Amino Acids (2007) 33: 631–638
DOI 10.1007/s00726-006-0487-7
Printed in The Netherlands

Amino Acids

The intramolecular $\delta^{15}N$ of lysine responds to respiratory status in *Paracoccus denitrificans*

B. S. Pan, C. J. Wolyniak, and **J. T. Brenna**

Cornell University, Division of Nutritional Sciences, Ithaca, NY, U.S.A.

Received May 3, 2006
Accepted November 15, 2006
Published online January 26, 2007;

Summary. Presented here is the first experimental evidence that natural, intramolecular, isotope ratios are sensitive to physiological status, based on observations of intramolecular $\delta^{15}N$ of lysine in the mitochondrial mimic *Paracoccus denitrificans*. *Paracoccus denitrificans*, a versatile, gram-negative bacterium, was grown either aerobically or anaerobically on isotopically-characterized ammonium as sole cell-nitrogen source. Nitrogen isotope composition of the biomass with respect to source ammonium was $\Delta^{15}N_{cell-NH_4} = \delta^{15}N_{cell} - \delta^{15}N_{NH_4} = -6.2 \pm 1.2‰$ for whole cells under aerobic respiration, whereas cells grown anaerobically produced no net fractionation ($\Delta^{15}N_{cell-NH_4} = -0.3 \pm 0.23‰$). Fractionation of ^{15}N between protein nitrogen and total cell nitrogen increased during anaerobic respiration and suggests that residual nitrogen-containing compounds in bacterial cell membranes are isotopically lighter under anaerobic respiration. In aerobic cells, the lysine intramolecular difference between peptide and sidechain nitrogen is negligible, but in anaerobic cells was a remarkable $\Delta^{15}N_{p-s} = \delta^{15}N_{peptide} - \delta^{15}N_{sidechain} = +11.0‰$, driven predominantly by enrichment at the peptide N. Consideration of known lysine pathways suggests this to be likely due to enhanced synthesis of peptidoglycans in the anaerobic state. These data indicate that distinct pathway branching ratios associated with microbial respiration can be detected by natural intramolecular $\Delta\delta^{15}N$ measurements, and are the first in vivo observations of position-specific measurements of nitrogen isotope fractionation.

Keywords: Intramolecular isotope ratio – $^{15}N/^{14}N$, $\delta^{15}N$ – *Paracoccus denitrificans* – Nitrogen metabolism

Introduction

Variations in isotopic fractionation arising from discrimination between heavy and light isotopes of carbon and nitrogen have been used extensively to monitor natural processes. The selectivities of biochemical reactions impart a unique position-specific isotopic signature reflecting pathways that form and degrade the bonds to the each atomic position within molecules. This phenomenon has been exploited most commonly in vitro in order to examine specific biochemical reaction kinetics and mechanisms (Weiss et al., 1988). For an organism, these observations imply that each stereochemically unique position within every unique chemical species in the organism has an isotope composition that reflects physiological state (Brenna, 2001). Isotope ratios have been treated primarily as static for a given organism, with little attention to the influence of physiological state. However, in vivo differences in isotopic fractionation among inputs and outputs in branched pathways define the isotope ratio of specific positions with weightings reflecting their relative contributions. A change in physiological state could influence a change in pathway branching ratios, which in turn predicts that isotope ratios at specific positions change with the relative inputs and outputs of different reactions (Monson and Hayes, 1982; Schmidt, 2003). One study of respiratory-state and carbon fractionation (Teece et al., 1999) showed that lipid $^{13}C/^{12}C$ at the compound-specific level is sensitive to aerobic vs. anaerobic state because of the shift in substrate usage and subsequent partitioning. To date, no reports of purely physiological fractionation of position-specific isotope ratios have been reported for any element, nor have there been studies of non-source related physiological N-fractionation at the compound or position-specific levels.

Many fields employ natural isotopic variability as a routine tool, such as forensic geographical sourcing (Slater, 2003), analysis of food webs in human and animal diets (Deniro and Epstein, 1981), and plant species differentiation (Oleary, 1981). The vast majority of isotope ratio measurements are reported at the bulk and compound-

specific levels, thus obscuring intramolecular isotopic information. However, isotopic selectivity is clearly an intramolecular phenomenon, and the ability to measure position-specific isotope distributions would permit sensitive analysis of metabolic processes within organisms, and potentially open a vast endogenous record of metabolic states. For instance, neural DNA (Slatkin et al., 1985; Spalding et al., 2005a) and tooth collagen (Spalding et al., 2005b) are both laid down during the formation of those tissues in humans, and are maintained through life. The ability to precisely detect intramolecular isotope signatures in these long-lived molecules and to relate them to physiological state would provide a new tool for evaluating the long-term consequences of physiological state during formative development.

Amino acids, in particular, are excellent candidates as isotopic markers since their biosynthetic and catabolic pathways have been largely deduced, and they play key roles in many pathways of intermediary metabolism. Abelson and Hoering (1961) first demonstrated systematic, position-specific variation in the carboxyl-group $\delta^{13}C$ of various amino acids across several species of photosynthetic organisms. Subsequent studies that analyzed the C and N isotope content of amino acids from fungi and bacteria grown on different nutrient sources also report this characteristic distribution, controlled by the mechanisms of assimilation (Macko et al., 1987). To date, there have been no reports of in vivo measurements of position-specific $\delta^{15}N$, owing in part to a lack until only recently of methods (Sacks and Brenna, 2005) for nitrogen position-specific isotope analysis (N-PSIA). This new method has been applied to lysine metabolism in a model bacterium as the first test in any organism of whether intramolecular $\delta^{15}N$ distribution is indicative of physiological state.

Paracoccus denitrificans, a gram-negative, heterotrophic bacteria bearing resemblance to mitochondria (John and Whatley, 1975), is well-suited as a model organism for studies of metabolic isotopic partitioning and fractionation. It grows well aerobically or anaerobically on easily characterized single-carbon (e.g., CH_3OH) and single-nitrogen sources (Bamforth and Quayle, 1978), so that all input C and N is isotopically uniform and known. When present in the media, NH_4^+ is the sole cell-nitrogen source in both the anaerobic state, in which nitrate or nitrite serve as the terminal electron acceptors (Chang and Morris, 1962), and the aerobic state, in which NH_4^+ inhibits metabolism of N from nitrate (Verhoeven, 1956). Additionally, stringent anoxic conditions are necessary for anaerobic growth, thus providing a clear shift in physiologically states (Baumann et al., 1996).

Materials and methods

Cell growth and preparation

P. denitrificans (ATCC 17741) were obtained from the American Type Culture Collection (Rockville, MD) and conditioned to grow exclusively on ammonia and methanol as sole N and C sources. The defined medium for the aerobic and anaerobic physiological states consisted of (in g/L) K_2HPO_4, 1.23; $K_2H_2PO_4$, 0.4; KNO_3, 3.03; NH_4Cl, 0.4; $MgSO_4 \cdot 7H_2O$, 0.4; CH_3OH, 1.6; trace-elements solution, 2 ml/L (Bamforth and Quayle, 1978). The modified Vishniac and Santer trace-elements solution (Vishniac and Santer, 1957) contained (in g/L) EDTA, 50.0; $ZnSO_4$, 2.2; $CaCl_2$, 5.5; $MnCl_2 \cdot 4H_2O$, 5.06; $FeSO_4 \cdot 7H_2O$, 5.0; $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 1.1; $CuSO_4^+ \cdot 5H_2O$, 1.57; $CoCl_2 \cdot 6H_2O$, 1.61 (Barford et al., 1999; Vishniac and Santer, 1957). Media for the anaerobic state was made anoxic by sparging with a steady stream of N_2 for 30 min and then kept tightly capped. One liter batch preparations of cells were grown at 34 °C and 250 revolutions per minute (RPM) in an incubated shaker (Labline; Max Q 5000; Melrose Park, IL), with periodic monitoring at O.D. 600 nm. (LKB Biochrom; Ultrospec 4050; Cambridge, UK) Cells were harvested in log phase and washed 3 times in ammonia-free phosphate buffer to remove residual N from cell surfaces. Centrifugation (Beckman Coulter; Avanti J-E; Fullerton, CA) followed by freeze-drying was performed before sample storage at −80 °C. Average freeze-dried weight for 1 L batches was 0.24 ± 0.07 mg/mL, with no significant differences between physiological states.

Sample preparation and hydrolysis

Free amino acids were prepared by standard liquid-phase protein hydrolysis (Fountoulakis and Lahm, 1998) of dry biomass using a Pico-Tag Workstation (Waters; Milford, MA). Briefly, 50 mg of biomass was suspended in 20 ml 6 N HCl, 0.1% phenol; the ambient atmosphere in hydrolysis vessels was replaced with nitrogen gas via several purges. Acid hydrolysis proceeded at 145 °C for 4 h, HCl was removed in vacuo, and dried hydrosylates were resuspended in 10 ml distilled H_2O. Solution pH was adjusted to 8 with KOH and insoluble matter was removed by centrifugation.

Dried bacterial pellets for both the aerobic and anaerobic states were characterized in triplicate for amino acid content (Molecular Structure Facility at the University of California, Davis, CA).

Enzymatic reactions

Lysine oxidase (EC 1.4.3.14; Sigma-Aldrich) was prepared as a 2500 unit/mL suspension in distilled H_2O, where 1 unit will catalyze the deamination of 1 μM of NH_4^+ from lysine per minute at 37 °C at pH 8. Ten microliters of freshly prepared lysine oxidase stock solution was added to 1 ml of hydrosylate and incubated in a water bath at 37 °C for 24 h. Enzymatic deamination was stopped by freezing reactions at −20 °C until derivatization.

N-ethoxycarbonyl ethyl ester (ECEE) derivatization of amino acids

Amino acids hydrosylates and enzymatic reactions were made amenable for analysis by a derivatization procedure modified from Husek (1991) and Fiamegos et al. (2004). Amino acids (600 μl; approximately 2.5 mM per amino acid) were diluted with 400 μL of ethanol-pyridine (4:1), followed by acidification with 10 μL of 2 N HCl and addition of 10 μL of an internal standard, norvaline (250 mM). Reaction mixtures were treated with 50 μL of ethyl chloroformate (ECF), capped, and mixed vigorously by vortexing and then sonicated for 15 min. Derivatization products were extracted into 1 ml of chloroform (containing 1% ECF). Chloroform was removed under a gentle stream of nitrogen and dried residue was resuspended in 50 μL chloroform prior to isotopic analysis.

Gas chromatography-combustion/reduction-IRMS of amino acid derivatives

Minor modifications were made to our previously reported system. Briefly, the ceramic column and metal fittings utilized in the previous combustion furnace were replaced with a glass capillary in order to reduce leaks and minimize background contamination from ambient air. Samples were injected, splitless, using an autosampler (Varian, Inc. 8200; Walnut Creek, CA) into a GC (Varian 3400CX). For routine analysis of amino acids, a moderately polar, thick-film, 15 m × 0.53 mm × 3 μm VB-1701 capillary column (VICI; Houston, TX) was utilized. A second, relatively non-polar, 60 m × 0.32 mm × 5 μm capillary column (Agilent J&W Scientific; Palo Alto, CA) was required for the separation of the deaminated lysine analogue, δ-aminovaleric acid (DAVA). For both columns, GC oven temperature was ramped from 110 to 285 °C at a rate of 10 °C/min and held for 5 min, followed by a final ramp of 50 °C/min to 300 °C and a hold of 5 min. Head pressures were set at 5 and 25 psi for the VB-1701 and the DB-1 column, respectively, which corresponded to a flow of 2.5 mL at the open-split. An actuated rotary valve controlled by the GC was used to divert solvent flow and facilitated periodic O_2 recharging of the combustion furnace (Sacks and Brenna, 2005).

Analyte peaks exiting the rotary valve were combusted in a capillary furnace consisting of oxidized Cu wire threaded into a 0.53 mm ID glass column housed in a ceramic tube. The combustion furnace was heated resistively to 950 °C by a Fibercraft unit (Thermcraft; Winston-Salem, NC). An external reduction furnace, similarly heated to 550 °C, was connected through an additional rotary valve, which diverted O_2 away from the reduction furnace and the downstream IRMS during recharge. Water and carbon dioxide byproducts were trapped by passing a glass capillary through a Dewar filled with liquid N_2 before the open-split (Sacks and Brenna, 2005).

Bulk isotope analysis of standards

Elemental analysis on whole cells and standards was performed on an NC2500 Carlo Erba Elemental Analyzer (EA) coupled to a Finnigan MAT Delta Plus IRMS (Bremen, Germany). Samples and standards (approximately 1 mg) were weighed in triplicate into tin cups (Costech; Valencia, CA). Conditions for EA analysis were 1000 °C over Cr_2O_3 and CuO for combustion and 650 °C over copper fillings for reduction.

Isotope ratio notation and statistics

Isotope measurements in IRMS are typically reported in delta notation with respect to established standards. For N analysis, the $^{15}N/^{14}N$ of the sample is compared to $^{15}N/^{14}N$ of N_2 in air.

$$\delta^{15}N_{sample} = [(R_{sample} - R_{air})/R_{air}] \times 1000;$$

where

$$R_x = [^{15}N_x/^{14}N_x]$$

Amino acid compositions from aerobic and anaerobic states were compared with a pairwise t-test with Bonferroni correction for multiple comparisons. Duncan's Multiple Range tests (alpha = 0.05) were performed on the means of the isotope compound-specific amino acids determinations.

Results and discussion

Three batches of *P. denitrificans* were each grown under either aerobic or anaerobic respiration. Under anaerobic conditions, NO_3^- serves as a terminal electron acceptor and is reduced to N_2 via a series of nitrate and nitrite reductases coupled to the electron transport chain (Bamforth and Quayle, 1978). Two nitrate reductases, a periplasmic nitrate reductase expressed constitutively and another membrane-bound reductase expressed under oxygen limitation, are active during denitrification (Baker et al., 1998; Baumann et al., 1996). Previous studies of nitrate utilization by *P. denitrificans* demonstrate negligible conversion of NO_3^- to NH_4^+ (Verhoeven, 1956). Energy conservation under anaerobic growth is impaired relative to aerobic growth owing to a loss of 2 electrons to nitrate reductases in the periplasm (Craske and Ferguson, 1986). Consequently, growth on methanol under aerobic and anaerobic conditions proceeded slowly and cells exhibited a longer lag-phase under anaerobic versus aerobic conditions, as reported in several studies (Bamforth and Quayle, 1978; Chang and Morris, 1962; Cox and Quayle, 1975).

Bulk physiological $^{15}N/^{14}N$ fractionation

Bulk fractionation of $^{15}N/^{14}N$ of the ammonium by the whole cells was $\Delta^{15}N_{cell-NH_4} = -6.2 \pm 1.2‰$ in the aerobic state, but there was no significant net fractionation in the anaerobic state ($-0.31 \pm 0.23‰$). No statistically significant differences in the prevalence of amino acids were found between states. Mass-balance calculations for protein $\delta^{15}N$ using the amino acid compositions from anaerobic cells showed a $+3.2 \pm 0.48‰$ enrichment in ^{15}N relative to total biomass, in agreement with a previous reports of approximately +3.5‰ (Macko et al., 1987), while aerobic cells showed a lower degree of enrichment ($+1.38 \pm 1.19‰$). As proposed by Werner and Schmidt (2002) this observation is likely explained by enhanced ^{15}N depletion in N-containing cell wall components in the anaerobic state, consistent with the present data (Table 1). The expression of membrane-associated nitrate reductases during anaerobic respiration and the corresponding changes to membrane chemistry is a likely source for this difference in fractionation between states.

Table 1. $\delta^{15}N$ of whole cells grown aerobically and anaerobically

	$\delta^{15}N$	
	Aerobic	Anaerobic
Total biomass	-6.2 ± 1.2	-0.3 ± 0.23
Protein fraction	-4.8 ± 0.17	2.9 ± 0.42
$\Delta\delta^{15}N_{protein\text{-}biomass}$	$+1.4 \pm 1.2$	$+3.2 \pm 0.48$

$\delta^{15}N$ of protein fraction is calculated from weighted-average of $\delta^{15}N$-CSIA amino acids. Difference between total biomass and protein indicates $^{15}N/^{14}N$ enrichment in proteins, or $^{15}N/^{14}N$ depletion in cell membranes

Studies of microbial fractionation of differing dietary amino acids (Macko and Estep, 1984) or N-source (Macko et al., 1987) have demonstrated a marked relationship between C/N source and bulk fractionation, however, the contribution of physiology to this isotopic fractionation is not well-understood. Incorporation of NH_4^+ in *P. denitrificans* proceeds via two pathways (Mikes et al., 1991), with glutamate dehydrogenase (GDH) and glutamine synthetase (GS) operating typically at mM and μM concentrations of NH_4^+, respectively. Hoch et al. (1992) established that at mM NH_4^+ concentrations, transport of N is passive and that intracellular and extracellular pools approach isotopic equilibrium. Under conditions of rapid equilibrium and excess source N, isotopically-selective barriers such as cell membranes do not play a significant role in overall fractionation. The rising trend in ^{15}N enrichment between states can be attributed either to loss of isotopically light NH_3 into the media, or to the previously observed in vitro pH-dependent fractionation of NH_4^+ by GDH reported by Weiss et al. (1988). Isotopic fractionation by GDH was reported from −18 to +10‰ at pH 5.8 and pH 9.2, respectively. Calculations of ^{15}N fractionation in *P. denitrificans* using a linear approximation ($r^2 = 0.993$) of observed ^{15}N kinetic isotope effects (KIE) for GDH correspond to values of $\Delta^{15}N_{cell-NH_4} = -8.1‰$ for aerobic cells (average $pH = 7.1 \pm 0.04$) and $\Delta^{15}N_{cell-NH_4} = -0.7‰$ (average $pH = 7.95 \pm 0.24$) for anaerobic cells, in good agreement with this study.

Compound-specific fractionation of ^{15}N

The quantitative compound-specific analysis of amino acids first requires the cleavage of peptide bonds to free amino acids from protein. Traditional acid hydrolysis deaminates Gln and Asn to Glu and Asp, respectively, resulting in two combined pools of N-peptide for Glu/Gln (Glx) and Asp/Asn (Asx). Gln/Asn are synthesized from the addition of sidechain nitrogen to pre-formed Glu and Asp, therefore the contribution of N-peptide from Gln/Asn which has been degraded to Glu/Asp is not likely to greatly affect the nitrogen isotope ratio of Glx/Asx, since they are essentially the same. Additionally, the fractionation of N isotopes during incomplete peptide-bond hydrolysis has been previously reported (Silfer et al., 1992), highlighting a significant fractionation that decreases with increasing temperature. While consideration of this potential source of fractionation is merited, acid hydrolysis of proteins is generally quantitative and furthermore, protein from both respiratory states was hydrolyzed and would share any systematic fractionation that may have occurred.

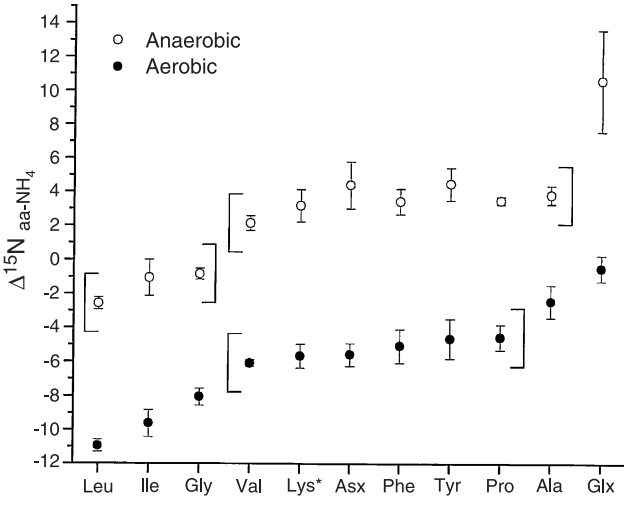

Fig. 1. Amino acid isotopic compositions expressed with respect to source ammonium, $\Delta^{15}N_{aa-NH_4} = \delta^{15}N_{amino\ acid} - \delta^{15}N_{NH_4}$. Brackets indicate overlapping ranges of non-significant differences in means analyzed using Duncan's Multiple Comparisons. *Represents average $\delta^{15}N$ of the two N in Lys

Illustrated in Fig. 1 are the

$$\Delta^{15}N_{aa-NH_4} = \delta^{15}N_{amino\ acid} - \delta^{15}N_{NH_4}$$

of 11 proteinogenic amino acids from both physiological states. The general distribution shows a characteristically high isotope ratio for Glx in both states, consistent with its role as a primary N-donor for metabolically related amino acids. This overall enrichment of ^{15}N in Glx with respect to other amino acids is in agreement with that reported by Macko et al. (1987) in regard to manipulating source substrates, although the specific impact of respiratory state on the distribution of ^{15}N in amino acids in a micro-organism has not been previously reported. The variability and distribution of ^{15}N in the amino acids fall within a smaller range compared to previous reports, which span a range of approximately 10‰. The remaining amino acids fell within a narrow range of ^{15}N depletion relative to total Glx, with an average difference of $\Delta^{15}N_{aa-Glx} = -4.2 \pm 0.61$ and $-7.0 \pm 0.80‰$ for aerobic and anaerobic states, respectively, followed by Gly, Ile, and Leu. Under aerobic conditions, Ala was distinguished from other amino acids with respect to Glx. The distinct fractionation of Ala in the aerobic state suggests differing Ala metabolism, possibly resulting from increased Ala deamination to produce pyruvate or an alternative N-source other than Glu as was demonstrated by Macko et al. (1987) with Asp serving as the predominant N-source for blue–green algae under certain conditions. Additionally, as a critical component in bacterial peptidoglycan synthesis, the relative branching ratios between the contributions of Ala to protein synthesis, as

opposed to cell-wall synthesis in either state, also affects the $^{15}N/^{14}N$ with respect to other amino acids.

Kinetic isotope effects during transamination are a likely source for ^{15}N fractionation in amino acids. Incorporation of N during amino acid biosynthesis relies on transamination from one amino acid to another and is largely independent of C amino acid metabolism. The direct transfer of free ammonium to 2-oxoglutarate by GDH to form glutamate (Glu) is the first step in NH_3 assimilation, and regeneration of 2-oxoglutarate by transamination from Glu to analogous oxo-amino acids is a reoccurring mechanism for amino acid metabolism, including the synthesis of Ala, Val, Ile, Leu, Tyr, Phe, Asp, Lys, Ser, and several others. Directly or indirectly, all N in amino acids originates from Glu.

The KIE for the enzyme aspartate aminotransferase (AspAT; EC 2.6.1.1), responsible for nitrogen exchange between Glu and Asp, have been reported in vitro as $\beta = 1.0083$ for the transamination of glutamic acid and $\beta = 1.0017$ for the reverse transamination reaction of aspartic acid ($\beta = k_1^{14}N/k_1^{15}N$) (Macko et al., 1986). The present results, $\Delta^{15}N_{Asx-Glx} = -5.1 \pm 1.0‰$ and $\Delta^{15}N_{Asx-Glx} = -6.2 \pm 3.1‰$, within the respective aerobic and anaerobic states, and are in agreement with these previous KIE. The magnitude of this in vivo difference in isotope ratios suggests significant transaminations in both directions between Glu and Asp, possibly due to feedback inhibition of transaminases by, among other reasons, their products. Over 20 transaminases, including aromatic-amino-acid transaminase (ArAT; E.C. 2.6.1.57) and branch-chain-amino-acid transaminase (BCAT; E.C. 2.6.1.42) are responsible for site-specific N transfer and are likely to exhibit similar isotopic fractionations which can be seen in the cascade of isotope signals seen in this paper and in previous reports by Macko et al. Amino acid nitrogen metabolism may be further complicated by the promiscuity of the major transaminases for various substrates. AspAT has been reported to exhibit activity on L-Tyr, L-Phe, and L-Trp (Mavrides and Orr, 1975) and in *P. denitrificans*, ArAT has also been shown to have other specificities aside from aromatic amino acids (Oue et al., 1997). The relative contribution of Asp and Glu to the citric acid cycle, which is suppressed in the absence of oxygen (Nakano et al., 1998), is also probable source of fractionation.

Relative in vivo physiological rates of transaminations between amino acids are not well-studied. In studies of N compound-specific analysis (CSIA) of amino acids, amino acid isotope profiles vary widely between organisms but are remarkably consistent within organisms, regardless of growth medium. The similarity of $^{15}N/^{14}N$ amino acid profiles between the aerobic and anaerobic state suggest that amino acid metabolism is, at least in part, preserved, independent of anaerobic/aerobic physiological state. One major source of fractionation appears to be at the bulk-level, where isotopic fractionation of ammonium during assimilation governs the relatively consistent bulk isotopic shift between the amino acid ^{15}N enrichment between states. However, changes within the profiles between states, such as those observed for Ala, may reveal sensitive biomarkers of physiological state, and merit further study.

Position-specific fractionation of Lys ^{15}N

Compound-specific measurement for Lys $\Delta^{15}N_{Lys-NH_4}$ was $-5.7 \pm 0.71‰$ in the aerobic state and $+3.2 \pm 0.95‰$ in the anaerobic state (Fig. 1). These values are a mean of $\delta^{15}N$ for the two N positions in Lys. The intramolecular isotopic signature of Lys-N in both respiratory states emphasizes the different pathways for nitrogen incorporation into the Lys (Fig. 2). There was no significant difference in the $\delta^{15}N$ for the peptide and sidechain N ($\Delta^{15}N_{p-s}$) in the aerobic state. However, under anaerobic conditions, the $\delta^{15}N$ of the two positions diverged, with the peptide-N of Lys becoming enriched in ^{15}N with respect to sidechain-N by $\Delta^{15}N_{p-s} = +11.0 \pm 2.0‰$. The sidechain (amido) N was depleted compared to source N, while the peptide (amino) N was remarkably enriched compared to source N. This finding dramatically illustrates the degree to which compound-specific measurements mask intramolecular variability.

The intramolecular $\Delta^{15}N_{p-s}$ for Lys must originate from a shift in the branching of the pathways for synthesis or degradation of the respective N sources. The accepted pathway for Lys synthesis in gram-negative bacteria

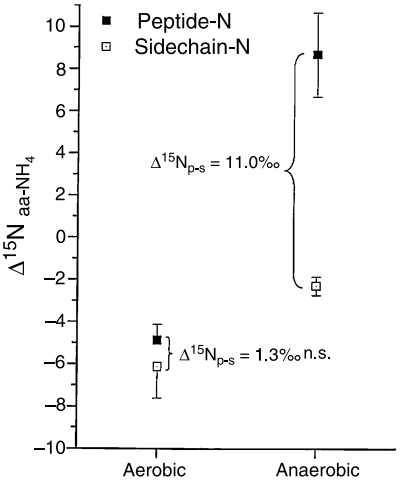

Fig. 2. Intramolecular distribution of $^{15}N/^{14}N$ in Lys in the aerobic and the anaerobic states, expressed with respect to source nitrogen ($\Delta^{15}N_{aa-NH_4} = \delta^{15}N_{amino\ acid} - \delta^{15}N_{NH_4}$). *n.s.* Not significant

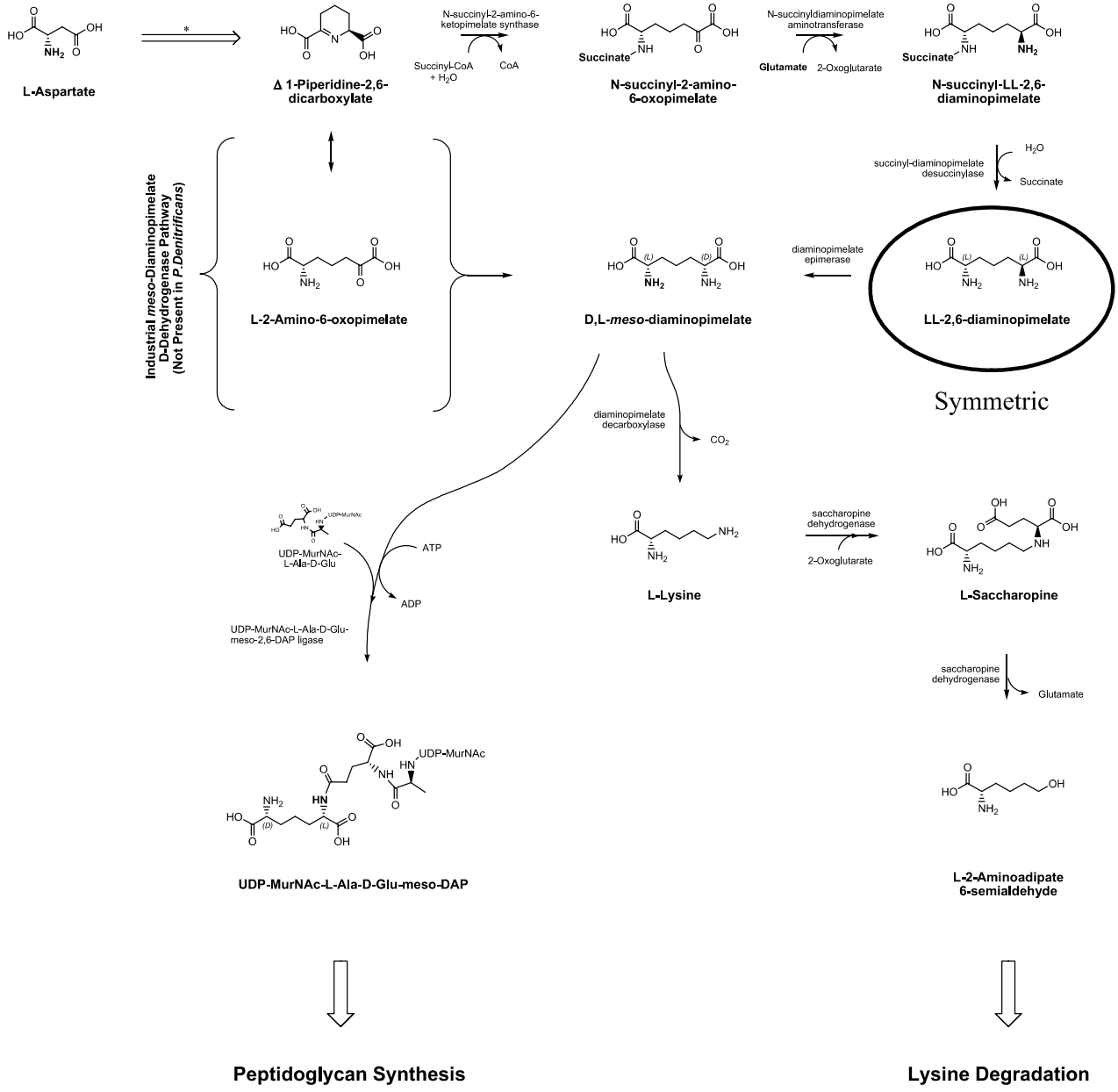

Fig. 3. Biosynthesis of lysine in bacteria indicating branching points. *Represents several critical steps, including regulatory phosphorylation by aspartate kinase, reduction with NADH, and condensation with pyruvate. Brackets indicate an alternate pathway unique to industrially-important bacteria utilized in Lys production. Isotope values expressed with respect to source ammonium ($\Delta^{15}N_{aa-NH_4} = \delta^{15}N_{amino\ acid} - \delta^{15}N_{NH_4}$). The N in the symmetric intermediate LL-2,6-diaminopimelate are biochemically indistinguishable and thus isotopically equivalent ($\Delta^{15}N_{p-s} = \delta^{15}N_{peptide} - \delta^{15}N_{sidechain} \sim 0‰$) Figure assembled from the KEGG: Kyoto Encyclopaedia of Genes and Genomes (http://www.genome.jp/kegg/)

(Schrumpf et al., 1991) begins with Asp, followed by condensation with pyruvate and transamination from Glu (Fig. 3). However, the origin of the $\Delta^{15}N_{p-s}$ cannot be in these steps because of the presence of a symmetric intermediate after the formation of both N–C bonds, in which the two N positions cannot be distinguished. Cleavage of succinate from N-succinyl-LL-2,6-diaminopimelate by succinyl-diaminopimelate desuccinylase yields LL-2,6-diaminopimelate (LL-2,6-DAP), a molecule with C_2 symmetry, which renders the two N indistinguishable and eliminates any differences in $\Delta^{15}N_{p-s}$ that develop during bond formation to this point.

The remaining steps are a stereoisomerization by diaminopimelate epimerase to yield, DL-*meso*-diaminopimelate (DL-*meso*-DAP), followed by decarboxylation. The isomerization step must pass through a transition state involving the bond to the precursor to the sidechain-N and thus could induce an isotopic fractionation, but the

mechanism does not appear to involve bond cleavage. The final decarboxylation step can induce fractionation only through a secondary isotope effect and thus is a very unlikely candidate step to induce a large isotope effect.

Importantly, DL-*meso*-DAP is a substrate at a pathway branch point, serving also as a substrate for synthesis of precursors for bacterial peptidoglycan (Koo and Blanchard, 1999), and presents a possible explanation for observed intramolecular fractionation of Lys-N. Preferential incorporation of isotopically light N from the l-stereocenter of DL-*meso*-DAP into the peptide moiety of uridine nucleotides by UDP N-Acetylmuramyl-L-alaynyl-D-glutmate:*meso*-2,6-diaminopimelate ligase (UDP-MurNAc-L-Ala-D-Glu-*meso*-2,6-DAP ligase) (Mizuno and Ito, 1968) would result in an enrichment of ^{15}N at L-stereocenters of the remaining DL-*meso*-DAP pool. The final decarboxylation of the D-stereocenter of this pool would yield Lys with peptide-N enriched in ^{15}N. While no isotopic studies have been reported for UDP-MurNAc-L-Ala-D-Glu-*meso*-2,6-DAP ligase, enzymatic KIE favor ^{14}N incorporation under physiological conditions. Thus, it is plausible that the difference in $\Delta^{15}N_{p-s}$ in the Lys between aerobic and anaerobic cells corresponds to a higher rate of bacterial peptidoglycan synthesis, possibly owing to adaptations in membrane chemistry to accommodate incorporation of the membrane-bound nitrate reductase which is expressed solely under anaerobic nitrate respiration. This explanation is consistent with the observation of increased fractionation between protein and total biomass in the anaerobic state, leaving depleted $^{15}N/^{14}N$ in cell membranes relative to aerobically grown cells (Table 1).

In contrast to these in vivo findings, the previous studies of intramolecular N fractionation in Lys from commercial distributors indicate depletion in most samples for peptide-N relative to sidechain-N (average $\Delta^{15}N_{p-s} = -7.5‰$) (Sacks and Brenna, 2005). The most commonly used microorganism for the commercial production of Lys, *Corynebacterium glutamicum*, employs an additional parallel pathway (Fig. 3, left) for synthesis of *meso*-DAP dependent on ammonium availability (Schrumpf et al., 1991; Sonntag et al., 1993). In this alternative route, which can occur with, or in lieu of (Shaw-Reid et al., 1999), the major pathway shared by *P. denitrificans* and most other bacteria, Asp is preserved as source N for the L-stereocenter of Lys while the sidechain-N originates from free ammonium. The alternative pathway is thus an explanation for the difference of the present in vivo results and observations made previously (Sacks and Brenna, 2005).

The final possible source of intramolecular fractionation of Lys is through an irreversible degradation to acetyl-CoA (Fig. 3). This pathway, which also operates in humans, consists of a step-wise transfer of N through multiple intermediates from the sidechain and the peptide-N of Lys to 2 respective molecules of 2-oxoglutarate. Selectivity for ^{14}N in the formation of saccharopine, the first irreversible step in the degradation of sidechain-N, would leave residual enrichment of ^{15}N in this position. The data do not support a significant amount of fractionation in this step, since the $\delta^{15}N$ for both N atoms of Lys in the aerobic state are identical to Asx, an N precursor, and sidechain-N under anaerobic conditions are actually lighter, suggesting that deamination at this position occurs at lower levels relative to other amino acids.

Intramolecular ^{15}N measurements of amino acids allow for an unprecedented specificity in assigning and characterizing sources of N-fractionation and proportionation. While CSIA of Lys revealed no differential metabolic branching with respect to physiology, intramolecular measurements reveal significant fractionation between sidechain- and peptide-N in the anaerobic state and demonstrate the utility of combination isotopic analysis in discerning metabolic status.

Bulk, compound, and position-specific high-precision $\delta^{15}N$ measurements of amino acids were conducted on *P. denitrificans* grown under aerobic and anaerobic physiological states. Reported here is the first use of N isotope ratios as sensitive indicators of metabolic states within an organism as well as the first in vivo measurement of intramolecular nitrogen isotope ratios of an amino acid. While alternative techniques such as isotopomer metabolic flux analysis (Christensen and Nielsen, 1999) exist as powerful tools for diagnosis and elucidation of metabolic branching under controlled conditions, these methods are unable to exploit the ubiquitous natural variability in isotope ratio induced by metabolism without addition of highly-enriched substrates required to enable detection by molecular mass spectrometers.

Natural isotopic variability requires no tracer intervention and thus is a powerful approach to the study of systems without perturbation, other than for sampling. Intramolecular isotope ratios reflect aspects of organismal biology, specifically environmental influences that are not available from the genome, epigenetics, or metabolomics. Isotope ratios necessarily reflect the net fractionation through all pathways leading to product synthesis and from degradation. The specific intramolecular isotopic fractionation at metabolic branching points in an organism is a composite of all inputs and outputs, and thus is very complex. Nonetheless, the use of multiple levels of isotopic characterization reveals consistent profiles that are indicative of physiological state. Ongoing studies on the metabolic isotopic response of *P. denitrificans* to other

environmental parameters such as osmoregulation and pH stress are underway and will improve the understanding of metabolic fractionation, ultimately leading to principles that can be extended to multicellular organisms.

Acknowledgements

This work was supported by NIH grant GM49209. BP acknowledges support from NIH training grant DK07158. We thank Gavin Sacks, Art Kasson, and Stephen Zinder for helpful discussions.

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Authors' address: J. Thomas Brenna, Cornell University, Savage Hall, Division of Nutritional Sciences, Ithaca, NY 14853, U.S.A.,
Fax: +1-607-255-1033, E-mail: jtb4@cornell.edu