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Subtle modification of isotope ratio proteomics; an integrated strategy for expression proteomics

Julian P. Whitelegge [a,*], Jonathan E. Katz [b], Katianna A. Pihakari [c], Rebecca Hale [a], Rodrigo Aguilera [d], Stephen M. Gómez [e], Kym F. Faull [a], Dmitrii Vavilin [f], Willem Vermaas [f]

[a] *The Pasarow Mass Spectrometry Laboratory, Departments of Psychiatry and Biobehavioral Sciences, Chemistry and Biochemistry and The Neuropsychiatric Institute, and The Molecular Biology Institute, University of California, Los Angeles, CA 90095, USA*
[b] *Department of Chemistry and Biochemistry, University of California, Los Angeles, USA*
[c] *Cedars-Sinai Medical Center, Los Angeles, CA, USA*
[d] *University of Southern California, Los Angeles, CA, USA*
[e] *Lovelace Respiratory Research Institute, Albuquerque, NM, USA*
[f] *School of Life Sciences, Arizona State University, Tempe, AZ, USA*

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Abstract

Use of minor modification of isotope ratio to code samples for expression proteomics is being investigated. Alteration of ^{13}C abundance to ~2% yields a measurable effect on peptide isotopic distribution and inferred isotope ratio. Elevation of ^{13}C abundance to 4% leads to extension of isotopic distribution and background peaks across every unit of the mass range. Assessment of isotope ratio measurement variability suggests substantial contributions from natural measurement variability. A better understanding of this variable will allow assessment of the contribution of sequence dependence. Both variables must be understood before meaningful mixing experiments for relative expression proteomics are performed.

Subtle modification of isotope ratio (~1–2% increase in ^{13}C) had no effect upon either the ability of data-dependent acquisition software or database searching software to trigger tandem mass spectrometry or match MSMS data to peptide sequences. More severe modification of isotope ratio caused a significant drop in performance of both functionalities. Development of software for deconvolution of isotope ratio concomitant with protein identification using LC-MSMS, or any other proteomics strategy, is underway (Isosolv). The identified peptide sequence is then be used to provide elemental composition for accurate isotope ratio decoding and the potential to control for specific amino acid biases should these prove significant. It is suggested that subtle modification of isotope ratio proteomics (SMIRP) offers a convenient approach to in vivo isotope coding of plants and might ultimately be extended to mammals including humans.

Keywords: *Synechocystis* sp. PCC 6803; Cyanobacteria; Stable isotopes; Isotope coding; Proteomics; Proteome

1. Introduction

Proteomics seeks to monitor the flux of protein through a biological system under variable developmental and environmental influences as programmed by the genome (Whitelegge, 2002). Consequently, it is necessary to measure changes in protein abundance and turnover rate as faithfully as possible. In the absence of non-invasive technologies, the majority of proteomics approaches involve destructive sampling at various time points to obtain 'snapshots' that periodically report the genomes's product. Thus, quantitation has become the major challenge facing the field as it matures. Because of the variability of day-to-day measurements of protein quantities, a common feature of quantitative proteomics

* Corresponding author. Department of Chemistry, 405 Hilgard Avenue, Los Angeles, CA 90095, USA. Tel.: +1-310-794-5156; fax: +1-310-206-2161.
E-mail address: jpw@chem.ucla.edu (J.P. Whitelegge).

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is the use of stable isotope coding to distinguish control and experimental samples in a mixture that can be profiled in a single experiment (Gygi et al., 1999; Conrads et al., 2003). In vivo pulse-chase experiments with stable isotopes allow for measurement of protein turnover rates (Pratt et al., 2002; Whitelegge et al., 2002). Coding with stable isotopes can be achieved by growth of an organism in depleted/enriched media or by chemically modifying proteins after extraction from the organism. Current stable isotope strategies seek full isotope exchange, that is to swap all ^{14}N for ^{15}N, ^{12}C for ^{13}C, or ^{16}O for ^{18}O, such that a peptide's mass is altered by several Daltons. These methods are expensive because of the need for high isotope purity. Furthermore, the fact that two peptide isotope distributions replace one leads to a practical loss of separation space in the mass spectrometer demanding more efficient peptide separations. Moreover, the second isotope distribution may trigger MSMS in proteomics experiments wasting mass spectrometer time.

Small fluctuations of isotope ratios occur in living organisms as a result of metabolic bias and nutrition (Meier-Augenstein, 1999). Here we investigate use of modification of isotope ratio for expression proteomics. The modification introduced will be gross compared to natural isotopic variability yet subtle compared with strategies that seek full exchange. Isotope ratio is calculated for specific peptides based upon their isotopic distributions obtained by high-resolution mass spectrometry. This requires either an estimate of elemental composition based upon mass and average amino acid elemental composition (averagine) or the precise elemental composition based upon the peptide sequence determined by tandem mass spectrometry and protein identification algorithms, as in typical proteomics experiments. In this paper, we present conclusions based upon modest elevation of the $^{13}C/^{12}C$ ratio in *Synechocystis* sp. PCC 6803 cultures. Carbon was supplied as CO_2/HCO_3^- (from bicarbonate) and entered the metabolic cycle via carbon fixation.

2. Results

The $^{13}C/^{12}C$ isotope ratio of *Synechocystis* sp. PCC 6803 grown in culture was altered modestly via manipulation of the source of CO_2 for photoautotrophic growth (bicarbonate). Soluble proteins were separated from the membrane fraction after mechanical disruption of the cells. Membrane proteins were precipitated with acetone, dissolved in formic acid and subjected to analysis by liquid chromatography electrospray-ionization

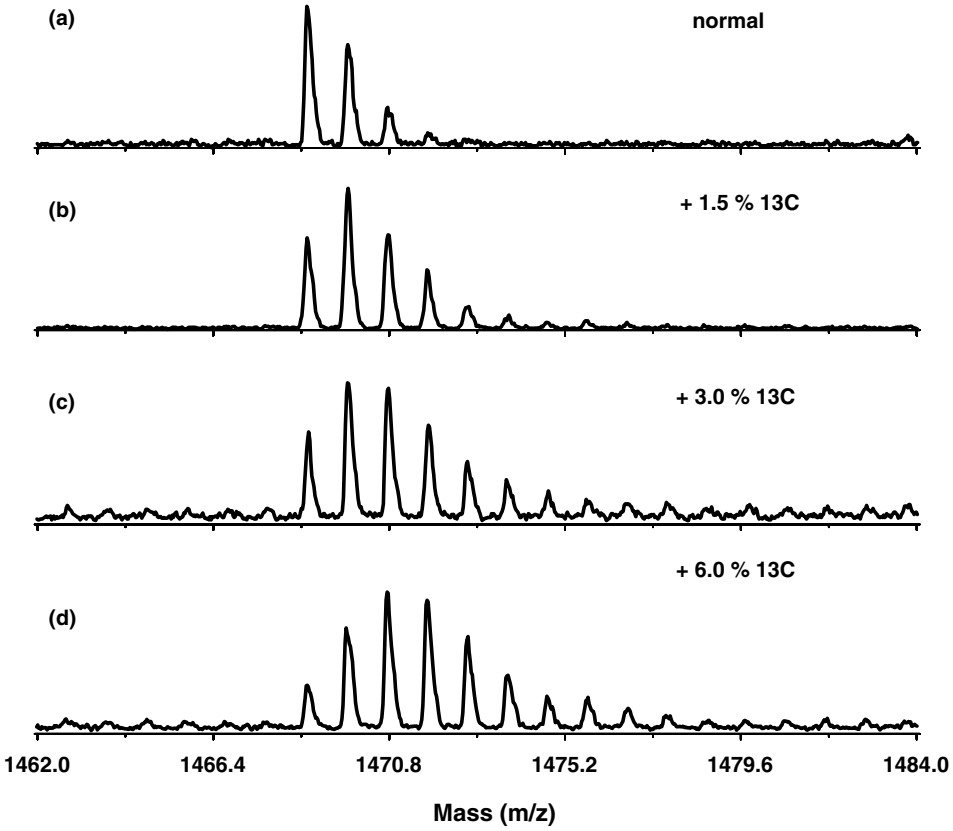

Fig. 1. Altered isotope profiles of peptides from cells grown in media with altered $^{13}C/^{12}C$ ratio. MALDI-TOF spectra of a tryptic peptide from phycocyanin a obtained from cultures of *Synechocystis* sp. PCC 6803 grown under elevated ^{13}C. The spectra show increased abundance of isotopic species containing ^{13}C. The relative abundance of these isotopomers allows computation of $^{13}C/^{12}C$ ratio (Table 1).

mass spectrometry with fraction collection (LC-MS+). Fractions were reduced, alkylated and digested with trypsin prior to MALDI-TOF analysis. Fig. 1 compares the isotopic distribution for a peptide derived from phycocyanin A from control and the ^{13}C supplemented cultures. The change in isotopic distribution is readily apparent from the spectra and $^{13}C/^{12}C$ ratio was calculated from peak heights and areas using the Isosolv algorithm (Table 1). There was a general decrease in signal to noise in the higher supplementation samples (Figs. 1(c) and (d)) with signals appearing at every unit across the mass range. This makes identification of the monoisotopic peak problematic and points to the benefits of *subtle* modification of isotope ratio. Fig. 2 shows the same peptide from μLC-MSMS analyses of reduced, alkylated and trypsinized proteins from the *Synechocystis* samples. For this experiment we used the zoom scan feature of the ion-trap mass spectrometer operating in data-dependent acquisition mode (LCQ-DECA, ThermoElectron) for our typical protein identification experiment, where ions are excluded from MSMS analysis when a zoom scan (10 Da width) shows them to be singly charged. Both single and doubly charged ions are shown, again displaying the clearly altered isotopic distributions, as in Fig. 1. Note that at higher ^{13}C supplementation the isotopic envelope for the single peptide was widened considerably, contributing to loss of separation space in the mass spectrometer; again *subtle* modification of $^{13}C/^{12}C$ ratio appears desirable. The doubly charged ions showed broadly comparable isotopic distributions to the singly charged ions with greater experimental variability apparent. The $^{13}C/^{12}C$ ratios calculated for these spectra are shown in Table 1 and compared to the results from MALDI-TOF. In the case of MALDI and zoom scans on singly charged ions by ion trap the $^{13}C/^{12}C$ ratios calculated are sufficient to distinguish the samples from each other, that is, they have been successfully isotope coded. However, the performance was less satisfactory for the doubly charged ions. Considerable variability was observed between zoom

Table 1
$^{13}C/^{12}C$ isotope ratios of peptides derived from their isotopic distribution[a]

% ^{13}C added[b]	MALDI-TOF[c]	Ion-trap[d] $M+H^+$ (height[e])	Ion-trap[d] $M+H^+$ (area[e])	Ion-trap $M+2H^+$ (height[e])	Ion-trap $M+2H^+$ (area[e])
0	0.0093	0.0114	0.0115	0.0105	0.0117
	0.0107	0.0141	0.0116	0.0092	0.0076
	0.0099	0.0134	0.0112	0.0143	0.0124
	0.0104	0.0136	0.0114	0.0119	0.0105
Mean	0.0101	0.0131	0.0114	0.0115	0.0106
SD	0.0006	0.0012	0.0002	0.0022	0.0021
1.5	0.0199	0.0178	0.0167	0.0148	0.0125
	0.0196	0.0180	0.0150	0.0117	0.0125
	0.0200	0.0164	0.0156	0.0164	0.0136
	0.0199	0.0172	0.0156	0.0175	0.0167
Mean	0.0199	0.0174	0.0157	0.0151	0.0138
SD	0.0002	0.0007	0.0007	0.0025	0.0020
3	0.0272	0.0288	0.0250	0.0244	0.0230
	0.0261	0.0281	0.0266	0.0277	0.0224
	0.0274	0.0305	0.0253	0.0225	0.0218
	0.0293	0.0276	0.0247	0.0241	0.0222
Mean	0.0275	0.0288	0.0254	0.0247	0.0224
SD	0.0013	0.0013	0.0008	0.0022	0.0005
6	0.0399	0.0358	0.0346	0.0362	0.0328
	0.0400	0.0355	0.0319	0.0396	0.0330
	0.0324	0.0344	0.0383	na[f]	na
	0.0398	0.0341	0.0315	na	na
Mean	0.0380	0.0350	0.0341	0.0379	0.0329
SD	0.0038	0.0008	0.0031	0.0024	0.0001

[a] The Isosolv algorithm (experimental) was used to calculate $^{13}C/^{12}C$ ratio for the peptide FLSSTELQIAFGR (amino acids 18–30 phycocyanin A; Q54715; elemental composition C67 H106 N17 O20; calculated $M+H^+$ 1468.7794 Da.

[b] Targeted ^{13}C addition.

[c] Sum of 300–500 laser flashes.

[d] LCQ-DECA single zoom scan data.

[e] Use of peak height versus area for calculation of isotope ratio.

[f] Not available.

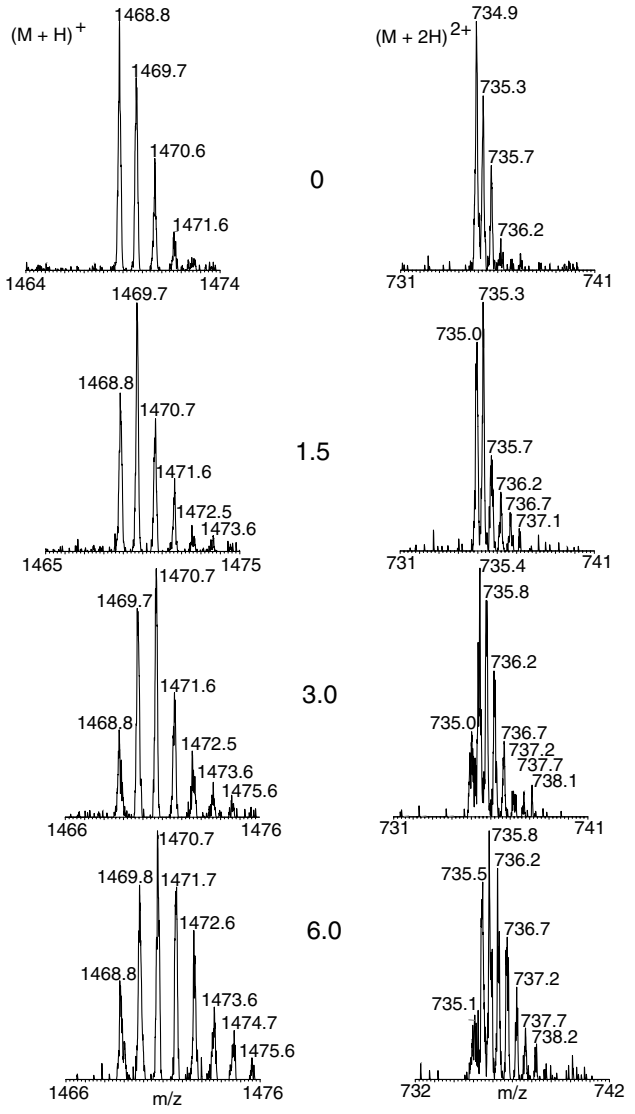

Fig. 2. Altered isotope profiles of peptides from cells grown in media with altered $^{13}C/^{12}C$ ratio: Ion-trap ESI. ESI spectra recorded on an ion-trap mass spectrometer showing spectra of the same peptide as in Fig. 1. These are single scans from a 'triple-play' protein identification experiment. The relative abundance of the isotopomers yielding $^{13}C/^{12}C$ ratio potentially provide relative expression measurement in parallel with protein identification.

scans on the same peptide (Table 1) presumably resulting from experimental variability of isotopomer capture in the ion-trap experiment. Use of peak area as opposed to height reduced measurement variability. Increasing the number of scans would improve statistics but is undesirable in the context of the proteomics experiment. Similarly, improvements in instrument performance with respect to accuracy, resolution and sensitivity will all improve reproducibility. We are currently evaluating the new generation of linear ion-traps and Fourier-transform instruments in this respect. The discrepancy between measured isotope ratio and the theoretically targeted ratio conferred by the bicarbonate is under investigation, but may be due to exchange of CO_2 with the atmosphere during the experiment. The cultures cannot be sealed due to the need to allow photosynthetically produced O_2 to escape.

It is conceptually elegant that the isotope coding strategy be compatible with existing protein identification protocols. Thus, we examined the protein identification performance in the μLC-MSMS experiments used for Fig. 2 (MSMS on the doubly charged ions only). Table 2 summarizes performance of the experiment when interrogated with Sequest for protein identifications. Note that the lower ^{13}C supplemented samples were unaffected with respect to peptides/proteins identified while a noticeable decline was observed for the highest ^{13}C sample. The widening of the isotopic envelope may lead to less frequent triggering of the MSMS experiment as maximum signal intensities drop. Also MSMS spectra may fail to yield Sequest hits as higher mass *b* and *y* fragments become too large to fall within tolerance limits for database matching. In Fig. 3, the MSMS spectra of the same 1468 Da peptide from Phycocyanin A (doubly charged parent) are compared for the control and the 3.0% supplementation samples. Note that while Sequest returned strong correct identifications for both peptides, the dominant larger *b* and *y* fragments in the 3.0% sample exhibit the mass of the first ^{13}C isotope rather than the monoisotopic mass. It is concluded that Sequest remains efficient until the

Table 2
Protein identification performance under altered $^{13}C/^{12}C$ ratios[a]

% ^{13}C added	Tryptic peptides (Xcorr[b] > 2.3)	Tryptic peptides (Xcorr > 4.0)	Peptides Phycocyanin B	ΔCn[c] Phycocyanin B
0	43	11	17	164.26
1.5	40	15	15	142.26
3.0	40	17	16	160.26
6.0	25	10	11	110.27

[a] Protein identification used Sequest (ThermoFinnigan) to match experimental tandem mass spectrometry data to a database of translated *Synechocystis* sp. PCC 6803 open reading frames. 'No enzyme' is selected so that all possible peptide sequences are screened. The results of a representative experiment are shown.

[b] The cross-correlation coefficient (Xcorr) provides a measure of how well a tandem mass spectrum matches that predicted for a particular peptide. Searches that yield tryptic peptide matches with Xcorr > 2.3 are generally significant matches. Searches that yield tryptic peptide matches with Xcorr > 4.0 are nearly always highly significant matches with good signal to noise.

[c] The delta correlation (ΔCn) is a measure of how well a number of peptide matches identify a specific protein.

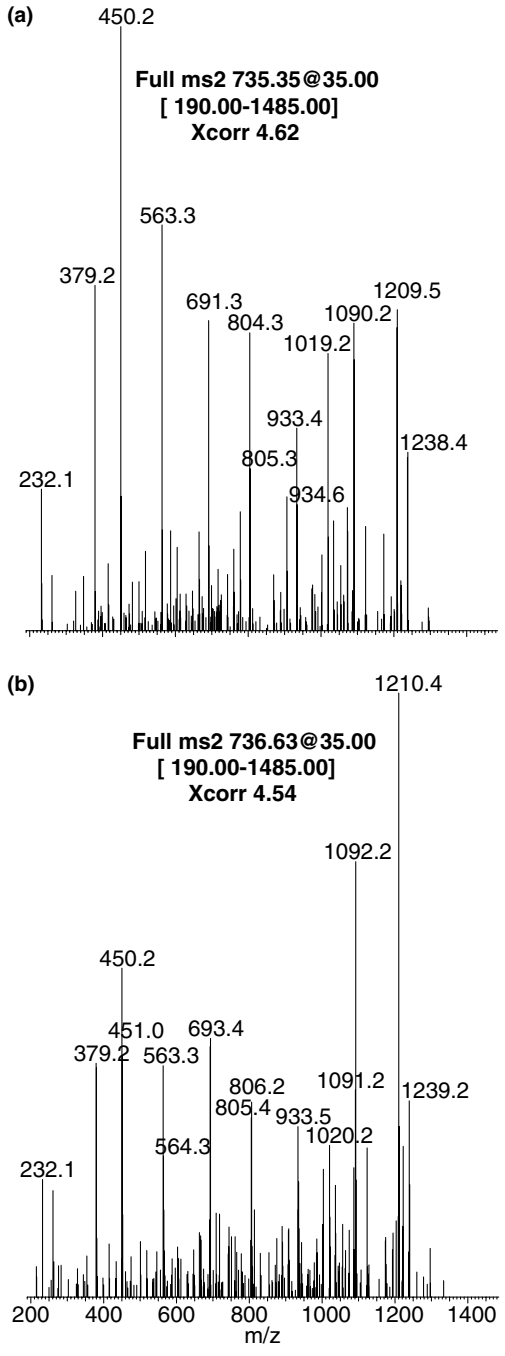

Fig. 3. Performance of protein identification by tandem mass spectrometry under conditions of modified $^{13}C/^{12}C$ isotope ratio. Tandem mass spectra from the 'triple-play' experiment shown in Fig. 2 are shown for control (a) and 3% samples (b), both derived from the doubly charged parent. Note the shift of higher mass *b* and *y* fragments in (b). Despite the increased abundance of ^{13}C isotope containing species, the protein identification experiment was still successful. Performance was compromised in the 6% sample (not shown). Successful peptide identification allows the use of elemental composition in calculation of $^{13}C/^{12}C$ ratio.

monoisotopic species is sufficiently diminished in abundance at the highest ^{13}C supplementation. It is concluded that the standard performance of Sequest tolerates subtle modification of isotope ratio in ranges that are useful for isotopic coding (+0.75–1.5% supplementation; 750–1500‰).

3. Discussion

Subtle modification of isotope ratio proteomics has been investigated as an alternative isotope coding strategy. Isotope coding was introduced to allow the distinction between two samples in a single mass spectrometry experiment in order to achieve improved relative quantitation since run-to-run variability is eliminated (Tao and Aebersold, 2003). Both chemical modification (Gygi et al., 1999) and in vivo labeling approaches (Pasa-Tolic et al., 1999) have been successful. These approaches have nearly always sought complete isotope substitution such that pairs of chemically identical species are separated by several atomic mass units dependent upon the protocol employed. A number of disadvantages have emerged, including isotope effects upon retention during liquid chromatography (Zhang et al., 2001), loss of separation space in the mass spectrometer and expense. This led us to investigate subtle modification of isotope ratio in order to code peptides by their isotopomer distributions. We chose to modify $^{13}C/^{12}C$ because carbon is the most abundant element in peptides and proteins, though the strategy could also employ $^{15}N{:}^{14}N$ or $^{18}O{:}^{16}O$ manipulation. Cargile (Cargile et al., 2004) used pulse labeling with ^{13}C to measure protein turnover kinetics although the use of high atom percentages of ^{13}C lead to dramatically extended isotope distributions that in the proteomics context would result in dramatic loss of separation space and, as is apparent in the figures presented, the appearance of peaks at every unit across the mass spectrum. The data presented in Figs. 1 and 2 show similar features when the isotope ratio is altered too dramatically and emphasize the benefits of *subtle* modification of the isotope ratio. Based upon the data presented we suggest that doubling the $^{13}C/^{12}C$ ratio provides adequate isotope coding with minimal extension of the isotopomer distribution. Others have pointed out the benefits of ^{13}C depletion (Marshall et al., 1997; Pasa-Tolic et al., 1999) and we are currently assessing this strategy in the context of SMIRP.

For SMIRP to be useful in the context of expression proteomics it will be necessary to control a number of variables such that significant changes in relative expression can be measured with quantifiable error. As the results show, there is significant variability associated with using relative isotopomer abundance for calculation of isotope ratio. The origin of this variability is most likely due to variability of relative isotopomer measurement by the mass spectrometer. Variability among different peptides as a result of their specific

elemental compositions combined with metabolic bias, and natural variability of specific peptide isotope ratio based upon changes in flux that alter metabolic bias might also be significant in the context of proteomics. Measurement variability can be addressed by increased sampling and the use of mass spectrometers with improved sensitivity and resolution while the later natural variability must be explored in detail in future research. While presenting a considerable challenge, natural variability may prove a goldmine of information with respect to cellular flux.

Soon after the development of isotope ratio mass spectrometers, it was shown that ^{13}C was naturally depleted in plants and that the extent of depletion varied among plants (Nier and Gulbransen, 1939; Murphy and Nier, 1941). ^{13}C isotope depletion measurements were integral to the discovery of C4 photosynthesis and crassulacean acid metabolism CAM; C3 plants are ^{13}C depleted by about 30‰ compared to 10–15‰ in C4 and 10–25‰ for CAM plants. [1] There are several origins of ^{13}C depletion including thermodynamic fractionation by the carboxylation activity of ribulose-bisphosphate carboxylase/oxygenase (RUBISCO, 29‰ depletion) and various physical contributions related to solubility, diffusion and hydration of CO_2 in water combined with biological bias from enzymes that impinge upon these properties. Thus, the subtle alterations to isotope ratio used for proteomics experiments must be substantially greater than these natural variations and the potential for biological isotope bias to introduce variability must be considered. While isotope fractionation during small molecule flux is quite well understood, less is known about the process with respect to protein synthesis. Recent literature has reported minor variations in isotope ratio for different amino acids from specific protein sources as well as positional effects within amino acids (Jim et al., 2003; Sacks and Brenna, 2003). ^{18}O bias has been reported in plants and has the potential to interfere with calculation of accurate $^{13}C/^{12}C$ ratios when the second "^{13}C" isotope peak is included in calculation of isotope ratio (Schmidt et al., 2001). Minor contributions from D/H and $^{15}N/^{14}N$ fractionation might also contribute minor variability.

We acknowledge that decoding isotope ratio from peptide isotope distributions will not approach the accuracy that is achieved by today's dedicated isotope ratio mass spectrometers. Unfortunately, these instruments reduce the sample to CO_2 (and water) so that any structural or sequence information is lost. Use of peptide isotopomer distribution eliminates this problem at the cost of accuracy of isotope ratio measurement.

Unlike the labeling of bacteria and plants, in which the entirety of the carbon content can be derived from CO_2 with concomitant isotopic labeling of all the polypeptides, the labeling of the polypeptides of animals is not as simple. The most straightforward way to manipulate the carbon isotopic ratio in animals would be by incorporating ^{13}C-enriched amino acids into the diet. Unfortunately, because of the differential flux of free amino acid pools, it is quite possible that polypeptides will not be homogeneously labeled. For example, the concentrations of the branched chain, aromatic and sulfur containing amino acids in humans are only minimally affected by dietary supplement (Millward and Rivers, 1988). A more direct comparison was done that shows that supplementation by both leucine and lysine at similar concentrations leads to a 2-fold increase in lysine concentration over that of leucine even though both amino acids are found in similar abundance in proteins and would therefore be expected to have the same metabolic flux (Bergstrom et al., 1990). Possibly most troublesome in terms of complexity of performing polypeptide analysis based on partial ^{13}C labeling via dietary amino acids is that many of the amino acids that are biochemically close to core metabolic pathways would be expected to undergo rapid isotopic shuffling. This prediction has been shown to be true on some occasions. For example, alanine and glutamate can undergo a transamination to become pyruvate and α-ketoglutarate, respectively; in a study of the incorporation of dietary labeled amino acids in chicken feed into egg protein only 11% of the alanine and 7% of the glutamate were incorporated without metabolic transformation (Berthold et al., 1991). Additionally, as might be expected, methionine partially behaved as an amino acid containing one less carbon due to methionine's role in methyl transfer biochemistry. These differences in flux suggest that studies in higher eukaryotes might be best performed by labeling the amino acids that show the lowest turnover. For relative expression proteomics of plants and animals it is clearly necessary to grow the organisms on isotopically modified media for as much of their life as possible to ensure uniform labeling. Pulse-chase turnover measurements will be complicated by the factors discussed above but offer the opportunity to integrate proteomics with metabolomics and global flux analysis; that is, systems biology.

3.1. Concluding remarks

Once the variability of decoding isotope ratio from peptide isotopomer distribution is understood, subtle modification of isotope ratio proteomics will provide an inexpensive and convenient means of isotope coding for

[1] 30‰ depletion changes the $^{13}C/^{12}C$ ratio from 0.0111 to 0.0108 (Ehleringer et al., 1993). In classical isotope ratio mass spectrometry the sample's isotope ratio is measured relative to a standard such that a relative rather than an absolute result is reported.

relative expression comparison in mixed samples and also for protein turnover measurement. Here we have shown that protein identification experiments are not affected by subtle modification of the isotope ratio. Thus, we envisage the incorporation of software to decode isotope ratio into protein identification packages such that relative expression/turnover information is reported concomitant with peptide identification. The elemental composition for the measured sequence could be used to improve accuracy of isotope ratio decoding and thus accuracy of relative expression/turnover calculation. SMIRP technology can be applied to any conceivable proteomics experiment including 2D gels, MuDPIT (Washburn et al., 2001), accurate mass and time tags (Strittmatter et al., 2003) and SILAC (Ong et al., 2002).

4. Experimental

4.1. Cell growth

Synechocystis sp. PCC 6803 cells were grown autotrophically in liquid BG-11 medium (Rippka et al., 1979) buffered with 5 mM *N*-tris-hydroxymethyl-2-aminoethanesulfonic acid (TES)-NaOH (pH 8.0) and supplemented with filter-sterilized mixture of $NaH^{13}CO2$ (Cambridge Isotope Laboratories; 99% ^{13}C) and $NaH^{12}CO2$ (Sigma, 1.1% of ^{13}C). The bicarbonate mixture was added to the freshly autoclaved BG-11 medium, which contained essentially no dissolved CO_2, and the bottles with the medium were sealed until further usage. The added $NaH^{13}CO_3$ was calculated to be 0%, 1.5%, 3.0%, or 6.0% of the total $NaHCO_3$ taking into account natural ^{13}C abundance ($\sim$1 $^{13}C/100$ ^{12}C) of the standard BG-11medium (20 mg/l). Cultures were started by inoculating 50 ml of the medium (in a 150 ml flask) with a small amount of cells followed by incubation at 28 °C with light intensity of 50 μmol photons/m^2 s while shaking at 100 rpm on a rotary shaker. Every 2–3 days the cultures were transferred to larger flasks and diluted with fresh BG-11 medium containing an appropriate amount of $NaH^{13}CO_3$. The cultivation continued until the cell cultures reached $OD_{730} = 0.5$ in a total volume of 500 ml. After that, cells were harvested by centrifugation, washed with thylakoid buffer (50 mM MES–NaOH at pH 7.0, 5 mM $CaCl_2$, 5 mM $MgCl_2$, 10 mM NaCl, 15% v/v glycerol, and 0.5% v/v DMSO), and then frozen in liquid nitrogen.

4.2. Protein preparation

Cells were thawed rapidly and placed on ice. Protease inhibitors (Sigma P8465; 50 μl/1 ml aliquot) were added prior to transfer of the cell suspension to tubes containing glass beads (0.1 mm; 1.0–1.2 g) pre-cooled on ice. Cells were broken using a micro-beadbeater (Biospec Products) on its maximum setting (4–5 × 30 s). Cells were cooled on ice between each treatment. Cell breakage efficiency was assessed by extraction of cells in acetone (10 μl cells plus 1 ml 80% acetone), agitation and centrifugation; chlorophyll was only extracted after cell breakage yielding a blue pellet. The broken cell suspension was diluted 10-fold with ice cold thylakoid buffer containing protease inhibitors and decanted to pre-cooled centrifuge tubes. Unbroken cells were removed (500 rpm SS34; 1 min) prior to transfer to clean tubes and sedimentation of the membranes (20,000 rpm SS34; 30 min). The supernatant was retained for soluble proteins and the pellet was resuspended in thylakoid buffer, homogenized (Teflon/glass) and stored at −80 °C.

4.3. Liquid chromatography electrospray-ionization mass spectrometry with fraction collection

Samples of *Synechocystis* membranes were analyzed by LCMS+ (Whitelegge et al., 2002). Membrane fraction proteins (300–600 μg) were precipitated at the interface of an aqueous chloroform/methanol phase separation (Wessel and Flugge, 1984) as described (Whitelegge et al., 1999). Precipitated proteins were recovered after removal of the aqueous phase and addition of methanol. Precipitated samples were dried at atmospheric pressure for 2 min (25 °C) and dissolved in 90% formic acid (100 μl) immediately prior to HPLC. Reverse phase chromatography (RPC) of intact proteins was performed as described previously (Whitelegge et al., 2002; Whitelegge, 2003, 2004) using a macroporous polymeric support (PLRP/S, 300 A, 5 μm, 2 × 150 mm; Polymer Labs) at 100 μl/min (40 °C). The column was previously equilibrated in 95% A, 5% B (A, 0.1% TFA in water; B, 0.05% TFA in acetonitrile/isopropanol, 1:1, v/v) and eluted with a compound linear gradient from 5% B at 5 min after injection, through 40% B at 30 minutes and to 100% B at 150 min. The eluent was passed through a UV detector (280 nm) prior to a liquid-flow splitter (inserted between HPLC detector and mass spectrometer) that made it possible to collect fractions concomitant with electrospray-ionization mass spectrometry (ESI-MS). Fused silica capillary was used to transfer liquid to the ESI source ($\sim$50 cm) or fraction collector ($\sim$25 cm). The split fractions were collected into micro-centrifuge tubes at 1 min intervals. ESI-MS was performed as described (Whitelegge et al., 1998) using a triple quadrupole instrument (API *III*, Applied Biosystems). Orifice voltage was ramped from 60 to 120 over the mass range acquired (600–2300) and the instrument scanned with a step size of 0.3 amu and 1 ms dwell. Data were processed using MacSpec 3.3, Hypermass or BioMultiview 1.3.1 software (Applied Biosystems).

4.4. Trypsin digestion

Selected fractions collected during LCMS+ were reduced, alkylated and treated with trypsin (Promega sequencing grade modified by reductive methylation). To aliquots of fractions (10 μl) was added DTT (15 μl, 10 mM in 50 mM ammonium bicarbonate; 30 min, 24 °C) then iodoacetamide (15 μl, 55 mM in 50 mM ammonium bicarbonate; 20 min, 24 °C) and finally trypsin (12.5 μl, 6 ng/μl in 50 mM ammonium bicarbonate; 3 h, 37 °C). After incubation, samples were dried by centrifugal evaporation and stored at −20 °C prior to analysis by μLC-MSMS.

4.5. Mass spectrometry. MALDI-TOF (matrix-assisted laser desorption ionization time-of-flight mass spectrometry)

Dried reaction mixtures were re-dissolved in 5 μl of 70% acetic acid and analyzed (0.2 μl plus 0.5 μl matrix) by matrix-assisted laser desorption ionization (MALDI) coupled to delayed extraction time-of-flight MS in the reflector mode (Voyager DE STR, Applied Biosystems) using α-cyano-4-hydroxycinnamic acid as matrix (10 mg/ml solution in water/acetonitrile/TFA 30/70/0.1) and internal/external calibration with bovine insulin. Manufacturer supplied default settings optimized for peptides less than 6000 Da were used for all samples.

4.6. μLC-MSMS (micro-liquid chromatography with tandem mass spectrometry)

Samples were analyzed by μLC-MSMS with data-dependent acquisition (LCQ-DECA, ThermoFinnigan, San Jose, CA) after dissolution in 5 μl of 70% acetic acid (v/v). A reverse phase column (200 μm × 10 cm; PLRP/S 5 μm, 300 Å ; Michrom Biosciences, San Jose) was equilibrated for 10 min at 1.5 μl/min with 95% A, 5% B (A, 0.1% formic acid in water; B, 0.1% formic acid in acetonitrile) prior to sample injection. A linear gradient was initiated 10 min after sample injection ramping to 60% A, 40% B after 50 min and 20% A, 80% B after 65 min. Column eluent was directed to a coated glass electrospray emitter (TaperTip, TT150-50-50-CE-5, New Objective) at 3.3 kV for ionization without nebulizer gas. The mass spectrometer was operated in 'triple-play' mode with a survey scan (400–1500 *m*/*z*), data-dependent zoom scan and MSMS. Individual sequencing experiments were matched to a custom *Synechocystis* sequence database using Sequest software (ThermoFinnigan).

4.7. Calculation of isotope ratio from peptide isotope distribution (Isosolv)

For a carbon isotope distribution the probability of having '*n*' ^{13}C's given '*X*' total carbons and a ^{13}C probability of '*P*' is described as follows:

$$\mathrm{prob}(n) = \mathrm{combin}(X, n) * P^n * (1 - P)^{(X-n)}.$$

When given an elemental composition Isosolv uses this for estimation of $^{13}C/^{12}C$ ratio. When given a molecular weight, the number of carbons is estimated by dividing the molecular weight by 110 (the average mass of an amino acid) and multiplying by 4.94 (the average number of carbons per amino acid). Then, given a measured isotopic distribution, the ^{13}C probability can be determined by calculating the difference between the measured distribution and the theoretical distribution for an arbitrary ^{13}C abundance. The estimated ^{13}C abundance parameters are then incrementally altered until the error between the theoretical distribution and the calculated distribution has been minimized thus yielding the ^{13}C probability in the measured spectrum. The version of Isosolv used in this communication includes natural minor contributions of D, ^{15}N, $^{17/18}O$ only.

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