Accounting for Metabolic Processes that Affect In vivo Labeling of Cell Culture using Isotopic Forms of Amino Acids in Media

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Overview

Supplementing culture media with stable isotopic forms of essential amino acids is a simple and direct way to achieve 100% labeling using the natural metabolic machinery of the cell. MS-based quantitative methods such as SILAC (Stable Isotopic Labeling by Amino Acids in Culture) use in vivo mass labeling to highlight sets of proteins affected by drug treatment, or to disentangle networks of interacting proteins and their respective cascade mechanisms. However, it must be remembered that the free amino acids themselves are subject to catabolism. In studying the membrane proteome of breast carcinoma we have observed significant partitioning of mass labels introduced with Arginine vehicle among Proline isotopic forms. Here, we how amino acid metabolism affects the repartitioning of metabolic labels and its impact on the search for biomarkers of cancer.

Introduction

Unlike carbohydrates and lipids, excess amino acids are not stored in cells but are degraded continually in conjunction with protein turnover. The α amino group is removed first and the resulting carbon skeleton cycled as a feedstock or fuel through one of the major metabolic pathways. For example, the carbon skeletons of several five-carbon amino acids (proline and arginine among them) enter the citric acid cycle through several steps that convert them to glutamate, which is oxidatively deaminated to α-ketoglutarate.

Likewise, the biosynthetic pathways that give rise to individual amino acids are intertwined because the amino acids themselves have one important feature in common: their carbon skeletons are derived from three central metabolic pathways; glycolysis (*pyruvate, phosphoenolpyruvate, 3 phosphoglycerate*), pentose phosphate pathway (*ribose 5-phosphate and erythrose 4-phosphate*) and intermediates of the citric acid cycle (*α-ketoglutarate, oxaloacetate*).

The ubiquitous housekeeping chores that catabolize amino acids into fuel and feedstock for the synthesis of many crucial biomolecules may seem to limit the use of Arginine as a vehicle for metabolic labeling of cultured cells. To date however, the only process leading to loss of sequence specific incorporation of ^{13}C has been attributed to the conversion of arginine to proline. In work on human cancer cells maintained in DMEM media labeled with heavy isotopic forms of Arginine, we found the conversion of Arginine to Proline quite prevalent, accounting for approximately 12% of the total Proline. Nevertheless, a correction for the loss of ^{13}C label to another compartment is effected by simple addition. The fact that labeled carbon from Arginine partitions no further than Proline suggests a low activity of the Glutamate γ-semialdehyde dehydrogenase enzyme.

Methods

Using SILAC approach, we labeled normal epithelial and malignant breast cells, isolated from the same patient with either light or heavy Lysine and Arginine. Two populations of cells were proliferated for at least six doubling times in light or heavy media and were then combined in a 1:1 ratio based on cell count. Cells were lysed in hypotonic buffer and a simple cell fractionation method was used to obtain crude membranes. Membrane proteins were dissolved in SDS sample buffer and partially resolved by 1D SDS-PAGE. Protein bands were divided into 40 sections, digested with trypsin and the recovered peptides were analyzed by MALDI-TOF or nESI LC-MS/MS (Q/TOF API-US, Waters). Protein identifications were performed with the Mascot search algorithm and quantification was achieved by manual analysis of corresponding peptide pairs.

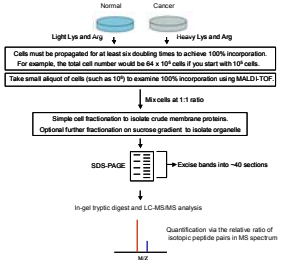

Figure 1. Experimental strategy of SILAC for quantification. Two populations of cells are propagated for at least six doubling times in medium containing either light or heavy arginine before aliquot of cells are taken to examine 100% incorporation. Cells labeled with light or heavy arginine are then combined at 1:1 ratio. Complexes are analyzed by SDS-PAGE. Protein bands are excised from the gel and digested with trypsin, followed by the analysis of MALDI-TOF or LC-MS/MS. Relative quantification is achieved by calculating the ratio of intensity of isotopic peptide pairs.

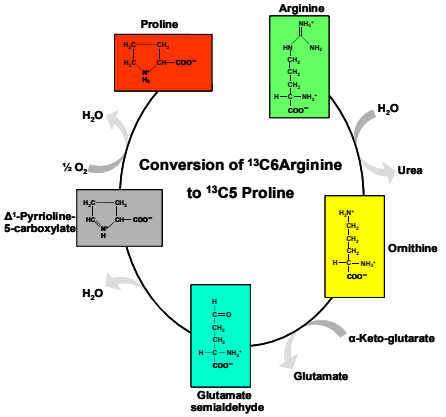

Figure 2. Schematic mechanism of ^{13}C arginie conversion to ^{13}C Proline

Results and Discussion

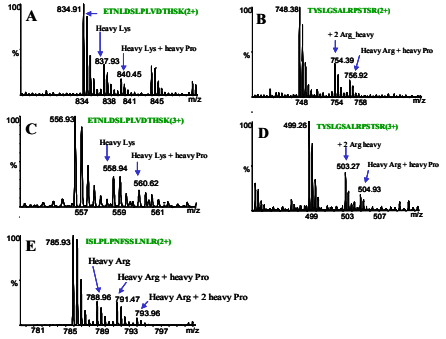

Figure 3. Evidence of conversion of Arginine to Proline. Normal and breast cancer cells were proliferated for at least doubling times in either light Lys and Arg or heavy [U-13C6]Lys and [U-13C6]Arg, and were then combined at 1:1 ratio based on cell count. Cell mixture was lysed in hypotonic buffer. After removing nuclei, crude membrane was obtained. Membrane pellets were dissolved in SDS sample buffer and analyzed by SDS-PAGE. Protein bands were excised from gel and digested with trypsin. Peptide extract was analyzed by Q-TOF. Note that heavy Arg is 6 Da larger than light Arg and satellite peaks are observed for Proline-containing peptides as Arg can be converted into Proline via α-Ketogluterate intermediate. Small satellite peak will appear at [M+2.5] Da if the peptide is doubly charged and at [M+1.6] Da if triply-charged since Proline has five carbons. The correct amount of peptides with heavy labels would be the sum of peptides with heavy Lys and/or Arg and peptides with heavy Lys and/or Arg plus heavy Proline. It would get very complicated when peptides contain multiple Prolines. Therefore, it is not recommended to use Proline-containing peptides for quantification when labeling with heavy Arg.

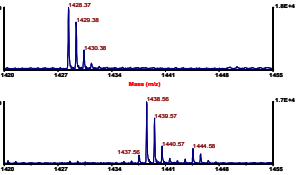

Figure 4. MALDI-TOF spectrum of Arg-containing peptide. Normal and breast cancer cells were cultured in either light Lys and Arg or heavy [U-13C6]Lys and [U-13C6, 15N4]Arg for at least six doubling times. Aliquots of cells were lysed separately and analyzed by SDS-PAGE. Protein bands were excised side by side, in-gel digested with trypsin, followed by the analysis of MALDI-TOF. In this case, heavy Arg is 10 Da larger than light Arg. Note that additional peak with 6 Da larger than heavy Arg-containing peptide was observed because heavy [U-13C6, 15N4] Arg was converted to heavy [U-13C5, 15N1] proline.

Significant hits:

gi|627367	desmoyokin - human (fragments)
gi|34536452	unnamed protein product [Homo sapiens]
gi|1477646	plectin [Homo sapiens]
gi|535177	AHNAK-related protein [Homo sapiens]
gi|21749456	unnamed protein product [Homo sapiens]
gi|24899202	KIAA2019 protein [Homo sapiens]

Table 1. Membrane proteins, especially matrix proteins, are often enriched in prolines. For example, all the tryptic peptides recovered from desmoyokin contains at least one proline. Conversion of Arg to Pro should be taken into consideration when quantification is performed.

Query	Observed	Mr(expt)	Mr(Calc)	Delta	Score	Expect	Peptide Sequence
1	407.20	812.39	812.39	0.00	15	31	FSM*PGFK
4	415.20	828.39	828.38	0.01	(15)	37	FSM*PGFK + Oxidation (M)
8	431.72	861.43	861.41	0.02	4	4.8e+002	MPE*NK
11	451.24	900.46	900.46	0.00	54	0.0045	ADDVSGPK
12	451.25	900.48	900.46	0.02	(53)	0.0054	ADDVSGPK
17	534.29	1066.56	1066.58	-0.02	13	72	GPEVDLKGPR
18	536.79	1071.56	1071.56	-0.01	45	0.041	MPSLEISAPK
21	550.79	1099.56	1099.56	0.00	29	1.3	MPDVDSVPK
27	577.84	1153.66	1153.65	0.01	52	0.0086	LNVGAPDVTLR
29	585.82	1169.63	1169.63	-0.00	77	1.9e-005	VSGPDLDLNLK
30	593.82	1185.63	1185.64	-0.01	64	0.00039	ISMPDVGLNLK
33	606.81	1211.60	1211.60	-0.00	58	0.0018	GEGPDVDVSLPK
37	620.33	1238.64	1238.61	0.02	53	0.0045	GEGPDVDVNLPK
38	620.35	1238.68	1238.69	-0.01	63	0.00046	LPTGQSGPEIK
41	627.32	1252.63	1252.63	0.00	71	7.8e-005	GEGPEVDVNLPK
45	634.33	1266.65	1266.65	0.01	70	0.00012	AEGPEVDVNLPK
46	634.34	1266.67	1266.65	0.02	(37)	0.24	AEGPEVDVNLPK
47	637.82	1273.63	1273.66	-0.03	(18)	17	ISMPDIDLNLK + Oxidation (M)
48	637.83	1273.65	1273.66	-0.01	51	0.0091	ISMPDIDLNLK + Oxidation (M)
49	637.84	1273.66	1273.66	-0.00	(8)	1.7e+002	ISMPDIDLNLK + Oxidation (M)
52	643.31	1284.60	1284.60	0.00	53	0.0046	GEGPEVDMNLPK
54	646.32	1290.63	1290.61	0.02	45	0.029	GEGPEFDVNLSK
58	708.88	1415.75	1415.73	0.03	51	0.0068	GPQVSSALNLDTSK
60	737.38	1472.74	1472.75	-0.01	58	0.0015	ISMSEVDLNVAAPK
62	745.38	1488.75	1488.75	-0.00	98	1.5e-007	ISMQDVDLSLQSPK
63	750.87	1499.73	1499.73	0.00	52	0.0053	IEGEMQVPDVDIR
64	750.89	1499.77	1499.76	0.00	36	0.25	ISAPNVDFNLEGPK
65	753.37	1504.73	1504.74	-0.02	(40)	0.088	ISMQDVDLSLQSPK + Oxidation (M)
66	758.88	1515.75	1515.72	0.02	(24)	3.6	IEGEMQVPDVDIR + Oxidation (M)
75	805.92	1609.83	1609.83	-0.00	82	5.6e-006	VNVEAPDVNLEGLGGK
76	819.42	1636.82	1636.83	-0.01	47	0.02	VGVEVPDVNEGPEGK
78	821.40	1640.79	1640.79	0.00	61	0.00066	VDTNAPDLSLEGPEGK
84	827.91	1653.81	1653.81	0.00	82	6.1e-006	VDEAPDVSLEGPEGK
87	834.91	1667.81	1667.83	-0.02	57	0.0015	VDVEVPDVSLEGPEGK
88	837.93	1673.85	1673.83	0.03	(43)	0.04	VDVEVPDVSLEGPEGK + Lys_heavy
90	841.42	1680.82	1680.82	0.00	30	0.81	VDVDVPDVNEGPDAK
91	844.43	1686.84	1686.82	0.02	(25)	2.6	VDVDVPDVNEGPDAK + Lys_heavy
94	850.40	1698.78	1698.78	-0.00	70	9.5e-005	MDAEVPDVNEGPDAK
99	856.45	1710.88	1710.88	0.00	45	0.025	VDETPNLEGTLTGPR

Conclusions

Although the glycogenic amino acid Arginine is metabolized to creatine and is a source of stress-induced NO, these reactions don't affect the labeling efficiency of proteins in the presence of excess heavy isotopic forms of Arg. However, the inter conversion of Arginine and Proline results in the presence of an additional satellite peak that shadows proline-containing peptides arising from heavy labeled media. Because they retain five 13C atoms, these heavy isotopic forms of proline-containing peptides appear at 2.5 and 1.6 m/z units, respectively, above the double and triple charge states of the most abundant form of the Arg-labeled peptide. However if Arg, uniformly double-labeled at both N and C is used, heterocyclic Proline arising from the pathway of Figure 2 appear 6 Da heavier than their naturally abundant congeners. That is to say, although the incorporation of Arg is essentially complete, the conversion of Proline and Arginine splits that mass signal stemming from proline-containing peptides of the heavy-labeled cell state into two channels. Accordingly, the protein expression ratio determined from proline-containing peptides may be brought into line with the remaining peptides by summing the peak intensities of these mass channels after summing the chromatogram over the elution window.

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