutamine. Phenylalanine and tyrosine incorporated higher amounts of M+2 from the [U-^{13}C, U-^{15}N] glutamine diet than from the [U-^{13}C, U-^{15}N]asparagine diet. The possibility of extremely small amounts of intact carbon chain incorporated into other amino acids cannot be ruled out as we did not carry out isotopomer analysis for all the amino acids if the percentage was very small (less than 2%). Even if this did happen, the contribution to total amino acid content is likely to be negligible. In the case of glycine (Fig. 4L), there was no evidence of ^{13}C incorporation from any of the [U-^{13}C, U-^{15}N]-labeled dietary amino acids.

Compared with the other measured amino acids (Fig. 2), proline was unusual in that the FM+1 isotopomer from aphids that had been fed with the diets containing [U-^{13}C, ^{15}N]aspartic acid, [U-^{13}C, ^{15}N]glutamic acid and [U-^{13}C, U-^{15}N]glutamine was severalfold higher than FM (up to 800% of FM; Fig. 5A). However, the [U-^{13}C, U-^{15}N]asparagine diet showed FM+1 as only about 97% of FM. This suggested that either much of the FM+1 isotopomer came from (i) the higher conversion of ^{15}N-labeled proline from the respective keto-acids or (ii) incorporation of a single labeled ^{13}C from the universally labeled respective dietary amino acids. To determine whether the contribution of +1 mass comes from labeled nitrogen and/or carbon, we analyzed the contribution of +1 mass of aphids that were fed with [^{15}N]-labeled diets (Fig. 5B). In each case, the FM+1 isotopomer was much less abundant than with the [U-^{13}C, ^{15}N]aspartic acid, [U-^{13}C, ^{15}N]glutamic acid and [U-^{13}C, U-^{15}N]glutamine diets, indicating that the elevated FM+1 in aphids that were fed on universally labeled diet comes from the incorporation of a labeled carbon (^{13}C) in addition to labeled nitrogen (^{15}N). The relatively low proline FM+1 isotopomer from [U-^{13}C, U-^{15}N]asparagine (Fig. 5A) abundance suggests that only ^{15}N is incorporated and that the four dietary amino acids are utilized differently by the aphid–*Buchnera* system in proline biosynthesis.

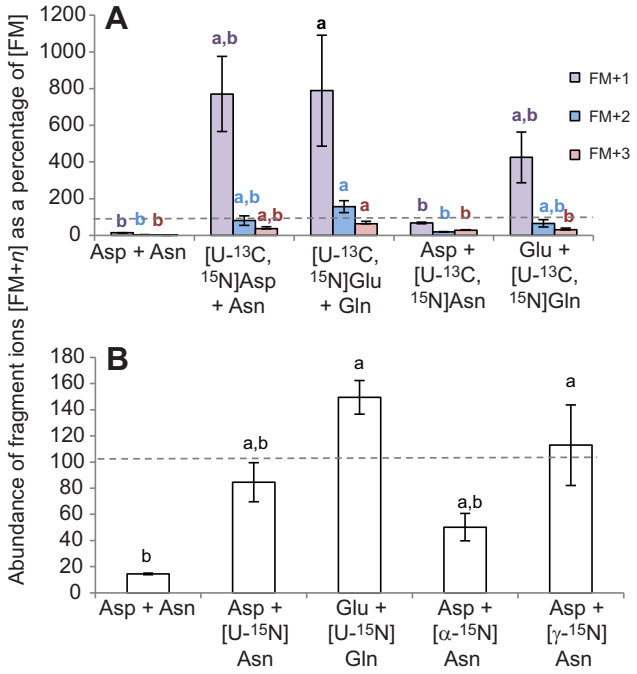

Fig. 5. Uptake and incorporation of dietary ^{13}C- and ^{15}N-labeled amino acids in *A. pisum*. (A) Isotopomers of proline, in the aphids fed with different labeled diets. Individual amino acids in the aphid diet of either a combination of aspartic acid and asparagine or glutamic acid and glutamine were replaced with amino acids that were universally labeled, i.e. [U-^{13}C, ^{15}N$_1$]aspartic acid, [U-^{13}C, ^{15}N]glutamic acid, [U-^{13}C, U-^{15}N]asparagine, or [U-^{13}C, U-^{15}N]glutamine. [U-^{13}C, ^{15}N$_1$]Aspartic acid and [U-^{13}C, U-^{15}N]asparagine show incorporation of one or more labeled components, but show no retention of fully labeled isotopomer carbons in proline. [U-^{13}C, U-^{15}N]Asparagine produced lesser amounts of FM+1 when compared with [U-^{13}C, ^{15}N$_1$]aspartic acid, [U-^{13}C, ^{15}N]glutamic acid, [U-^{13}C, U-^{15}N]asparagine, or [U-^{13}C, U-^{15}N]glutamine. We cannot distinguish whether only one ^{13}C or one ^{15}N is incorporated into the respective amino acids. (B) Isotopomers of proline in the aphids fed the diets containing ^{15}N-labeled asparagine and glutamine. Incorporation of ^{15}N showed a normal pattern of FM+1 increase, similar to other amino acids. This suggests that there is incorporation of labeled ^{13}C in excess amounts, but only from labeled aspartic acid, glutamic acid and glutamine, not from asparagine.

DISCUSSION

Metabolic models based on the presence and absence of amino acid biosynthesis genes in *A. pisum* and *Buchnera*, as well as amino acid uptake and labeling experiments, show that precursors for the biosynthesis of essential amino acids are transported from the aphid host to the bacterial endosymbionts (Brinza et al., 2010; Hansen and Moran, 2011; Poliakov et al., 2011; Russell et al., 2014; Sasaki and Ishikawa, 1995; Shigenobu et al., 2000; Wilson et al., 2010). Four amino acids, aspartic acid, asparagine, glutamic acid and glutamine, not only provide the majority of the nitrogen flow into the *Buchnera* endosymbionts for the synthesis of essential amino acids but also constitute the majority of the phloem amino acids in most plant species, including in *V. faba*, a natural host for *A. pisum* (Gündüz and Douglas, 2009). These observations, together with aphid feeding experiments using radioactive metabolites (Sasaki and Ishikawa, 1995), suggested that dietary amino acids can be moved intact from the insect gut to the bacterial endosymbionts to serve as precursors for the synthesis of other amino acids.

For most of the amino acids detected in our experiments, the predominant isotopomers were M+1 (Fig. 2), likely indicating incorporation of ^{15}N into amino acids through transamination of

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keto-acids. This is consistent with what is known about the amino acid biosynthesis pathways in *A. pisum* and *Buchnera* (Hansen and Moran, 2011; Wilson et al., 2010). Other than negligible amounts from vitamins, the only sources of nitrogen in our aphid diets were aspartic acid/asparagine and glutamic acid/glutamine, respectively, with differing relative amounts of ^{15}N and ^{14}N. The abundance of the M+1 isotopomer in the detected aphid amino acids after acid hydrolysis (Fig. 2) is generally consistent with the fraction of ^{15}N in the respective amino acid diets. However, there were some exceptions. For instance, we observed that the addition of ^{15}N to glycine, serine and valine was significantly higher for aphids fed on the [U-^{13}C, U-^{15}N]glutamine diet than for those on the [U-^{13}C, U-^{15}N]aspargine diet, suggesting that nitrogen from the glutamic acid/glutamine diet is incorporated differently to nitrogen from the aspartic acid/asparagine diet.

For many of the tested amino acids, the α-nitrogen from dietary asparagine was incorporated more efficiently than the γ-nitrogen (Fig. 3). Experiments with labeled precursors show that glutamine taken up by bacteriocytes is deaminated to form glutamic acid and ammonia, prior to uptake of the glutamic acid by *Buchnera* (Sasaki and Ishikawa, 1995). Although at least some of the released nitrogen was assembled into the synthesis of glutamic acid, likely from α-ketoglutarate, this is a less direct path for the incorporation of dietary nitrogen into essential amino acids. Metabolic modeling predicts a similar deamination of asparagine to aspartic acid, causing the release of the γ-nitrogen (Fig. S4A,B). Thus, this deamination reaction could explain the lower incorporation of the γ-nitrogen than the α-nitrogen of asparagine into other amino acids. It is likely that the [γ-^{15}N] is either excreted or is incorporated into other nitrogen-containing compounds, such as cholines and nucleic acids. For instance, unlike in the case of amino acids, there was no significant difference in the incorporation of the α-nitrogen and γ-nitrogen from asparagine into uracil and thymine (Fig. S3).

Although the majority of M+1 isotopomers of amino acids in our experiments were likely the result of transamination reactions incorporating ^{15}N, there is also evidence for the incorporation of single carbon atoms. For example, the relative abundance of the M+1 isotopomer for lysine was 86% for the [U-^{13}C, U-^{15}N]glutamine diet and 53% for the [U-^{15}N]glutamine diet (Figs 2, 3). This suggests that at least part of the M+1 isotopomer comes from labeled carbon being incorporated in the aphids fed on the [U-^{13}C, U-^{15}N]glutamine diet, though we cannot rule out the possibility that aphid physiology and amino acid biosynthesis are different on the two diets. There are other recent studies where the metabolic fate of universally carbon-labeled glutamine has been traced. These often show partial incorporation of carbons from glutamine into alanine, glutamine, glycine and valine and other amino acids, with M+1 being the most commonly observed isotopomer (Chen et al., 2011; Alonso et al., 2007).

In addition to the M+1 isotopomers, other isotopomers were found in the total amino acids of aphids fed on diets containing [U-^{13}C, U-^{15}N] amino acids (Fig. 4). For instance, although four carbons of asparagine or aspartate should be incorporated into isoleucine based on the shared biosynthesis pathways that are present in the *A. pisum–Buchnera* system (Russell et al., 2013; Wilson et al., 2010), in fact variable numbers of carbon atoms from dietary amino acids are incorporated (Fig. 4). This variable ^{13}C incorporation indicates that the majority of the carbon that is used for essential amino acid biosynthesis in *Buchnera* does not come directly from dietary amino acid sources. Thus, rather than being transported intact from the aphid gut to the bacterial endosymbionts, these dietary amino acids are catabolized and re-synthesized. Most likely, the carbon backbone of the detected amino acids was synthesized from products of the citric acid or pyruvate pathways, which would allow the incorporation of a variable number of ^{13}C atoms from dietary amino acids.

Further evidence for the catabolism and re-synthesis of dietary amino acids comes from the GC-MS analysis of total aspartic acid, asparagine, glutamic acid and glutamine in the aphids. The majority of these amino acids in the aphids were not the intact dietary forms. Only 4% of the aspartic acid/asparagine and 12% of the glutamic acid/glutamine showed the FM+4 or FM+5 molecular mass, respectively, that is expected after loss of the carboxyl group in the MS assays (Fig. 4). These numbers likely represent a slight overestimate of the actual uptake of intact amino acids by the aphids, because at the time when the aphids were collected, some amount of diet with the [U-^{13}C, ^{15}N]-labeled amino acids will be contained in the aphid gut and will be measured in our assays. Given the abundance of isotope-labeled amino acids in the diet (Table 1) and the roughly threefold increase in aphid mass in the course of the 5 day experiments (Fig. S2), one would expect approximately 51% of the aspartic acid/asparagine and 43% of the glutamic acid/glutamine to be fully isotope labeled if there were no catabolism and re-synthesis of the carbon backbone of these amino acids. Thus, the majority of the endogenous aspartic acid, asparagine, glutamic acid and glutamine in the aphids as a whole is the product of catabolism and re-synthesis, rather than direct uptake and incorporation of these amino acids from the diet. However, the ^{13}C in the amino acids of the aphid diet constitutes less than 10% of the total carbon in the diet, which contains 500 mmol l^{-1} sucrose and ∼150 mmol l^{-1} amino acids. Thus, although carbon from sugar can be incorporated into amino acids in aphids (Febvay et al., 1999), the observed ^{13}C incorporation in our experiments (Fig. 4) indicates carbon from dietary amino acids is utilized for endogenous aphid amino acid biosynthesis in aphids with greater efficiency than carbon from sucrose. This enrichment of carbon from amino acids in the aphid body most likely is the result of selective uptake from the gut. Whereas most dietary amino acids are taken up by the aphids, most of the sugar is excreted in the honeydew. Thus, there will be an enrichment of amino acid-derived carbon atoms in the aphid bodies relative to the diet from which they are feeding.

The variability of the aphid dietary amino acid content may explain the propensity for the *de novo* synthesis of even amino acids that are overabundant in the diet. The composition of phloem amino acids in plants depends on the type of nitrogen available to the roots, oxygen levels, plant symbionts, the time of day, and other environmental conditions (Coruzzi and Last, 2000; Liu et al., 2013; Oliveira et al., 2013). Moreover, several studies have demonstrated that aphid-infested plants have altered amino acid composition (Leroy et al., 2011; Liadouze et al., 1995; Sandström and Moran, 2001; Sasaki et al., 1990). Thus, the *A. pisum–Buchnera* system may have evolved to efficiently assimilate multiple available phloem nitrogen resources to produce other amino acids. Maximum flexibility in the utilization of dietary nitrogen might be provided through the catabolism and reassembly of dietary amino acids, as needed, rather than relying on the variable abundance of particular dietary amino acids for transport of metabolic precursors to the *Buchnera* endosymbionts.

Proline is unusual among the detected aphid amino acids in that there is an eightfold higher ratio of a single ^{13}C incorporated from dietary sources (Fig. 5). This could be an indication that proline has a higher turnover than other aphid amino acids, thereby allowing a higher labeling specificity. In addition to being an essential component of proteins, proline has an osmoprotective function in

many organisms, ranging from yeast to arthropods (Kostal et al., 2011; Takagi, 2008; Verslues and Sharma, 2010). As osmoregulation is a unique challenge faced by aphids feeding from phloem sap containing roughly 1 mol l^{-1} sucrose (Douglas, 2006), there may be aspects of proline metabolism and turnover that make synthesis of this amino acid different from that of other amino acids that were detected in our GC-MS assays.

Together, our results provide new insight into amino acid biosynthesis by *A. pisum* and *Buchnera* symbiosis. Notable findings include: (i) the catabolism and re-synthesis of dietary amino acids; (ii) the high level of *de novo* synthesis of non-essential amino acids, even when they are presumably overabundant in the diet; and (iii) the variable incorporation of different nitrogen sources into essential amino acids. The supply of precursors for synthesis of essential amino acids by *Buchnera* is clearly more complex than simply the transport of dietary amino acids from the gut to the endosymbionts. As all animals interact with microbes during their growth and development, the provisioning of amino acids by commensal microbes is a more general phenomenon. Thus, research on the shared amino acid biosynthesis of aphids and their bacterial endosymbionts may have implications beyond this specific biological system.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
M.H. designed the experiments, performed the research, analyzed the data and wrote the manuscript. G.J. designed the experiments, analyzed the data and wrote the manuscript.

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