Published in final edited form as:

NMR Biomed. 2011 February ; 24(2): 176–187. doi:10.1002/nbm.1569.

Delineation of substrate selection and anaplerosis in tricarboxylic acid cycle of the heart by ^{13}C NMR spectroscopy and mass spectrometry

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Abstract

^{13}C NMR and mass spectrometry (MS) provide complementary information regarding the ^{13}C labeling of intermediary metabolites. Currently, these two techniques are rarely used together because of the complexity of modeling the distribution of both positional and mass isotopomers. In this study, we developed a matrix-based model for the assessment of ^{13}C label distribution in the tricarboxylic acid cycle and related metabolites. The model was applied to the analysis of NMR- and MS-measured ^{13}C isotopomers for quantification of substrate utilization and anaplerotic fluxes in isolated perfused rat hearts. NMR and MS data were acquired from two groups of rat hearts perfused with substrates in complementary labeling patterns, i.e. the ^{13}C -PAL +GLC group (0.6 mM [$^{13}\text{C}_{16}$]palmitate + 5.5 mM glucose) and the PAL + ^{13}C -GLC group (0.6 mM palmitate +5.5 mM [$^{13}\text{C}_6$]glucose). Relative flux parameters were obtained by fitting the model to the NMR data, MS data and their combination, respectively. Our results suggest that, although both NMR and MS can provide accurate quantification of substrate selection in oxidative metabolism, the accuracy of estimation of anaplerotic fluxes relies on the combination of these two experimental methods.

Keywords

metabolomics; metabolic modeling; stable isotope; anaplerosis; ^{13}C isotopomer

INTRODUCTION

In the past decade, the development of NMR spectroscopy- and mass spectrometry (MS)-based methods has led to the emergence of a new discipline: metabolomics (1,2). The use of ^{13}C -labeled tracer molecules in combination with NMR and MS extends the ability of metabolic phenotyping to the tracing of the metabolic fate of labeled substrates, and therefore allows the assessment of metabolic fluxes. Frequently, flux information is

desirable for the elucidation of disease mechanisms and for the design of more specific therapies. In this regard, ^{13}C -based metabolomics offers unique information that traditional metabolic profiling methods cannot provide.

NMR and MS analyses of ^{13}C isotopomers yield complementary information regarding label distribution in tricarboxylic acid (TCA) cycle-associated intermediates. MS methods, often coupled with gas or liquid chromatography (GC or LC), allow the assessment of the mass isotopomer distribution (MID) of almost all TCA cycle intermediates that are not detectable by NMR because of its low sensitivity (3,4). However, ^{13}C NMR allows the more specific determination of the positional isotopomer distribution (PID) via the analysis of the multiplet spectra of glutamate, a metabolite that is in chemical exchange with the TCA cycle intermediate, α -ketoglutarate (5,6). Therefore, with complementary information on the isotopomer distribution, the combination of these two methods may allow a more accurate quantification of metabolic fluxes via several important pathways.

The determination of metabolic fluxes from NMR or MS experiments requires mathematical modeling of ^{13}C isotopomer distribution (3–5,7–10). These models frequently entail a large number of equations because of the large number of isotopomers and the complexity of metabolite labeling patterns associated with ^{13}C label scrambling and recycling. Previously, models (tcaSIM and tcaCALC) with algebraic equations have been developed for the estimation of substrate utilization and relative metabolic fluxes (5,6,11). These models were applied to NMR and MS data for parameter estimation (10). Other models have used probability and transition matrices to describe the cycling of ^{13}C labels (12). In the current study, we developed a ^{13}C isotopomer model using the isotopomer distribution matrix (IDM). The model was applied to the analysis of NMR and MS data. Using this model, we sought to evaluate the accuracy of NMR, MS and the combination of both methods in the quantification of substrate selection and anaplerosis in hearts perfused with ^{13}C -labeled substrates. Our results showed that, although either method alone yielded similar estimation of substrate selection, more accurate determination of anaplerosis could only be achieved by combining both methods.

MATERIALS AND METHODS

Heart perfusion

Hearts were excised from 11–13-week-old male Sprague-Dawley rats (Charles River Laboratory, Wilmington, MA, USA) that were heparinized (1000 units/kg) and anesthetized with intraperitoneal sodium pentobarbital (85 mg/kg). The isolated hearts were perfused in Langendorff mode with modified Krebs–Henseleit buffer equilibrated with 95% O_2 –5% CO_2 at 37°C. The buffer contained (in mM): NaCl, 118; KCl, 4.7; Mg_2SO_4 , 1.2; CaCl_2 , 2; KH_2PO_4 , 1.2; NaHCO_3 , 25; glucose, 5.5; sodium palmitate, 0.6. The sodium palmitate was bound to 3% fatty acid-free bovine serum albumin (Equitech-Bio Inc., Kerrville, TX, USA). Perfusion experiments were performed on two groups of hearts perfused with substrates of complementary labeling patterns. Hearts in the first group were perfused with [$^{13}\text{C}_{16}$]palmitate and unlabeled glucose (the ^{13}C -PAL +GLC group), and hearts in the second group were perfused with unlabeled palmitate and [$^{13}\text{C}_6$]glucose (the PAL + ^{13}C -GLC group). The complementary labeling of the perfusion substrates allowed the determination of palmitate and glucose oxidation through the TCA cycle. Further, with identical substrate concentrations, the two groups were expected to yield similar estimation of anaplerosis.

The left ventricular function was measured inline through a water-filled balloon inserted into the left ventricle. The balloon was connected to a pressure transducer (World Precision Instruments, Inc., Sarasota, FL, USA). The pressure signal was monitored and processed

with Chart 5 software (ADInstruments, Inc., Colorado Springs, CO, USA) for functional assessments. The influent and effluent O₂ contents were measured with an ABL77 blood gas and electrolyte analyzer (Radiometer America Inc., Cleveland, OH, USA) to calculate the oxygen consumption rate (13). After 30 min of perfusion with labeled substrates, hearts were freeze-clamped and stored at −80°C. Simulation studies using flux parameters published in the literature (14) suggested that the difference in ¹³C-labeling at 30 min and steady state was less than 5% (data not shown). All procedures for this study were approved by the Institutional Animal Care and Use Committee of Case Western Reserve University, Cleveland, OH, USA.

NMR spectroscopy

¹³C NMR spectroscopy of heart extracts was performed to determine the PID of glutamate. Briefly, the freeze-clamped tissues were fragmented and randomly divided for NMR and GC-MS analyses. Half of the heart tissue was homogenized and extracted with 6% perchloric acid. The extract was neutralized to pH 6.5–7.0 and lyophilized. The remaining powder was reconstituted in 0.5 mL D₂O. The sample was transferred to a 5-mm NMR tube. Proton-decoupled ¹³C NMR spectra were acquired on a Bruker (Bruker Biospin, Billerica, MA, USA) 800-MHz (or 900-MHz) spectrometer, corresponding to a field strength of 18.8 T (or 21 T), using a 5-mm inverted probe. Acquisition parameters were as follows: 45° pulse, 1.3 s interpulse delay, 8 K data matrix and 1024–3072 scan averages. The WALTZ-16 sequence was used for proton decoupling and was turned on only during signal acquisition to minimize the heating effect. This pulse sequence led to a significant nuclear Overhauser effect compared with spectra acquired with a 7.3-s interpulse delay. However, the quantified multiplet structure remained the same (data not shown). ¹H NMR spectra with water suppression were acquired with 90° pulses and 16 scan averages. The temperature was maintained at 25°C during spectrum acquisitions.

Spectral analysis used MestRec software (Mestrelab Research S.L. Santiago de Compostella, Spain). ¹³C NMR data were zero-filled to 32 K, exponentially filtered with 2-Hz line broadening and Fourier transformed to the frequency domain. Peak areas for each glutamate multiplet component were determined from line fitting of the ¹³C NMR spectrum with a Voigt lineshape, a mixed Lorentzian and Gaussian lineshape function. The relative peak area for each multiplet component was calculated by normalizing the fitted peak area to the total peak area of the corresponding glutamate carbon.

¹H NMR spectra were used to evaluate the ¹³C enrichment of alanine and lactate. The ¹³C enrichment of alanine (or lactate) was calculated by normalizing the peak area of the C3 proton from ¹³C-labeled alanine (or lactate) to the total peak area of the C3 proton from both labeled and unlabeled alanine (or lactate).

GC-MS

The ¹³C MIDs of α -ketoglutarate, succinate and malate were determined by GC-MS. Briefly, half of the powdered heart tissue was extracted with 8% sulfosalicylic acid containing 10 mM hydroxylamine-HCl. This treatment allowed the conversion of α -ketoglutarate to the more stable oxime derivatives. The supernatant was neutralized with KOH, saturated with NaCl and extracted by ethyl acetate. The resulting extracts were evaporated under N₂ gas. The residue was reacted with 100 μ L of trimethylsilyl at 70°C for 50 min to form the trimethylsilyl derivatives of the analytes. Samples were analyzed on an Agilent mass spectrometer (Palo Alto, CA, USA) linked to a gas chromatograph equipped with a Varian (Palo Alto, CA, USA) VF-5MS capillary column.

The mass isotopomer of metabolites containing i ^{13}C atoms was defined as M_i . The fractional enrichment (FE) of each mass isotopomer M_i was calculated as:

$$\text{FE}(M_i) = \frac{A_{M_i}}{\sum A_{M_i}} \quad [1]$$

where A_{M_i} is the peak area of M_i from ion chromatograms corrected for natural abundance (15).

Mathematical modeling

A novel matrix-based approach was employed to develop equations that describe the dynamic evolution of ^{13}C isotopomers. The IDM of a metabolite is defined as a matrix with each element representing the FE of a specific isotopomer. Thus, for a metabolite with n carbons, an IDM with 2^n elements provides a complete description of all of its isotopomers. The schematic representation of TCA with related anaplerotic pathways and the IDMs of key TCA and associated intermediates are shown in Fig. 1. Using '0' and '1' to represent ^{12}C and ^{13}C , respectively, each element in an IDM describes the labeling pattern of a specific isotopomer. For example, the four elements (00, 10, 01 and 11) in the IDM of acetyl-CoA describe the FE of unlabeled, C1-, C2- and C12-labeled acetyl-CoA, respectively. This IDM of acetyl-CoA is dependent on both the labeling patterns of the substrates and substrate selection.

The condensation of 2-carbon acetyl-CoA and 4-carbon oxaloacetate through citrate synthase gives rise to 6-carbon citrate. Using 16×1 and 1×4 vectors to describe the IDMs of oxaloacetate (OAA) and acetyl-CoA (AcCoA), respectively, a 16×4 matrix of citrate IDM (CIT) can be derived as:

$$\text{CIT} = \text{OAA} \times \text{AcCoA} \quad [2]$$

α -Ketoglutarate is generated from the decarboxylation of the C6 carbon in citrate. Examining the labeling pattern of CIT, the elimination of the last carbon in citrate renders the labeling pattern of the upper half and lower half of CIT identical. Therefore, the IDM of α -ketoglutarate (aKG), an 8×4 matrix, can be formulated as:

$$\text{aKG} = [I_8 \ I_8] \times \text{CIT} \quad [3]$$

where I_n represents an $n \times n$ identity matrix.

Two pathways lead to the generation of succinyl-CoA: the decarboxylation of C1 carbon in α -ketoglutarate and the anaplerosis through the propionyl-CoA-succinate pathway. Endogenous propionyl-CoA can be metabolically derived from branched-chain amino acids and/or odd-chain fatty acids. The IDM for α -ketoglutarate-derived succinyl-CoA ($\text{SucCoA}_{\text{aKG}}$) can be calculated as:

$$\text{SucCoA}_{\text{aKG}} = [I_4 \ I_4] \times \text{aKG} \quad [4]$$

The IDM for propionyl-CoA-derived succinyl-CoA ($\text{SucCoA}_{\text{Prp}}$) can be calculated from the Kronecker product ($\otimes$) of the 4×2 propionyl-CoA IDM (PrpCoA) and the 2×1 CO_2 IDM (CO_2):

$$SucCoA_{PRP} = PrpCoA \otimes CO_2 \quad [5]$$

Assuming the propionyl-CoA-succinate pathway has a relative flux of Y_{PS} relative to the TCA cycle, the succinate-CoA IDM (SucCoA) can be formulated with a linear combination of SucCoA_{aKG} and SucCoA_{PRP}:

$$SucCoA = \frac{SucCoA_{aKG} + SucCoA_{PRP} \cdot Y_{PS}}{1 + Y_{PS}} \quad [6]$$

As fumarate, malate and oxaloacetate are in rapid interconversion, it is assumed that the labeling patterns of these three intermediates are identical. The IDM for pyruvate-derived oxaloacetate (OAA_{PYR}) can be calculated from the Kronecker product of pyruvate IDM (PYR) and CO₂:

$$OAA_{PYR} = PYR \otimes CO_2 \quad [7]$$

Assuming that pyruvate carboxylation has a relative flux of Y_{PC} relative to the TCA cycle, the malate IDM (MAL) can be obtained by a linear combination of the IDM for succinate and OAA_{PYR}:

$$MAL = \frac{SucCoA(1 + Y_{PS}) + OAA_{PYR} \cdot Y_{PC}}{1 + Y_{PS} + Y_{PC}} \quad [8]$$

Further, a transpose-average operation on MAL is performed to account for the label scrambling as a result of the symmetry of succinate:

$$MAL = (MAL + MAL^T) / 2 \quad [9]$$

where superscript ‘T’ denotes the transpose operation.

Finally, a mapping operation converts MAL, a 4×4 matrix, to OAA, a 16×1 vector:

$$OAA = f(MAL) \quad [10]$$

The matrix formulation simplified equation development. The above nine matrix equations (eqns [2]–[10]) constitute a complete description of the isotopomer distribution in the TCA cycle. With an initial OAA representing unlabeled oxaloacetate, dynamic incorporation of the ¹³C label into the TCA cycle intermediates from substrate-derived acetyl-CoA was calculated using an iterative approach. Steady state was reached when the total absolute difference of OAA from two consecutive iterations was less than 10^{-7} . From these steady-state IDMs, model-simulated steady-state PID and MID can be calculated.

Determination of substrate selection and anaplerosis from data fitting

Model-derived isotopomer distribution was fitted to the experimental data for the determination of substrate selection and relative anaplerotic fluxes. Model-derived MIDs were calculated by summation of all the corresponding mass isotopomers. As the steady-state labeling patterns of α -ketoglutarate and glutamate are identical, model-derived

glutamate PID was obtained from the summation of the corresponding positional isotopomers in aKG, normalized by the ^{13}C enrichment of the corresponding carbon.

A MATLAB-based program, TCA-Matrix, was developed for data analysis. Pyruvate IDM, determined from the analysis of ^1H alanine spectra, and natural abundant propionyl-CoA IDM were used as the model inputs. The fitted parameters, including substrate selection (AcCoA) and anaplerosis through the propionyl-CoA-succinate pathway (Y_{PS}) and pyruvate carboxylation (Y_{PC}), were estimated by least-squares fitting of the model-derived isotopomer distributions to either NMR-determined PIDs alone, MS-determined MIDs alone, or a combination of both PIDs and MIDs, the dependent variables in model fitting. The parameter optimization used the Broyden–Fletcher–Goldfarb–Shanno quasi-Newton method with a cubic line search. A typical parameter estimation process took about 2–3 s to converge. Total relative anaplerosis was calculated as $Y_{\text{PS}} + Y_{\text{PC}}$.

The existence of natural abundance ^{13}C was incorporated into the IDMs of acetyl-CoA, pyruvate, propionyl-CoA and CO_2 in the fitting to NMR data. As MS data were corrected for natural abundance (15), ^{13}C natural abundance was not included in the fitting to GC-MS data.

To evaluate the errors of the fitted parameters for each individual dataset, the covariance matrix (COV) was calculated from the Hessian matrix (H) and the difference between the experimental and simulated data (ε_i) as:

$$\text{COV} = \frac{\sum_{i=1}^n \varepsilon_i^2}{n - p} \cdot \left(\frac{H}{2} \right)^{-1} \quad [11]$$

where n and p were the number of experimental data points and the number of fitted parameters, respectively. The diagonal elements of COV represented the variance of each fitted parameter. Consequently, the estimation error for each fitted parameter was calculated as the square root of the variance.

To further evaluate the accuracy of parameter estimation, the isotopomer distribution was simulated using the flux parameters from model fitting. The simulated isotopomer distribution was compared with that of experimentally measured isotopomers by linear regression analysis.

Statistical analysis

The data are presented as the mean $\pm$ standard deviation. Linear regression analysis was employed to compare experimental data with model-simulated data. Unpaired Student's t -test was employed for the comparison of functional data, oxygen consumption and total anaplerosis between the ^{13}C -PAL +GLC group and the PAL + ^{13}C -GLC group. One-way ANOVA was employed for the comparison of model fitted parameters using different experimental data. If statistical significance was detected, further comparison was performed using paired Student's t -test. $p < 0.05$ was considered to be statistically significant.

RESULTS

Animal characteristics, ventricular function and oxygen consumption

The animal characteristics, functional and oxygen consumption data are summarized in Table 1. The animal characteristics, including age, body weight, heart weight and heart-to-body weight ratio, were all similar between the two groups ($n = 8$ for the ^{13}C -PAL +GLC group and $n = 9$ for the PAL + ^{13}C -GLC group). All functional parameters, including heart

rate, systolic (P_{systole}) and diastolic (P_{diastole}) pressure, left ventricular developed pressure, rate pressure product, and maximum (dP/dt_{max}) and minimum (dP/dt_{min}) dP/dt , were statistically the same between the two groups. The oxygen consumption rate was also similar.

Alanine and lactate enrichment

Representative ^1H NMR spectra are shown in Fig. 2. For hearts perfused with $[^{13}\text{C}_{16}]$ palmitate and unlabeled glucose, both alanine and lactate were unlabeled (Fig. 2A). The J -coupling between the methyl protons and their vicinal protons is 7 Hz in both alanine and lactate. This coupling led to two doublets centered at 1.46 and 1.31 ppm for alanine and lactate methyl protons, respectively.

For hearts perfused with $[^{13}\text{C}_6]$ glucose and unlabeled palmitate, the glycolytic end-product of $[^{13}\text{C}_6]$ glucose led to ^{13}C -labeled lactate and alanine (Fig. 2B). The J -coupling between the methyl protons and ^{13}C -labeled methyl carbon is 130 Hz in both alanine and lactate. The proton-carbon coupling in alanine led to the splitting of the methyl proton peaks to 1.54 and 1.38 ppm, respectively. The proton-carbon coupling in lactate led to the splitting of the methyl proton peaks to 1.38 and 1.23 ppm, respectively. The ^{13}C -labeled alanine level was therefore calculated from its methyl proton peaks at 1.54 ppm, and the amount of unlabeled alanine was quantified using the resonance peaks at 1.46 ppm. Similarly, ^{13}C -labeled and unlabeled lactate levels were determined from its methyl proton peaks at 1.23 ppm (^{13}C -labeled) and 1.31 ppm (unlabeled), respectively. From these analyses, FEs of alanine and lactate were $66 \pm 16\%$ and $65 \pm 11\%$, respectively ($p = \text{nonsignificant}$).

Substrate utilization

Representative glutamate ^{13}C NMR spectra are shown in Fig. 3. For hearts perfused with ^{13}C -palmitate and unlabeled glucose, the fractions of C3 triplet, as well as C2 and C4 quartets, in ^{13}C -labeled glutamate were $> 50\%$, suggesting a large fraction of isotopomer with ^{13}C labeling in three adjacent carbons. By contrast, the C2 or C3 singlet only accounted for $< 10\%$ of the corresponding resonance peaks. Accordingly, the MS-determined MIDs from the ^{13}C -PAL +GLC group displayed high ^{13}C enrichment for all the TCA intermediates (Fig. 4). These results indicated high palmitate utilization.

By contrast with hearts from the ^{13}C -PAL +GLC group, hearts perfused with ^{13}C -glucose and unlabeled palmitate showed no detectable C3 triplet, but a large fraction of C2 and C3 singlet (Fig. 3B). In addition, an unexpected C4 singlet was consistently observed. GC-MS data showed a large fraction of unlabeled isotopomers in all the TCA cycle intermediates analyzed (Fig. 4). These data suggested that the utilization of exogenous glucose was low.

The utilization of ^{13}C -labeled exogenous substrates was determined quantitatively by fitting the model to NMR and/or MS data. The fitted parameters are listed in Table 2. The added utilization of exogenous glucose and palmitate was less than 100%, suggesting the oxidation of endogenous substrates. In the ^{13}C -PAL + GLC group, NMR, MS and NMR + MS methods yielded similar palmitate utilization with similar estimation error. In the PAL + ^{13}C -GLC group, NMR and NMR + MS methods also provided similar glucose utilization, whereas model fitting using MS data alone yielded lower glucose utilization ($p < 0.05$) and a larger estimation error.

Anaplerosis

Model-determined relative anaplerotic fluxes are listed in Table 3. In the ^{13}C -PAL +GLC group, precursors to both propionyl-CoA-succinate and pyruvate carboxylase pathways were unlabeled. As a result, fluxes through these two anaplerotic pathways cannot be

differentiated. Hence, total anaplerosis was estimated as a single lumped parameter. MS and NMR + MS methods yielded similar total anaplerosis, whereas NMR-determined total anaplerosis was lower than that by MS or NMR + MS ($p < 0.01$). Further, the model-simulated isotopomer distribution showed good agreement with experimental data regardless of the experimental data used in parameter estimation (Fig. 5A and Table 4).

With ^{13}C -glucose being oxidized to labeled pyruvate, the precursor to the pyruvate carboxylase pathway became labeled, whereas that to the propionyl-CoA-succinate pathway remained unlabeled in the PAL + ^{13}C -GLC group. This difference in precursor labeling allowed the differentiation of the two anaplerotic pathways. The relative pyruvate carboxylation fluxes (Y_{PC}) determined by fitting the model to NMR or NMR + MS data were similar, whereas Y_{PC} determined from MS data alone was slightly higher ($p < 0.05$ compared with NMR + MS). In addition, the estimation error was smallest when both NMR and MS data were used in model fitting.

The relative propionyl-CoA-succinate fluxes (Y_{PS}) determined from MS or NMR + MS data were similar. However, simulated PID using parameters fitted to MS data alone showed poor agreement with the NMR data (Fig. 5B). Moreover, Y_{PS} determined from NMR data alone was unrealistically large, although simulated PID showed good agreement with the NMR data (Fig. 5B). In addition, the estimation of Y_{PS} from NMR data was accompanied by large quantification errors.

Total anaplerosis, calculated as the sum of fluxes through the pyruvate carboxylation and propionyl-CoA-succinate pathways, was in agreement with that from the ^{13}C -PAL + GLC group when either MS or NMR + MS data were used in model fitting ($p = \text{nonsignificant}$). However, total anaplerosis determined by fitting to NMR data alone was also unrealistically large because of the inaccuracy in Y_{PS} estimation.

Error analysis

Further analysis was performed to account for the unrealistic propionyl-CoA-succinate flux estimation from NMR data. First, parameter estimation was performed by fitting the model to simulated NMR isotopomer data with 5% noise. Compared with the estimated glucose utilization and pyruvate carboxylation flux, the estimated propionyl-CoA-succinate flux showed a much wider range of variations in the presence of measurement noise (Fig. 6A). The contour map of the objective function as a function of the two anaplerotic flux parameters is shown in Fig. 6B. The extensive elongation of the isocontours of the objective function along the Y_{PS} axis suggests that the objective function is insensitive to changes in propionyl-CoA-succinate flux over a wide range of values. Although objective function for fitting MS data showed more sensitivity to both anaplerotic flux parameters, the oblong shape of the contour map indicated a high level of interdependence between the two parameters (Fig. 6C).

To identify the metabolite that is important to the determination of anaplerotic fluxes, we evaluated the effect of different combinations of NMR and MS data on the accuracy of parameter estimation. The contour map for the objective function of NMR + malate MID data showed both reduced interdependence between the two parameters and a limited range of variations (Fig. 6D). Accordingly, the variations and estimation errors were significantly reduced in the estimated glucose utilization and anaplerotic fluxes. The glucose utilization ($5.5 \pm 2.2\%$), relative pyruvate carboxylation flux ($4.0 \pm 2.2\%$) and relative propionyl-CoA-succinate flux ($7.6 \pm 5.3\%$), determined by fitting the model to NMR + malate MID data, were similar to those obtained by fitting to NMR + MS data. These results suggest that NMR, in combination with malate MID data from MS analysis, is sufficient to accurately determine the flux parameters. In contrast, the combination of NMR data with other MS-

measured TCA intermediates is less effective in improving the accuracy of parameter estimation (data not shown).

The accuracy of the estimated substrate utilization was also evaluated. The contour maps of the objective functions suggest that the estimation of substrate selection from NMR data is independent of the estimation errors of other parameters (Fig. 7A, B). However, the oblique contours for the GC-MS method indicate that the estimation of substrate selection and anaplerotic fluxes is interrelated (Fig. 7C, D). When both NMR and GC-MS data were used for parameter estimation, both glucose utilization and anaplerosis could be accurately determined (Fig. 7E, F). These analyses suggest that NMR alone, or the combination of NMR and GC-MS methods, will allow more robust and accurate estimation of substrate selection.

DISCUSSION

In this work, we have developed a mathematical model of the TCA cycle for the quantification of metabolic fluxes in isolated perfused heart using ^{13}C -labeled substrates. The superior simplicity in formality is the key feature of this model. With only nine equations, this model encompasses all possible isotopomers of key TCA cycle and associated metabolites, allowing flux quantification from both NMR and MS data. In the current study, substrate utilization and relative anaplerosis through both pyruvate carboxylation and propionyl-CoA-succinate pathways were determined by fitting the model to experimental NMR or GC-MS data. Sensitivity and error analyses were performed on the fitted parameters to evaluate the accuracy of parameter fitting. Our results demonstrated that the combination of NMR and MS data provided more accurate quantification of metabolic fluxes.

Palmitate and glucose utilization obtained from the fitting to NMR data, MS data or their combination was consistent in our study. Consistent with previous findings at similar physiological conditions, glucose utilization was low with palmitate as substrate (16,17). With only ^{13}C -labeled glucose present in the substrate, the low glucose utilization led to a glutamate C4 singlet, representing naturally enriched ^{13}C acetyl-CoA. However, the glutamate C4 singlet became indistinguishable when large amount of ^{13}C palmitate-derived acetyl-CoA entered the TCA cycle in hearts perfused with ^{13}C -labeled palmitate. Error analysis indicated that NMR and NMR + MS-determined glucose utilizations were accurate, both of which were independent of the estimation of anaplerosis. However, MS-determined glucose utilization and anaplerosis were interrelated, which may explain the larger quantification error for MS-determined glucose utilization.

Estimated anaplerosis showed a wide range of variations depending on the experimental data used for parameter fitting. In the ^{13}C -PAL +GLC group, NMR-determined total anaplerosis was significantly lower than that determined from MS or NMR + MS data, whereas, in the PAL + ^{13}C -GLC group, the NMR-determined total anaplerosis was unrealistically high. Further analysis showed that the estimation of propionyl-CoA-succinate flux using only NMR data was very sensitive to noise (Fig. 6A), which can be attributed to the insensitivity of the objective function to propionyl-CoA-succinate flux (Fig. 6B). The objective function for parameter estimation from MS data also showed an elongated shape with a strong interdependence between pyruvate carboxylation and propionyl-CoA-succinate flux (Fig. 6C). As a result, MS-determined anaplerosis also showed a large estimation error. In contrast, the combination of NMR and MS data can effectively reduce the parameter interdependence and increase the sensitivity of the objective function to changes in anaplerotic flux (Fig. 6D), leading to reduced parameter estimation error (Tables 2 and 3). The improved accuracy was also supported by the agreement in estimated total

anaplerosis between the two experimental groups. Further, our results suggest that the combination of NMR data and malate MID data alone is sufficient to constrain the estimation of the flux parameters.

Previous studies have compared NMR-calculated isotopomer distribution with that measured by GC-MS. Des Rosiers and colleagues (8,9) reported an underestimation of α -ketoglutarate/glutamate M0 derived from the analysis of NMR data. Sherry and Malloy (18) also observed a similar discrepancy between ^{13}C NMR-determined glutamate M0 and that measured by ^1H NMR. In the current study, GC-MS-measured α -ketoglutarate M0 was also consistently higher than that predicted by model simulation, regardless of the experimental data used in parameter estimation (Fig. 5A). These results suggest the possibility of the compartmentalization of metabolites that are not in exchange with the TCA cycle.

We have developed a novel mathematical framework for isotopomer modeling, that is the use of IDM. To date, several mathematical frameworks have been developed for the analysis of ^{13}C isotopomer distribution in the TCA cycle and related metabolites. Chance *et al.* (7) first published their pioneering work on a comprehensive kinetic model of ^{13}C labeling in the TCA cycle using vector representations of the isotopomer distribution. Later, analytical solutions were derived for the quantification of metabolic fluxes in the TCA cycle (5,19). These two methods involve a large number of equations that render the incorporation of new biochemical pathways difficult. Recent studies have employed matrix mapping approaches to describe the cycling of ^{13}C label in a complex metabolic network (20–23). However, the construction of mapping matrices is difficult to implement. In contrast, our current approach is more intuitive, and easy to implement, modify and expand.

The compactness of the matrix-based approach allows easy expansion of the model to describe metabolic compartmentalization or complex metabolic networks. Metabolic compartmentalization exists at both organ and cellular levels. In the current study, ventricular myocardium was considered as a metabolically homogeneous tissue that consists primarily of cardiomyocytes. However, for other tissues, such as the brain, the neuronal and glial cells have distinct metabolic profiles that warrant separate consideration (12,24). Under these circumstances, the current matrix-based approach may provide a simple solution by introducing separate IDMs for each metabolite in its corresponding compartment. In addition, this approach allows flexible expansion of the model to encompass other metabolic networks, such as gluconeogenesis and pentose-phosphate pathways. In general, such an approach can provide a promising tool for the analysis of isotope-based metabolomic data.

Acknowledgments

This work was supported by grants from the National Institutes of Health (R01 HL-73315 and HL-86935 to XY; R01 DK069752 and RoadMap grant 5R33DK070291 to HB).

Abbreviations used

^{13}C-PAL + GLC	$[^{13}\text{C}_{16}]$ palmitate and unlabeled glucose
COV	covariance matrix
FE	fractional enrichment
GC-MS	gas chromatography-mass spectrometry
IDM	isotopomer distribution matrix
MID	mass isotopomer distribution

MS	mass spectrometry
PAL + ^{13}C-GLC	unlabeled palmitate and [$^{13}\text{C}_{16}$]glucose
PID	positional isotopomer distribution
TCA	tricarboxylic acid

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Figure 1.

Schematic representation of the tricarboxylic acid (TCA) cycle (A) and the isotopomer distribution matrices (IDMs) for TCA cycle intermediates (B). Y_{PS} , relative anaplerotic flux through the propionyl-CoA-succinate pathway; Y_{PC} , relative pyruvate carboxylation flux. '0' and '1' in the IDMs represent ¹²C and ¹³C, respectively. Abbreviations for metabolites and related IDMs: AcCoA, acetyl-CoA; aKG, α-ketoglutarate; ALA, alanine; CIT, citrate; CO₂, CO₂; GLUT, glutamate; LAC, lactate; MAL, malate; OAA, oxaloacetate; OAA_{PYR}, pyruvate-derived oxaloacetate; PrpCoA, propionyl-CoA; PYR, pyruvate; SucCoA, succinyl-CoA; SucCoA_{aKG}, α-ketoglutarate-derived succinyl-CoA; SucCoA_{PRP}, propionyl-CoA-derived succinyl-CoA.

Figure 2. Representative ^1H NMR spectra in the $^{13}\text{C}_{16}$ palmitate + unlabeled glucose (^{13}C -PAL + GLC) group (A) and unlabeled palmitate + $^{13}\text{C}_6$ glucose (PAL + ^{13}C -GLC) group (B).

Figure 3.

Representative ^{13}C NMR spectra of glutamate C2 (left panels), C4 (middle panels) and C3 (right panels) from the $[^{13}\text{C}_{16}]$ palmitate + unlabeled glucose (^{13}C -PAL +GLC) group (A) and unlabeled palmitate + $[^{13}\text{C}_6]$ glucose (PAL + ^{13}C -GLC) group (B). Quantification of the corresponding positional isotopomers is presented at the bottom of each spectrum. Open and filled circles represent ^{12}C and ^{13}C , respectively. Gray circles indicate either ^{12}C or ^{13}C .

Figure 4. Representative natural abundance-corrected ^{13}C mass isotopomer distribution determined by gas chromatography-mass spectrometry (GC-MS).

Figure 5.

Comparison of model-simulated isotopomer distribution with experimental data. (A) [$^{13}\text{C}_{16}$]Palmitate + unlabeled glucose (^{13}C -PAL +GLC) group; (B) unlabeled palmitate + [$^{13}\text{C}_6$]glucose (PAL + ^{13}C -GLC) group. Positional isotopomer distributions (PIDs) and mass isotopomer distributions (MIDs) in the left, middle and right panels are simulated from parameters determined by model fitting to NMR, mass spectrometry (MS) and NMR + MS data, respectively. Full lines represent the identity line. Filled and open circles represent ^{13}C -labeled and unlabeled isotopomers, respectively. aKG, α -ketoglutarate; MAL, malate; succinate.

Figure 6.

Error analysis of fitted anaplerosis. (A) Variations in fitted parameters with 5% noise. (B–D) Contour maps of the objective functions of parameter fitting to NMR-determined positional isotopomer distribution (PID) data (B), mass spectrometry (MS)-determined mass isotopomer distribution (MID) data (C) and PID + malate MID data (D). The values on the isocontours are displayed as the logarithmic value to base 10.

Figure 7.

Error analysis of fitted substrate utilization. Contour maps of the objective functions of parameter fitting to NMR-determined positional isotopomer distribution (PID) data (A, B), mass spectrometry (MS)-determined mass isotopomer distribution (MID) data (C, D) and NMR + malate MID data (E, F). The values on the isocontours are displayed as the logarithmic value to base 10.

Table 1

Animal characteristics, functional data and oxygen consumption rate

Parameter	$[^{13}\text{C}_{16}]$ palmitate + glucose	Palmitate + $[^{13}\text{C}_6]$ glucose
Age (weeks)	14.0 ± 0.4	14.2 ± 0.5
Body weight (g)	342 ± 20	346 ± 32
Heart weight (g)	1.54 ± 0.19	1.66 ± 0.17
Heart weight/body weight (%)	0.45 ± 0.06	0.48 ± 0.04
Heart rate (bpm)	292 ± 39	297 ± 34
P_{systole} (mmHg)	90 ± 13	92 ± 10
P_{diastole} (mmHg)	10 ± 4	12 ± 6
Left ventricular developed pressure (mmHg)	80 ± 11	82 ± 6
Rate pressure product (bpm3mmHg)	21800 ± 1500	23300 ± 1800
dP/dt_{max} (mmHg/s)	3550 ± 270	3750 ± 480
dP/dt_{min} (mmHg/s)	-2420 ± 280	-2100 ± 260
Oxygen consumption rate ($\mu\text{mol}/\text{min}/\text{g}$)	4.5 ± 0.7	5.0 ± 1.7

Table 2

Substrate utilization (%)

Method of parameter estimation	$[^{13}\text{C}_{16}]$ palmitate + glucose		Palmitate + $[^{13}\text{C}_6]$ glucose	
	Palmitate	Error	Glucose	Error
NMR	76.6 $\pm$ 8.7	2.0 $\pm$ 0.9	5.0 $\pm$ 2.1	0.9 $\pm$ 0.4
Mass spectrometry (MS)	75.1 $\pm$ 8.1	2.5 $\pm$ 1.5	2.5 $\pm$ 1.8 ^a	1.9 $\pm$ 0.6 ^a
NMR + MS	77.0 $\pm$ 8.0	1.9 $\pm$ 0.7	4.6 $\pm$ 1.6	0.5 $\pm$ 0.2

^a $p < 0.05$ compared with NMR and NMR + MS.

Table 3

Anaplerosis (%)

Group	Method of parameter estimation	Pyruvate carboxylation	Error	Propionyl-CoA-succinate pathway	Error	Total anaplerosis	Error
¹³ C-PAL +GLC	NMR	—	—	—	—	8.1 ± 2.0 ^d	2.0 ± 1.0
	MS	—	—	—	—	13.2 ± 2.6	2.5 ± 1.5
	NMR + MS	—	—	—	—	12.4 ± 2.4	2.2 ± 0.8
PAL + ¹³ C-GLC	NMR	4.2 ± 2.9	1.1 ± 1.3	51 ± 41 ^a	34 ± 24	55 ± 40 ^a	—
	MS	7.1 ± 2.4 ^b	3.1 ± 1.0	5.2 ± 8.6	8.6 ± 2.2	12.4 ± 8.8	—
	NMR + MS	3.9 ± 1.9	0.5 ± 0.3 ^c	8.5 ± 8.1	4.9 ± 1.5 ^d	12.4 ± 9.7	—

¹³C-PAL +GLC, [¹³C₁₆]palmitate + unlabeled glucose; PAL + ¹³C-GLC, unlabeled palmitate + [¹³C₆]glucose.

^a $p < 0.05$ compared with MS and NMR + MS.
^b $p < 0.05$ compared with NMR and NMR + MS.
^c $p < 0.01$ compared with MS.
^d $p < 0.01$ compared with NMR.

Table 4

Comparison between experimental and simulated isotopomer distributions by different approaches [NMR, mass spectrometry (MS) and NMR + MS]

		¹³ C ₁₆ palmitate + unlabeled glucose			Unlabeled palmitate + [¹³ C] ₆ glucose		
		Simulated			Simulated		
		Experimental	NMR	MS	Experimental	NMR	MS
Glutamate PID	C2S	0.07 ± 0.02	0.03 ± 0.02 ^a	0.04 ± 0.02 ^b	0.35 ± 0.05	0.36 ± 0.04	0.30 ± 0.02 ^b
	C2D12	0.26 ± 0.05	0.26 ± 0.05	0.30 ± 0.05	0.38 ± 0.06	0.36 ± 0.05	0.29 ± 0.04 ^a
	C2D23	0.11 ± 0.02	0.10 ± 0.01	0.11 ± 0.01	0.13 ± 0.05	0.14 ± 0.04	0.20 ± 0.03 ^a
	C2Q	0.57 ± 0.06	0.61 ± 0.09	0.55 ± 0.08	0.14 ± 0.07	0.14 ± 0.04	0.21 ± 0.02 ^a
	C3S	0.08 ± 0.03	0.07 ± 0.04	0.09 ± 0.04	0.68 ± 0.06	0.67 ± 0.07	0.56 ± 0.04 ^a
α-Ketoglutarate MID	C3D	0.38 ± 0.07	0.38 ± 0.07	0.41 ± 0.06	0.32 ± 0.06	0.31 ± 0.07	0.42 ± 0.04 ^a
	C3T	0.53 ± 0.10	0.55 ± 0.12	0.50 ± 0.10	0.00 ± 0.00	0.02 ± 0.00	0.02 ± 0.01
	C4S	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.20 ± 0.06	0.17 ± 0.05	0.31 ± 0.18 ^b
	C4D45	0.35 ± 0.05	0.34 ± 0.06	0.40 ± 0.06	0.77 ± 0.06	0.76 ± 0.05	0.59 ± 0.18 ^b
	C4Q	0.65 ± 0.05	0.66 ± 0.06	0.60 ± 0.06	0.15 ± 0.03	0.05 ± 0.03	0.07 ± 0.03
Succinate MID	M0	0.18 ± 0.03	0.03 ± 0.02 ^a	0.05 ± 0.02 ^a	0.84 ± 0.05	0.85 ± 0.09	0.81 ± 0.05
	M1	0.03 ± 0.02	0.04 ± 0.03	0.05 ± 0.02	0.06 ± 0.05	0.06 ± 0.05	0.09 ± 0.02
	M2	0.16 ± 0.06	0.17 ± 0.04	0.21 ± 0.03	0.07 ± 0.03	0.08 ± 0.03	0.07 ± 0.02
	M3	0.16 ± 0.03	0.21 ± 0.04 ^b	0.21 ± 0.02 ^b	0.02 ± 0.01	0.01 ± 0.01	0.02 ± 0.00
	M4	0.22 ± 0.02	0.23 ± 0.01	0.23 ± 0.01	0.00 ± 0.01	0.00 ± 0.00	0.00 ± 0.00
Malate MID	M5	0.28 ± 0.12	0.32 ± 0.11	0.26 ± 0.09	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	M0	0.18 ± 0.06	0.11 ± 0.02 ^b	0.17 ± 0.06	0.80 ± 0.06	0.90 ± 0.09 ^b	0.84 ± 0.04
	M1	0.10 ± 0.04	0.09 ± 0.04	0.09 ± 0.04	0.10 ± 0.03	0.05 ± 0.06	0.10 ± 0.03
	M2	0.19 ± 0.03	0.20 ± 0.03	0.22 ± 0.03	0.08 ± 0.02	0.05 ± 0.03	0.06 ± 0.01
	M3	0.25 ± 0.02	0.26 ± 0.02	0.26 ± 0.02	0.02 ± 0.01	0.00 ± 0.01	0.00 ± 0.00
	M4	0.28 ± 0.07	0.34 ± 0.10	0.27 ± 0.08	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	M0	0.18 ± 0.03	0.11 ± 0.02 ^a	0.16 ± 0.02	0.78 ± 0.04	0.88 ± 0.10 ^b	0.81 ± 0.04
	M1	0.11 ± 0.04	0.09 ± 0.04	0.09 ± 0.04	0.11 ± 0.02	0.05 ± 0.06 ^b	0.09 ± 0.03
	M2	0.21 ± 0.02	0.20 ± 0.03	0.22 ± 0.02	0.08 ± 0.02	0.04 ± 0.03 ^a	0.06 ± 0.01
							0.06 ± 0.01 ^a

	$^{13}\text{C}_{16}$ palmitate + unlabeled glucose				Unlabeled palmitate + $^{13}\text{C}_6$ glucose			
	Simulated				Simulated			
	Experimental	NMR	MS	NMR + MS	Experimental	NMR	MS	NMR + MS
M3	0.25 ± 0.01	0.26 ± 0.02	0.26 ± 0.01	0.26 ± 0.01	0.03 ± 0.01	0.02 ± 0.01	0.04 ± 0.01	0.03 ± 0.00
M4	0.25 ± 0.07	0.34 ± 0.10	0.27 ± 0.08	0.30 ± 0.08	0.01 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00

MID, mass isotopomer distribution; PID, positional isotopomer distribution.

^a $p < 0.01$ between simulation and experimental data.

^b $p < 0.05$ between simulation and experimental data.