

## A <sup>13</sup>C Isotopomer Model for Accurate NMR Quantification of Substrate Selection and Anaplerosis

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<sup>13</sup>C NMR spectroscopy provides unique positional information regarding the carbon labeling in glutamate, which has been used to study substrate selection and anaplerosis in various metabolic systems. Current <sup>13</sup>C NMR analysis usually assumes only one site of anaplerotic influx, and does not differentiate flux distribution between pyruvate carboxylation and anaplerosis at succinate. Recent studies with gas chromatography-mass spectroscopy (GC-MS) have shown that pyruvate carboxylation contributes to 3~8% of citrate synthesis [11], and plays an important role in the maintenance of citric acid cycle (CAC). With <sup>13</sup>C labeled pyruvate either directly from the substrate or as glycolytic end product from <sup>13</sup>C labeled glucose, CAC can be labeled with <sup>13</sup>C via anaplerosis through pyruvate carboxylation. Therefore, neglecting the difference between these two anaplerotic fluxes may lead to inaccurate flux determination. Moreover, NMR and GC-MS provides complimentary information regarding <sup>13</sup>C labeling. The combination of NMR and GC-MS may lead to more accurate quantification of metabolic fluxes through the complex metabolic networks. However, the integrative study of NMR and GC-MS has been hindered by the lack of a unified mathematical model. In this work, we developed a unified <sup>13</sup>C isotopomer model for <sup>13</sup>C NMR and GC-MS analysis. Based on this model, we proposed an approach for the quantification of pyruvate carboxylation flux. We also compared NMR and GC-MS in quantification of substrate selection and anaplerosis.

## Mathematical modeling

The model was based on the work of Malloy et al [2]. The model diagram was shown in Fig 1.  $Y_S$  was relative anaplerosis at succinate, and  $Y_{PC}$  was relative pyruvate carboxylation. Total anaplerosis can be calculated as the summation of  $Y_S$  and  $Y_{PC}$ . PYR  $^{13}\text{C}$  enrichment was needed for  $Y_{PC}$  calculation, which can be estimated from that of LAC (or ALA) due to the fast equilibrium between PYR and LAC (or ALA). All possible isotopomer information for AcCoA, CIT, aKG, SUC, OAA (MAL/FUM) were considered, i.e. an intermediate of  $n$  carbon would have  $2^n$  isotopomers. Metabolite pools of FUM, MAL and OAA were combined, since they were in fast exchange. The symmetric properties of SUC and FUM were taken into account. We employed a matrix representation of isotopomer distribution, which greatly facilitated the model development. The model only contained only 8 equations and was solved iteratively.  $^{13}\text{C}$  natural abundance of 1.1% was taken into account.

## Methods

**Heart perfusion:** Hearts from male rats of 13~14 weeks of age were perfused in Langendorff mode with Krebs-Henseleit bicarbonate (KHB) buffer containing 5.5 mM glucose and 0.6 mM sodium palmitate with 3% bovine serum albumin for 30 min. Two groups of experiments are done under the same conditions with palmitate (group A, n=8) or glucose (group B, n=7) replaced by [ $^{13}\text{C}$ ]palmitate or [ $^{13}\text{C}$ ]glucose.

**NMR spectroscopy:** Freeze-clamped hearts were extracted with 6% perchloric acid. The extract was neutralized to pH 6.5–7.0 and was then lyophilized. The resulting powder was dissolved in 0.5 ml D<sub>2</sub>O. High resolution <sup>1</sup>H and <sup>13</sup>C-NMR spectra were collected using Bruker 800 MHz or 900 MHz NMR spectrometer using inverted probes. Positional isotopomer distribution (PID) was determined from glutamate <sup>13</sup>C-NMR spectrum.

probes. Positional isotopomer distribution (MID) was determined from glutamate  $^{13}\text{C}$  NMR spectrum. **GC-MS:** Frozen heart was extracted with 8% sulfosalicylic acid, treated with methoxylamine-HCl, and then extracted with ethyl acetate. TMS derivatives of aKG, SUC and MAL were prepared and analyzed by an Agilent mass spectrometer linked to a gas chromatography. Natural abundance was corrected. The mass isotopomer distribution (MID) was calculated as the molar fraction of all mass isotopomers, in which the unlabeled isotopomer (M0) was included.

## Results

Groups A ([U-<sup>12</sup>C] glucose and [U-<sup>13</sup>C] palmitate) and group B ([U-<sup>13</sup>C] glucose and [U-<sup>12</sup>C] palmitate) displayed similar heart rate (281±20 v.s. 292±42 BPM), left ventricular developed pressure (95±8 v.s. 81±11 mmHg), rate pressure product (22,900±1,600 v.s. 21,600±1,500 BPM·mmHg) and O<sub>2</sub> consumption rate (5.20±1.47 v.s. 4.66±0.75 μmol/min/g dry weight).

NMR experiments of the two groups were employed to quantify the substrate selection,  $Y_S$  and  $Y_{PC}$ . For group A, PYR was not labeled. Therefore, anaplerotic fluxes through SUC and pyruvate carboxylation ( $Y_S$  and  $Y_{PC}$ ) were indistinguishable. Hence, total anaplerosis ( $Y_S+Y_{PC}$ ) was determined, which was  $0.08\pm0.02$ . The contribution of  $[1,2-^{13}\text{C}]$ AcCoA to citrate synthesis ( $F_{12}$ ), representing palmitate utilization, was  $0.76\pm0.09$ . For group B,  $^1\text{H}$  NMR spectroscopy showed that the  $^{13}\text{C}$  enrichment of LAC and ALA was similar,  $66\pm10\%$  v.s.  $69\pm15\%$  ( $P>0.6$ ). PYR enrichment was estimated from that of LAC due to its higher signal-to-noise ratio. Under this condition, if  $Y_{PC}$  is not taken into count, the model does not fit the data very well (Fig 2 left). Moreover, the total anaplerosis was estimated to be  $-0.01\pm0.07$ , which was significant different from those calculated from group A ( $p<0.01$ ). Using total anaplerotic flux ( $Y_S+Y_{PC}$ ) determined from Group A as the constraint for data fitting, model-fitted  $F_{12}$ , representing glucose utilization, was  $0.05\pm0.02$ , and  $Y_{PC}$  was  $0.034\pm0.020$ , and there was good agreement between model-simulated isotopomer distribution and NMR-measured isotopomer distribution (Fig. 2 right).

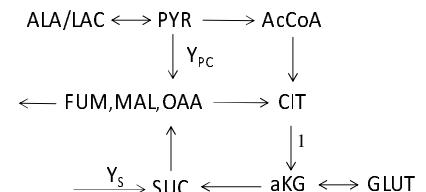
In group A, flux determination from NMR or GC-MS methods were compared. The two methods yielded same palmitate utilization,  $0.76 \pm 0.09$  from both methods.  $Y_S + Y_{PC}$  determined from GC-MS data was higher than from NMR data:  $0.13 \pm 0.03$  vs  $0.08 \pm 0.02$ . Compare to model simulated isotopomer distribution, GC-MS measured MID showed a large fraction of M0 isotopomer:  $18 \pm 4\%$  vs  $9 \pm 4\%$ . Fig. 3 shows the comparison of experimental data and model simulations.

## Conclusions

A unified  $^{13}\text{C}$  isotopomer model for isotopomer analysis of NMR and GC-MS data was developed. Substrate utilization, pyruvate carboxylation and total anaplerosis was determined from NMR experiments with two substrate utilization determined from either NMR and GC-MS data was similar. However, greater anaplerotic

## References

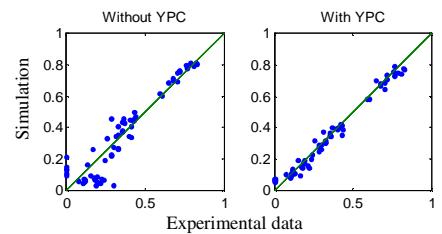
## References



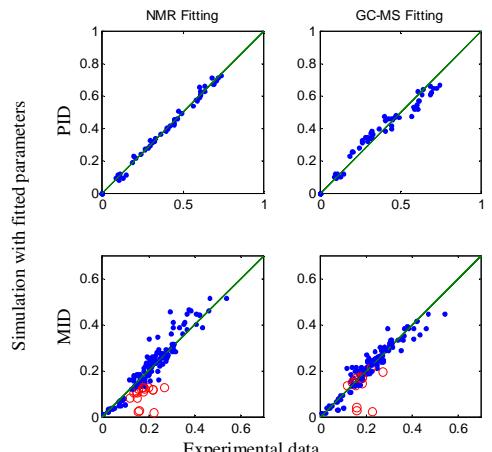
**Fig. 1. Schematic representation of CAC.**

Fig. 1. Schematic Representation of CAC.

AcCoA: acetyl-CoA; CIT: citrate; MAL: malate. aKG:  $\alpha$ -ketoglutarate; SUC: succinate; FUM: fumurate; MAL: malate; OAA: oxaloacetate; PYR: pyruvate; ALA: alanine; IAC: lactate; GLUT: glutamate



**Fig. 2. Comparison of experimental data and simulated data for group B.**



**Fig. 3. Comparison of experimental data and simulated data with fitted parameters for group A. Red circle: M0.**

complementary labeling groups. Our results suggest that flux was estimated by GC-MS analysis