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Investigating amino acid utilization by soil microorganisms using compound specific stable isotope analysis

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ABSTRACT

Amino acids comprise a large proportion of soil organic nitrogen (N). Microorganisms can take up amino acids as intact molecules or as ammonium and α-keto acids after enzymatic deamination. The objective of this study was to trace double labeled (^{13}C,^{15}N) glycine and L-leucine from soil solution into microbial biomass using compound specific stable isotope analysis. The two amino acids were utilized rapidly by soil microorganisms, with the half-life of glycine and leucine in soil solution being 2.9 and 5.0 h, respectively. The highest concentration of added glycine and leucine in the microbial biomass was measured after 4 h and corresponded to 10 and 13% of the glycine and leucine added, respectively, showing that a part of the added amino acids was taken up as intact molecules. Based on the results from an accompanying isotope pool dilution experiment, at least 15% of the glycine and 50% of the leucine was taken up as intact molecules. Our results suggest that glycine mainly served as carbon (C) source, while leucine provided C as well as N for the soil microbial community. Labeled keto acids were detected in soil solution and in the microbial biomass; however, their concentration corresponded only to a small fraction of the C added with the amino acids. The fact that 30 and 65% of glycine and leucine N was mineralized within 12 h, while never more than 5% of the amino acid C was recovered in the form of keto acids highlights how quickly keto acids were utilized by soil microorganisms. The combination of compound specific stable isotope analysis and chloroform fumigation extraction proved to be a valuable tool to trace amino acids and keto acids in soil solution and the microbial biomass.

1. Introduction

Amino acids play a central role in the soil nitrogen (N) cycle, as a large proportion of the N in soil organic matter is in the form of proteinaceous material (Nannipieri and Paul, 2009). Microorganisms can take up a variety of mineral and organic molecules to satisfy their N requirement. While ammonium (NH_4^+) is generally the preferred source of N, a variety of amino acid transporters have been identified in bacteria and fungi (Sanchez and Demain, 2008; Geisseler et al., 2010). Finding direct evidence for the uptake of intact amino acids by soil microorganisms has been challenging, because microorganisms produce deaminating enzymes which may be actively excreted into soil solution or released from decaying cells. These enzymes can remain active in soil for some time, breaking down amino acids into NH_4^+ and α-keto acids, which may then be taken up separately by the microbial biomass (Braun et al., 1992; Klose and Tabatabai, 2002). Different approaches to assess the direct uptake of amino acids by soil microorganisms have been used. To better understand the processes affecting the fate of N in soils, a good understanding of the complex processes influencing the microbial uptake of organic N is needed.

In previous studies, we determined the mineralization of added ^{15}N-labeled amino acids using a pool dilution approach (Geisseler et al., 2009, 2012). By measuring the decrease in amino acid concentration in soil solution and their N mineralization rate, we concluded that the difference between amino acid utilization and mineralization was due to uptake of intact molecules by the microbial biomass (Geisseler et al., 2012). However, the method provided only indirect evidence for the uptake of intact amino acids. In addition, ammonium released after the uptake of intact amino acids and intracellular deamination also contributes to mineralization. Therefore, the proportion of amino acids taken up directly may be underestimated with this approach.

Another common approach to determine the uptake of amino acids by soil microorganisms is to apply double-labeled (^{13}C and ^{15}N) amino acids and measure the ^{13}C and ^{15}N concentration in

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the microbial biomass after an incubation period. A significant linear correlation between the ^{15}N and ^{13}C enrichment of the microbial biomass is considered a strong indication for the uptake of intact amino acids (e.g. Clemmensen et al., 2008; Andresen et al., 2011). However, this approach does not distinguish between the uptake of intact amino acids and the separate uptake of NH_4^+ and keto acids from the labeled amino acids.

Compound specific isotope analysis is another approach to determine the fate of amino acids in soil. This approach has been used to determine the gross rate of amino acid production in soil (Wanek et al., 2010) and to trace the C and N from added amino acids through various soil amino acid pools (Knowles et al., 2010). We hypothesize that a significant amount of amino acids are taken up as intact molecules by soil microorganisms. To test this hypothesis, we used gas chromatography (GC) coupled with ion-trap mass spectrometry (MS) to perform compound specific stable isotope analysis in combination with chloroform fumigation to determine the fate of added amino acids.

2. Material and methods

2.1. Soil and residue samples

Soil samples were collected in summer 2013 at the UC Davis Russell Ranch Sustainable Agricultural Facility site. Samples were taken from the top 20 cm of the profile from a field planted to corn before the sidedress N application. The soil is mapped as Rincon silty clay loam (fine, montmorillonitic, thermic Mollic Haploxeralf; Soil Survey Staff, 1997). The field-moist soil was passed through a 4-mm sieve and stored at 4 °C. The soil had a pH of 7.2 (determined in a 1:2 soil:water solution; Thomas, 1996) and contained 12.3 g C kg^{-1} dry soil and 1.1 g N kg^{-1} dry soil (analyzed by dry combustion on a Costech EAS 4010 elemental analyzer; Bremner, 1996; Nelson and Sommers, 1996). Percentages of sand, silt, and clay were 15, 53 and 32%, respectively (pipet method; Gee and Bauder, 1986), and the microbial biomass N was 56.4 mg kg^{-1} dry soil (chloroform fumigation extraction method followed by the alkaline persulfate oxidation method and colorimetric analysis of nitrate; Brookes et al., 1985; Cabrera and Beare, 1993; Doane and Horwath, 2003).

2.2. Microcosm experiment

Samples for the different analyses were prepared by weighing field-moist soil, equivalent to 6 g oven dry soil, into 40 mL glass vials. For the different analyses, 0.64 mL of solutions containing NH_4^+, glycine and L-leucine dissolved in DI water were added (see following paragraphs for amounts and treatments). With this addition, the gravimetric moisture content was brought to 28 g g^{-1} dry soil, which corresponded to 50% water filled pore space. The vials were then placed into a 12-L plastic container with a lid, lined with moist paper towels to minimize evaporation, and incubated at room temperature (22 °C). After 0, 0.5, 1, 2, 4, 12, and 24 h, four random replicates per treatment were destructively sampled. Ammonium and amino acids were extracted by adding 30 mL of 0.5 M potassium sulfate (K_2SO_4) to the soil samples (Mulvaney, 1996). Samples were shaken for 1 h on a reciprocal shaker and the suspension filtered (Fisherbrand, Q5).

2.2.1. Amino acid-N mineralization analysis

The following treatment solutions containing N were added to samples at the beginning of the incubation to analyze amino acid-N mineralization by pool dilution: (a) $(^{15}NH_4)_2SO_4$ (40 atom%); (b) $(^{15}NH_4)_2SO_4$ (40 atom%) + unlabeled glycine and unlabeled L-leucine; (c) $(NH_4)_2SO_4$ + ^{13}C,^{15}N-glycine and unlabeled L-leucine; (d) $(NH_4)_2SO_4$ + unlabeled glycine and ^{13}C,^{15}N-L-leucine. 10 mg N kg^{-1} dry soil were added in the form of NH_4^+ and 5 mg N kg^{-1} dry soil in the form of each glycine and L-leucine. The atom% ^{15}N of both amino acids was >95% and the atom% ^{13}C at the C_2 position was 99%. The treatment solutions were applied uniformly to 4 replicates ($n = 4$) using a syringe with needle. Glycine and L-leucine were chosen because they have the lowest and highest C to N ratios of all the aliphatic protein-forming amino acids. The samples of treatment (a) were extracted immediately with (K_2SO_4) as described below. The remaining treatments (b–d) were extracted after the predetermined incubation time.

2.2.2. GC–MS amino acid analysis

For the GC–MS analysis, two samples were prepared for each incubation time and replicate ($n = 4$). The solutions added with the DI water contained $(NH_4)_2SO_4$ (10 mg N kg^{-1} dry soil), ^{13}C,^{15}N-glycine, and ^{13}C,^{15}N-L-leucine (5 mg N kg^{-1} dry soil each). The addition of each amino acid corresponded to 0.33 mmol kg^{-1} dry soil. One sample was extracted immediately at the end of the incubation as described above; the other was chloroform fumigated first for 24 h. A standard chloroform fumigation extraction method (Brookes et al., 1985) was used with one adjustment: Preliminary analyses showed that the concentration of double-labeled amino acids in the fumigated samples was lower than in the corresponding control samples, while the concentration of single-labeled amino acids was increased. A series of tests indicated that aminotransferases released during fumigation were responsible for this reaction (Geisseler and Horwath, submitted for publication). In order to inactivate these enzymes, 0.6 mL of a 5% sodium dodecyl sulfate (SDS) solution were added to the samples prior to fumigation. With this addition, the gravimetric soil moisture content was raised to 0.38 g g^{-1}. After the fumigation, the samples were extracted as described above.

All extracts from the above experiments were kept in the fridge at 4 °C and analyzed within two days. Preliminary test showed that the amino acid concentration in the samples did not change during this time.

2.3. Nitrogen mineralization from amino acids

Nitrogen mineralized from amino acids was determined at all sampling dates using a mirror image pool dilution approach (Equations (1) and (2)) described by Watkins and Barraclough (1996).

First, the gross rate of N mineralization (m) was calculated using Equation (1).

$$A_t^* = \frac{A_0^*}{\left(1 + \frac{\theta t}{A_0}\right)^{(m/\theta)}} \quad (1)$$

Where A_0 and A_t are the NH_4^+ pool at time 0 and t, respectively. The ^{15}N excess of the NH_4^+ pools is indicated by *. The rate at which the pool size changes (θ) is given by $(A_t - A_0)/t$. Values for A_0 and A_0^* were derived from the samples which were immediately extracted (treatment a), while A_t and A_t^* were determined on the samples to which unlabeled amino acids and $^{15}NH_4^+$ were applied (treatment b).

The value for m from Equation (1) is substituted into Equation (2) together with the size and ^{15}N excess of the NH_4^+ pool in the samples to which labeled amino acids and NH_4^+ were applied (treatments c and d).