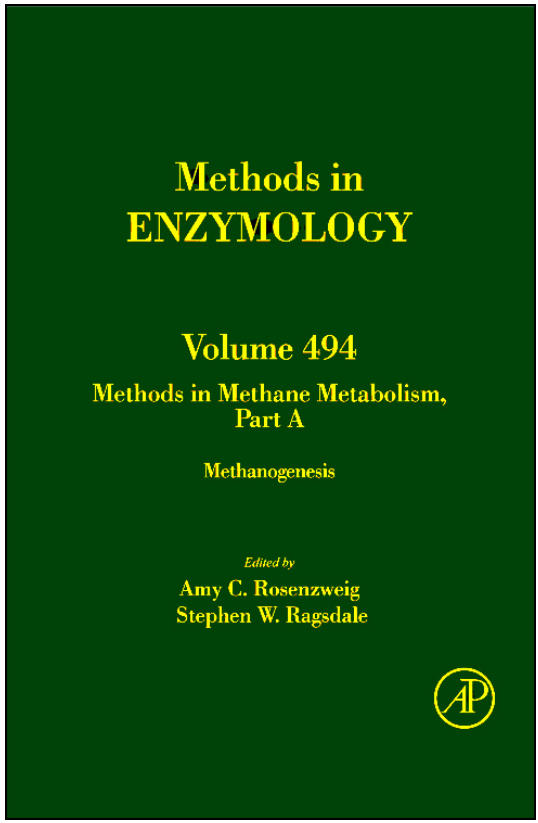

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CHAPTER FOURTEEN

Measuring Isotope Fractionation by Autotrophic Microorganisms and Enzymes

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Contents

Abstract

Physical, chemical, and biological processes commonly discriminate among stable isotopes. Therefore, the stable isotope compositions of biomass, growth substrates, and products often carry the isotopic fingerprints of the processes that shape them. Therefore, measuring isotope fractionation by enzymes and cultures of autotrophic microorganisms can provide insights at many levels, from metabolism to ecosystem function. Discussed here are considerations relevant to measuring isotope discrimination by enzymes as well as intact cells, with an emphasis on stable one-carbon isotopes and autotrophic microorganisms.

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1. Introduction

Many biologically relevant elements occur as multiple stable (nonradioactive) isotopes in nature. Carbon, which is the focus of this chapter, exists primarily as ^{12}C, with approximately 1% as ^{13}C. Given that both isotopes have the same valence electrons, both can form the same compounds and are readily assimilated into biological materials. However, covalent bonds with ^{12}C atoms are typically more labile than those with ^{13}C, due to lower zero-point energies for bonds with the heavier isotope, which result in larger activation energies (Cook, 1998; Melander and Saunders, 1980). The relative rates of ^{12}C and ^{13}C reaction vary subtly from process to process, and can be used to identify as well as quantify biological and abiotic activities (Hayes, 1993).

Studies using ^{12}C and ^{13}C to describe processes use a reasonably standard terminology to describe isotopic compositions, as well as the relative rates of their reactions. The relative amounts of ^{12}C and ^{13}C are described as isotope ratios ($R = {}^{13}C/{}^{12}C$). Given that R values are cumbersome (e.g., currently, $R_{atmCO_2} \sim 0.011145$; CDIAC, ORNL) and typically differ by <0.001, stable carbon isotopic compositions are usually reported as $\delta^{13}C$ values:

$$\delta^{13}C = \left(\frac{R_{sample}}{R_{std}} - 1\right) \times 10^3‰ \tag{14.1}$$

(Hayes, 1993), in which R_{std} is the isotope ratio of the PeeDee Belemnite standard. Differences in relative rates of reaction of ^{12}C and ^{13}C are expressed as kinetic isotope effects ($\alpha = {}^{12}k/{}^{13}k$). For most processes, α values are close to 1; as with R values, kinetic isotope effects are usually reported as fractionation factors (ε values) to magnify their differences from one another:

$$\varepsilon = (\alpha - 1) \times 10^3‰ \tag{14.2}$$

Enzymatic reactions fractionate carbon to varying degrees. For example, carboxylation by Calvin–Benson–Bassham (CBB) cycle carboxylase ribulose 1,5-bisphosphate carboxylase/oxygenase (RubisCO) has a rather large fractionation factor compared to other carboxylases. RubisCO from spinach has an ε value of 29‰ (Roeske and O'Leary, 1984), while maize phosphoenol carboxylase fractionates substantially less ($\varepsilon = 2.9‰$; O'Leary *et al.*, 1981). It is important to note that enzymes isolated from different organisms can have markedly different fractionation factors. For example, RubisCO from other organisms fractionate less than the spinach enzyme (18–24‰; Guy *et al.*, 1993; Robinson *et al.*, 2003; Roeske and O'Leary, 1985; Scott, 2003; Scott *et al.*, 2007).

Enzyme fractionation factors affect the isotopic composition of an organism's biomass, and can be used to infer metabolism *in situ*. Due to the relatively large fractionation factors by RubisCO enzymes, microorganisms using the CBB cycle for carbon fixation often have ^{13}C-depleted biomass. Cultures of these organisms have biomass $\delta^{13}C$ values of -11 to $-26‰$ relative to dissolved inorganic carbon (DIC $= CO_2 + HCO_3^- + CO_3^{-2}$; Madigan *et al.*, 1989; Pardue *et al.*, 1976; Quandt *et al.*, 1977; Ruby *et al.*, 1987; Sakata *et al.*, 2008). Autotrophic methanogens using the Wood–Ljungdahl pathway for CO_2 reduction and uptake fractionate even more than CBB autotrophs, with biomass values that are -22 to $-38‰$ relative to DIC (Fuchs *et al.*, 1979; Londry *et al.*, 2008). Autotrophic microorganisms using either the reductive citric acid cycle or hydroxypropionate cycle fractionate CO_2 to a lesser degree, with $\delta^{13}C$ values that are -1 to $-12‰$ relative to DIC (Hügler *et al.*, 2005; Quandt *et al.*, 1977; vanderMeer *et al.*, 2001a,b; Williams *et al.*, 2006). Given the differences in isotope discrimination by cultured autotrophs that use different pathways, it is sometimes possible to infer the autotrophic pathway that microorganisms are using *in situ* based on biomass $\delta^{13}C$ values. For example, Yellowstone mats with a mixed chloroflexus/cyanobacterial community have $\delta^{13}C$ values that are more positive than would be expected for CBB autotrophs, consistent with organic carbon input from the hydroxypropionate cycle present in chloroflexi (vanderMeer, 2007).

2. Enzyme-Level Studies

2.1. General assay considerations

To measure an enzyme's kinetic isotope effect, the enzyme and substrate(s) are incubated together under physiologically relevant conditions of pH, temperature, and cofactor presence, and the reaction is monitored by recording the stable isotope composition, as well as the concentration, of substrate(s) and/or product(s). Either a single-timepoint or a multiple-timepoint approach can be taken. For the single-timepoint approach, the enzyme is incubated with a vast overabundance of substrate and the reaction is terminated before the concentration of substrate is significantly diminished, or its isotope composition significantly impacted. The stable isotope compositions of the substrate and product are compared and used to calculate the kinetic isotope effect directly:

$$\alpha = \frac{R_s}{R_p} \tag{14.3}$$

where R_s and R_p are the isotope ratios of the substrate and product, respectively.

For the multiple-timepoint approach, the enzyme incubation is assembled as a closed system with respect to substrates and products. The reaction is sampled over a timecourse and both the concentrations and stable isotope compositions of target compounds are measured; kinetic isotope effects are calculated using the Rayleigh distillation equation (RDE) to account for the cumulative effect of isotope discrimination on the composition of the reaction substrate and product (Fig. 14.1). If the isotope composition and concentration of the substrate are being measured, the appropriate version of the RDE is

$$\frac{R_{st}}{R_{si}} = \left(\frac{C_{st}}{C_{si}}\right)^{1/\alpha - 1} \tag{14.4}$$

where R_{st} is the isotope composition and C_{st} is the concentration of substrate at a particular timepoint t, and R_{si} and C_{si} describe these quantities at the beginning of the reaction. The corresponding RDE when measuring the isotope ratio of accumulated product instead of substrate is

$$\frac{R_{pt}}{R_{si}} = \frac{\left[1 - \left(C_{pt}/C_{si}\right)^{1/\alpha}\right]}{\left[1 - C_{pt}/C_{si}\right]} \tag{14.5}$$

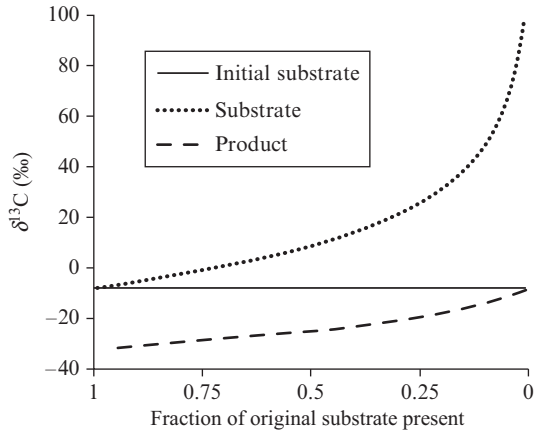

Figure 14.1 Isotopic composition of the substrate and the product as a reaction with a kinetic isotope effect progresses as a closed system. For this example, $\varepsilon = 25‰$ ($\alpha = 1.025$). Changes in the $\delta^{13}C$ value of the substrate and the product were modeled with the Rayleigh distillation equation (Eq. (14.4)). As the reaction progresses, due to selective consumption of the lighter isotope (^{12}C in this case), the remaining substrate is ^{13}C-enriched (and therefore has a more positive $\delta^{13}C$ value).

where R_{pt} and C_{pt} are the isotope ratio and concentration of accumulated product at timepoint t, and C_{si} is the initial concentration of substrate (see (Mariotti *et al.*, 1981) for review and algebra, but note that he calculates α as R_p/R_s).

The multiple-timepoint approach has the advantage of providing an estimate of α with associated error from a single incubation as it is sampled at multiple timepoints, which can provide "quality control" for reactions in which it is anticipated that isotope fractionation will be constant despite changes in C_{st}. Under these conditions, should isotope fractionation change over the timecourse of the reaction, it will suggest the necessity of further scrutiny either of reaction conditions or the assumption of a constant fractionation factor; such a "checkpoint" is absent from a single-timepoint incubation. Though this approach provides an estimate of error for α, it must be noted that this error estimate is with respect to this single experiment. Replicate incubations should be run, and the estimate of error should be based on α values from independent incubations (see below for calculations).

2.2. Setting up the enzyme assay

It is necessary to prepare the target enzyme in such a manner that its activity is as high as possible; high enzyme activity will result in large changes in substrate concentration and isotope ratios, which in turn yield data with more favorable signal-to-noise ratios. Ideally, purified enzyme will be used, as this will minimize the chance that the observed isotope fractionation is due to enzymes other than the one under study. However, in many cases, enzymes lose activity during the purification process and it is necessary to use partially purified enzymes or cell extracts. If this is the case, it is necessary to run control experiments to ensure that the observed reaction is indeed catalyzed by the target enzyme. For example, parallel incubations can be run, in which the target enzyme is inactivated (via the absence of a substrate or critical cofactor), and activity, if any is observed, is quantified.

With respect to the reaction vessel and conditions, a few commonsense considerations must apply. Given that incubation duration can sometimes extend to hours to allow the reaction to proceed sufficiently, it is necessary for the incubation to be sterile. The reaction vessel must be well mixed over the course of the reaction, as diffusion itself has a kinetic isotope effect (for CO_2, $\varepsilon = 0.7‰$ at 25 °C; O'Leary, 1984). Since the kinetic isotope effect for the enzyme is calculated from some combination of the isotope ratios of the substrate, product, and their concentrations, the reaction must be a closed system with respect to substrate (and product, if measured). Addition of substrate to supplement an ongoing reaction must be avoided, as it will likely change the isotope ratio of the substrate pool available to the enzyme,

which in turn will impact the isotope ratio of the product, and therefore the α value calculated from that experiment.

Incubations with gaseous substrates and/or products must be undertaken with particular care. In this case, given the necessity of a closed system, as well as the kinetic isotope effect associated with diffusion, the reaction vessel must be fabricated from materials that are impermeable to the substrate or product (whichever is being measured to estimate the kinetic isotope effect). Furthermore, single-phase incubations (e.g., an aqueous assay without gaseous headspace) are optimal because two-phase incubations require an assumption of chemical and isotope equilibrium between the gas and aqueous phases, quantification of target compound in both phases, and application of the equilibrium isotope effect for dissolution (for CO_2, $\varepsilon = 1.1‰$ at 25 °C; Mook *et al.*, 1974) when calculating the kinetic isotope effect for the enzyme.

If the kinetic isotope effect of an enzyme will be calculated from the concentration and isotopic composition of one of the components of the DIC system, it is absolutely essential to keep the system in chemical and isotopic equilibrium. Abiotic interconversion of CO_2 and HCO_3^- is relatively slow, as full chemical and isotopic equilibration takes a few minutes at room temperature (Zeebe and Wolf-Gladrow, 2003). The interconversion of CO_2 and HCO_3^- also has an equilibrium effect of

$$\varepsilon_{b,c} = \left[\frac{R_{CO_2}}{R_{bic}} - 1\right] \times 10^3 = \left(\frac{-9.866 \times 10^3}{T}\right) + 24.12 \qquad (14.6)$$

(Mook *et al.*, 1974), in which T is the temperature in Kelvin. This equilibrium isotope effect results in dissolved HCO_3^- having a $\delta^{13}C$ value approximately 9‰ more enriched than dissolved CO_2 at 25 °C. When assaying for inorganic carbon assimilation or production, it is optimal to add carbonic anhydrase at sufficient activity to substantially outpace DIC consumption by the target enzyme. This will keep the DIC system in chemical and isotopic equilibrium.

2.3. Analytical concerns

Given the emphasis here on autotrophic processes (including methanogenesis), this section will focus on quantification issues with respect to DIC and methane, as well as the multicarbon products resulting from biological fixation of one-carbon compounds.

2.3.1. Dissolved inorganic carbon (CO_2, HCO_3^-, CO_3^{-2})

DIC can be quantified readily via infrared gas analyzer (Scott *et al.*, 2004b), gas chromatography (Dobrinski *et al.*, 2005), or membrane-inlet mass spectrometry (Girguis *et al.*, 2000, 2002; McNevin *et al.*, 2006) if samples are acidified

to quantitatively convert all HCO_3^- and CO_3^{-2} to CO_2. Solid-state sensors also exist for quantifying CO_2, though many sensors are plagued by a lack of substrate specificity, and cross-react with other compounds (Fergus, 2007, 2008; Stetter and Li, 2008). DIC can also be quantified enzymatically via a coupled phosphoenolpyruvate carboxylase/malate dehydrogenase system (Arnelle and O'Leary, 1992), but this method is less reliable at DIC concentrations below approximately 1 m*M* (K. Scott, unpublished data).

Typically, enzymes will consume or produce only one species of DIC, which presents special issues in substrate or product quantification. It is possible to quantify CO_2 independently from HCO_3^- and CO_3^{-2} via membrane-inlet mass spectrometry and simultaneously quantify the abundances of $^{12}CO_2$ and $^{13}CO_2$ (McNevin *et al.*, 2006). Often, however, the concentrations of CO_2, HCO_3^-, and CO_3^{-2} are calculated from the DIC concentration and pH, using the appropriate dissociation constants and the Henderson–Hasselbach equation.

It is particularly important to select dissociation constants for the carbonate system carefully, as they are very sensitive to temperature, pressure, and ionic strength (Zeebe and Wolf-Gladrow, 2003). At the low-to-moderate ionic strengths present in most enzyme assays, the dissociation constant for the protonation of bicarbonate can be calculated from

$$\mathrm{p}K_\mathrm{a} = -\log\frac{[\mathrm{H}^+][\mathrm{HCO_3}^-]}{[\mathrm{CO_2}]} = \left[0.175\log\left(\frac{1}{I}\right)\right] + \left[0.2957\log\left(\frac{1}{^\circ\mathrm{C}}\right)\right] + 6.3572 \tag{14.7}$$

where I is the ionic strength of the incubation and °C is the incubation temperature in Celsius (Yokota and Kitaoka, 1985). If incubations have ionic strengths and compositions similar to seawater, the CO2SYS program can be used to calculate pK values (Lewis and Wallace, 1998). CO2SYS is available in a variety of formats from Oak Ridge National Laboratories at http://cdiac.ornl.gov/ftp/co2sys/.

Isotope ratios of DIC can be measured via conversion of DIC to CO_2, usually by adding phosphoric acid. Once the conversion is complete, the isotope ratio of the CO_2 can be determined via isotope ratio mass spectrometry, provided the instrument is sufficiently sensitive to measure natural abundance levels of ^{13}C. Newer technologies, for example, tunable laser diode systems and integrated off-axis absorption spectroscopy, provide resolution comparable to isotope ratio mass spectrometers (ca. 0.2‰; Baer *et al.*, 2002; Mihalcea *et al.*, 1997, 1998; Nagali *et al.*, 1996; Webber *et al.*, 2000). Because of the low cost and high performance of these instruments, they may soon displace isotope ratio mass spectrometers as the standard analytical methodology.

To calculate the isotopic composition of a particular component of DIC, it is necessary to take both the pK_a and the equilibrium fractionation factor for the interconversion of the different forms into account. For example, to calculate the isotope ratio of CO_2 from the isotope ratio of DIC at a pH where the dominant form of DIC is HCO_3^-, one would use Eq. (14.6), with the approximation that $R_{DIC} \sim R_{HCO_3^-}$.

Prolonged waiting periods between sample collection and DIC quantification and/or isotope ratio determination should be avoided if possible. This is particularly true if the samples have been acidified to quench the reaction and convert the DIC to CO_2. If the sample container is permeable to CO_2, such as might occur in plastic vessels, the sample will become ^{13}C-enriched due to the kinetic isotope for CO_2 diffusion (O'Leary, 1984), and/or will become contaminated with atmospheric CO_2. This can also be problematic when using glass anaerobic serum vials or autosampler analytical vials (e.g., exetainers), as the rubber stoppers used with these vials can retain inorganic carbon, causing carryover if the septa are reused. Thus, if analysis must be delayed, control experiments should be run with a standard to check whether the storage protocol significantly affects DIC concentration or isotopic composition. One fail-safe storage protocol for CO_2 is cryodistillation with a vacuum line and sealing in glass ampoules (Scott *et al.*, 2004b).

2.3.2. Methane

Unlike DIC, there is one chemical species of methane. As a consequence of its inherent stability (five atoms joined by covalent bonds), there is no abiotic oxidation or reduction to other compounds at typical laboratory conditions. For example, the abiotic oxidation of methane to carbon dioxide without ignition typically requires metal catalysts at elevated temperatures (Foger and Ahmed, 2005; Park *et al.*, 2000). Unlike DIC, methane is not highly soluble in water, nor can it be "trapped" by altering chemical conditions, for example, pH. It too can be absorbed into polymers and, for similar reasons as described above, methane stored for later analyses must be kept in appropriate vessels. Storage within glass ampoules that use low permeability rubber stoppers is acceptable in the short term. As with DIC, cryodistillation and storage in glass ampoules are preferred. Thus, storing methane requires one to consider its modest reactivity, limited solubility, and rapid diffusivity.

Methane concentrations can be readily quantified via infrared gas analyzer (Bartlome *et al.*, 2007; Borjesson *et al.*, 2007; Court and Sephton, 2009; Griffith *et al.*, 2008; Kassi *et al.*, 2008; Kim *et al.*, 2009; Uotila and Kauppinen, 2008), gas chromatography (Behrens *et al.*, 2008; Bock *et al.*, 2010; Court and Sephton, 2009; Fisher *et al.*, 2006; Harrison *et al.*, 2000; Jacq *et al.*, 2008; Kampbell and Vandegrift, 1998; Pedersen *et al.*, 2005; Tang *et al.*, 2006; Valentin *et al.*, 1985), solid-state amperometric gas sensors (Fergus, 2007, 2008; Stetter and Li, 2008), and membrane-inlet or isotope

ratio mass spectrometry (Beckmann and Lloyd, 2001; Benstead and Lloyd, 1994; Fisher *et al.*, 2006; Girguis *et al.*, 2003, 2005; Hemond *et al.*, 2008; Lloyd *et al.*, 2002; Mastepanov and Christensen, 2008; Panikov *et al.*, 2007; Schluter and Gentz, 2008; Schluter *et al.*, 2009; Thomas *et al.*, 1995; Tortell and Long, 2009), without acidification or any pretreatment. Some analytical methods, for example, infrared spectroscopy, are best conducted on dry gas to avoid the interference of water with the absorption signal. No widespread biological assays for rapidly quantifying methane concentrations exist, though some studies have used biological methane oxidation as an index for methanogenesis (Fitzgerald, 1996; Owen *et al.*, 1979).

Methane isotope ratios are measured in the same manner as for DIC. Unlike DIC, there is no cause for concern regarding pH, as it has no measureable effect of methane at the relevant time scales. In some instances, such as when measuring trace amounts of methane, the gas is combusted in a high-temperature column with oxygen and a catalyst, and the resulting DIC is "base-trapped" and concentrated for isotope ratio determination. However, the advent of higher sensitivity continuous flow isotope ratio mass spectrometers now enables parts per billion measurements of atmospheric methane with automated chromatographic preconcentration (e.g., Brass and Röckmann, 2010).

It is worth nothing that biological methane oxidation (aerobic and anaerobic) is often quantified by measuring the production of labeled DIC from labeled methane. In such cases, accurate quantification is best enabled by "base-trapping" the DIC (as carbonate) and sparging the solution with high-purity nitrogen, argon, or helium to eliminate any dissolved methane. Vaccum may also be used, but air should never be used to sparge, as atmospheric carbon dioxide will accumulate within the fluids and potentially influence the measurements. In such cases, all the aforementioned concerns regarding DIC concentration and stable isotopic measurements must be considered, in particular, pH and temperature. As above, two-phase systems should be avoided to minimize complexity.

While radioisotopic DIC is commonly available, radioisotopic methane is more difficult to commercially acquire. Many laboratories produce their own radioisotopic methane from biological methanogenesis. If such is the case, care must be taken to purify the resulting methane prior to use as a tracer. Residual labeled biological and abiotic constituents (e.g., volatile fatty acids, DIC) can confound the data, yielding artifically inflated rates of methane usage. Here again, distillation of methane or impurities using chemical or physical traps is recommended prior to use in experimentation.

2.3.3. Multicarbon compounds

If measuring an enzyme kinetic isotope effect requires determining the isotope ratio of the single-carbon unit removed or added to a multiple-carbon compound, simple combustion of this multiple-carbon compound

can result in less precise results, as was the case for early attempts to measure isotope fractionation by RubisCO. RubisCO adds a CO_2 molecule to the five-carbon sugar ribulose 1,5-bisphosphate, generating a transient six-carbon intermediate, which spontaneously converts into two molecules of phosphoglycerate. Isotope fractionation was initially calculated by comparing the isotope ratio of the CO_2 substrate to that of the phosphoglycerate produced. Since only one of the six carbons in the product resulted from RubisCO activity, it was necessary to subtract the isotope ratio of five-sixth of the sample to obtain the isotope ratio of the carbon of interest. This difficulty was remedied by purifying the phosphoglyceric acid product, enzymatically releasing the carbon atom fixed by the carboxylase, and measuring its isotope ratio (Roeske and O'Leary, 1984, 1985). If this approach is used, it is critical that the product be quantitatively decarboxylated, as many decarboxylases also have kinetic isotope effects.

3. Culture/Cell-Level Studies

To measure isotope discrimination by intact cells, many of the same considerations apply as for enzyme-level studies. However, one really critical consideration is that, if the reaction of interest occurs within the cell, or in a semi-enclosed extracellular space (e.g., a bacterial periplasm), it is likely that the isotopic composition of the substrate differs from that of the bulk medium. Accordingly, it is important when publishing values from whole-cell assays to acknowledge and explore this possibility when comparing values from enzyme-level assays.

3.1. General assay considerations

Isotope discrimination by cell cultures is measured by growing cells under conditions favorable to the process being studied, and monitoring the stable isotope composition and concentration of target compounds. As for enzyme-level studies, either a single-timepoint or multiple-timepoint approach is possible, and Eqs. (14.3)–(14.5) can be used to describe these systems. For a single-timepoint assay, a culture would be grown under conditions where the concentration and isotope composition of substrate were not substantially diminished, and α would be calculated directly from Eq. (14.3). For this approach, the best system to use would be a chemostat, as cells are growing under steady-state conditions (substrate concentration and composition will be constant). Such measurements are possible in batch culture (e.g., in a flask), but conditions (and therefore the physiological state of the cells) are continually changing in such cultures. Given that cellular

responses to changing conditions include changes in nutrient demand, transporter arsenal, and metabolic pathways, isotope discrimination is likely to vary considerably over time in a batch culture. Indeed, carbon isotope fractionation by methanogens is inversely proportional to demand (Londry *et al.*, 2008; Penning *et al.*, 2005; Valentine *et al.*, 2004).

Though problematic for single-timepoint studies, batch culture could be used for multiple-timepoint assays. If, as for enzyme isotope discrimination experiments, the culture is a closed system with respect to the target compound(s) being analyzed, it is possible to apply the RDE to such a system. This approach would be particularly attractive if isotope discrimination is small; tracking the cumulative effect of a small isotope effect as substrate is consumed will magnify the difference between the substrate and the product. As described above, however, such an approach must be used with caution, as physiological changes in the cells resulting from changing culture conditions (e.g., nutrient depletion or end-product accumulation) are likely to result in changing isotope fractionation.

3.2. Setting up the culture assay

It is ideal for cultures used for cell-level studies to be axenic, to ensure that the measured activity is due to the target organism. However, mixed-culture studies are of course more appropriate for processes that require crossfeeding.

When designing the experiment, it is helpful to consider all possible sources and sinks of the target compounds to be measured. For example, the growth vessel should be selected with care to ensure that the target compounds are not contaminated with atmospheric inputs, and conversely cannot be depleted via diffusion from the growth vessel. Additionally, inocula should be small to minimize the effect of prior cultivation conditions on the isotopic composition of substrate and biomass. Furthermore, if isotope discrimination is to be estimated by comparing the isotope composition of substrate to products, it is important to capture, quantify, and analyze all substrates and products (e.g., both biomass and secreted compounds).

For isotope discrimination studies in which cells are cultivated in a chemostat and a single timepoint is taken, it is necessary to verify steady-state conditions. For example, one could measure whether biomass, substrate, and/or product concentrations are constant for a reasonable interval before and after sampling.

Another commonsense consideration for cultivation experiments, be they batch- or chemostat culture based, is that the vessel must be well stirred to prevent localized nutrient depletion as well as undue influence from the kinetic isotope effect associated with diffusion.

3.3. Analytical concerns

3.3.1. Headspace analyses

It is common, with gaseous substrates such as methane and CO_2, to measure headspace gas composition and calculate dissolved gas parameters from these analyses. However, this calculation assumes isotopic and chemical equilibrium between the two phases. This assumption is problematic, as diffusion is a slow process with a kinetic isotope effect; even at low cell densities, organisms can exert large multipermil effects on the dissolved substrate. Any assertion of chemical and isotopic equilibrium between the phases of a two-phase culture must be verified with measurements. Reliance on headspace analysis is likely a major contributor to the heterogeneity in isotope fractionation observed for culture studies.

3.3.2. Biomass

Cells that are to be used for isotopic analyses should be washed upon harvesting to remove compounds that could skew estimates of biomass isotopic composition (e.g., DIC). If possible, it is helpful to design the growth medium to minimize the presence of compounds that could compromise the interpretation of the biomass values. For example, if the $\delta^{13}C$ value of biomass in a methanogen is going to be compared to the $\delta^{13}C$ of the DIC in the growth medium, it is wise to use inorganic buffers to prevent the influence of the isotopic signal from an organic buffer (e.g., TRIS or HEPES) that may be difficult to completely remove from cells harvested from a culture.

Biomass must be combusted prior to mass spectrometry, and it is important to verify complete combustion, as early combustion products are likely to be ^{12}C-enriched due to differences in bond energy between ^{12}C and ^{13}C. Complete combustion can be verified either by comparing to an organic standard combusted in parallel with a sample, or detected gravimetrically.

4. Calculations

To calculate a kinetic isotope effect from a multiple-timepoint assay using the RDE, it is simplest to use a linearized form of Eq. (14.4). It has been common practice to simply natural log-transform the RDE

$$\ln\left(\frac{R_{st}}{R_{si}}\right) = \left(\frac{1}{\alpha} - 1\right) \ln\left(\frac{C_{st}}{C_{si}}\right) \tag{14.8}$$

However, this form of the equation places more emphasis on the initial measurement than on the other timepoints, since each timepoint is divided by the initial one (C_{si}, R_{si}), which can skew the estimate of α (McNevin

et al., 2006; Scott *et al.*, 2004a). A better approach is to regress the natural log of the substrate isotope ratio on the natural log of the substrate concentration:

$$\ln(R_{st}) = \left(\frac{1}{\alpha} - 1\right)\ln(C_{st}) + \ln\left[\frac{R_{si}}{C_{si}^{1/\alpha-1}}\right] \tag{14.9}$$

If the product is monitored, it is not possible to linearize Eq. (14.5) to estimate α. Nonparametric bootstrapping could be used instead. To obtain an unbiased estimate of α, if N datapoints are taken, the R and C values from each datapoint could be used to calculate N estimates of α. One might be concerned about simply using the mean and sample standard error for such a derived quantity, because the sampling distribution may well not be normal. To derive a bootstrap estimate, a set of N-1 of these α values could be chosen at random (sampling with replacement), saving the mean of these (α'). If this procedure were repeated a large number (1000 or 5000) of times, the mean of these α' values would be an unbiased estimate of the mean α. A 95% confidence interval would be given by finding the smallest and largest 2.5% of the α' values.

If the substrate or product of interest is one of the components of the DIC system, it is necessary to modify these equations to include DIC equilibrium isotope effects. For example, if the substrate for an enzyme reaction is CO_2, and the concentration and isotopic composition of DIC are being monitored, Eq. (14.9) must be modified to calculate α. If

$$K = \frac{R_{HCO_3^-}}{R_{CO_2}} \tag{14.10}$$

and the reaction conditions are circumneutral so that HCO_3^- is the dominant form of DIC present, combining Eq. (14.9) and Eq. (14.10) results in

$$\ln(R_{DICt}) = \left[\frac{1}{\alpha K} - 1\right]\ln(DIC_t) + \ln\left[\frac{R_{DICi}}{DIC_i^{(1/\alpha K)-1}}\right] \tag{14.11}$$

where R_{DICt} and DIC_t are the isotope ratio and concentration of DIC at a timepoint, R_{DICi} and DIC_i are the corresponding values for the DIC initially present in the reaction (Scott *et al.*, 2004b).

These linear forms of the RDE assume that α is constant over the course of the reaction. As mentioned above, some enzymes do not fractionate to the same degree at all concentrations of the substrate, and cells in a closed system often fractionate differently when growth conditions change. If this is the case, it is not appropriate to apply these equations.

The best estimate of α, and the error associated with it, will be obtained with multiple independent reactions or cultures. Often, different reactions will have different numbers of datapoints, and different amounts of error associated with their individual α values. Taking the average of the collected α values weighs more-informative reactions (more datapoints) equally with less-informative ones (fewer datapoints). To elucidate the best approach to take when substrate concentrations and isotope compositions are measured, simulated datasets with realistic measurement error in DIC quantification and R_{DIC} were used to find the method of combining datasets that had the best probability of generating α values and associated error estimates that covered the "true value" of α (Scott *et al.*, 2004a). If the error is the same for each individual dataset, combining the reactions into a single regression with dummy variables for each reaction reliably generated accurate estimates of α (Scott *et al.*, 2004a). For example, to combine data from two independent reactions, Eq. (14.9) would be modified to

$$\ln(R_{st}) = \left(\frac{1}{\alpha} - 1\right)\ln(C_{st}) + \ln\left[\frac{R_{si1}}{{C_{si1}}^{1/\alpha-1}}\right] + \left(\ln\frac{R_{si1}}{{C_{si1}}^{1/\alpha-1}} - \ln\left[\frac{R_{si2}}{{C_{si2}}^{1/\alpha-1}}\right]\right)D2 \tag{14.12}$$

in which R_{si1} and C_{si1} are the isotope ratio and concentration of substrate initially present in reaction 1, R_{si2} and C_{si2} are the corresponding values for the second reaction, and $D2$ is a dummy variable, and $=0$ for reaction 1, and $=1$ for reaction 2. If the error is not equal between reactions, it is better to combine them using Pitman estimators (Scott *et al.*, 2004a). As Pitman estimators are a novelty beyond the discipline of statistics, and space does not permit a full description here, the reader is referred to (Scott *et al.*, 2004a), and a Matlab program to combine datasets using this method is available online at http://kmscott.myweb.usf.edu/.

If product concentrations and isotope compositions are being monitored instead, an extension of the approach suggested for a single experiment of this nature is suggested here. The populations of α' values calculated from each experiment could be compared via single-factor ANOVA. If these populations are statistically indistinguishable, they could be pooled into a single population from which the overall average α and 95% confidence interval could be determined.

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