Multiple Bond ^{13}C-^{13}C Spin-Spin Coupling Provides Complementary Information in a ^{13}C NMR Isotopomer Analysis of Glutamate

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Most ^{13}C nuclear magnetic resonance (NMR) isotopomer analyses relate a metabolic index of interest to populations of ^{13}C isotopomers as reported by one-bond ^{13}C-^{13}C spin-spin couplings. Metabolic conditions that produce highly enriched citric acid cycle intermediates often lead to ^{13}C NMR spectra of metabolites such as glutamate that show extra multiplets due to long-range couplings. It can be demonstrated from ^{13}C NMR spectra of hearts perfused with mixtures of acetate plus propionate that multiplets in glutamate C2 arising from $^{3}J_{25}$ coupling provide a direct readout of acetyl-CoA fractional enrichment (F_{C1} and F_{C3}), while multiplets in glutamate C5 arising from $^{2}J_{35}$ and $^{3}J_{25}$ couplings quantitatively reflect enrichment of the anaplerotic substrate. Magn Reson Med 42:197–200, 1999. © 1999 Wiley-Liss, Inc.

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In the last two decades, ^{13}C nuclear magnetic resonance (NMR) has been established as a powerful tool for studies of intermediary metabolism, both in vitro and in vivo (1–6). ^{13}C NMR is able to distinguish between singly and multiply labeled metabolite isotopomers due to splitting of carbon resonances by nearest neighbor, one-bond, ^{13}C-^{13}C spin-spin couplings (7–9). In a typical ^{13}C isotopomer analysis (4,5,10,11), metabolic information is obtained by quantifying groups of isotopomers that are generated during metabolic turnover of either a single ^{13}C-enriched substrate or mixture of enriched substrates through the Krebs citric acid cycle. Glutamate is most commonly used for a ^{13}C isotopomer analysis because it is present in high concentrations in most tissues (12) and it directly mirrors ^{13}C turnover in the Krebs citric acid cycle (2). A sensitivity analysis has shown that fractional ^{13}C enrichment of acetyl-CoA and relative anaplerotic flux can be accurately determined using ^{13}C NMR isotopomer methods based upon one-bond couplings (5,10). However, for tissues such as liver, carboxylation of pyruvate, or other 3-carbon metabolites can dominate total entry of ^{13}C-enriched car-

bons into the cycle while acetyl-CoA may not be significantly enriched (13–15). Under these circumstances, a conventional ^{13}C isotopomer analysis based on one-bond ^{13}C-^{13}C spin-spin scalar couplings may provide less satisfactory results. For example, anaplerosis can be directly measured using the glutamate C2 multiplets when [1,2,3-^{13}C$_3$]propionate is available (14), but this analysis does not measure the ^{13}C fractional enrichment of the anaplerotic substrate (14,15). However, as we demonstrate here, it is often possible to detect multiple-bond ^{13}C-^{13}C spin-spin couplings in tissue extracts and this extra information can provide data that is helpful in the elucidation of more complex metabolic situations (9).

In this study, rat hearts were perfused with mixtures of [1,2,3-^{13}C$_3$]propionate and different acetate isotopomers (unlabeled, [1-^{13}C]-, [2-^{13}C]- or [1,2-^{13}C$_2$]acetate) to obtain specific mixtures of glutamate isotopomers. The ^{13}C NMR spectra of glutamate derived from these tissues demonstrate that multiple-bond couplings ($^{n}J_{CC}$, $n > 1$) can be enhanced in the spectrum by judicious choice of sample pH. For example, the glutamate C2 resonance can have from 9 to 27 components depending on the population of isotopomers enriched at C1, C3, and C5. Similarly, glutamate C5 is split by both C2 and C3, which creates a complex ^{13}C multiplet in the C5 resonance. This extra information allows further quantification of subgroups of ^{13}C isotopomers, thereby permitting a direct evaluation of anaplerosis.

MATERIALS AND METHODS

Heart Perfusions

Hearts from anesthetized, adult rats were rapidly excised and arrested in ice-cold Krebs-Hensleit buffer. Hearts in groups I–IV were perfused in the Langendorff mode with one of four different mixtures of propionate (2 mM) and acetate (5 mM): I, [1,2,3-^{13}C$_3$]propionate; unlabeled acetate; II, [1,2,3-^{13}C$_3$]propionate; [2-^{13}C]acetate; III, [1,2,3-^{13}C$_3$] propionate; [1-^{13}C]acetate; and IV. [1,2,3-^{13}C$_3$]propionate; [1,2-^{13}C$_2$]acetate. All ^{13}C-enriched materials were purchased from Cambridge Isotope Laboratories (Cambridge, MA). All other chemicals were analytical grade and used without purification (Sigma, St. Louis, MO).

^{13}C NMR

^{13}C NMR spectra of heart extracts were acquired using a one-pulse sequence (45° pulse) on a 5 mm broadband probe in a 14.1 T Unity INOVA spectrometer (Varian Instuments, Palo Alto, CA). Fourier transformation, line fitting, and

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spectral simulations were performed using either the PC-based software NUTS (Acorn NMR, Fremont, CA) or the Varian software. A sample of [U-$^{13}C_5$]glutamate (5 mM in 300 mM NaCl) was used to determine the pH sensitivity of all one-bond and multiple-bond ^{13}C-^{13}C coupling constants.

RESULTS AND DISCUSSION

It is well known that the ^{13}C chemical shifts of glutamate are sensitive to pH, but it is perhaps less widely appreciated that the ^{13}C linewidths of glutamate derived from a tissue highly enriched in ^{13}C are also pH sensitive. This effect is partly due to multiple-bond ^{13}C-^{13}C spin-spin couplings that are sensitive to the protonation state of glutamate. Although some multiple-bond coupling constants have been reported for glutamate (9), we examined the pH dependence of these couplings to determine whether there is a particular pH where long-range spin-spin interactions can be enhanced and thereby used in a ^{13}C isotopomer analysis. The pH dependence of all one-bond and multiple-bond couplings in uniformly labeled ([U-$^{13}C_5$]) glutamate is summarized in Table 1. All one-bond ($^1J_{CC}$) and two-bond ($^2J_{CC}$) coupling constants are essentially independent of pH, while a significant increase ($\cong$30%) is seen in the three-bond $^3J_{25}$ coupling at high pH (above the pK$_a$ of the α-amino group, $\geq$ 10.8). Thus, if one is interested in extracting isotopomer data from the information encoded by $^3J_{25}$, one should record the ^{13}C glutamate spectrum at high pH to maximize this coupling. With this extra information, groups of isotopomers with ^{13}C at either C2 or C5 (but not both) can be distinguished from isotopomers having ^{13}C at both C2 and C5.

In most situations ^{13}C NMR spectra from tissues extracts are acquired near neutral pH (7.0–7.4). Even here, $^3J_{25}$ can be detected in the C2 resonance whenever both carbons are highly enriched in ^{13}C. To demonstrate the influence of ^{13}C isotopomer populations on the appearance of glutamate C2, hearts were perfused with mixtures of [1,2,3-$^{13}C_3$] propionate plus either unlabeled acetate, [2-^{13}C]acetate, [1-^{13}C]acetate, or [1,2-$^{13}C_2$]acetate. These mixtures were chosen to produce a high level of ^{13}C enrichment in glutamate C1, C2, and C3 plus either no enrichment in C4 or C5 (group I), enrichment in C4 only (group II), C5 only (group III), or both C4 and C5 (group IV) while maintaining
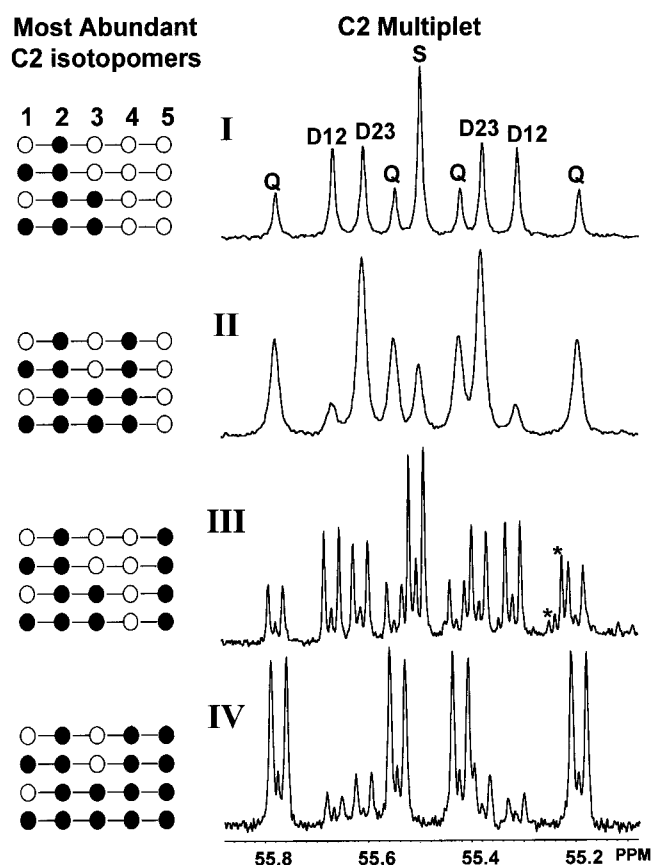

FIG. 1. Glutamate C2 resonances from ^{13}C NMR spectra of extracts of rat hearts perfused with mixtures of propionate and acetate. From top to bottom, all hearts were perfused with [1,2,3-$^{13}C_3$]propionate and either unlabeled acetate (group I), [2-^{13}C]acetate (group II), [1-^{13}C]acetate (group III), or [1,2-$^{13}C_2$]acetate (group IV). In group I, no long-range ^{13}C-^{13}C coupling is possible and a "typical" 9-line C2 resonance is observed. In group II, a small $^2J_{24}$ coupling ((1 Hz) increases the apparent line width of all nine lines contributing to C2. In groups III and IV, three-bond $^3J_{25}$ coupling is easily resolved. The spectra of groups I and II hearts were collected at pH 7.4 while those from groups III and IV hearts were acquired at pH 10.8 to enhance $^3J_{25}$ coupling. The resonances labeled with an asterisk are from metabolites other than glutamate.

constant metabolic conditions. The glutamate C2 resonances from ^{13}C NMR spectra of extracts of hearts perfused to steady state with the above mixtures are compared in Fig. 1. The top spectrum shows a "typical" nine-line C2 resonance as it appears under most metabolic situations. This complex multiplet, reflecting one-bond couplings in all four possible ^{13}C isotopomers in glutamate carbons 1–3, consists of a singlet (S), two doublets (D12 and D23) with differing one-bond coupling constants, and a doublet-of-doublets or quartet (Q). In the spectrum of a group II heart, the relative intensities of the C2 multiplets change compared with a group I heart because ^{13}C entering the cycle as [2-^{13}C]acetyl-CoA enters glutamate C2 after two turns of the cycle and alters the distribution of ^{13}C isotopomers in that position. Furthermore, the linewidth of each multiplet component is increased due to unresolved two-bond coupling between C2 and C4 ($^2J_{24} \cong$1 Hz). In the spectrum of a group III heart, each of the 9 lines that comprises glutamate C2 is further split by three-bond coupling between C2 and

Table 1
One-Bond and Multiple-Bond ^{13}C-^{13}C Couplings in Glutamate at Neutral and High pH

One-bond coupling constants	pH 6–8	pH 10–12
One bond		
J_{12}	53.3	53.1
J_{23}	34.8	34.5
J_{34}	34.5	34.4
J_{45}	51.3	51.3
Multiple bond		
J_{13}	~1	~1
J_{14}	2.3	2.3
J_{24}	~1	~1
J_{35}	~1	~1
J_{25}	3.3	4.5

C5 ($^3J_{25} \cong 4.5$ Hz), yielding a 27-line C2 resonance. Thus, a direct distinction between subgroups of glutamate isotopomers (those having ^{13}C at carbons 1, 2, or 3 versus those having ^{13}C at 1, 2, or 3 plus 5) is possible by deconvolution of the 27 lines contributing to C2. In the spectrum of a group IV heart, carbons 4 and 5 are both highly enriched, and the spectrum is now heavily dominated by the [U-$^{13}C_5$]glutamate isotopomer. Thus, even though the metabolic conditions for these four groups of hearts were identical, their ^{13}C spectra differ dramatically in appearance.
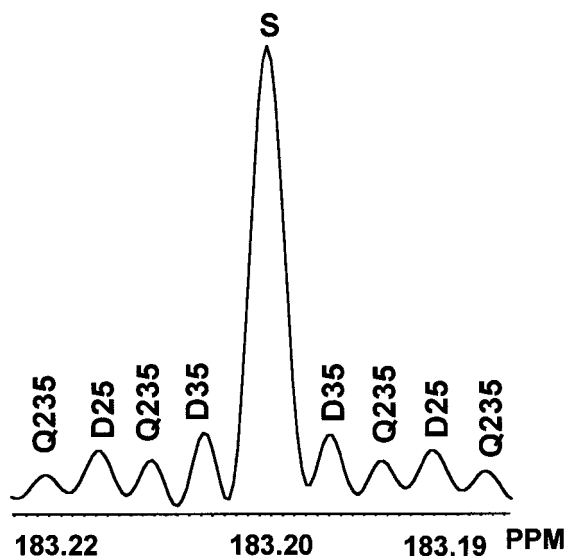
The glutamate C5 resonance from the spectrum of a group III heart (no labeling in C4) also shows $^2J_{35}$ and $^3J_{25}$ couplings (Fig. 2). The central singlet (S) reflects those isotopomers having an enriched ^{13}C in C5 but not in C4, C3, or C2 (^{13}C enrichment at C1 does not affect the spectrum). The small doublet (D35) surrounding the large central singlet comes from $^2J_{35}$ and hence represents isotopomers with ^{13}C at both C3 and C5, but not at C2. A second, slightly less intense doublet (D25) surrounding the central singlet arises from $^3J_{25}$ coupling. This reflects all isotopomers having an enriched ^{13}C at both C2 and C5 but not C3. Finally, a doublet-of-doublets or quartet (Q235) reflects all isotopomers having ^{13}C at all three (C2, C3 and C5) positions.

How can one take advantage of the extra information provided by long-range coupling in these spectra? Two examples can be illustrated. First, in the spectrum of a group III heart, each of the normal nine-lines is further split into three lines reflecting the presence (the two outer lines) or absence (the central line) of $^3J_{25}$ coupling. The area of the outer lines within each set of three lines provides a direct measure of the fraction of acetyl-CoA derived from [1-^{13}C]acetate, given the symbol F_{C1}. In this case, F_{C1} averaged 0.91 ± 0.01 for the nine separate measurements. A similar direct analysis of glutamate C2 in the spectrum of the group IV heart provided the fraction of acetyl-CoA derived from [1,2-$^{13}C_2$]acetate (F_{C3}). In this case, F_{C3} averaged 0.94 ± 0.02. These values (0.91 and 0.94) are typical for hearts perfused with acetate plus propionate (5). Second, given the analytical distribution of C5 isotopomers listed below the spectrum of Fig. 2, one can easily see that D25 + Q235 provides a direct measure of C2 fractional enrichment (C2F = 0.19) while D35 + Q235 provides a measure of C3 fractional enrichment (C3F = 0.19). Given that C2F = C3F = 0.19, one can use previously published equations (10,14) and the spectrum shown in Fig, 1 (group III) to evaluate total anaplerosis (*y*) and the fraction of anaplerotic substrate derived from [1,2,3-$^{13}C_3$]propionate (AS$_{123}$).

$$y = (C2D23 - C2D12)/C2D12 =$$

$$(0.267 - 0.238)/0.238 = 0.12 \quad [1]$$

$$AS_{123} = C2F (1 + 2y)/2y = 0.19 (1.244)/0.244 = 0.98 \quad [2]$$

The result shown in Eq. [1] indicates that anaplerosis was relatively high in these hearts, similar to previously reported values for acetate plus propionate perfused hearts (5). The result shown in Eq. [2] indicates, as expected, that all of the anaplerotic substrate was derived from [1,2,3-$^{13}C_3$]propionate. As was pointed out above, this value is not always well determined in a complete ^{13}C isotopomer analysis using multiplet data from all three protonated carbon resonances (C2, C3, and C4) and non-linear least squares fitting of the data (13). The rather simple analysis demonstrated here is limited only by the accuracy of the C2 and C5 multiplet area measurements.

The results presented in this study have several implications. If glutamate C5 is highly enriched, the NMR spectrum should be run at high pH to maximize $^3J_{25}$ coupling and the glutamate C2 resonance should be fit to 27 lines rather than the usual 9. The doublet contribution to each set of "triplets" in the glutamate C2 resonance arising from $^3J_{25}$ coupling provide a direct measure of the fraction of acetyl-CoA with a ^{13}C-enriched carbonyl carbon (either singly or doubly enriched acetyl-CoA). Furthermore, information provided by multiple-bond coupling in the glutamate C5 resonance can be used to estimate the contribution of labeled anaplerotic substrate. Thus, relationships based upon multiple-bond coupling data may allow analysis of even more complicated metabolic models than currently possible using conventional ^{13}C isotopomer methods. Finally, the central line in the C2S resonance of Fig. 1 is the only resonance that could have a contribution from natural abundance levels of glutamate, so a comparison of the S/D ratio for this central "triplet" compared with the remaining eight "triplets" provides a direct indication of the contribution of natural abundance glutamate to the spectrum.

^{13}C - Multiplet		% Isotopomer
S		70.2
D35		9.60
D25		9.52
Q235		9.75

FIG. 2. Glutamate C5 resonance in a ^{13}C NMR spectrum of a group III heart ([1,2,3-$^{13}C_3$]propionate + [1-^{13}C]acetate). Deconvolution of the C5 multiplet areas provided a direct readout of the ^{13}C isotopomer populations shown. The circles labeled with a ? mean that C1 could either be enriched with ^{13}C or unenriched.

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