PHYSIOLOGICAL NITROGEN ISOTOPIC RESPONSES IN *PARACOCCUS DENITRIFICANS*

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Bruce Sean Pan

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Bruce Sean Pan, Ph.D.

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The natural abundances of stable isotopes in our environment serve as intrinsic tracers that are incorporated into biological compounds and create unique isotopic signatures that record molecular history. Use of stable isotopes in the investigation of biochemical, geological, and forensic applications has become increasingly popular in the past two decades, in large part due to the recent development of requisite instrumentation capable of measuring the subtle variations of naturally occurring isotope ratios. Progress towards a universal model describing the behavior of isotopes in physiology has seen little consolidation due to the empirical nature of isotopic response. Elucidation of underlying mechanisms requires tightly controlled experiments with careful isotopic characterization of specific physiological states.

We exploit the environmentally-relevant and nutritionally versatile bacteria, *Paracoccus denitrificans*, to assess the fractionation of nitrogen in the metabolism of amino acids as a means to deduce physiological state. The manipulation of respiratory state and osmotic stress yields distinctive distributions of relative isotopic enrichment which is indicative of changes in metabolic pathways. We report the first *in vivo* position-specific measurement of the poly-nitrogenous

amino acid, Lysine, and note a dramatic deviation in isotope ratios between sidechain and peptide nitrogen, which is correlated with respiratory state. In aerobic cells, the lysine intramolecular $\Delta\delta^{15}N$ was negligible, but in anaerobic cells it was a remarkable $\Delta\delta^{15}N = +11.0‰$, driven predominantly by enrichment at the peptide N. With regard to osmotic stress, we report a linear response with salt concentration in nitrogen isotope ratios of several amino acids, indicating an isotopic enrichment in amino acids which exhibit known osmoprotective properties. Bulk $^{15}N/^{14}N$ increased by $\Delta\delta^{15}N = +5.603 \pm 0.79‰$ from 1% to 1.75% NaCl and significant linear trends of $\Delta\delta^{15}N$ values with salt concentration between 1% and 1.75% ($p<0.05$) were found for five amino acids, Ala ($\Delta\delta^{15}N = 4.66 \pm 0.23‰$), Gly ($\Delta\delta^{15}N = 4.03 \pm 0.84‰$), Val ($\Delta\delta^{15}N = 3.59 \pm 0.32‰$), Pro ($\Delta\delta^{15}N = 6.13 \pm 0.76‰$), and Asx ($\Delta\delta^{15}N = 5.27 \pm 0.56‰$). These findings illustrate the sensitivity of isotope ratios in amino acids to physiological state and establish a premise for diagnostic techniques to assess metabolism.

BIOGRAPHICAL SKETCH

Born in Taiwan in the year 1977 to loving parents, Long-Far and Shu-Ling Pan, Bruce moved to the United States when he was three. His early days of child were filled with hot, humid, Texas afternoons and fond memories of sneaker-melting asphalt. After moving to New Jersey and attending Montville Township High School, Bruce enrolled in Cornell University, in lovely Ithaca, New York, as an undergraduate in the fall of 1995. As is with the case with many Cornellians, he failed to reach critical escape velocity when he graduated in 1999 with a degree in Cellular and Molecular Biology and re-enrolled as a graduate student in the field of Nutritional Biochemistry. He joined the Brenna lab in the summer of 2001, where he spent many happy days fixing computers and performing interesting scientific research.

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I would like to thank my advisor, Tom Brenna, for providing me with the opportunity and tutelage to conduct research and develop as a scientist. His patience and support have been generous and appreciated.

To my family and friends, I am indebted to for their limitless love and kindness. I also owe thanks to my fellow peers at the Brenna Lab for their indispensable assistance throughout my graduate career. We acknowledge and are grateful for the support of National Institute of Health Training Grant number DK07158.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AP	Atom Percent
APE	Atom Percent Excess
BSIA	Bulk-Specific Isotope Analysis
CSIA	Compound-Specific Isotope Analysis
DI-IRMS	Dual Inlet Isotope Ratio Mass Spectrometry
EI	Electron Impact
EIE	Equilibrium Isotope Effect
FID	Flame Ionization Detector
GC	Gas Chromatography
GC-C-IRMS	Gas Chromatography-Combustion-Isotope Ratio Mass Spectrometry
GC-C-R-IRMS	Gas Chromatography-Combustion-Reduction-Isotope Ratio Mass Spectrometry
IRMS	Isotope Ratio Mass Spectrometry
KIE	Kinetic Isotope Effect
LC	Liquid Chromatography
LN	Liquid Nitrogen
MS	Mass Spectrometry, Mass Spectrometer
m/z	Mass-to-Charge
PDB	PeeDee Belemnite
PSIA	Position-Specific Isotope Analysis
Spl	Sample

CHAPTER 1

Introduction

1.1 Stable Isotopes at Natural Abundance

The ubiquity of stable isotopes in our surroundings and their differing re-activities is an often overlooked source of information regarding the molecular history of organic compounds. Natural variations in the stable isotope distribution of biological compounds result from discriminations in a variety of predictable factors which govern isotopic fractionation, including source effects, formation processes, enzyme selectivity, reaction and equilibrium constants, and local environmental conditions prevalent at the time of bond breakage or formation. For the better part of a century now, the heavy stable isotopes of biologically-relevant elements such as ^{2}H, ^{13}C, ^{15}N, ^{18}O, and ^{34}S have been used as intrinsic tracers for analysis of chemical and physical processes beginning from single reactions at the atomic scale and expanding to elemental isotope analysis of extra-terrestrial debris. While the majority of studies before 1960 were enveloped by the field of isotope geochemistry, later applications of isotope ratio mass spectrometry (IRMS) extend the versatility of this method to agriculture, health, and environmental research. Even more recently, highly-precise IRMS, boasting precisions up to seven significant figures, has been utilized for in-depth analysis of physiological fractionation by biological life and to differentiate between species or to examine metabolic pathways within an organism. Continued advances in the design of IRMS and associated equipment, especially

the development of automated on-line sample preparation devices, novel off-line enzymatic preparations, and continuous-flow analysis, have facilitated the introduction and utilization of IRMS in numerous new fields.

1.2 Applications for Isotope Ratio Mass Spectrometry

For each and every position, stable isotopes record two potential types of information. Where physical and (bio)chemical reactions fractionate stable isotopes, the resulting isotopic distributions reflect molecular pathways and process information [1]. Additionally, the characteristic origins of samples are also recorded in stable isotope distributions. These two factors contribute such that the source material sets an isotopic baseline that can be subsequently shifted by biochemical or physiological isotopic fractionation.

The routine measurement and application of carbon and nitrogen isotope ratios fall predominantly into three categories; 1) Studies of fractionation altered only by natural processes such as in geochemical, archaeological, and physiological studies [2, 3]. 2) Forensic applications, including the detection of contaminants based on unlikely isotopic signatures, such as in the adulteration of food [4] and in human substance abuse [5], or the assignment of terrestrial or extra-terrestrial origin to samples, such as isotope ratios in meteorites [6]. 3) The purposeful administration and subsequent measurement of compounds containing enriched isotope ratios to label and trace chemical, environmental, or physiological fates of compounds of interest, which is commonly employed in tracer experiments [7-9].

The utilization of stable isotopes in tracer studies is replete in the literature and comprises the bulk of isotopic measurements applied to biochemistry to date. Studies of physiological fractionation, however, are far more infrequent despite the merit of isotope ratio measurements in evaluating metabolism, due in large part to a general lack of awareness of the phenomenon [1, 10-12]. Moreover, existing studies of isotopic fractionation are greatly imbalanced in favor of C as opposed to N because of the relative difficulty in measuring the latter. Consequently, N fractionation in response to changes in physiology is poorly documented and not understood. Understanding of the nature of physiological fractionation requires tightly controlled parameters and specific aims, since a large number of factors contribute to the net fractionation of isotopes.

1.3 Isotopic Effects and Physiological Fractionation

The impact of isotopic substitution on the rate or equilibrium constant of a reaction whose participants are otherwise chemically identical is known as the kinetic or thermodynamic isotope effect, respectively. Consequently, physiological fractionation necessarily surfaces as subtle variations of isotopomers of compounds in complex systems, such as micro-organisms, travel through metabolic pathways. While the net effect of physiological fractionation can be obscured by simultaneous opposing shifts towards enrichment and depletion, the relative flux through branching points can manifest a characteristic and consistent isotopic response. This frequently-observed effect *in vitro* is the basis for utilizing isotopic ratios to

differentiate between biological specimens, such as the characteristically isotopic differences between C3 and C4 plants. A plausible and meaningful explanation for these fractionations is elucidated by examination of known underlying pathways and unexpected fractionations have been shown to predict the existence of unknown pathways [12].

1.3.1 Thermodynamic Isotope Effects

The effect of isotopic substitution on the equilibrium constant of two otherwise identical reactions is known as the thermodynamic (or equilibrium) isotope effect. These effects are evident in reversible reactions where chemical exchanges occur between molecules as they approach equilibrium. In the following simplified reaction:

$$A + B \rightleftharpoons C \tag{1}$$

The thermodynamic isotope effect is the ratio K^{light}/K^{heavy} of the equilibrium constant for the reaction in which A contains the heavy isotope when compared to that in which A contains the light isotope.

$$A^{light} + C^{heavy} \rightleftharpoons A^{heavy} + C^{light} \tag{2}$$

One example of this effect is the exchange of carbon between CO_2 in air and dissolved bicarbonate in the ocean [13], where bicarbonate equilibrated with CO_2 in air is significantly enriched in ^{13}C relative to CO_2. The potential energy surfaces of isotopic molecules are identical

to a large degree of approximation, therefore thermodynamic isotope effects must arise predominantly from a mass-dependent effect on the nuclear motions of reactants and products. The general rule for equilibrium isotopic fractionations is that heavy isotopes concentrate in the molecules where their bond strengths are greatest. While these effects are common in systems with lengthy residence times and free-exchange of atoms, they are not often the cause of significant fractionations in biological reactions, where enzymatically-catalyzed reactions are largely unidirectional and complex feed-back mechanisms affect isotopic discriminations predominantly through a kinetic isotope effect.

1.3.2 Kinetic Isotope Effects

Perturbations in rate constants due to preferential isotopic discriminations are referred to as a kinetic isotope effects. In the following simplified reaction:

$$A + B \rightarrow C \qquad (3)$$

The effect of isotopic substitution on rate is expressed as the ratio of k^{light}/k^{heavy}, where the numerator and the denominator refer to independent rates in which the molecules of reactant A contain heavy and light isotopes, respectively.

The cause of these changes in rate constants can be partially attributed to the impact of isotopically distinct vibrational states (Figure 1.1), where:

$$A + B \rightleftharpoons [TS]^{\ddagger} \rightarrow C \qquad (4)$$

kinetic isotope effects can also be expressed as the respective change in the equilibrium constants between the reactants and the transition state in otherwise identical molecules.

Measurements of biological kinetic isotope effects for many enzymes have been made [14-16] and are likely to be a significant source of metabolic fractionation. The enzymes and barriers that an organic molecule encounters as it proceeds through assimilation, anabolism, and finally catabolism, necessarily imprint an isotopic fingerprint corresponding to pathways traveled by these compounds. A comparison across species of organisms, such as C3 or C4 plants (Figure 1.2) which utilize differing routes of carbon assimilation, demonstrate that isotope ratios can serve to differentiate between species. Consequently, changes in response to physiological state within an organism in these pathways would also yield characteristic isotope ratios, although direct evidence for this phenomenon have only recently been reported.

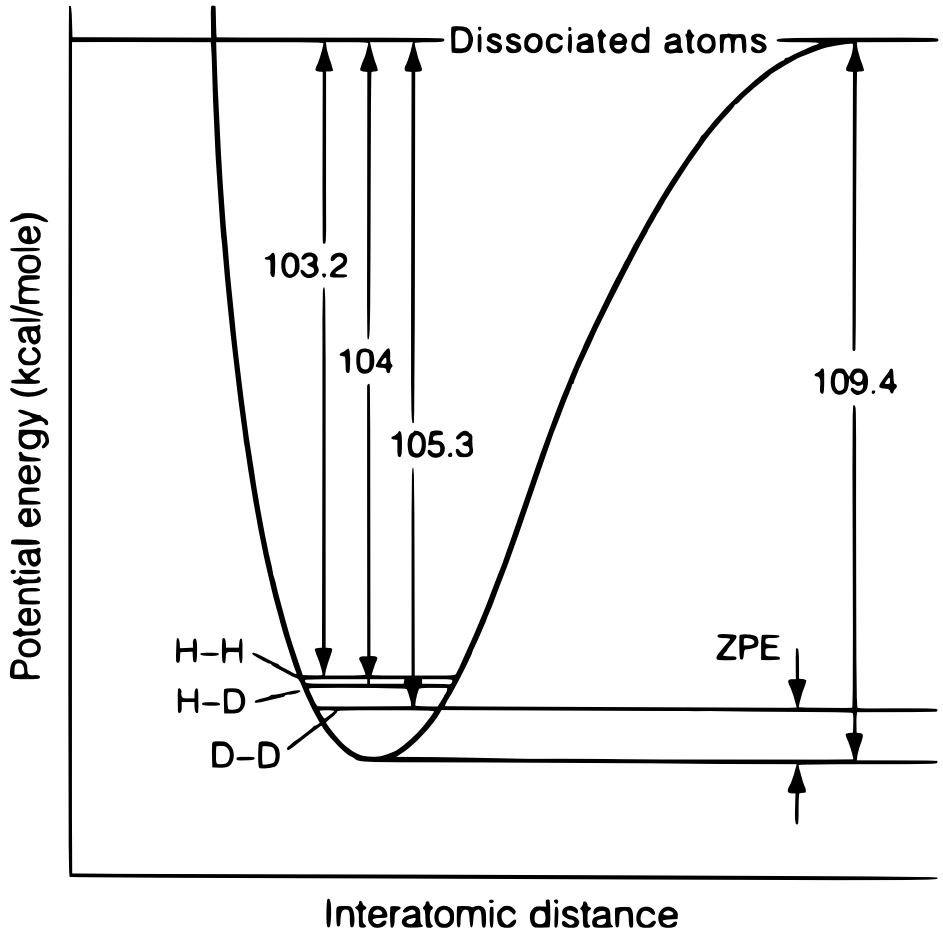

Figure 1.1 – A generic Morse curve representing vibrational states for Hydrogen and Deuterium isotopes. Differences in Zero Point Energy (ZPE) between isotopically distinct bonds result in unique activation energies for each transition state. Consequently, bond cleavage between heavy atoms typically progresses at a slower rate than their lighter isotopic counterparts†.

† Adapted from O'Neil, J.R., 1986. Theoretical and experimental aspects of isotopic fractionation. In: J.W. Valley, H.P. Taylor and J.R. O'Neil (Eds), Stable Isotopes in High Temperature Geological Processes. Reviews in Mineralogy, Volume 16

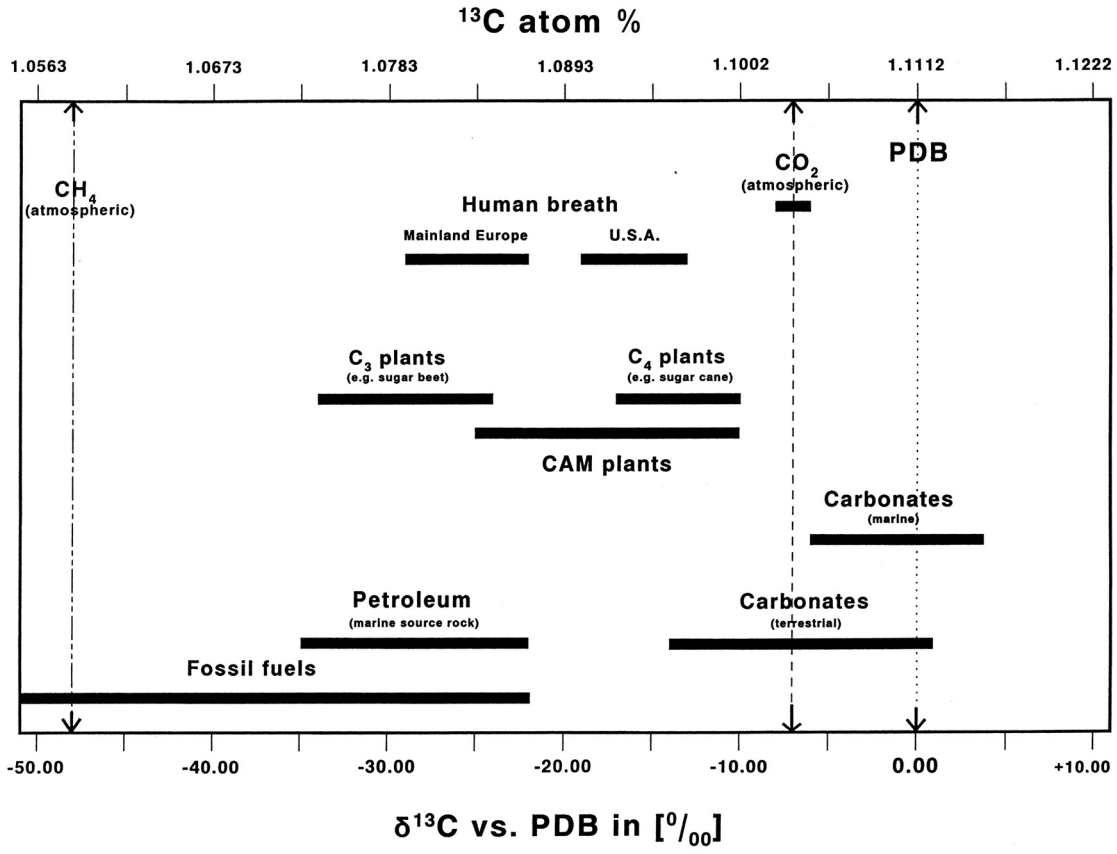

Figure 1.2 - Some typical examples of natural $\delta^{13}C$ values grouped according to origin along the scale of ^{13}C abundance†.

† Taken from Meier-Augenstein, W., *Applied gas chromatography coupled to isotope ratio mass spectrometry.* Journal of Chromatography A, 1999. **842**(1-2): p. 351-371.

1.4 Notations & Units of Measurement

1. Isotope measurements in IRMS are typically reported in delta notation with respect to established standards. The conventional delta notation suggested by Urey in 1948 [17], based on the idea of Nier [18] in 1946, reports in permil (per thousand) units and is abbreviated as ‰. The general form of the equation used to express isotope ratios for nitrogen at natural abundance is:

$$\delta^{15}N_{air} = \frac{R_{sample} - R_{air}}{R_{air}} \times 1000 \qquad R_x = [{}^{15}N_x / {}^{14}N_x] \quad (5)$$

The availability and constant abundance of N ($^{15}N/^{14}N$ = 0.3664%) [19] in air makes it an ideal standard for nitrogen isotopic analysis. For carbon, a natural source of ^{13}C-enriched carbonate (Pee Dee Belemnite (PDB); $^{13}C/^{12}C$ = 0.0112372) [20] named for its origins from a river in South Carolina serves as the international standard. The delta notation is a practical format and emphasizes relative precision by eliminating the unchanging, leading, digits common to comparisons of $^{15}N/^{14}N$ and $^{13}C/^{12}C$ ratios at natural abundance. While this notation is suitable for representing values ascertained at natural, un-enriched, levels, it is inappropriate when working with degrees of adulteration usually employed in tracer studies.

2. Measurements of enriched distributions of isotopes such as those used in tracer experiments are expressed as fractions, a formulation more suitable for these ranges of isotopic enrichment. In

these measurements, F is presented as the quantity of ^{15}N divided by the total nitrogen in the sample and is presented in equation 6.

$$F = \frac{^{15}N}{^{14}N + ^{15}N} \tag{6}$$

Other related expressions of isotopic enrichment include atom percent (AP), shown as

$$AP = F * 100 \tag{7}$$

as well as atom percent excess (APE) given by equation 8

$$APE = (F_s - F_b) * 100 \tag{8}$$

where F_s and F_b are the fractions corresponding to sample and baseline, respectively.

1.5 Isotopic Standards

Comparisons of isotopic data between laboratories are achievable due to the adoption of international standards against which all measurements are internally calibrated. This allows for compensations for mass discriminating effects which may fluctuate with time and from instrument to instrument. For the five principle light elements of biological relevance, there are originally four accepted

isotopic standards. Hydrogen and oxygen are calibrated against Standard Mean Ocean Water (SMOW). PDB serves as the standard for carbon as well as an alternative standard for oxygen. Nitrogen is calibrated against atmospheric air and Canyon Diablo meteorite (CD) is employed as the standard for sulfur.

Although the original supplies of both SMOW and PDB have been exhausted, other standards which are either compared to the original or calibrated to secondary standards have risen to replace them. These standards are distributed by the National Institute of Standards and Technology (NIST) and the International Atomic Energy Agency (IAEA) and utilized by individual investigators for calibrating working "in-house" standards. Vienna SMOW (V-SMOW), which is comprised of various waters to match the isotopic composition of the original SMOW, serves as the standard for O and H analyses, and Vienna PDB (VPDB) a conceptual reference scale derived from the original PDB and a reference sample of limestone, has been adopted for C and O measurements.

1.6 Isotope Ratio Mass Spectrometry

The ability to measure isotope ratios at high precision, generally regarded as standard deviations in the range of 4-6 significant figures, allows for meaningful analysis of natural isotopic abundances. Isotope Ratio Mass Spectrometry (IRMS), as opposed to molecular and elemental mass spectrometry (MS) which scan across several masses to identify characteristic fragment ions to ascertain structure, utilizes the simultaneous measurement of only select few streams of ions in

order to enhance precision. Briefly, gases admitted to the instrument, which are isotopically representative of the original sample, are ionized in the source and subsequently condensed into an ion stream. A magnetic sector separates the stream based on m/z ratios and the streams are collected in Faraday cups (Figure 1.3)

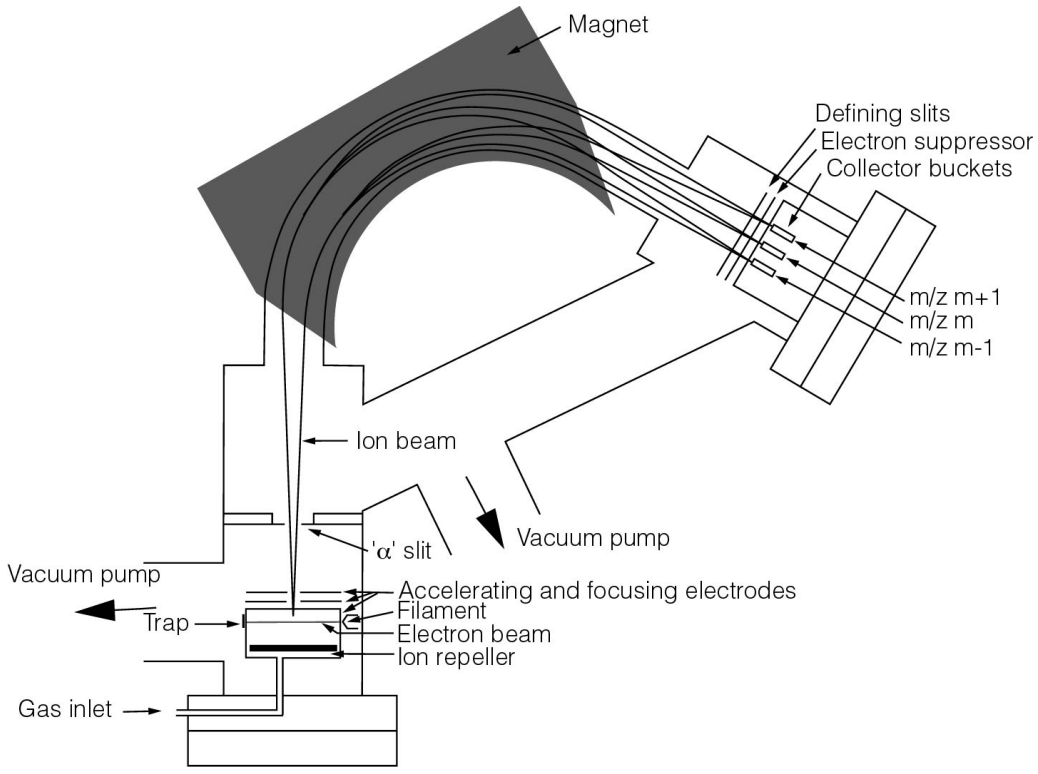

Figure 1.3 - A detailed schematic of an Isotope Ratio Mass Spectrometer (IRMS) featuring ion source, magnetic sector, and collector array.†

† Taken from Platzner, I.T., 1997. *Modern Isotope Ratio Mass Spectrometry*. 1997, New York: J. Wiley.

1.6.1 Ion Source

The tight electron impact (EI) ion source of an IRMS is optimized for sample residency time which in turn enhances ionization probability and facilitates the transmission of signal from simple gases entering the source into ions which are ultimately directed to the end-array of Faraday Cups (FC) employed in collecting IRMS signals (Figure 1.3, right). Ionization of gases proceeds via electron displacement by an electron beam emitted from a hot wire filament (W or ThO_2) which travels a tight helical path that is directed across the source chamber and perpendicular to the gas stream. Ions are focused by a series of electromagnetic lenses and delivered to the analyzer via a combination of an ion repeller plate (Figure 1.3, bottom) and a large potential difference held between the trap and an aperture leading to the analyzer. The ionization efficiency in a tight EI source is roughly three orders of magnitude higher in an IRMS source than in a conventional organic mass spectrometer source, able to generate one molecule in 10^3 as opposed to one molecule in 10^6, respectively. These high ionization efficiencies are required for optimal measurement of isotope ratios at natural abundance.

1.6.2 Isotope Ratio Analyzer

The analyzer of an IRMS is a robust single magnetic sector, ideally suited for high-precision analysis due to its ability to produce flat-topped peaks and maintain high transmission and stability (Figure 1.3, top). While the analyzer is limited in regards to mass resolution, due, in part to ion space-charge saturation effects and the physical

constrains of the detector, it is unequalled in its reliability and consistency in separating molecules in ion streams based on their respective mass/charge ratios, following the equation:

$$\frac{m}{z} = \frac{(Br)^2}{2V} \tag{9}$$

where r is the radius of the circle, m the mass of the ion, z the charge of the ion, and V the voltage at which it was accelerated into the magnetic field of strength B. The magnetic sector is separated from the ion source by a slit of fixed width through which the collimated ion beam must pass, resulting in an appropriate working resolution of about $m/\Delta m$ = 100 for masses of interest [21]. A high-vacuum environment is maintained inside the analyzer to reduce collisions between the ion stream and ambient particles.

1.6.3 Signal Detection and Data Acquisition

In IRMS, the role of ion detection is fulfilled by Faraday cups (FC) (Figure 1.3, right) whereas Electron Multipliers (EM) are typically used in common organic mass spectrometers. Some exceptions such as Ion Cyclotron Resonance (ICR) [22] or advanced RF Ion Traps [23], utilize non-destructive ion detection, allowing multiple measurements of the same ion-population, while the ions in FC and EM are destructively measured; ions directly collide and are neutralized by the detectors. The versatility of non-destructive methods of ion detection, however, comes at the cost of sensitivity, accuracy, and precision, all of which are high priorities in IRMS. In regards to destructive techniques of ion

detection, while FCs are less sensitive than EMs, they are ideally suited for IRMS applications and superior for several reasons; 1) the extremely high count rates required for high-precision measurements are in the normal operating range of FCs but would rapidly degrade EMs which rely on delicate, non-reusable, metal plates or coatings to generate secondary electron cascades. 2) FCs can linearly detect large currents. 3) FCs are durable, can typically last the entire lifetime of an instrument, and require slightly simpler electronics than EMs.

Modern-day implementations of IRMS instruments utilize individual FCs for each mass. The simultaneous measurement of multiple ion beams eliminates variability in analytical conditions that cause correlated changes in signal intensity, such as filament brightness. The design of FCs is relatively simple; the cups are long and narrow to prevent escape of secondary electrons generated on ion impact, aided also by electron suppressor plates near the entrance of each collector. Dedicated amplifiers positioned close to the cups (to minimize signal path) adjust the currents to account for the relative abundance of lighter isotopes. The amplified beams are then digitized by high-linearity voltage to frequency converters (VFCs). Continuously integrated ion currents are sent to the data acquisition computer at predefined intervals as counts.

1.7 Dual-Inlet Isotope Ratio Mass Spectrometry

Several front-end apparatuses have been developed for IRMS techniques. The dual-inlet IRMS (DI-IRMS) system (Figure 1.4) utilizes

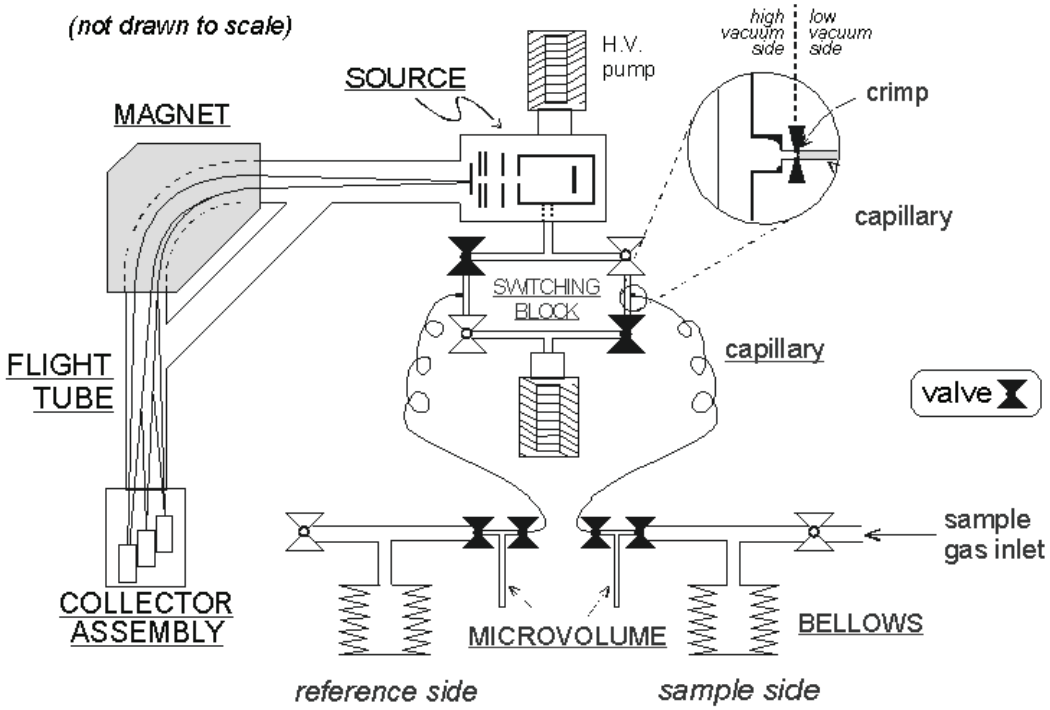

Figure 1.4 - A representation of the dual-inlet IRMS. Isotopically representative gases are directed to the ion source via a series of switches and pressure sensors. †

† Stable Isotopes in Ecophysiology. <http://www.uwyo.edu/terra>

two ballasts containing sample and analyte, respectively, which are matched in parameters such as temperature, path-length, and pressure. By treating both sample and reference gas in an identical manner, accounting for all factors affecting isotopic fractionation, DI-IRMS is able to achieve the highest precision and accuracy available to current IRMS techniques, with precisions as high as SD (^{13}C) < 0.02‰. Despite the accuracy of measurement, the requirement for a tedious isotopically-accountable conversion and purification of samples to gases admissible to IRMS (CO_2, N_2 ...) makes routine DI-IRMS impractical. For reasons mainly concerning the complexity of use, earlier inlet designs such as the DI-IRMS are rare and have been largely replaced by alternative techniques.

1.8 Continuous-Flow Isotope Ratio Mass Spectrometry

The development of a system for high-throughput routine measurement of isotope ratios was pioneered in 1976 by Sano [24] who interfaced a scanning organic MS with a gas chromatography (GC) device and a combustion furnace for detection of ^{13}C-labeled metabolites. The concept of continuous flow revolves around the use of an inert carrier gas that continually sweeps separated organic compounds in effluents from the GC to the ion source. Sano utilized an MS devoted to scanning masses 44/45 and was therefore able to calculate carbon isotope ratios. A similar design was introduced in 1978 by Mathews and Hayes [25] who employed a single collector MS with improvements to upstream sample treatment, mainly, the passing of combustion products through a Nafion® tube to remove H_2O from

the effluent stream and also the fine-tuning of the MS, both of which ultimately enhanced the precision of isotope measurements. The advancements in this system allowed it to differentiate between some samples at natural variability, but consistent performance was not realized until the development of a GC-combustion-IRMS equipped with dual simultaneous collectors [26]. This compromise between the precision and accuracy of DI-IRMS methods and the practical applications of CF-IRMS was able to achieve precisions better than 1‰ on nanomoles of sample while incorporating automation. Other instrument designs have since been reported, but this particular manifestation most resembles the most popular modern, commercial CF-IRMS instruments.

1.8.1 Elemental Analysis

An overview of IRMS techniques would not be complete without addressing the most commonly employed application for isotope ratio analysis, Elemental Analyzer-IRMS (EA-IRMS). Elemental isotope ratio values are especially useful for soil forensic sciences and are routinely used to examine food webs [27]. Bulk isotope analysis of sample and source materials allows for measurement of net physiological fraction and represents the most basic level of isotopic data. The demand for automated rapid analysis has resulted in the development of specialized commercial instruments designed for on-line high-precision isotope ratios for bulk analysis of solid and nonvolatile liquid samples. EA-IRMS is a CF-IRMS quantitative technique that allows for routine and simultaneous measurement of $^{13}C/^{12}C$ and $^{15}N/^{14}N$

(and sulfur) in samples as well as their relative compositions. Briefly, samples weighed out in disposable tin or silver cups are dropped into an oxidatively-charged reactor and quantitatively combusted into CO_2 and N_2 products. Evolved gases are separating on a packed GC column and detected non-destructively by thermal conductivity before introduction to the IRMS. Optimal flows for packed columns are typically in the range of 20-60 mL/min, which is significantly higher than those for most IRMS ion sources (0.1-0.3 mL/min), which dilutes the number of particles in the effluent that actually enter the ion source. Consequently, EA-IRMS usually requires milligram quantities of sample sizes in contrast to the nanogram quantities sufficient for most other IRMS techniques.

1.9 Levels of Isotopic Interest

There are three levels of isotopic data available to scientists: bulk, compound-specific (CSIA) and position-specific (PSIA). Isotopic information at all three levels contributes to our understanding of chemical and physiological fractionation. Bulk isotope ratios are necessarily the average isotope ratios of constituent compounds, which are in turn the average isotope ratios of the elements they are comprised of. This inherent relationship between levels of specificity can be exploited to derive isotopic data and metabolically-related information. CSIA analysis is becoming routine, whereas PSIA methods are still in their infancy, with only a handful of reports to date.

1.9.1 Compound-specific Isotope Analysis

The routine carbon CSIA analysis of samples proceeds via Gas Chromatography-Combustion-IRMS (GCC-IRMS). Resembling the systems first developed by Barrie [26] and Hayes [25], CSIA systems for analysis of carbon have been commercially available since 1990 [21]. Shortly after, the extension of CSIA to nitrogen methods was demonstrated by Brand [28-30], and included the addition of an in-line reduction furnace (Figure 1.5). Due to certain logistical limitations, including the relatively low abundance of nitrogen in biological compounds (~5% N as opposed to ~60% C), decreased ionization efficiencies (70% that of carbon), and twice the stoichiometric requirement (2 N per N_2 as opposed to 1 C per CO_2), N-CSIA analysis is less commonly reported than carbon and requires much larger sample sizes.

Systems for C and N analysis share the majority of components in common. The use of a GC for separation of compounds is an essential component of on-line CSIA. Consequently, high performance of the GC, valid choice of GC column, and appropriately matched flows, are basic requirements for proper separation and isotope ratio analysis of compounds of interest; sample peaks eluted from the column must be distinct and have base-line separation for consistent and accurate assessment of isotope ratios.

Samples are typically injected into a heated split/splitless liner, rapidly volatilized into component compounds, and separated based on a customized temperature ramp and affinity for the specialized phase of the GC column. The combustion furnace, which is

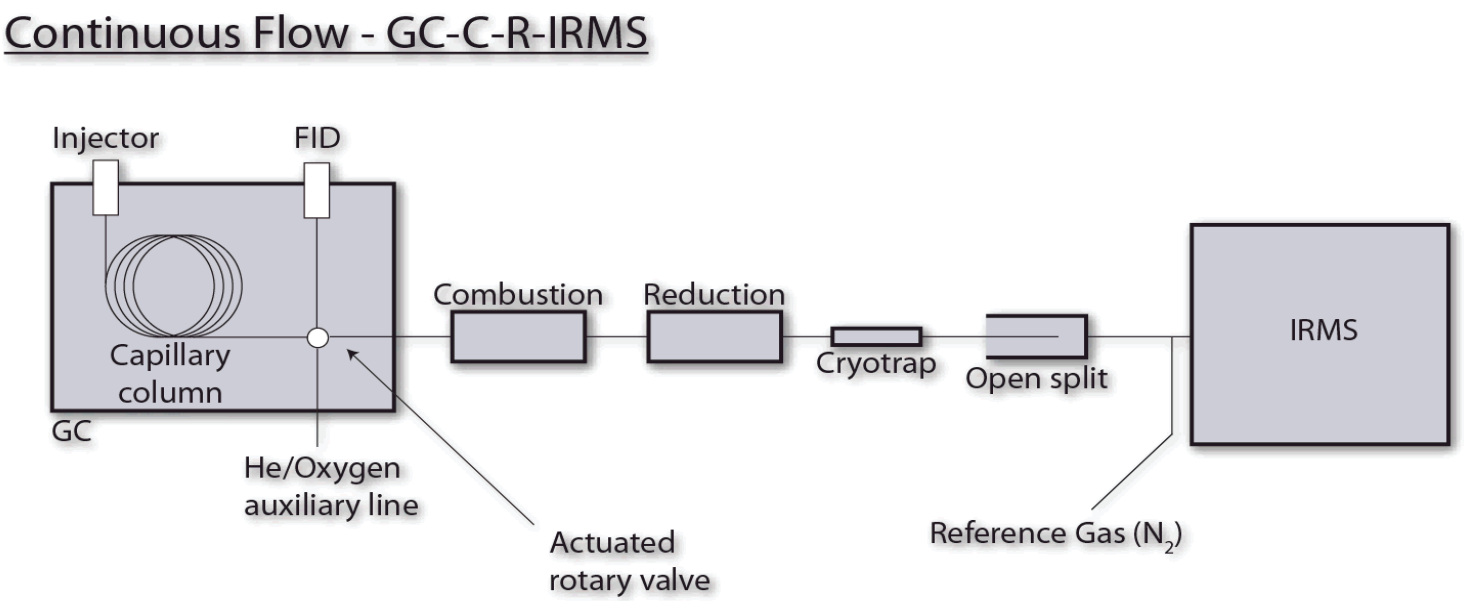

Figure 1.5 - Schematic of a Gas Chromatography-Combustion-Reduction-IRMS (GC-C-R-IRMS) for Nitrogen CSIA analysis.

responsible for oxidizing compounds to CO_2 and N_2, respectively, for C and N analysis, is packed with Cu and Pt wires, charged with O_2, and held resistively at 850°C. At this temperature, CuO exists in equilibrium with Cu and O_2, while Pt aids as a combustion catalyst.

$$2CuO \xrightarrow{850°C} 2Cu(s) + O_2 \quad (10)$$

Organic compounds separated by the column are swept into the furnace by the He carrier gas and quantitatively combusted to gases amenable to IRMS. Incomplete combustion is detrimental to precision and accuracy due to interferences with ions of the same mass. For N analysis, the incomplete combustion of carbon-containing compounds leads to generation of CO which contaminates the *m/z* 28 and 29 signal, since the relative abundance of the rare ^{13}C isotope (1.08%) is much higher than that of ^{15}N (0.732%). To minimize this phenomenon, large solvent peaks, which greatly shorten the life-cycle of a furnace, must be diverted away from the combustion furnace and the furnace must be periodically recharged with O_2. An actuated rotary valve facilitates these procedures.

The presence of H_2O resulting from combustion is also problematic for C analysis, since it protonates CO_2 to produce HCO_2^+, which interferes with analysis at *m/z* 45. For C and N analysis, water is typically removed with selectively-permeable Nafion® tubing or a cryotrap of liquid nitrogen. An additional furnace for reduction, constructed similarly to the combustion furnace, enhances the

accuracy and precision of N analysis by completely reducing the N-oxides formed by combustion to N_2.

$$2Cu(s) + O_2 \xrightarrow{600°C} 2CuO(s) \quad (11)$$

At 600°C, the equilibrium of Cu with O is shifted such that O in compounds is adsorbed into the metal. The liquid nitrogen cryotrap in N analysis performs the additional function of removing residual CO from the effluent. For C and N analysis respectively, calibrated CO_2 and N_2 (Figure 1.5; right), are released into the source at pre-determined intervals where peaks do not appear as an internal standard.

1.9.2 Position-specific Isotope Analysis

The intramolecular distribution of isotopes can reflect aspects of metabolism that are unattainable by other techniques. Specific fractionation factors introduced by enzymes can imprint an isotopic signature indicative of branching or alternative pathways. The topic of position-specific analysis (PSIA) and metabolism will be discussed in further detail in chapter 2, but a brief overview of existing techniques will be presented here. Due to tedious off-line reactions, the realm of PSIA has seen little progress in the past decades.

The beginnings of the field are perhaps best grounded in the discovery of a non-statistical distribution of carbon isotope ratios in the carboxyl groups of amino acids first observed by Abelson and Hoering in 1961 [31]. These measurements were obtained by

selectively analyzing the carboxyl carbons as a byproduct of the popular colorimetric ninhydrin reaction used to determine amino acid and protein concentration. Deniro and Epstein [32] later demonstrated a physiologically-induced isotopic fractionation pattern in fatty-acids that was attributed to a kinetic isotope effect for the enzyme, pyruvate dehydrogenase, in the Embden-Meyerhof pathway. In 1991, Rossman and Schmidt [10] were able to identify the relative carbon isotope content in each position of glucose from a C_4 plant and a C_3 plant and revealed a characteristic and reproducible ^{13}C distribution in glucose from either origin. With respect to N, Friedman and Bigeleisen pioneered early work [33] in N-PSIA by analyzing the intramolecular distribution of ^{15}N in N_2O at m/z 45 and 44, which has been recently extended by others [34, 35]. Since then, only two reports of position-specific N analysis, an abstract [36] and a recent publication by our laboratory [37], have been made.

Measuring intramolecular distributions of any isotope is often tedious, requiring time-consuming off-line procedures. One of the first successful adaptations of a completely on-line, automated procedure was developed by Corso and Brenna [38] involving the partial pyrolysis of analyte molecules and their subsequent separation on a second GC before introduction to the IRMS. Effluent exiting the second GC could also be diverted to an organic MS for identification of fragment compounds. By reassembling pyrolytic fragments, the authors were able to ascertain position-specific data mapped to the origin of each fragment. Further studies have broadened the application of this system to position-specific carbon analysis of amino acids [37, 39].

Recently, a novel enzymatic technique for the direct analysis of specific N in compounds was reported [37] which represents the first *in vitro*, position-specific, measurement of N in biologically-relevant compounds. Although this technique is an off-line adaptation to the N-CSIA instrumentation, it addresses the obvious need for N-specific techniques which offer complementary isotopic information to the routine measurement of C-CSIA.

1.10 Summary

The field of isotope ratio mass spectrometry has proven to be suitable for a broad range of applications. The ability to measure isotope ratios at natural abundance allows for exploitation of intrinsic tracers, but careful attention must be paid to the nature and implications of observed isotope ratios. The basis for this thesis is to demonstrate the existence of an *in vivo* isotope response in a model system and also show that isotope ratios can be indicative of a change in physiological state. Isotope ratios in various positions in molecules record the identity of their source as well as minute fractionations incurred along biosynthesis. The second chapter will outline the current known theory of non-statistical distributions of isotopes in biology, and provide specific insight as to the source of N-fractionation in amino acids. Chapters three and four represent two studies of *in vivo* physiological N-fractionation as a result of changing environment, specifically, responses to respiratory state and osmoregulation. These findings represent the first intramolecular reports of purely biologically-induced N isotope fractionation. The exploration of non-

source related changes in isotope ratios is also novel and plays a crucial role in exposing the underlying mechanisms of physiological fractionation. The last chapter of this thesis will outline the development of future applications for N isotopic information, including the validity of utilizing isotope ratios in humans to detect metabolic disorders and other potential uses.

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CHAPTER 2

Fundamentals of Physiological Isotopic Fractionation

2.1 Introduction

The phenomenon of isotopic fractionation creates distributions of isotopes in reactants and products from any chemical or physical reaction. Known mostly from empirical evidence, the exact causes of these distributions are not known, but the often characteristic enrichment or depletion in compounds is routinely exploited in forensic sciences [1-3] and a number of other fields. Utilization of isotopic signatures are sometimes limited due to a lack of isotopic benchmarks with which to compare new data, but it is widely accepted that physiological isotopic fraction cannot be arbitrary and must be the expression of some logical order [4]. Records of isotope ratios in biological molecules are necessarily governed by the defined contribution of both extrinsic and intrinsic influences and subject to physical laws. While several practical interpretations of existing experimental data can account for observed fractionations on a case-by-case basis, a general theory combining all aspects of fractionation for the prediction of isotopic patterns has not been developed. The scope of this chapter will encompass some fundamentals in physiological isotope fractionation with an emphasis on potential and known sources of isotope effects. The majority of given examples will involve carbon and nitrogen metabolism, although, for completeness, fractionation of other biologically relevant elements will be considered as well.

2.2 Thermodynamic and Kinetic Modeling

One of the earliest theories explaining the distribution of isotope ratios in living organisms was developed by Galimov [5], who, for many biological compounds, observed a correlation between $\delta^{13}C$ and reduced partition function ratios (RPFRs). RPFRs, also known as a β factors, are thermodynamic equilibrium constants which represent the relative preference of bound isotopes in molecules. Galimov employed a semi-empirical increment method [6] to perform practical calculations of carbon isotope distributions in binding partners. As mentioned in Chapter One, the general rule for a thermodynamic isotopic effect, also known as an equilibrium isotope effect (EIE), is that isotopes become most abundant in positions where their bond strengths are greatest.

$$^{13}CO_2(g) + H^{12}CO_3^-(aq) \rightleftharpoons {}^{12}CO_2(g) + H^{13}CO_3^-(aq) \qquad (1)$$

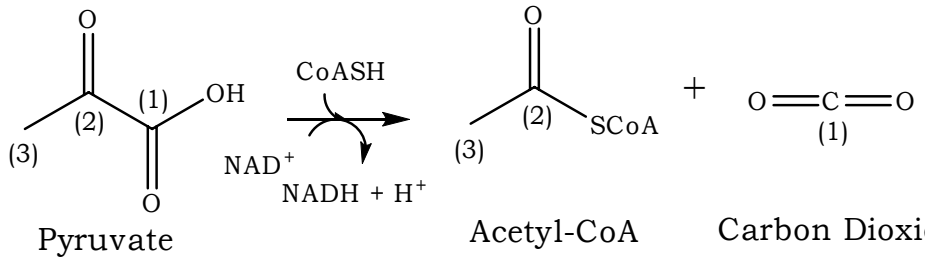
$$K = 1.0092\ (0°C) \quad / \quad 1.0068\ (30°C)$$

Equation 1 illustrates the preferential partitioning of isotopes to one molecular form versus another in a freely-equilibrated reaction, in this example, the preferential partitioning of heavy ^{13}C to bicarbonate in lieu of CO_2 between ocean water and air [7], as a manifestation of the EIE. The characteristic relationship between isotopic enrichment in various molecules and the calculated thermodynamic RPFRs led Galimov to conclude that the isotopic distribution of carbon within and between biological molecules obeyed a “thermodynamic order” and

that the reason for observed non-statistical isotopic distribution in molecules was predominantly a realization of an EIE, with a relatively small component of kinetic isotope effect (KIE). In order for this to be true, one must assume that, for any reaction utilizing enzymes, some "microscopic reversibility of the intra-complex substrate-product transition" must exist, since the expression of a thermodynamic effect relies heavily on reversibility and the potential to achieve or mimic equilibrium conditions.

Although a thermodynamic model for isotopic distributions adequately explains much of the intermolecular data collected by Galimov, large deviations from the correlation curve between β factors (EIEs) and observed $\delta^{13}C$ isotope ratios have also been reported [8]. Additionally, a publication refuting the validity of the assumption of reversibility of the substrate-product transition state has recently surfaced [9]. Using elementary chemical transformations in enzyme-substrate complexes, Buchachenko demonstrated that the thermodynamic isotope effect is only simulated and proposed that its propagation is actually better explained by kinetic isotope effects.

An example of a reaction which demonstrates a kinetic isotope effect is presented in equation 2 [10, 11]. For KIEs, the fractionation factor is equal to the ratio of the isotope-specific rate constants. The decarboxylation of pyruvate by the Pyruvate Dehydrogenase proceeds more quickly when the lighter ^{12}C is in the C-2 position than when ^{13}C is present.



(2)

$$\left(\frac{^{12}k}{^{13}k}\right)_{C\text{-}2} = 1.0213$$

Consequently, as the reaction proceeds, acetyl-CoA that is generated from pyruvate is isotopically depleted in ^{13}C in the C-2 position while its parent compound, pyruvate, shows increasing $^{13}C/^{12}C$ isotope ratios in the C-2 position. The measurement of these KIEs helped to explain the well-known relative ^{13}C depletion in lipids when compared to other classes of biological compounds [12]. In light of these findings, the contribution of EIEs to the physiological fractionation of isotopes is uncertain, but, regardless of its cause, there is an obvious expression of a thermodynamic order, simulated or real, at some level.

The incorporation of a kinetic model for physiological isotope fractionation has become increasingly popular as *in vitro* measurements of KIEs for metabolically-important enzymes are reported. Recent studies have shown that thermodynamic order is not often reached, especially not for intramolecular isotope distributions [13], where measured KIEs provide a more suitable model for observed isotope ratios. Other investigations highlighting the significance of KIEs [11, 14, 15] in directing isotopomer distributions support this theory and stress the importance of carbon budgets and their effect

within metabolic networks. The flux of carbon in biosynthetic networks is necessarily tied to its availability and the relative demand in branching pathways.

2.2.1 Special Considerations for Nitrogen Fractionation

Strategies for the evaluation of nitrogen isotopic fractionations differ from those for carbon, mainly because the majority of biological compounds contain only one nitrogen, limiting the number of candidates for intramolecular measurement. On the other hand, the relative simplicity of nitrogen metabolism may make evaluation more straightforward. Additionally, modeling of nitrogen isotopic fractionation may differ from carbon due to the mechanism in which nitrogen is utilized. The predominant fate of nitrogen in bacteria is amino acid-bound nitrogen, either free or in protein, making amino acids especially good candidates for nitrogen isotopic analysis. Whereas synthesis of carbon chains of amino acids typically proceeds via sequential chains of reactions, addition of nitrogen occurs almost exclusively by reversible step-wise transamination. Specifically, Glu, whose peptide amino group is the nitrogen donor for almost all other amino acids, plays a critical role as an nitrogen reservoir, collecting and distributing nitrogen to amino acids according to demand. These transaminations are catalyzed by a family of transaminases, some with low or multiple specificities [16]. The frequency of transaminations may mimic the free-exchange of atoms between molecules at equilibrium and introduces the potential for EIEs to play a large role in

nitrogen isotopic fractionation, although this has not yet been observed.

2.2.2 Reversibility and Openness in Biological Systems

Although the terms, *reversible*, *irreversible*, *open*, and *closed*, have strict thermodynamic definitions, their applicability to biological systems can be ambiguous. For instance, the direction catalyzed by enzymatic reactions is usually dictated by complex feedback mechanisms. Also, while biological systems such as cells are by strict definition, *open*, the selective permeability of biological membranes may require special consideration. Some open systems are at steady-state, with inputs and outputs balanced such that the contents of a cell remain constant. Other systems not at steady-state may be accumulating pools of reactants or products in preparation for phase-specific metabolism. To account for the complexity of biological reactions, we will adopt the term, *unidirectional*, to describe a process that, under normal circumstances, occurs only in one direction. Also, we will accept that biological systems are generally open, but that certain local environments such as organelles may be more appropriately analyzed as closed.

2.2.3 Calculations at Steady-State

The predicted behavior of isotope ratios necessarily changes as a function of certain parameters, one of which can be the assumption of steady-state conditions. Under steady-state conditions (Figure 2.1), the inventory of the biological cell is strictly maintained and the flux of

reactant entering the cell is equal to the formation of P and Q. Steady-state conditions allow for mass-balance calculations to be made with regard to fractionation, since the flow and isotopic composition of carbon or nitrogen flowing into the system is constant. Additionally, all compounds entering the cell have the same residence time once steady-state is established.

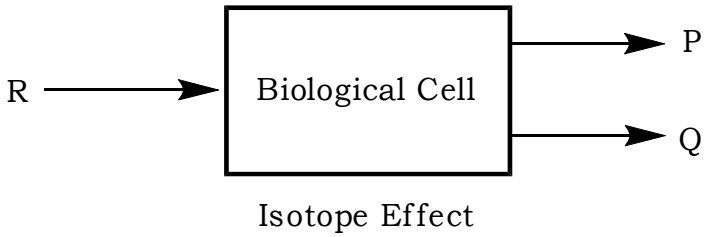


Figure 2.1 – A schematic of biological cell, an open system with input R and products P and Q. At steady-state, the contents of the cell are constant, and the rate of P and Q exiting the cell equals the rate of R entering the cell.

Assumptions of steady-state are not always valid; the initial establishment of the biological cell is frequently not under steady-state conditions, with pools of intermediates accumulating to proper intracellular concentrations. Additionally, organisms may proceed through several episodes of steady-state with respect to physiology. The demonstration of changes in fractionation patterns based on phases of growth (lag, exponential, stationary, and death) was first documented by Summons [17] and supports the concept of unique

modes of fractionation based on physiological state. This particular finding stresses the need to evaluate all organisms under study in the same phase of growth in order to discern changes due to non-growth related factors.

Isotope propagation under steady-state conditions can be expressed with the following equation:

$$\delta_{P'} = \delta_{R'} - \varepsilon_{P/R} \quad (3)$$

where $\delta_{P'}$ and $\delta_{R'}$ represent the instantaneous isotopic composition (isotope ratio) of the products and reactions, respectively, and ε represents the observed or calculated isotope effect. Equation 3 includes an approximation which is accurate to a large degree for calculations of carbon and nitrogen which typically have small physiological fractionation factors, but is inappropriate for analysis of hydrogen, which can exhibit very large isotope effects ($\varepsilon_{P/R}$ > 200 ‰).

2.3 Isotopic Complexity in Metabolic Networks

A useful exercise to illustrate the propagation of isotopic fractionation in metabolism is to evaluate an idealized network of reactions under steady-state conditions and assess potential factors affecting the distribution of isotopomers. Because not all reactions are catalyzed by enzymes, the contribution of EIEs or KIEs in metabolic pathways may be significantly different. An overview of rudimentary networks and potential biological pathways that behave accordingly

may help establish the conceptual building blocks from which complex biological fractionations arise.

2.3.1 Irreversible Unbranched Metabolism

Equation 4 represents the beginning of a sequence of theoretical reactions with known fractionation factors. Each letter signifies nitrogen in a specific position in reactants and products.

$$\overset{\delta_A}{\mathbf{A}} \xrightarrow[\mathbf{1}]{\delta_1,\ \varphi_1} \overset{\delta_B}{\mathbf{B}} \xrightarrow[\mathbf{2}]{\delta_2,\ \varphi_2} \overset{\delta_C}{\mathbf{C}} \qquad \begin{matrix} \varepsilon_1 = 0 & \varepsilon_2 = 15‰ \end{matrix} \tag{4}$$

δs with alphabetical subscripts are the designated isotope ratios of their respective, lettered, N position in compounds. Numbered arrows represent reactions and corresponding δs with numerical subscripts represent the instantaneous isotope ratio of the nitrogen being transmitted. φs represent the flux of nitrogen (moles/time) in each reaction and finally, εs represent the isotope effect associated with the specific numbered transformation. In this sequence of reactions, reaction 1 has no isotope effect (ε_1=0 ‰), so the $\delta^{15}N$ of position B will be identical to that of A. Let us assume that A represents a non-depletable reservoir of source N whose isotope ratio does not change and has the composition, for sake of convenience, $\delta^{15}N_A$ = 0 ‰. The isotope ratio of δ_1, the instantaneous isotope ratio of nitrogen being transmitted to position B, as well as δ_B, the isotope ratio of position B, must also equal 0 ‰.

In the reaction 2, which converts reactant B to C, a 15 ‰ isotope effect is known. From the equation 3, we have:

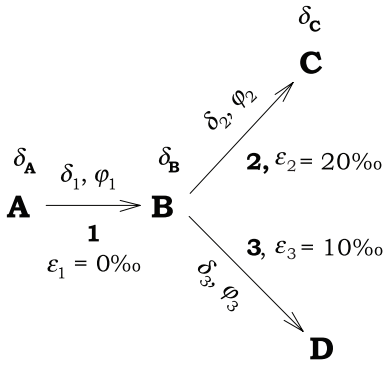
$$\delta_2 = \delta_B - 15\ ‰ \quad (5)$$

At steady-state, the instantaneous isotope ratio of transmitted nitrogen, δ_1 and δ_2, must be equal (δ_1, δ_2 = 0 ‰) due to conservation of mass, since the flow through each reaction is uniform. However, as stated above, the initial isotopic composition of δ_B is equal to that of δ_A = 0 ‰, therefore, at initial non-steady-state conditions, δ_2 must equal -15 ‰. This irregularity in isotopic ratios between starting conditions and steady-state conditions is resolved in the interim phase by a rise in δ_B as lighter isotopes are more quickly removed from this position. δ_B will continue to rise until δ_2 reaches a steady-state value of 0 ‰, bringing isotope ratios into agreement with mass-balance. Logically, the amount of time required to reach steady-state conditions is proportional to the quantity of the intermediate, in this case, the compound containing position B, and inversely proportional to the overall flux of nitrogen (φ) through the system. To summarize, at steady-state, the isotopic compositions are δ_A = 0 ‰, δ_B = +15 ‰, and δ_C = 0 ‰, in this sequence of reactions. The distribution of these isotope ratios makes evident the importance of choosing appropriate biomarkers for measurement. Under steady-state conditions, the isotope ratios of positions A and C show no change, whereas δ of position B would show a marked shift.

Examples of biological pathways which conform to irreversible non-branched metabolism may include the glutamine synthetase reaction for the fractionation of nitrogen in NH_3 [18], bacterial assimilatory sulfate reduction [19], and the action of lyases which remove NH_3 or H_2O from compounds [20]. Under typical physiological conditions, the enzymes responsible for these conversions all operate unidirectionally. Successive chains of unidirectional reactions will continue to fractionate intermediates while yielding no fractionation in the final sink product, in our example, position C.

2.3.2. Irreversible Branched Metabolism

Equation 6 represents a theoretical branching pathway with isotope effects associated with reactions 2 and 3. The reaction converting position A to B remains unchanged from the previous formula (Equation 4) and has no observed fractionations. Branching at position B results in the creation of two products, C and D, each with a different isotope effect.



(6)

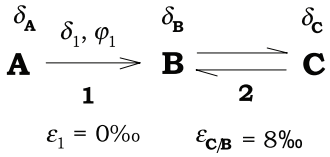
Evaluating each branch individually, the isotopic behavior of this system is similar to the relationship described by Equation 4.

Therefore, under steady-state conditions in which all nitrogen is flowing through only reaction 2, or reaction 3, we would find δ_2 = 0 ‰, δ_B = 20 ‰ and δ_3 = 0 ‰, δ_B = 10 ‰, respectively. The relative contribution of isotope effects to fractionation is linear under steady-state conditions, thus if the flow is being split evenly between the two branches, we find δ_B = 15 ‰, and δ_2 = -5 ‰ and δ_3 = 5 ‰. This relationship demonstrates how the relative flux of nitrogen or carbon through branches can modify the isotope ratio of their respectively transmitted isotopes, resulting in different distributions of downstream fractionations.

Metabolic pathways are replete with branching due to the sharing of common substrates by multiple enzymes. The physiological mechanisms which regulate enzyme expression and activity necessarily impact the propagation of an isotope effect. Nitrogen fractionation in amino acids appears to behave in accordance with this branching model. Transamination of amino groups from Glu in competing synthesis of Asp and Ala, catalyzed by respective transaminases, for instance, appears to leave marked enrichment of nitrogen in Glu [15, 21, 22]. An additional complication may arise with transaminases with multiple specificities [16, 23], since competition for available enzymes may also play a role in dictating nitrogen fractionations. Under *in vivo* conditions, pathways of synthesis may combine several aspects of model components, making the interpretation of isotopes difficult.

2.3.3. Reversible Reactions in Metabolism

The equilibration and exchange of elements with isotopes in compounds present in the local cell-environment is typically slower than corresponding rates of enzymatic-exchange, which can impart a KIE. Consequently, attainment of thermodynamic equilibrium with good agreement with EIEs is not frequently observed [24].



(7)

Equation 7 represents a sequence of reactions in which atoms present in positions B and C achieve equilibrium and agree with predicted EIEs. Under steady-state conditions, the pools of compound B and C are in equilibrium and will always differ by the fractionation factor dictated by $\varepsilon_{C/B}$, resulting in δ_C = 0 ‰ and δ_B = -8 ‰. The relative scarcity of nitrogen in biological compounds makes this scenario unlikely for nitrogen fractionation; however, equilibrated distributions of oxygen isotopes in glucose have been observed. It has been proposed that oxygen equilibrations between carbonyl groups of glucose intermediates with water lead to an average ^{18}O-enrichment in glucose of 27 ‰ through an equilibrium isotope effect [25]. However, recent reports [26] indicate that the agreement of average ^{18}O-abundances with the equilibrium isotope effect may be the overlap of highly deviating positional ^{18}O-abundances, indicating that the original source of oxygen for those positions may be preserved.

2.4 Non-Steady-State Conditions in Closed-Systems

The fractionation of isotopes between standing pools of reactants and products in closed-systems at conditions other than steady-state results in differential distributions of isotopes due to the consumption of reactants. The isotopic relationship between the reactants and the products over the duration of a reaction, starting with only reactants and ending with a 100% yield of products, is represented by "The Rayleigh Equation",

$$f^{\varepsilon_{P/R}} = \frac{R_R}{R_{R,0}} \tag{8}$$

where f represents the extent of the reaction, $\varepsilon_{P/R}$ represents the isotope effect associated with the reaction, and R_R and $R_{R,O}$ signify the isotopic composition of the residual reactant and initial reactant, respectively.

Isotopic literature utilizes many different approximations of this equation, which was originally developed to study the fractional distillation of mixed liquids, to highlight the exponential relation that describes the partitioning of isotopes between two reservoirs as one reservoir decreases in size. Studies which analyze isotopic fractionations *in vitro* often employ this method to elucidate fractionation factors. The relationship between the cumulative

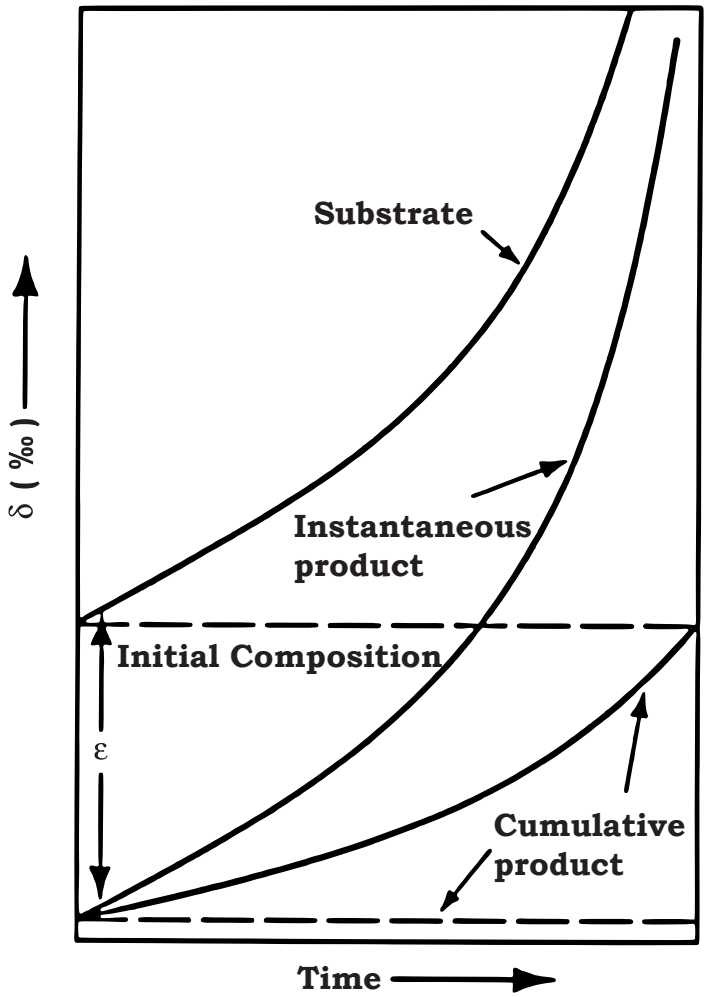


Figure 2.2 A visual representation of the relationship between δs of substrate, instantaneous product, cumulative product, and initial composition of reactants over time. Dotted-lines represent isotopic abundance in an open-system, where the supply of reactant is infinite†.

†Adapted from 1. Hogberg, P., *Tansley review No 95 - N-15 natural abundance in soil-plant systems.* New Phytologist, 1997. **137**(2): p. 179-203.

product, instantaneous product and residual substrate is shown visually in Figure 2.2 [27]. This figure demonstrates how Rayleigh-type fractionations affect the compositions of residual substrate, instantaneous product, and cumulative product (curved lines) during a closed-system kinetic reaction. The static difference, controlled by ε, between substrate and product in steady-state open-system reactions such as those described in preceding sections, is represented by dotted lines. However, under conditions where the supply of reactants is not infinite (e.g. nutrient limitation), the isotope ratios of products eventually approaches the isotope ratio of the reactants as they are metabolized, resulting in indiscernible fractionation between source and product.

2.5 Conclusions

Predictive modeling of isotopic fractionation by biological organisms is necessarily complex. The sizeable number of chains and branches in metabolism, each with specialized enzymes with potential isotope effects, results in distributions of isotopes that can be characteristic for particular physiological states. As demonstrated in this chapter, the behavior of isotopes is correlated to the individual components of metabolism. Under non-equilibrium conditions, many enzymes impart unique isotopic fractionations on their substrates through a kinetic isotope effect. On the other hand, when free-exchange of functional groups between intermediate compounds and solvents is allowed to occur, the pattern of fractionation may be in better agreement with equilibrium fractionation factors. Physiological fractionation is further

complicated by transient phases of growth where the concentration and isotope ratio of intermediates are continually changing. The importance of timing as well as proper choice of intermediate for analysis must be carefully considered. For nitrogen analysis, in particular, amino acids are ideal candidates due to their central and widespread involvement in nitrogen metabolism. Improved understanding of physiological fractionation can only occur under controlled conditions where the specific cause and metabolic adaptation to local environmental conditions can be isolated.

Recent advances in mass spectrometric techniques [28] allow for an unprecedented level of amino acid nitrogen isotopic analysis. Expanding on these methods, we have evaluated the *in vivo* fractionation of nitrogen, under different physiological conditions, in amino acids in a bacterial model at the bulk, compound, and position-specific level. Consideration of known pathways has lead to plausible and interesting explanations for observed, characteristic fractionations, and provides additional support for the usage of isotope ratios as sensitive indicators of physiological state.

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CHAPTER 3

The Intramolecular $\delta^{15}N$ of Lysine Responds to Respiratory Status in *Paracoccus denitrificans**

3.1 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, particularly in the environment. The isotopic selectivity of (bio)chemical reactions imparts a unique position-specific isotopic signature reflecting pathways that form and degrade the bonds to each specific atom within molecules. This phenomenon has been exploited most commonly *in vitro* to examine specific biochemical reaction kinetics and mechanisms [1]. For an organism, these observations imply that each stereochemically unique position within every unique chemical species in the organism has an isotope ratio that may reflect physiological state [2]. Isotope ratios have been treated primarily as static for a given organism, with little attention to any 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 contribution. A change in physiological state is equivalent to a change in pathway branching ratios, which in turn predicts that isotope ratios at specific positions change

* Submitted to Amino Acids, 2006

with the relative inputs and outputs of different reactions [3]. One study of respiratory-state and carbon fractionation [4] 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. 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 physiological N-fractionation at the compound or position-specific levels.

Many biological fields employ natural isotopic variability as a routine tool, such as forensic geographical sourcing [5], analysis of food webs in human and animal diets [6], and plant species differentiation [7]. The vast majority of isotope ratio measurements are reported at the bulk and compound-specific levels, thus diluting 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 state. For instance, neural DNA [8, 9] and tooth collagen [10] 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 first demonstrated systematic, position-specific variation in carboxyl-group $\delta^{13}C$ of various amino acids across several species of photosynthetic organisms [11]. Subsequent studies analyzing C and N isotope ratios in amino acids from fungi and bacteria grown on different nutrient sources also report this characteristic distribution of isotopes, controlled by the mechanisms of C and N assimilation [12]. To date, there have been no reports of *in vivo* measurements of position-specific $\delta^{15}N$, in part because until our recent report of methods for four amino acids [13], there have been no methods available for N-PSIA analysis, apart from an abstract [14]. We have applied this method 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 [15] bacteria bearing resemblance to mitochondria [16], is well suited as a model organism for studies of metabolic isotopic fractionation. It grows well aerobically or anaerobically on easily characterized single-C (e.g., CH_3OH) and single-N sources [17], 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 [18], and the aerobic state, in which NH_4+ inhibits fixation of N from nitrate [19]. Additionally, stringent anoxic conditions are necessary for anaerobic growth, thus providing a clear shift in physiologically states [20].

We present the first experimental evidence that natural, intramolecular, isotope ratios are sensitive to physiological status, using the example of intramolecular $\delta^{15}N$ of lysine in the mitochondrial mimic *Paracoccus denitrificans*. *P. denitrificans*, a versatile, gram-negative bacterium, was grown either aerobically or anaerobically on isotopically-characterized ammonia as sole cell-nitrogen source. Nitrogen isotope ratio of the biomass with respect to source nitrogen was $\delta^{15}N_{NH4}$ = -6.22 ± 1.18‰ for whole cells under aerobic respiration, while cells grown anaerobically produced no net fractionation ($\delta^{15}N_{NH4}$ = -0.31 ± 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 $\Delta\delta^{15}N$ is negligible, but in anaerobic cells was a remarkable $\Delta\delta^{15}N$ = + 11.0‰, driven predominantly by enrichment at the peptide N. Consideration of known lysine pathways suggest this to be due to enhanced synthesis of peptidoglycans in the anaerobic state. These data indicate that isotopically distinct pathway branching ratios associated with microbial respiration can be detected by natural intramolecular $\delta^{15}N$ measurements, and are the first *in vivo* position-specific measurements of nitrogen isotope fractionation.

3.2 Experimental Section

3.2.1 Cell Growth and Preparation

P. denitrifricans ATCC 17741 were obtained from the American Type Culture Collection (Rockville. Md.) and conditioned to grow on ammo-

nia and methanol as sole N and C sources exclusively. The defined medium for the aerobic and anaerobic physiological states consisted of (in grams per liter) 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 liter^{-1}. The modified Vishniac and Santer trace-elements solution [21, 22] contained (in grams per liter) 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.[21, 22] Media for the anaerobic state was made anoxic by sparging with a steady stream of N_2 for 30 minutes and then kept tightly capped. 1L batch preparations of cells were grown at 34°C and 250 RPM (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.

3.2.2 Sample Preparation and Hydrolysis

Free amino acids were prepared by standard liquid-phase protein hydrolysis [23] of dry biomass using a Pico-Tag Workstation (Waters; Milford, MA). Briefly, 50 mg of biomass was suspended in 20 ml 6N HCl, 0.1% Phenol, and ambient atmosphere in hydrolysis vessels was replaced with nitrogen gas via several purges. Acid hydrolysis proceeded at 145°C for 4 hours, 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 submitted in triplicate for amino acid analysis to the Molecular Structure Facility (MCF) at the University of California, Davis (UC Davis; Davis, CA).

3.2.3 Enyzmatic Reactions

Lysine Oxidase (EC 1.4.3.14) was purchased from Sigma-Aldrich as a lyophilized powder and prepared as a 2500 unit/mL suspension in distilled H_2O, where 1 unit will catalyze the deamination of 1 μM of NH_3 from Lysine per minute at 37°C at pH 8. 10 μl 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.

3.2.4 N-Ethoxycarbonyl Ethyl Ester (ECEE) Derivitization of Amino Acids

Amino acids in hydrosylates and enzymatic reactions were made amenable for analysis by a derivatization procedure modified from Husek [24] and Fiamegos [25]. 600 μl of amino acids were diluted with 400 μl of ethanol-pyridine (4:1), followed by acidification with 10 μl of 2N 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 minutes. 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 analysis by GCC-IRMS.

3.2.5 Gas Chromatography-IRMS of Amino Acid Derivatives

Minor modifications were made to our previously reported [13] system. Briefly, 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 x 0.53 mm x 3 μm VB-1701 capillary column (VICI; Houston, TX) was utilized. A second, relatively non-polar, 60 m x 0.32 mm x 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°C 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 psi 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.

Analyte peaks exiting the rotary valve were combusted in a continuous-capillary furnace consisting of oxidized Cu wire packed 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), which was partially embedded in the GC oven-wall. An external reduction furnace, similarly heated to 550°C,

was made using Valco vespel ferruels and zero dead volume connectors and was connected through an additional rotary valve, which diverted O_2 away from the reduction furnace and the downstream IRMS during recharge. H_2O and carbon byproducts were trapped by passing glass capillary through a dewar filled with liquid N_2 before the open-split. Automated sampling and subsequent data processing were performed as previously reported [13].

3.2.6 Bulk Isotope Analysis of Standards

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

3.2.7 Isotope Ratios

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.

$$\delta^{15}N_{air} = (R_{spl} - R_{air})/R_{air} \times 1000 \qquad Rx = [^{15}N_x/^{14}N_x]$$

where R_{spl} is the isotope ratio of the sample and R_{air} = 0.0036782

3.2.8 Statistics

Comparison of amino acid compositions from amino acid analysis of aerobic and anaerobic states were performed with a pairwise t-test with Bonferronni correction for multiple comparisons. Duncan's Multiple Range tests (alpha = 0.05) were performed on the compound-specific means of amino acids, measured via GC/IRMS, within each state to establish groups of interest.

3.3 Results and Discussion

Three batches of *P. Denitrificans* grown under either aerobic or anaerobic respiration were prepared. 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 [17]. Two nitrate reductases, a periplasmic nitrate reductase expressed constitutively and another membrane-bound reductase expressed under oxygen limitation, are active during dinitrification [20, 26]. Previous studies of nitrate utilization by *P. denitrificans* demonstrate negligible conversion of NO_3 to NH_4 [19]. Energy conservation under anaerobic growth is impaired relative to aerobic growth due to a loss of 2 electrons to nitrate reductases in the periplasm [27]. 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 [17, 18, 28]. Average freeze-dried weight for 1 L batches was 0.243 ± 0.07 mg/ml, with no significant differences between physiological states.

3.3.1 Bulk Physiological $^{15}N/^{14}N$ Fractionation

Table 3.1 shows that bulk fractionation of $^{15}N/^{14}N$ of whole cells is $\delta^{15}N_{NH4}$ = -6.22 ± 1.18‰ in the aerobic state, but there was no significant net fractionation in the anaerobic state ($\delta^{15}N_{NH4}$ = -0.31 ± 0.23‰). Statistical analysis (t-test) with Bonferroni correction for multiple comparisons, performed on percent compositions of amino acid in biomass hydrosylates (UC Davis), indicate no statistically significant differences in the prevalence of amino acids between states. Mass-balance calculations for protein $^{15}N/^{14}N$ using known available amino acid compositions from anaerobic cells show $\delta^{15}N_{NH3}$ = +3.19 ± 0.48‰ enrichment in ^{15}N relative to total biomass, in agreement with a previous reports of approximately $\delta^{15}N_{NH3}$ = +3.5‰ [12], while aerobic cells show a lower degree of enrichment ($\delta^{15}N_{NH3}$ = + 1.38 ± 1.19‰). As proposed by Werner and Schmidt [29] this observation is likely explained by enhanced ^{15}N depletion in N-containing cell wall components in the anaerobic state, consistent with data in our Table 3.1. 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. Studies of microbial fractionation of differing dietary amino acids [30] or N-source [12] 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.

Table 3.1 – δ^{15}N-BSIA of whole cells grown aerobically and anaerobically. δ^{15}N of protein fraction is calculated from weighted-average of δ^{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.

	Aerobic			Anaerobic		
	δ^{15}N			δ^{15}N		
Total Biomass	-6.22	±	1.18	-0.31	±	0.23
Protein Fraction	-4.84	±	0.17	2.88	±	0.42
$\Delta\delta^{15}N_{Protein\text{-}Biomass}$	+1.38	±	1.19	+3.19	±	0.48

Fixation of NH_4^+ in *P. denitrificans* proceeds via two pathways [31], with glutamate dehydrogenase (GDH) and glutamine synthetase (GS) operating typically at mM and μM concentrations of NH_4^+, respectively. Hoch [32] established that at mM concentrations of NH_4^+, transport of N is passive and that intracellular and extracellular pools of NH_3 approach isotopic equilibrium. Under these conditions of rapid equilibrium and excess source N, isotopically-selective barriers such as cell membranes do not play a significant role in overall fractionation, and the rising trend in isotopic ^{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 [33]; isotopic fractionation by GDH was reported from -18 ‰ to +10‰ at pH 5.8 and pH 9.2, respectively. Calculations of $^{15}N/^{14}N$ fractionation in *P. denitrificans* using a linear approximation ($r^2 = 0.993$) of observed ^{15}N kinetic isotope effects for GDH correspond to values of $\delta^{15}N_{NH4} = -8.07$‰ for aerobic cells (average pH = 7.06 ± 0.04) and $\delta^{15}N_{NH4} = -0.70$‰ (average pH = 7.95 ± 0.24) for anaerobic cells, in good agreement with our results.

3.3.2 Compound-Specific Fractionation of $^{15}N/^{14}N$

Figure 3.1 illustrates the $^{15}N/^{14}N$ of 11 proteinogenic amino acids from both physiological states. The general distribution of $^{15}N/^{14}N$ in amino acids shows a characteristically high isotope ratio for Glu in both states, consistent with its role as a primary N-donor for metabolically related amino acids. This overall enrichment of ^{15}N in Glu with respect to other amino acids is in agreement with that reported by

Macko [12], 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. Furthermore, 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 ‰. Our remaining amino acids fall within a narrow range of ^{15}N depletion relative to Glu, with an average difference of $\Delta\delta^{15}N_{Glx}$ = -4.15 ± 0.61 ‰ and -7.03 ± 0.80 ‰ for aerobic, and anaerobic states, respectively, followed by Gly, Iso, and Leu. Under aerobic conditions, Ala is distinguished from other amino acid with respect to Glu. The distinct fractionation of Ala in the aerobic state suggests differing Ala metabolism, possibly resulting from increased Ala deamination to pyruvate or an alternative N-source other than Glu. As a critical component in bacterial peptidoglycan synthesis, the relative branching ratios between the contributions of Ala to protein synthesis, as opposed to cell-well synthesis in either state, also affects its $^{15}N/^{14}N$ with respect to other amino acids. Kinetic isotope effects (KIE) during transamination are a likely source for N fractionation in amino acids. Incorporation of N in 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 NH_3 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, Iso, Leu, Tyr, Phe, Asp, Lys, Ser, and several others. Directly or indirectly, all N in amino acids originates from Glu.

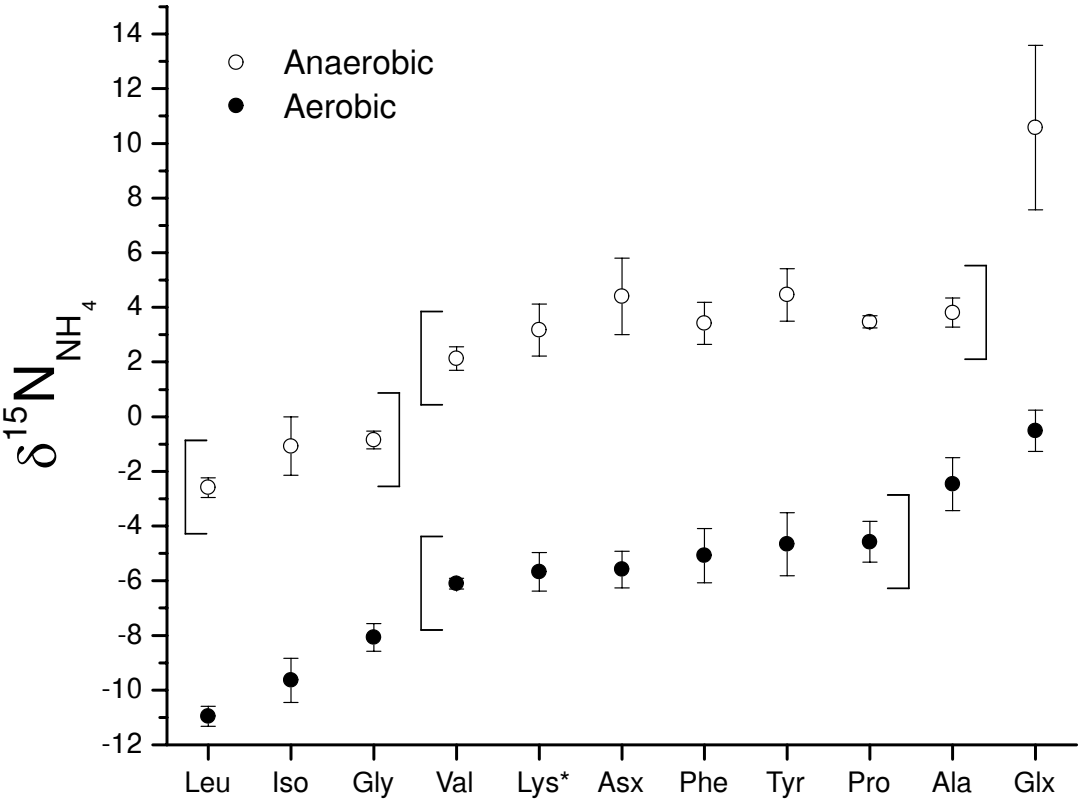

Figure 3.1 - Amino acid $^{15}N/^{14}N$ expressed with respect to source nitrogen, $\delta^{15}N_{NH4}$. Brackets indicate overlapping ranges of non-significant differences in means analyzed using Duncan's Multiple Comparisons. *Represents average $^{15}N/^{14}N$ of the two N in Lys.

KIE for the enzyme aspartate aminotransferase (AspAT; EC 2.6.1.1), responsible for NH_3 exchange between Glu and Asp, have been reported *in vitro* as $\beta = 1.0083$ for the transamination from Glu to Asp and $\beta = 1.0017$ for the reverse reaction ($\beta = k_1{}^{14}N/k_1{}^{15}N$) [34]. Our results, $\Delta\delta^{15}N_{Asx\text{-}Glx} = -5.07 \pm 1.04$ ‰ and $\Delta\delta^{15}N_{Asx\text{-}Glx} = -6.18 \pm 3.10$ ‰, within respective aerobic and anaerobic states, and are in agreement with kinetic isotope effects for this enzyme. 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 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. The complexity of amino acid nitrogen metabolism is further complicated by the promiscuity of the major transaminases. AspAT has been reported to exhibit activity on l-Tyr, l-Phe, and l-Trp [35] and in *P. Denitrificans*, ArAT has also been shown to have other specificities aside from aromatic amino acids [36]. The relative contribution of Asp and Glu to the citric acid cycle, which is suppressed in the absence of oxygen [37], is also a probable source of fractionation.

Relative *in vivo* physiological rates of transaminations between amino acids are not well-studied. In studies of N-CSIA of amino acids, amino acid isotope profiles vary widely between organisms but are surprisingly consistent within organisms, regardless of growth medium. The similarity of $^{15}N/^{14}N$ amino acid profiles between the aero-

bic 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 NH_3 during assimilation governs the relatively consistent bulk isotopic shift between 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.

3.3.3 Position-Specific Fractionation of Lys $^{15}N/^{14}N$

Compound-specific measurement for Lys $\delta^{15}N_{NH3}$ was -5.67 ± 0.71‰ in the aerobic state and + 3.18 ± 0.95‰ in the anaerobic state (Figure 3.1). These values are a mean of $\delta^{15}N_{NH3}$ for the two N positions in Lys. Figure 3.2 shows intramolecular isotopic signature of Lys-N in both respiratory states. There is no significant difference in $\Delta\delta^{15}N_{NH3}$ in the aerobic state. However, under anaerobic conditions, the $\delta^{15}N_{NH3}$ of the two positions diverge, with the peptide-N of Lys becoming enriched in ^{15}N with respect to sidechain-N by $\Delta\delta^{15}N_{NH3}$ = + 10.98 ± 2.0‰. The sidechain (amido) N is depleted compared to source N, while the peptide (amino) N is remarkably enriched compared to source N. This finding dramatically illustrates the degree to which compound-specific measurements mask intramolecular variability.

The intramolecular $\Delta\delta^{15}N_{NH3}$ for Lys must originate in a shift in the branching of the pathways for synthesis or degradation of the respect-

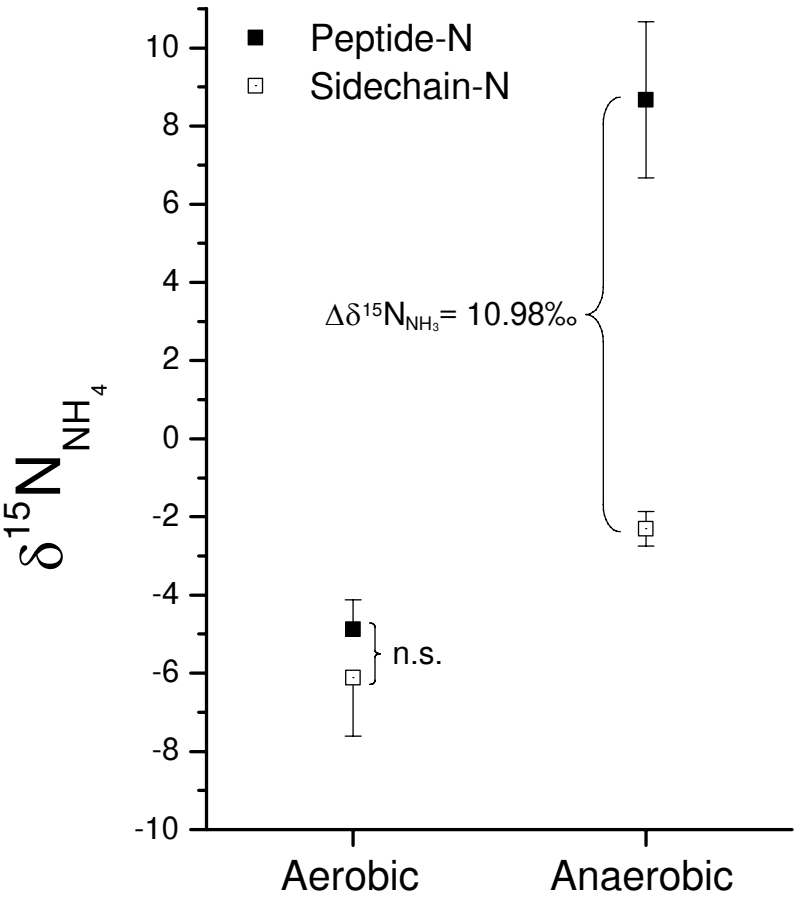

Figure 3.2 - Intramolecular distribution of $^{15}N/^{14}N$ in Lys in the aerobic and the anaerobic states, expressed with respect to source nitrogen ($\delta^{15}N_{NH4}$).

tive N. The accepted pathway for Lys synthesis in gram-negative bacteria [38] is presented in Figure 3.3, and begins with Asp, followed by condensation with pyruvate and transamination from Glu. However, the origin of the $\Delta\delta^{15}N_{NH3}$ 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 $\Delta\delta^{15}N_{NH3}$ 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 [38], 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-

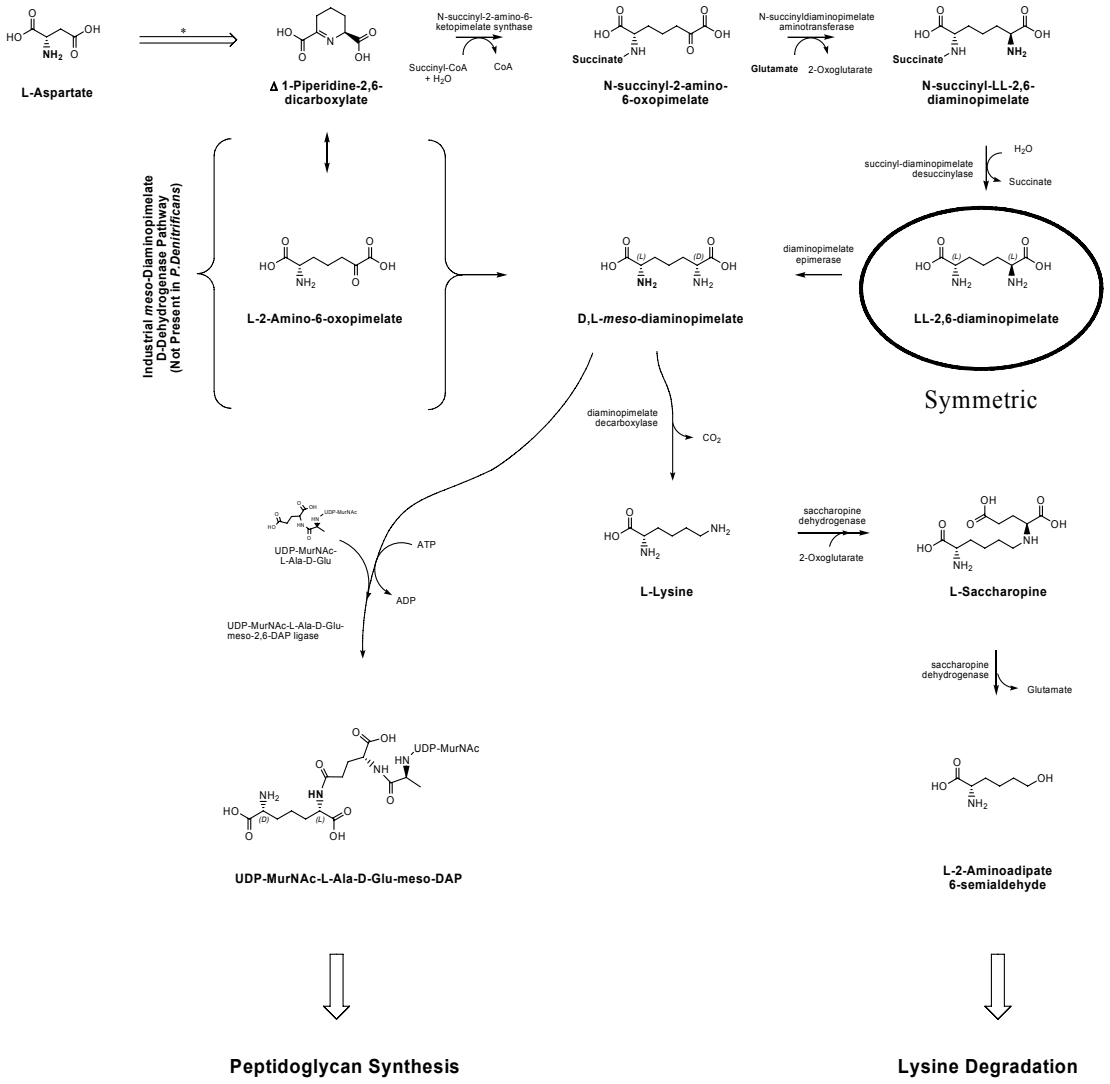

Figure 3.3 - Biosynthesis of Lysine in bacteria indicating branching points. *Represents several critical steps, including regulatory phosphorylation by aspartate kinase, reduction with NADH, and condensation with pyruvate. Brackets indicate an alternate pathway unique to industrially-important bacteria utilized in Lys production. The N in the symmetric intermediate LL-2,6-diaminopimelate are biochemically indistinguishable and thus isotopically equivalent ($\Delta\delta^{15}N_{NH3} = 0$ ‰).

Ala-D-Glu-*meso*-2,6-DAP ligase) [39] 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 kinetic isotope effects typically favor lighter ^{14}N incorporation under physiological conditions. We hypothesize that the difference in Lys $\Delta\delta^{15}N_{NH3}$ between aerobic and anaerobic cells corresponds to a higher rate of bacterial peptidoglycan synthesis, possibly due 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 our 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, shown in Table 3.1.

In contrast to these *in vivo* findings, our previous studies of intramolecular N fractionation in Lys from major industrial distributors indicate enrichment in most samples for sidechain-N relative to peptide-N (average $\Delta\delta^{15}N$ = 7.5 ‰) [13]. The most commonly used microorganism for the industrial production of Lys, *Corynebacterium glutamicum*, employs an additional parallel pathway (figure 3.3, left) for synthesis of *meso*-DAP dependent on ammonium availability [38, 39]. In this alternative route, which can occur with, or in lieu of [40], 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 ammonia. The alternative pathway is

thus a likely explanation for the difference of our present *in vivo* results and observations made in the context of methods development.

The final possible source of intramolecular fractionation of Lys is its irreversible degradation to acetyl-CoA, also shown in Figure 3.3. This pathway, which also operates in humans, consists of a step-wise transfer of N through multiple intermediates from first, the sidechain-, and second, the peptide-N, of Lys to 2 respective molecules of 2-oxoglutarate. Isotopic-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. Our data does not support a significant amount of fractionation in this step, since $\delta^{15}N_{NH3}$ for both N of Lys in the aerobic state are identical to Asp, 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.

3.4 Conclusions

Intramolecular $^{15}N/^{14}N$ measurements of amino acids allow for an unprecedented specificity in assigning and characterizing sources of N-fractionation. While CSIA of Lys-N 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 state.

Bulk, Compound, and Position-specific high-precision $\delta^{15}N$ measurements of amino acids were conducted on *P. denitrificans* represent-

ing aerobic and anaerobic physiological states. We report 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 [41] 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 applies to natural systems without perturbation, other than for sampling. Intramolecular isotope ratios reflect aspects of organismal biology, specifically environmental influences, that is not available from the genome, epigenetics, or metabolomics. Isotope ratios necessarily reflect the net fractionation through all pathways leading to product synthesis and degradation. The specific intramolecular isotopic fractionation at branch points in an organism is a composite of all inputs and outputs, and thus is likely to be 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 our understanding of metabolic fractionation, ultimately leading to principles that can be extended to multicellular organisms.

3.5 References

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CHAPTER 4

Salt-induced Nitrogen Isotopic Fractionation of Amino Acids by *Paracoccus denitrificans*

4.1 Introduction

Isotopic fractionation during incorporation of naturally occurring isotopes by biochemical and physical processes records in every position of every molecule an isotopic signature that reflects source and process information at the time of synthesis. This information becomes increasingly difficult to interpret as the complexity of reaction networks increases. As molecules flow through pathways, individual fractionations at specific branches can potentially be obscured by opposing shifts in later ones, thereby confounding the significance of isotope ratios. In the case of biological organisms, isotopic fractionations can occur at several steps such as the active or passive transport of metabolites and their subsequent assimilation. Modern high-precision mass spectrometry techniques enable scientists to evaluate isotope ratios in the range of natural abundance at the bulk, compound, and position-specific level, but an incomplete understanding of underlying mechanisms prevents exploitation of these intrinsic tracers to determine physiological state. The utilization of amino acids as a resource for meaningful isotopic information was first highlighted in a ground-breaking examination of carbon isotopes at carboxyl positions in amino acids across several species of organisms [1]. Affirmation of metabolically-predicted patterns of enrichment paved the way for numerous studies of isotopic

fractionation in various classes of biological compounds in several organisms [2-8], but the utility of isotope ratios as a measure of physiology is still inconclusive despite an increasing body of knowledge.

A systematic rise in $\delta^{15}N$ with increasing trophic levels is commonly exploited in ecology to study food webs [9, 10]. Aspects of N fractionation have been utilized in archaeology and geochemistry [11, 12], extensively in ecology to examine the global N cycle and recently as a powerful tool to study nitrogen metabolism in plants [13]. However, the majority of reports which aid in elucidating the underlying mechanisms of physiological fractionation of N in organisms investigate the fractionation with respect to differing N sources or availability [2, 3, 5, 14, 15], whereas the influences of non-input related environmental stresses are not as well-characterized. The significance of a purely physiological isotopic response by modification of parameters unrelated to source-N lies in the potential exploitation of $^{15}N/^{14}N$ isotope ratios in ascertaining information about the physiological state of an organism in addition to source. While the robustness and sensitivity of isotope ratios to deducing source and physiology is uncertain, isotopic fractionation must follow a logical system. This applies to the steady-state "isotome" of an organism as well as the cumulative changes in isotope ratios in intermediates in response to environmental stresses. Inter- and intra-molecular distributions of isotopes in biologically relevant molecules are the net expression of fractionations that are incurred throughout biosynthesis and degradation [6, 16, 17], but large variability in isotopes ratios both

within and between species are common. The utilization of isotope ratios for metabolic identification requires a benchmark of isotope ratios under controlled conditions as a basis for comparison. The individual and combined contributions of stresses must first be understood before the cumulative physiological response of an organism manifested in changes in isotope ratios can be interpreted. Where information regarding the environment or conditions under which an organism is grown is incomplete, knowledge of existing isotopic responses can potentially aid in its reassembly.

The complexity of forces governing physiological fractionation in a cell, including the individual contributions of kinetic and thermodynamic isotope effects [18] at various stages of growth, the impact of closed versus open-system thermodynamics, and the largely irreversible nature of enzymatically-catalyzed reactions [6, 17, 18], makes defining a general model difficult. Precise measurements of isotope ratios in metabolic intermediates such as amino acids in simplified systems where sources are easily characterized allows for necessary consideration of physiological effects.

One example of an environmental factor that is often overlooked is the impact of membrane stress on isotopic fractionation. Hyper-saline waters far exceeding the salt-concentrations of marine environments once covered much of the Earth and the physiological response of microbes and higher-level organisms as they are subjected to rapid changes in external solute concentrations is well-conserved. The N isotopic response to increasing external solutes such as NaCl, the major solute responsible for changes in osmolarity in the natural

environment [19], has been directly measured only a handful of times [20-22] with conflicting reports and only at the bulk-tissue level. An assessment of isotopic response to salt-stress in a simpler organism such as bacteria is a logical and convenient step in understanding how the metabolic changes in response to salt/solute stress might affect isotope ratios. Additionally, an increased understanding of the physiological responses to osmotic stress in prokaryotes provides a means to deduce potential sources of fractionation. Amino acids are potentially good candidates for sensitive biomarkers towards hyperosmotic-shock due to the wide-spread strategy of balancing high ionic concentration in the media by *de novo* synthesis or uptake of organic osmotic, "compatible" solutes, many of which are amino acids. We examined the physiological isotopic response of *Paracoccus denitrificans*, a nutritionally versatile gram-negative bacteria which is capable of growing on simple, isotopically characterized substrates, to changes in external osmolarity by varying the concentration NaCl in the media.

We report evidence for a salinity-induced response in $\delta^{15}N$ isotope ratios at the bulk and compound-specific level in the environmentally relevant bacteria, *Paracoccus denitrificans*. Amino acids extracted from cells conditioned to grow on single-N and single-C sources and varying degrees of salt concentration were analyzed for N isotope content. Bulk $^{15}N/^{14}N$ increased by $\Delta\delta^{15}N = + 5.60 \pm 0.79‰$ from 1% to 1.75% NaCl. Significant linear trends of $\Delta\delta^{15}N$ values with salt concentration between 1% and 1.75% ($p<0.05$) were found for five amino acids, Ala ($\Delta\delta^{15}N = 4.66 \pm 0.23‰$), Gly ($\Delta\delta^{15}N = 4.03 \pm 0.84‰$),

Val ($\Delta\delta^{15}N$ = 3.59 ± 0.32‰), Pro ($\Delta\delta^{15}N$ = 6.131 ± 0.76‰), and Asx ($\Delta\delta^{15}N$ = 5.27 ± 0.56‰). Studies of $\delta^{15}N$ isotopic fractionation in plants with respect to salt show conflicting responses at the bulk level while compound-specific measurements have not been previously reported. These findings highlight the potential impact of non-source related factors in the environment on physiological fractionation and the complexity of mechanisms associated with the fractionation of N isotopes. An examination of osmoregulation pathways in bacteria provides some potential sources for this pattern of fractionation.

4.2 Experimental Section

4.2.1 Cell Growth and Preparation

P. denitrificans ATCC 17741 obtained from the American Type Culture Collection (Rockville. Md.) were conditioned to grow on ammonia and methanol as sole N and C sources exclusively and three concentrations of salt, 1%, 1.3% and 1.75%. Defined medium consisted of (in grams per liter) 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 $liter^{-1}$. The modified Vishniac and Santer trace-elements solution [23, 24] contained (in grams per liter) 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 [23, 24]. All media was adjusted to pH 7 with KOH before autoclaving and 1L batch preparations of cells were grown in parallel at 34°C and 250 RPM (Labline; Max Q 5000; Melrose Park, IL), with periodic monitoring at O.D. 600 nm (LKB Biochrom; Ultrospec 4050; Cambridge, UK).

Bacteria 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) and freeze-drying were performed before sample storage at -80◦C.

4.2.2 Sample Preparation and Hydrolysis

Free amino acids were prepared by standard liquid-phase protein hydrolysis [25] of dry biomass using a Pico-Tag Workstation (Waters; Milford, MA). For each hydrolysis, 50 mg of biomass was suspended in 20 ml 6N HCl, 0.1% Phenol, and ambient atmosphere in hydrolysis vessels cycled with nitrogen gas. Acid hydrolysis proceeded at 145°C for 4 hours, 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 before derivatization.

4.2.3 N-Ethoxycarbonyl Ethyl Ester (ECEE) Derivitization of Amino Acids

Amino acids in hydrosylates and enzymatic reactions were prepared for analysis by a derivatization procedure modified from Husek[26] and Fiamegos [27]. 600 μl of amino acids were diluted with 400 μl of ethanol-pyridine (4:1), followed by acidification with 10 μl of 2N 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 minutes. 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 analysis by GCC-IRMS.

4.2.4 Gas Chromatography-IRMS of Amino Acid Derivatives

Chromatographic separation was performed using our system as previously reported [28]. Briefly, samples were injected, splitless, using an autosampler (Varian, Inc. 8200; Walnut Creek, CA) into a GC (Varian 3400CX). Amino acids were separated on a moderately polar, thick-film, 15 m x 0.53 mm x 3 μm VB-1701 capillary column (VICI; Houston, TX). GC oven temperature was ramped from 110°C 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 psi and 25 psi for the VB-1701 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.

Analyte peaks exiting the rotary valve were combusted in a continuous-capillary furnace consisting of oxidized Cu wire packed 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), which was partially embedded in the GC oven-wall. An external reduction furnace, similarly heated to 550°C, was made using Valco vespel ferruels and zero dead volume connectors and was connected through an additional rotary valve, which diverted O_2 away from the reduction furnace and the

downstream IRMS during recharge. H_2O and carbon byproducts were trapped by passing glass capillary through a dewar filled with liquid N_2 before the open-split. Automated sampling and subsequent data processing were performed as previously reported [28].

4.2.5 Bulk Isotope Analysis

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

4.2.6 Isotope Ratios

Isotope measurements in IRMS are 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.

$$\delta^{15}N_{air} = (R_{spl} - R_{air})/R_{air} \times 1000 \qquad R_x = [^{15}N_x/^{14}N_x]$$

where R_{spl} is the isotope ratio of the sample and $R_{air} = 0.0036782$

4.2.7 Statistics

Linear regressions with error as weight were performed to determine linearity of isotopic response. ANOVA analysis was performed for significance of differences between amino acids.

4.3 Results and Discussion

Paracoccus denitrificans was grown on single mono-atomic C and N sources, CH_3OH and NH_4, respectively, and varying concentrations of NaCl. The limit of salt-tolerance for *P. Denitrificans* was previously reported to be approximately 5 % (0.8 M) NaCl in complex medium [29], classifying this species as halotolerant. Another promiment member of the genus, *Paracoccus halonitrificans*, is classified as halophillic, requiring at least 3 % NaCl to grow. For growth on CH_3OH and NH_4 we found concentrations above 1.75 % (300 mM) to have a large inhibitory effect on growth rates, possibly due to the combined stress of decreased relative energy efficiency [30] when metabolizing CH_3OH and the lack of accessible osmoprotectants in the media. After experimentation on a practical range of salt concentrations, bulk and compound-specific measurements of two batches of cells grown under conditions of 1%, 1.3%, and 1.75% NaCl were performed.

The relative distribution of isotope ratios among amino acids and bulk isotope results are shown in Figure 4.1. The profiles are similar for the three salt conditions and reflect the participation of individual amino acids in transamination reactions or their demand in metabolic processes. These differences are thought to be mainly caused by

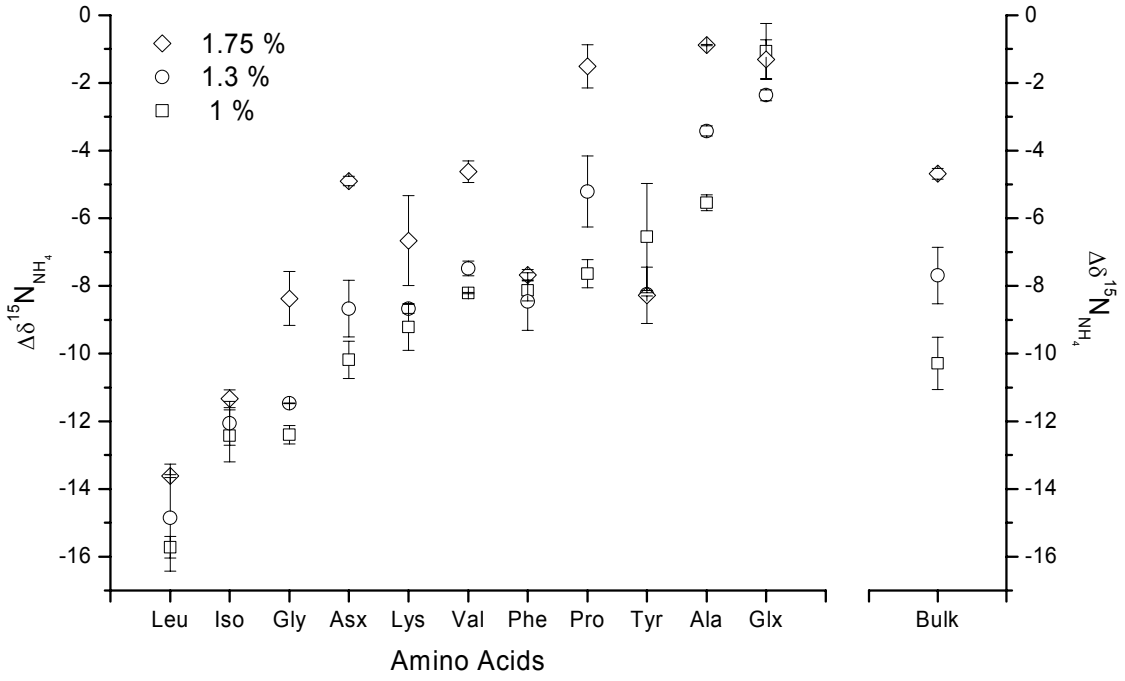

Figure 4.1 - Left: Nitrogen isotope ratios of amino acids of bacteria grown in different concentrations of NaCl, 1%, 1.3%, and 1.75%. Amino acid $^{15}N/^{14}N$ is expressed with respect to sole source nitrogen in the media, NH_4Cl. Amino acids are plotted from lowest to highest relative isotope ratio, $\delta^{15}N_{NH4}$, at 1 % NaCl. Right: Bulk N-isotope ratios for dried biomass of bacteria at three concentrations of salt.

kinetic isotope effects exhibited by the various transaminases [3, 7, 31] that are responsible for reversibly transferring NH_3 from Glu, the main reservoir of N for amino acid metabolism, to other amino acids. The observed enrichment of $^{15}N/^{14}N$ in Glu with respect to other amino acids is in line with this role, although the isotope ratios for Glx, Ala, and Pro at 1.75% NaCl are not different, suggesting increasing demand for Ala and Pro with respect to Glx near the upper limit of salt-tolerance.

$^{15}N/^{14}N$ isotope ratios for all measured amino acids, with the exception of Glx, Tyr, and Phe were correlated to increases in external NaCl. Significant linear trends ($p<0.05$) were found for five of the eleven measured amino acids and at the bulk level with respect to salt concentration (Figure 4.2). Pro was found to have the highest slope (S=8.172) while Val showed the lowest (S=4.207). Asx had $\Delta\delta^{15}N$ values and slopes closest to those of bulk, indicating that the two may be used as proxies for one another under these conditions. Previous measurements (data not shown) of bulk cells grown with no additional salt ($\Delta\delta^{15}N$ = -6.22±1.18‰) fell within the values that corresponded to 1.3% (~220 mM) and 1.75% (300 mM) salt. These findings suggest that the isotopic response is not linear across the entire range of tolerable salt-concentrations and may imply that the activation of pathways responsible for osmoregulation causes a dramatic shift in isotope ratios and presumably in the physiology of *P. denitrificans* beginning at low levels of salt. Activities of common enzymes with respect to salt-concentration in several species of salt-tolerant organisms have also

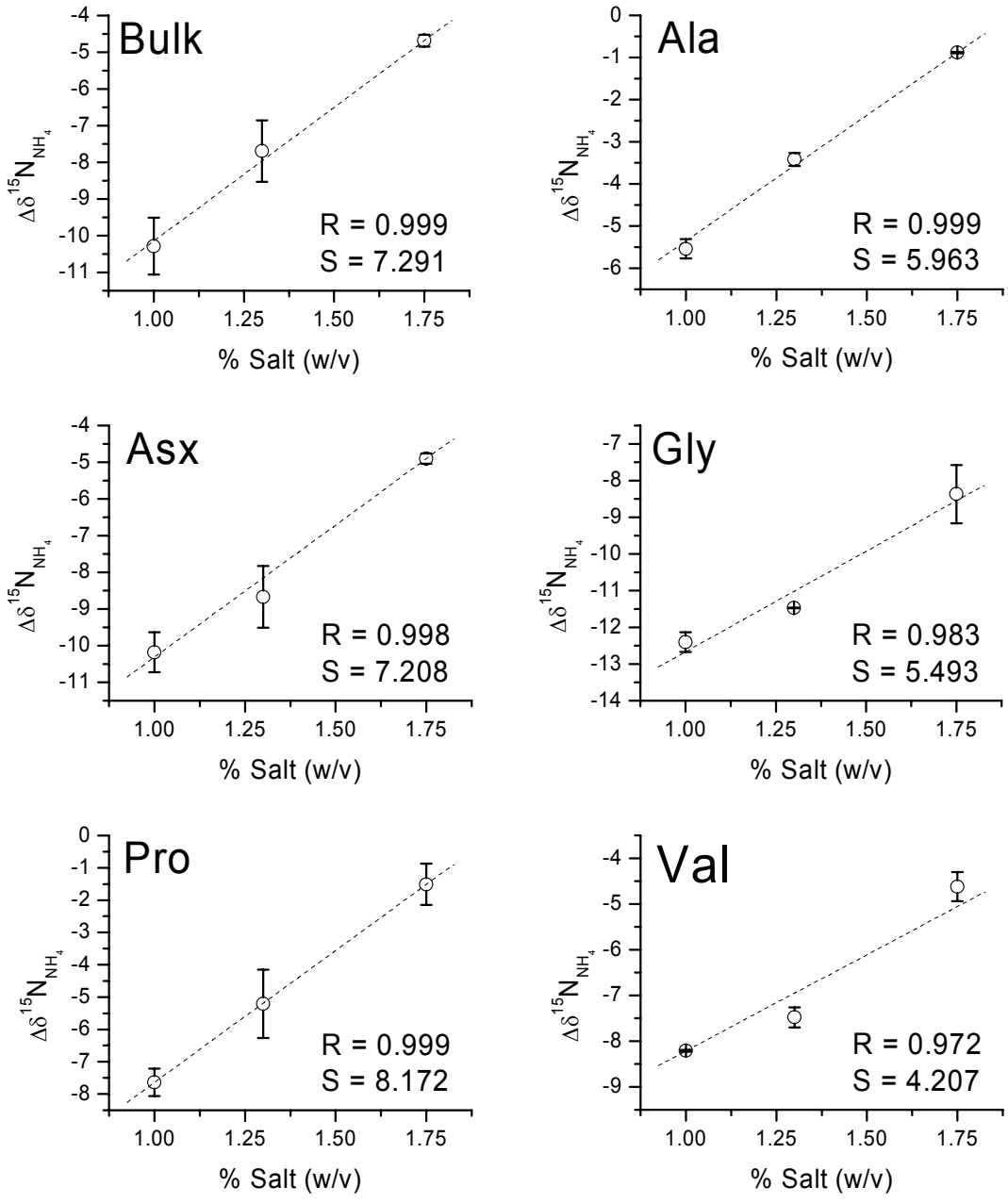

Figure 4.2 - Nitrogen isotope ratios of amino acids showing a strong linear correlation to NaCl concentration. Pro and Ala are known osmoprotectants capable of being transported from the media or synthesized *de novo*.

exhibited non-linear responses [32]. Consequently, the linearity of isotopic fractionation at higher concentrations of salt may be due to upregulation of existing osmoprotective pathways as opposed to the activation of new pathways.

4.3.1 Osmoregulation

Strategies utilized by organisms to maintain ideal growing conditions under external solute stress are shared across many diverse organisms. The accumulation of compatible solutes, biological molecules that can be present at high concentration without interfering with metabolic processes, be it through active-transport from external media or increase in *de novo* synthesis is universally-conserved in osmoregulation. In gram-negative bacteria like *Escherichia coli*, regulation of the periplasm can add an additional layer of complexity to hyperosmotically-induced changes. Changes in the composition of membrane lipids also occur as a result of increased salinity, comprised predominantly of the substitution of anionic lipid in favor of zwitterionic lipids [33]. Osmoregulation typically consists of two distinct phases, an acute phase in response to hyperosmotic shock, involving the uptake of K+ and an appropriate counter-ion, followed by a chronic persistent phase after physiological pathways for adaptation have been implemented [34]. The osmoregulatory persistent phase triggers the preferential active transport of a number of compatible-solutes or *de novo* synthesis of osmoprotectants in the events that none can be procured from the environment and the organism is capable. The most common compatible solutes, which

can vary depending on species, fall into four predominant categories, ions such as K+, sugars and sugar-polyol derivatives like trehalose, amino acid and amino acid derivatives including Glx, Ala, and Pro, and ectoines [35].

4.3.2 Chloride-Dependence and Synthesis of Osmoprotectants

The ability for organisms to grow in the presence of elevated salt is largely dependent on other details of the local environment [19]. We observed the salt-tolerance threshold for *P. denitrificans* to be lower in cells grown on minimal synthetic media than previously reported tolerances for these bacteria in complex media [29]. A requirement for chloride ions in the media at even moderate levels of salt (less than 400 mM NaCl) for growth of *P. denitrificans* [29] suggests that these bacteria employ a strategy of accumulating of intracellular K^+ and Cl^- to maintain solute equivalence as a component of this organism's adaptation to salt. The ability of *P. denitrificans* to recruit K^+ at both a high velocity and against a large concentration gradient adds plausibility to this explanation [36]. There have not been previous reports of K+ intake and N-isotope responses in amino acids, although induction of necessary transport proteins may raise overall amino-acid production levels. Additionally, overexpression of Glu as an alternative counter-ion to K+ is often employed in gram-negative bacteria in response to salt [19]. In *E. coli*, increased synthesis of Glu occurs within minutes of K+ uptake [37]. We detect no significant differences the N-isotope ratio of Glx with increasing salt concentration. It is possible that the amount of Glu in the cell, which

can be increased to concentrations greater than 90% of free amino acids in the cell [38] is high enough that its role as N-donor to other amino acids and subsequent products is overwhelmed by its need as an osmoprotectant. At concentrations greatly exceeding the other amino acids, even relatively large shifts in downstream amino acid synthesis requiring transamination of N from Glu would not greatly affect the overall N-isotope ratio of the residual pool.

In Gram-negative bacteria, the active transport of external osmoprotectants into the cell greatly enhances their ability to survive in high salt environments and is the preferred method of accumulation. In the absence of osmoprotectants in the media, bacteria must rely on *de novo* synthesis, which, of the common compatible solutes, includes only trehalose and the amino acids, Ala, Pro, and Glx [34, 35, 39, 40]. It is unlikely that the synthesis of the carbohydrate, trehalose, which contains no N, would have a large impact on N isotope ratios given that the metabolisms of N and C in the cell are largely independent. The linear relationship between the isotope ratios of Pro and Ala and salt, however, suggests that these two amino acids could be prominent players in this organism's response to hyper-salinity. Near the limit of salt-tolerance at 1.75% (300 mM NaCl), isotope ratios of Pro and Ala approach those of Glx, indicating that the continual synthesis of these amino acids during exponential growth is occurring at a high rate.

4.4 Conclusions

An isotopic response was observed in the $^{15}N/^{14}N$ isotope ratios of amino acids extracted from the bacteria, *P. denitrificans*, which may reflect the accumulation of amino acids, specifically, Pro and Ala, as a factor of osmoregulation. It is possible that other causes of increased amino acid production or protection from catabolism may have a similar result in increasing amino acid N-isotope ratios. By changing only the salt concentration in the media, we were able to illicit an isotopic response which is indicative of a change in overall physiological state. This information, in conjunction with measurements of physiological isotopic responses to other biological stresses, represents a critical component of the developing field of physiological isotope fractionation.

The *in vivo* physiological fractionation of isotopes is a complex system of fluxes which simultaneously affect the resulting net isotope ratios observed in measured intermediates. For N, this phenomenon has assisted in the reconstruction of food webs and greatly increased the understanding of N metabolism in plants and N cycle rates [41]. While it has been shown that environmental conditions concerning both source and, to a far lesser degree, non-source-related parameters, have an effect on isotopic fractionation, specific examples of changes in physiological fractionation as a result of minute changes in environment are rare. We show an isotopic response with respect to salt-tolerance and osmolarity in environmentally relevant bacteria over a relatively small but common range of concentrations, showing that N-isotope ratios in amino acids are extremely sensitive to physiology.

The linearity of response in the bulk level and in five amino acids suggests that physiological changes over this range behave predictably and may be accounted for. Measurements of this type are crucial to increasing our understanding of N-fractionation as a means of inferring physiological state.

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CHAPTER 5

Strategies and Potential Applications for Physiological Fractionations of Isotopes at Natural Abundance

5.1 Introduction

Current paradigms in biology are dominated by fields such as proteomics and genomics which allow for simultaneous measurement of a multitude of factors affecting physiology. The complex interaction between the genome and proteome is the basis for biological development and adaptation. Given the nature of physiological isotopic fractionation, it is logical that subtle, associated, changes in metabolism may yield characteristic isotopic signatures indicative of physiological state. Furthermore, the persistence of long-lived molecules in the body [1-3] provides an additional resource for assessing historical molecular information unattainable by otherwise equivalent techniques. These avenues of knowledge have yet to be explored, but they represent a wealth of information that is becoming increasingly available for study. The results and conclusions of this thesis demonstrate that physiological adaptations to various stresses affect the distribution of $^{15}N/^{14}N$ isotope ratios at natural abundance in amino acids of micro-organisms in a characteristic manner, suggesting that these procedures can serve as potential diagnostic methods.

The development of mass spectrometric techniques to enhance isotope ratio analysis has resulted in an unprecedented capability to exploit the presence of intrinsic, natural tracers in our environment. Modern geological, biochemical, physiological, and nutritional research

are replete with the use of enriched isotopic tracers [4-6] to provide qualitative and quantitative information on absorption, synthesis, degradation, and turnover of intermediates. This level of isotopic enrichment necessarily obscures the potential observation of physiological fractionation of isotopes at natural abundance, which are frequently too subtle to be observed by the even most sensitive of mass discriminators. The advent of compound-specific analysis (CSIA) has drastically decreased the often complicated and tedious procedures previously required for consistent measurements of isotopes. Consequently, the field of isotope ratio mass spectrometry (IRMS) and the numerous areas of specializations which rely on IRMS have seen mutual benefit from these advancements. Our report of the first *in vivo* position-specific measurement (PSIA) of nitrogen fractionation in amino acids provides a means to a previously unattainable level of isotopic detail. Underlying mechanisms of physiological fraction continue to be elucidated as fractionations in bacteria, plants, and animals are reported and analyzed. The logical extension of physiological fractionation of isotopes at natural abundance to studies of human metabolism may produce new diagnostic techniques to assess physiology.

5.2 Isotopic Analysis of Amino Acids In Humans

Measurement of isotope ratios at levels of natural abundance in humans has been performed relatively few times, largely due to limitations of mass spectrometric techniques for nitrogen. Of recent note, Petzke and Metges demonstrated a relationship between source

of dietary protein, animal or vegetable, and N-amino acid isotope ratios in hair for humans [7-9]. Spalding showed a dramatic correlation between ^{14}C in genomic DNA and historic atmospheric levels, which can be used to establish the time point when the DNA was synthesized and cells were born [3]. These studies focus on the recording of source information into tissues and intermediates, but fail to regard the contribution of physiological fractionation to the distribution of amino acid isotope ratios. Amino acids, in general, can be prominent players in gene expression [10], and the effects of amino acid metabolic disorders are widespread. It is reasonable to assume that N-isotope ratios of amino acids in human plasma and proteins, which record the metabolic pathways through which they are formed and degraded, may yield meaningful information regarding specific disease states.

5.2.1 Plasma Amino Acids

The analysis of free amino acids in plasma does not require acid hydrolysis. With respect to our existing techniques, this results in the beneficial side-effect of being able to differentiate between Glu/Gln and Asp/Asn, two pools of amino acids whose identities are obscured by traditional acid hydrolysis. Access to Gln and Asn, specifically, provides two additional candidates for intramolecular N analysis, for which isotopic methods already exist [11]. The N-sidechain of Gln is the exclusive NH_3 donor for synthesis of Cytosine Tri-phosphate (CTP) and a partial donor of NH_3, along with Asp in the construction of the purine precursor, inosine monophosphate (IMP). The metabolism and catabolism of Asn is of particular interest for treatment of certain

forms of leukemia [12]. Due to the critical involvement of these two amino acids in metabolism and disease, the intramolecular distribution of $^{15}N/^{14}N$ in Gln and Asn may be a significant biomarker for relevant metabolic disorders.

5.2.2 Amino Acids in Proteins

The metabolism and fate of individual amino acids differ according to their essentiality, as not all amino acids can be synthesized *de novo*. Additionally, the function of some amino acids is unique in proteins, with some serving predominantly as structural building blocks while others serve crucial roles in regions of catalytic activity. The behavior and function of protein-bound Lys and the synthesis of carnitine, a non-standard amino acid required for transport of fatty acids into the mitochondria for oxidation [13, 14], highlights a specific use for Lys and its intramolecular distribution of N, specifically in proteins; carnitine cannot be synthesized from free Lys. Although Lys is an essential amino acid which is only available from the diet, its explicit catabolism in protein may be insightful and suggests that ideal strategies for elucidation of N-amino acid metabolism should evaluate free- and protein-bound amino acids separately.

5.3 Conclusions

The extension of N-amino acid isotopic analysis as a potential diagnostic tool for human disease requires elucidation of the underlying mechanisms of physiological N-fractionation. The ability to measure isotope ratios at natural abundance provides an opportunity

to study isotopic fractionation in an unlimited number of compounds, although, from the standpoint of metabolism, as well as consideration of current predictive, fractionation models, some intermediates are arguably better candidates than others. Nevertheless, there is no doubt that more studies of isotopic fractionation, specifically for N, are required before an appropriate model can be formulated. We report an isotopic response in N-amino acids at the compound- and position-specific level with regard to various physiological stresses in micro-organisms. These findings represent a critical step in the endeavor to extend principles of isotopic fractionation to higher life-forms.

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