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Studying Enzymes by In Vivo ^{13}C Magnetic Resonance Spectroscopy

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1. Introduction

Magnetic resonance spectroscopy (MRS) allows noninvasive detection of specific biologically relevant molecules in vivo. It has become a very useful and versatile tool for both clinical and basic science studies because it can measure concentrations of many important endogenous and exogenous molecules such as the putative neuronal marker *N*-acetylaspartate [1], the ^{19}F -containing selective serotonin reuptake inhibitor Prozac [2], glycogen[3], and adenosine triphosphate [4]. For an endogenous molecule, its concentration measured by MRS is usually the result of a complex balance among various metabolic fluxes with each of the fluxes controlled by a host of different enzymes. By introducing exogenous ^{13}C -labeled substrates certain metabolic pathways can be measured using ^{13}C MRS (e.g., the glutamate-glutamine cycling flux in the brain [5,6]).

In addition to concentrations and metabolic fluxes, an exceptional feature of MRS is its ability to measure the rate of an exchange reaction catalyzed by a specific enzyme in vivo using the technique of magnetization (or saturation) transfer. When kinetically relevant reporter molecules are spin labeled and spin transferred, their exchange rate can be quantified based on the competition between chemical exchange and the longitudinal relaxation time (T_1). The theory of chemical exchange magnetization transfer was developed by chemists more than fifty years ago [7–12]. The phenomenon of in vivo enzyme-specific chemical exchange magnetization transfer was discovered approximately thirty years ago for adenosine triphosphate (ATP)-related exchange reactions [13] including the exchange reactions catalyzed by creatine kinase [14] and the invertebrate-originated arginine kinase [14]. The ability of noninvasively extracting information from specific enzymes using in vivo MRS is highly significant and has generated a great deal of enthusiasm [14–21]. In particular, creatine kinase-catalyzed magnetization transfer effect has been demonstrated to be a useful magnetic resonance reporter of gene expression [22]. Obviously, it would be highly desirable if more enzymes were accessible to in vivo MRS-based magnetization transfer spectroscopy methods.

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However, since the early discoveries of the above-mentioned enzymes involved in catalyzing the transfer of phosphate groups no new enzymes exhibiting detectable *in vivo* magnetization transfer effects had been found until our recent discovery of *in vivo* ^{13}C magnetization transfer effects [23,24].

Our interests in magnetization transfer started with the long-standing controversies on the rate of exchange between brain cytosolic glutamate/aspartate and mitochondrial α -ketoglutarate/oxaloacetate pools extracted from metabolic modeling of *in vivo* ^{13}C MRS data. We hypothesized that if this exchange rate is very rapid it should be directly measurable using magnetization transfer. This line of research first led to the discovery of the *in vivo* magnetization transfer effect catalyzed by aspartate aminotransferase (AAT), then by lactate dehydrogenase (LDH) [25], malate dehydrogenase (MDH) [26], and carbonic anhydrase (CA) [27]. We demonstrated that the chemical exchange processes of these enzymes could be measured by ^{13}C saturation transfer with ^{13}C detection [24–27] and/or ^{13}C saturation transfer with ^1H detection techniques [28]. We also found that the exchange between ^{13}C -labeled mitochondrial and cytosolic pools in brain is much faster than the tricarboxylic acid (TCA) cycle flux [29]. Here we endeavor to first give a brief overview of the early work in the field of *in vivo* ^{31}P magnetization transfer spectroscopy because it is beyond the scope of this article to comprehensively review all *in vivo* magnetization transfer studies conducted on ATP-related enzymes using ^{31}P MRS (the interested readers are referred to several excellent reviews on this topic [19–21]), Previous *in vitro* studies of enzyme systems using ^{13}C NMR spectroscopy are also discussed. Then we will present the theoretical analyses and the experimental methods associated with detecting *in vivo* ^{13}C magnetization transfer effects of a rapid chemical exchange process between small and large substrate pools, and review the current applications of *in vivo* ^{13}C magnetization transfer spectroscopy to the study of enzymes. The chemical shifts of chemicals involved in enzyme-specific ^{13}C magnetization transfer effects discovered so far are given in Table 1.

2. Overview

Creatine kinase (CK) has proven to be particularly amenable - in conjunction with ^{31}P magnetization transfer spectroscopy - for elucidating rapid chemical exchange processes. CK catalyzes the phosphate of phosphocreatine (PCr) exchanges with the adenosine triphosphate (ATP) reaction, and is a key enzyme for maintaining cellular energy supplies:

Magnetization transfer for the CK reaction is usually done by selective perturbation of the equilibrium magnetization of the ^{31}P spin at the γ -position in ATP (see above illustration for its chemical structure), and then measuring the effect on the signal strength of its exchange partner phosphocreatine. The experiment can be performed in several ways (for technical details see [14–16,18]). Traditionally, *in vivo* magnetization transfer experiments are done via a saturation transfer procedure that saturates the γ -ATP resonance with a long frequency-selective radio frequency pulse, and measures the reduction in PCr peak intensity. The unidirectional pseudo-first-order rate constant of $\text{PCr} \rightarrow \text{ATP}$ is quantified by the Bloch

equation modified for chemical exchange [14,15], with the additional knowledge of the T_1 of PCr in the absence of exchange. The exchange rate has been extensively measured in skeletal muscle [30–37], heart [30,32,38–44], and brain [32,45,46]. These investigations helped in understanding the relationship between CK flux and work load [31,34], developmental changes [39,40], individual iso-enzyme functions [35–37,44], CK flux alterations in various physiological [30,32,33,38,45,46], and pathological conditions [41–43].

Another successfully studied enzyme is the mitochondrial ATP synthase. By selective saturation of γ -ATP resonance and quantification of inorganic phosphate signal intensity reduction, the in vivo unidirectional pseudo-first-order rate constant of inorganic phosphate $\rightarrow$ ATP can be calculated. This kinetic parameter reflects mitochondrial functionality, which has been measured in rodent or/and human skeletal muscle under rest [47–50], aging [51] and diseased [52,53] conditions.

Early ^{13}C magnetization transfer spectroscopy work was performed only in vitro. The pseudo-first-order rate constants for the reactions catalyzed by carbonic anhydrase [54–61], alanine aminotransferase [62], and mutarotase [63] were investigated in prepared solutions. These investigations offered important insights into enzyme activities and regulations. However, translating in vitro measurements to in vivo measurements requires matching the natural intracellular environment of the investigated enzyme. Thus, results from in vitro studies may or may not reflect the enzyme activities associated with the living condition. Therefore, it is preferable to measure the pseudo-first-order rate constant for the reactions catalyzed by enzymes in vivo using noninvasive ^{13}C magnetization transfer spectroscopy techniques.

3. Theory and Experimental Methods

3.1. Theory

Magnetization transfer spectroscopy is sensitive to fast chemical exchange in an enzymatic reaction [14,16]. In this section, we will provide a theoretical analysis of saturation transfer during the fast exchange process occurring in a two-site model that contains small ($\ll \text{mM}$) and large substrate pools ($\geq \text{mM}$), a common theme in all currently discovered in vivo ^{13}C magnetization transfer systems, and quantitatively examine the effect of rapidly turning over small substrate pool, which mandates the use of relatively high radio frequency power for irradiation; it also allows a quasi-steady state approximation of a substrate's dynamic longitudinal relaxation process in the large substrate pool during the presence of the rapid exchange with the substrate in the small pool.

If we consider a two-site exchange system in which an exchange occurs between substrate A in a small pool and substrate B in a large pool (Fig.1), the irradiating radio frequency pulse is of constant amplitude (ω_1) applied along the x -axis in the radio frequency rotating frame at the resonant frequency of a selected ^{13}C spin at a certain resonant frequency of substrate A; the x , y , z magnetizations of the ^{13}C spin of substrate A (M_{xA} , M_{yA} , M_{zA}) and those of substrate B (M_{xB} , M_{yB} , M_{zB}) are described by the following Bloch-McConnell equations for the two-site exchange model [64–67]:

$$\frac{dM_{xA}}{dt} = -\frac{M_{xA}}{T_{2A}} - k_{AB}M_{xA} + k_{BA}M_{xB} \quad (1)$$

$$\frac{dM_{yA}}{dt} = \omega_1 M_{zA} - \frac{M_{yA}}{T_{2A}} - k_{AB}M_{yA} + k_{BA}M_{yB} \quad (2)$$

$$\frac{dM_{zA}}{dt} = -\omega_1 M_{yA} - \frac{M_{zA} - M_{0A}}{T_{1A}} - k_{AB} M_{zA} + k_{BA} M_{zB} \quad (3)$$

$$\frac{dM_{xB}}{dt} = -\Delta\omega M_{yB} - \frac{M_{xB}}{T_{2B}} + k_{AB} M_{xA} - k_{BA} M_{xB} \quad (4)$$

$$\frac{dM_{yB}}{dt} = \Delta\omega M_{xB} + \omega_1 M_{zB} - \frac{M_{yB}}{T_{2B}} + k_{AB} M_{yA} - k_{BA} M_{yB} \quad (5)$$

$$\frac{dM_{zB}}{dt} = -\omega_1 M_{yB} - \frac{M_{zB} - M_{0B}}{T_{1B}} + k_{AB} M_{zA} - k_{BA} M_{zB} \quad (6)$$

where $\Delta\omega$ is the chemical shift difference between the ^{13}C spin of substrate B and the ^{13}C spin of substrate A, and T_{1B} , T_{1A} , T_{2B} , and T_{2A} are their corresponding T_1 and transverse relaxation times (T_2). The pseudo-first-order rate constants k_{AB} and k_{BA} are for the unidirectional substrate $B \rightarrow$ substrate A, and substrate A $\rightarrow$ substrate B, respectively.

In CK or ATP synthase-catalyzed reactions (e.g. $\text{ATP} \leftrightarrow$ phosphocreatine or inorganic phosphate $\leftrightarrow$ ATP) studied via traditional ^{31}P magnetization transfer spectroscopy, the relevant pool concentrations are relatively large and of similar magnitude (several mM), which allows the use of both saturation and inversion transfer methods [68,69]. In contrast, the concentration of substrate A is usually in a much smaller range than 1 mM in in vivo ^{13}C magnetization transfer. For example, in the reaction catalyzed by AAT, the concentration of α -ketoglutarate was reported to be $0.136 \mu\text{mol/g}$, which is approximately 75-fold more dilute than glutamate [70]. Therefore, the quasi-steady-state assumption (or small substrate pool assumption) [71] can be used:

$$\frac{dM_{xA}}{dt} \approx 0, \quad \frac{dM_{yA}}{dt} \approx 0, \quad \frac{dM_{zA}}{dt} \approx 0$$

At the steady state, and assuming (i) $k_{BA}M_{0B} = k_{AB}M_{0A}$ and (ii) $\omega_1 \ll \Delta\omega$, where M_{0A} and M_{0B} represent the thermal equilibrium magnetizations of the ^{13}C spins of substrate A and B under the condition of complete radio frequency saturation of the selected ^{13}C spin of substrate A, the unidirectional substrate $B \rightarrow$ substrate A rate constant k_{BA} can be determined from the steady-state magnetization of the observed spin (M_{zB}^{ss}), and its T_1 is measured while saturating the ^{13}C spin of substrate A (see references [14,72]):

$$k_{BA} = \frac{\Delta M_{zB}}{M_{0B} T_{1B}^{sat}} \quad \text{or} \quad k_{BA} = \frac{\Delta M_{zB}}{M_{zB}^{ss} T_{1B}} \quad (7)$$

where $\Delta M_{zB} = M_{0B} - M_{zB}^{ss}$ and $T_{1B}^{sat} = T_{1B} / (1 + k_{BA} T_{1B})$. Under conditions of incomplete radio frequency saturation of the ^{13}C spin of substrate A, the expression for k_{BA} [66] can be derived from the expanded Bloch-McConnell equations ((2), (3), ((5)) and ((6))):

$$k_{BA} = \frac{\left(1 + \frac{pq}{\omega_1^2}\right) \frac{\Delta M_{zB}}{M_{0B}}}{T_{1B}^{sat}}, \quad (8)$$

where

$$p = \frac{1}{T_{2A}} + k_{AB} - \frac{k_{AB}k_{BA}T_{2B}}{1 + k_{BA}T_{2B}}, \quad q = \frac{1}{T_{1A}} + k_{AB} - \frac{k_{AB}k_{BA}T_{1B}}{1 + k_{BA}T_{1B}}.$$

When $\omega_1 \sqrt{pg}$, Eq. (8) is reduced to Eq. (7) for the case of complete radio frequency saturation of the ^{13}C spin of substrate A.

Errors in determining k_{BA} can occur when the longitudinal magnetization of the ^{13}C spin of substrate B is significantly perturbed by ω_1 (e.g. radio frequency spillover, [73–75]). At large ω_1 , the dependence of k_{BA} on both ω_1 ($\sqrt{pg}$) and $\Delta\omega$ can be derived from the expanded Bloch-McConnell Eqs. (4)–(6) for the steady-state condition of substrate B:

$$k_{BA} = \frac{\Delta M_{zB}}{M_{0B}} \left(\frac{1}{T_{1B}^{sat}} + r \right) - r \quad (9)$$

where

$$r = \frac{\omega_1^2 T_{2B}^{sat}}{1 + \Delta\omega^2 T_{2B}^{sat}}, \quad T_{2B}^{sat} = \frac{T_{2B}}{1 + k_{BA}T_{2B}}.$$

When $\Delta\omega\omega_1 \sqrt{\frac{T_{1B}^{sat}}{T_{2B}^{sat}}}$, $r \approx 0$, and Eq. (9) is reduced to Eq. (7). Thus, Eq. (7) can be used to determine k_{BA} accurately using the traditional steady-state saturation transfer experiment under the conditions of $pq \ll \omega_1 \ll \Delta\omega \sqrt{\frac{T_{1B}^{sat}}{T_{2B}^{sat}}}$ which requires the complete saturation of the ^{13}C spin of substrate A in the small pool without perturbing the ^{13}C spin of substrate B in the large pool by ω_1 .

When calculating k_{BA} using Eq. (7), the experimentally measured T_{1B}^{sat} may also be affected by the off-resonance effect of ω_1 [74] on the ^{13}C spin of substrate B. When $\omega_1 1/T_{2B}^{sat}$ and under the influence of the off-resonance effect of ω_1 on the ^{13}C spin of substrate B, T_{1B}^{sat} [denoted as $(T_{1B}^{sat})'$] is given by [64,75,76]:

$$\frac{1}{(T_{1B}^{sat})'} \approx \frac{1}{T_{1B}^{sat}} \cos^2\beta + \frac{1}{T_{2B}^{sat}} \sin^2\beta \quad (10)$$

where $\beta = \arccos \frac{\Delta\omega}{\sqrt{\Delta\omega^2 + \omega_1^2}}$. Based on Eq. (10), the off-resonance effect of ω_1 in determining T_{1B}^{sat} also contributes to the overall error in k_{BA} .

The inversion transfer based magnetization transfer experiments require the acquisition of the ^{13}C signal from substrate A in the small pool that has a concentration below the detectable level. Therefore, the inversion experiment cannot be applied to the in vivo exchange process of an enzyme that involves a very large difference in the concentrations in the small and large substrate pools ($M_{0A} \ll M_{0B}$).

Under non-steady-state conditions, the magnetization of the small substrate pool is approximately in instantaneous equilibrium with the large substrate pool. Under conditions of complete radio frequency saturation of the ^{13}C spin of substrate A, and no radio frequency perturbation of the ^{13}C spin of substrate B, Eq. (6) describes a mono-exponential longitudinal

relaxation process for the ^{13}C spin of substrate B at a rate of $(T_{1B}^{\text{sat}})^{-1}$. When the ^{13}C spin of substrate A is not saturated (e.g. during acquisition of control spectra with ω_1 placed at an equal distance from the ^{13}C spin of substrate B but on the opposite side of the ^{13}C spin of substrate A), the dynamic behavior of the longitudinal relaxation of the ^{13}C spin of substrate B is described by the analytical solutions to the classic Bloch-McConnell equations for two-site exchange [68]. Significant insight into the longitudinal relaxation behavior of the ^{13}C spin

of substrate B can be obtained by applying the quasi-steady-state assumption ($dM_{zA}/dt \approx 0$), which reduces the ^{13}C spin of substrate B longitudinal relaxation process to being mono-exponential one at the rate of $(T_{1B}^{\text{nosat}})^{-1}$:

$$\frac{1}{T_{1B}^{\text{nosat}}} = \frac{1}{T_{1B}} + \frac{k_{BA}}{1 + k_{AB} T_{1A}} \quad (11)$$

because $M_{0A} \ll M_{0B}$ and $k_{BA} M_{0B} = k_{AB} M_{0A}$, when $k_{AB} k_{BA} \left(\frac{T_{1B}}{T_{1A}} \right) k_{BA}$, we have $T_{1B}^{\text{nosat}} \approx T_{1B}$. That is, in the absence of radio frequency saturation of the ^{13}C spin of substrate A, the longitudinal relaxation behavior of the ^{13}C spin of substrate B is approximately equivalent to that in the absence of exchange with the ^{13}C spin of substrate A; for a more specific theoretical analysis of ^{13}C magnetization transfer for in vivo exchange between α -ketoglutarate and glutamate of the AAT reaction, see [76].

3.2. Experimental Methods

3.2.1 ^{13}C Saturation Transfer with ^{13}C Detection—To perform an in vivo ^{13}C saturation transfer with ^{13}C detection experiment, a pulse sequence (as shown in Fig. 2) can be chosen. The ^{13}C magnetization transfer effect can be measured by either pulse- or surface-coil localization with interleaved acquisition. When saturation transfer spectra are acquired in the ^{13}C channel, saturation of the selected ^{13}C spin of substrate A is performed using continuous wave (CW) or a train of spectrally selective 2-ms Gaussian pulses. When control spectra are acquired, the saturating pulse is placed at an equal spectral distance from the observed ^{13}C spin of substrate B but on the opposite site of the saturated spin A. The saturated and control spectra are interleaved in every free induction decay acquisition. With the 90° excitation method, a 1ms adiabatic half-passage pulse (AHP) can be used for nonselective 90° ^{13}C excitation. A smaller flip angle rectangular hard pulse may also be chosen for ^{13}C excitation. In the ^1H channel, broadband $^1\text{H} \rightarrow ^{13}\text{C}$ nuclear Overhauser enhancement (NOE) is generated using a train of nonselective hard pulses with a nominal flip angle of 180° spaced at 100 ms apart during continuous wave ^{13}C radio frequency saturation. ^1H decoupling is achieved using a WALTZ (wideband alternating phase low-power technique for zero-residue splitting)-4 decoupling scheme based on a 400 μs nominal 90° rectangular pulse. The decoupling pulse train is executed for 106 ms at the start of data acquisition.

To determine the longitudinal relaxation time of the observed ^{13}C spin of substrate B, a T_{1B}^{sat}

or T_{1B} null experiment $\left(\exp\left(\frac{T_{1\text{null}}}{T_{1B}^{\text{sat}}}\right) + \exp\left(-\frac{TR - T_{1\text{null}}}{T_{1B}^{\text{sat}}}\right) = 2\right)$ is performed, where TR is the repetition time, and $T_{1\text{null}}$ is the time of nulling of the magnetization of the observed ^{13}C spin of substrate B. The inversion-recovery null of ^{13}C spin of substrate B at its resonance frequency with optional saturation of the ^{13}C spin of substrate A is measured using a 4ms hyperbolic secant inversion pulse (phase factor = 5, 1% amplitude truncation) with a long TR followed by direct, single-shot, and adiabatic 3-dimensional localization of ^{13}C -labeled spins.

When $TR \gg T_1$, a value for k_{BA} can be calculated using Eq. (7). If the total concentration of substrate B (both ^{12}C and ^{13}C labeled) is known, the flux of substrate B $\rightarrow$ substrate A can also be calculated using:

$$V_{BA} = k_{BA} [B]. \quad (12)$$

3.2.2. ^{13}C Saturation Transfer with ^1H Detection—To improve the sensitivity of ^{13}C saturation transfer spectroscopy, we developed an insensitive nuclei enhanced by polarization transfer (INEPT) based inverse ^{13}C -to- ^1H heteronuclear polarization transfer technique, to spatially detect localized ^{13}C magnetization transfer effects in vivo [28]. The advantages of examining the ^{13}C saturation transfer with a ^1H detection method are obvious; namely, (i) the long ^{13}C T_1 maximizes magnetization transfer; (ii) the wide ^{13}C chemical shift dispersion minimizes radio frequency spillover during saturation; (iii) the high sensitivity of proton detection.

The pulse sequence consists of radio frequency saturation of the selected ^{13}C spin of substrate A at its resonance frequency using a train of nominally 180° 2ms Gaussian pulses and simultaneous pre-saturation of ^1H signals to generate NOE enhancement and water suppression (see Fig. 3). The observed ^{13}C spin of substrate B is then transferred to proton using an inverse ^{13}C -to- ^1H INEPT-based polarization transfer technique [77,78]. The inverse INEPT-based sequence uses adiabatic refocusing of ^{13}C spins. After generation of the heteronuclear longitudinal two-spin order, outer-volume suppression and additional water suppression are applied. The longitudinal two-spin order is then converted into antiphase ^1H transverse magnetization, spatially localized, and refocused before detection. During data acquisition, WALTZ-4 decoupling of ^{13}C spins is applied for 192 ms.

3.2.3. Magnetic Resonance Hardware—A two-channel spectrometer is required for the type of in vivo ^{13}C saturation transfer experiments described above [24–29]. Our experiments were performed on a Bruker microimaging spectrometer (Bruker Biospin, Billerica, MA) interfaced to an 11.7 Tesla 89-mm bore vertical magnet (Magnex Scientific, Abingdon, UK). The magnet is equipped with a 57-mm inner diameter gradient (Mini0.5 (Bruker Biospin), with a maximum gradient strength of 3.0 G/mm and a rise time of 100 μs). The integrated radio frequency coils/head-holder systems were used for all experiments. The ^1H and ^{13}C radio frequency coils are co-planar. For the ^{13}C saturation transfer with ^{13}C detection method, the inner circular loop is the ^{13}C coil with an inner diameter of 11.1 mm and a conductor width of 2.8 mm. The outer circular loop is the ^1H coil with an inner diameter of 24.6 mm and a conductor width of 3.2 mm [25]. For the ^{13}C saturation transfer with ^1H detection method, the inner circular loop is the ^1H coil with an inner diameter of 15 mm and a conductor width of 2.5 mm. The outer rectangular loop is the ^{13}C coil with an inner width, length, and conductor width of 22.6, 25.0, and 2.5 mm, respectively [28]. The integrated radio frequency coils/head-holder system is capable of rat head fixation, body support, physiology maintenance, coil tuning, and radio frequency shielding. A detailed integrated radio frequency coils/head-holder system

design for in vivo rat brain studies using the vertical 89-mm bore magnet was described previously [79]. The coils were positioned approximately 0 – 1 mm posterior to bregma based on the separate position calibrations.

3.2.4. Animal Preparation—In our previously described experiment [24–29], a young male Sprague-Dawley rat (160 – 230 g) was orally intubated and ventilated with a mixture of 70% N₂O/30% O₂ and 1.5% isoflurane. One femoral vein was cannulated for the intravenous (i.v.) infusion of ¹³C-labeled glucose. A second femoral vein was cannulated for administration of other chemicals. One artery was cannulated for intermittent sampling of arterial blood to measure plasma gases and glucose concentrations using a blood analyzer (Bayer Rapidlab 860, East Walpole, MA). Rectal temperature was monitored and maintained at 37.5 ± 0.5°C using an external pump for heat exchange by water circulation (BayVotex, Modesto, CA). End-tidal CO₂, tidal pressure of ventilation, and heart rate were also monitored. After surgery, anesthesia was maintained using either by i.v. infusion of α-chloralose (initial dose: 80 mg/kg supplemented with a constant infusion of 26.7 mg/kg/h throughout the experiment), or orally ventilated with a mixture of 70% N₂O/30% O₂ and 1.5% isoflurane. For postmortem NMR measurements, a lethal dose of potassium chloride was injected into the femoral vein to induce cardiac arrest prior to spectroscopy data acquisition.

Three-slice (coronal, horizontal, and sagittal) scout rapid acquisition with relaxation enhancement (RARE) images (field of view (FOV) = 2.5 cm, slice thickness = 1 mm, TR/TE (echo time) = 200/15 ms, RARE factor = 8, data matrix = 128 × 128) were acquired to position the radio frequency coils/head-holder system so that the gradient isocenter approximately coincided with the coil center along the z-direction. The rat brain was shimmed using FASTMAP (Fast, Automatic Shimming Technique by Mapping Along Projections)/FLATNESS (Five Linear Acquisitions for up to Third-order, Noniterative, Efficient Slice Shimming) methods as described in our previous publication [80].

4. Aspartate Aminotransferase (AAT)

AAT is one of the most important aminotransferases. It catalyzes the interconversion of aspartate and α-ketoglutarate with glutamate and oxaloacetate at near equilibrium conditions [70]:

The C2 carbons of aspartate, α-ketoglutarate, oxaloacetate and glutamate are marked with a "*". AAT is widely distributed in red blood cells, liver, kidney, pancreas, cardiac muscle, skeletal muscle, and brain. Of these, the highest activity per unit weight [81] is expressed in the liver. AAT functions in tandem with MDH to transfer electrons from reduced nicotinamide adenine dinucleotide (NADH) across the inner mitochondrial membrane in the cells. Low levels of AAT are normally found in the blood, but its level and activity change significantly when body tissues or organs are diseased or damaged.

Clinically, determining AAT levels and their activity in biopsies, plasma, or serum is of considerable diagnostic value [82]. In particular, AAT activity in plasma or serum increases

markedly due to tissue damage and necrosis, and is a valuable marker used routinely to screen and diagnose diseases of the heart, liver, muscle, and biliary tract. However, because of its lack of organ specificity, measuring AAT activity in plasma or serum has significant limitations. In the brain, AAT activity in cerebrospinal fluid (CSF) and serum is of little diagnostic value for brain diseases because only mechanical or functional lesion of the blood-brain barrier will increase the enzyme activity in serum and CSF [83–86]. In studies of diseased brain tissues, Sherwin and colleagues found that AAT activity was significantly elevated in the excised human epileptic cortex [87,88]. Kish and colleagues further showed that AAT activity may be used to reliably distinguish actively spiking versus nonspiking human epileptic cortex [89]. In rat brain, the activity of AAT was found to be significantly increased in electroconvulsive shock-induced seizures [90,91]. Neurochemically, AAT is closely associated with the metabolism of the excitatory amino acid neurotransmitter glutamate [92]. The enzyme catalyzes the formation of glutamate for the synthesis of the inhibitory neurotransmitter γ -aminobutyric acid (GABA). The activity of AAT is comparable to the enzymes of the glycolytic and respiratory cycles [93].

AAT is found in both cytoplasm and mitochondrion and catalyzes a near-equilibrium reaction in the brain [94]. Both cytoplasmic and mitochondrial AAT isozymes, as well as MDH, are components of the malate-aspartate shuttle, which maintains the cytosolic and mitochondrial redox states in the brain [70]. AAT may also mediate the conversion of glutamate derived from the glutaminase reaction into aspartate [95].

The mechanism of AAT reaction is the so-called enzyme-substitution or ping-pong mechanism [96,97]. The general formulation of the reaction is based on two steps. First, a donor amino acid transfers its amino group to the pyridoxal cofactor covalently bound to the apoprotein, and forms a pyridoximine-enzyme-keto acid intermediate. Second, the cognate keto acid dissociates from the enzyme before binding a second keto acid, which acts as the acceptor of the amino group. In the AAT reaction, glutamate, which originates from its cognate α -ketoglutarate, is released from the pyridoxal-enzyme-glutamate complex before the binding of aspartate required for the full reaction. The amino group can be transferred to regenerate the pyridoxamine-enzyme complex for the next α -ketoglutarate after aspartate binding. The exchange between glutamate and α -ketoglutarate catalyzed by AAT does not need the presence of oxaloacetate or aspartate, and vice versa. This mechanism also allows a keto acid to accept the amino group from its cognate amino acid, catalyzing exchanges between α -ketoglutarate and glutamate and between oxaloacetate and aspartate, in addition to the full transamination reaction, which involves the transfer of amino groups between glutamate and aspartate. The reaction can thus be treated as the sum of two half-reactions:

As a result, the overall exchanges between the amino acids and their cognate keto acids are significantly faster than indicated by the Michaelis-Menten parameters of AAT [71,95].

Using ^{13}C saturation transfer with the ^{13}C detection method with radio frequency irradiation of the nuclear magnetic resonance (NMR)-invisible keto acids (α -ketoglutarate and oxaloacetate), we previously detected a ^{13}C magnetization transfer effect in both glutamate $\leftrightarrow$ α -ketoglutarate and aspartate $\leftrightarrow$ oxaloacetate half reactions [24]. Fig. 4 shows the ^{13}C magnetization transfer effect due to the exchanges between α -ketoglutarate and glutamate and between oxaloacetate and aspartate using the 90° excitation method, in α -chloralose-anesthetized rat brains infused with $[1,6\text{-}^{13}\text{C}_2]\text{glucose}$. The spectra were measured on the system with surface coils for localization (described above) [98].

In Fig. 4a, the continuous wave (CW) saturation pulse (nominal $B_{1sat} = 158$ Hz) was centered at the frequency of the carbonyl carbon of α -ketoglutarate at 206.0 ppm (see Table 1). When the control spectra were acquired, the CW saturation pulse was placed at an equal distance from glutamate C2 at 55.7 ppm (see Table 1) but on the opposite site of α -ketoglutarate C2. The saturation transfer spectra and the control spectra were interleaved. With the addition knowledge of T_{1Glu}^{sat} (1.3 ± 0.1 s, mean $\pm$ SD (standard deviation)) from the null experiment and the concentration of glutamate ($10.2 \mu\text{mol/g}$) based on analysis of brain perchloric acid extracts, the pseudo-first-order rate constant ($k_{Glu \rightarrow \alpha-KG}$) and the corresponding unidirectional glutamate $\rightarrow \alpha$ -ketoglutarate flux ($V_{AAT(Glu \rightarrow \alpha-KG)}$) were calculated using Eq. (7) and Eq. (12) to be $0.13 \pm 0.01 \text{ s}^{-1}$ (mean $\pm$ SD) and $78 \pm 9 \mu\text{mol/g/min}$ (mean $\pm$ SD), respectively [24]. Since the AAT reactions are near equilibrium in vivo, the unidirectional α -ketoglutarate $\rightarrow$ glutamate flux rate is also $\sim 78 \mu\text{mol/g/min}$.

The full mathematical description of the two-site exchange model described in Section 3.1 can be used to analyze saturation transfer errors due to incomplete radio frequency saturation, which takes into account of transverse magnetization-related exchange terms pq/ω_1^2 . To reduce the error in k_{BA} (i.e., $k_{Glu \rightarrow \alpha-KG}$) contributed from the pq/ω_1^2 term using Eq. (8), the saturation pulse $\gamma B_{1sat} > 13$ Hz was required for a less than 1% saturation transfer error in k_{BA} . In our investigation, the completeness of radio frequency saturation of α -ketoglutarate was verified by increasing γB_{1sat} from 158 to 628 Hz. No significant differences in $\Delta M_{Glu}/M_{0Glu}$ values were found using the different γB_{1sat} settings. The lowest γB_{1sat} field strength used in our study to saturate α -ketoglutarate was 158 Hz, which is far greater than required for its complete saturation. The estimated saturation transfer error in $\Delta M_{Glu}/M_{0Glu}$ is negligible at this power strength.

A complete ^{13}C saturation transfer experiment on the aspartate $\leftrightarrow$ oxaloacetate half reaction is more difficult to perform than the glutamate $\leftrightarrow \alpha$ -ketoglutarate half reaction. The concentration of oxaloacetate has been estimated to be $3.6\text{--}5.7 \times 10^{-3} \mu\text{mol/g}$ in cytoplasm [70,94], which is more than 1000 times lower than the concentration of α -ketoglutarate ($0.136 \mu\text{mol/g}$) [70]. In addition, a significant portion of oxaloacetate may be bound to enzymes [71], which shorten the apparent T_2 . These combined effects require a stronger saturation pulse to make a complete radio frequency saturation of oxaloacetate than those of α -ketoglutarate. Fig. 4b shows experimental results obtained with the CW saturation pulse (nominal $B_{1sat} = 790$ Hz) centered at the frequency of the carbonyl carbon of oxaloacetate at 201.3 ppm (see Table 1). Other experimental parameters were the same as in Fig. 4a. We tested the completeness of the radio frequency saturation of oxaloacetate C2 by setting γB_{1sat} at 200 Hz and 790 Hz. The same amounts of magnetization transfer ($\Delta M_{Asp}/M_{0Asp}$) were obtained, confirming the nearly complete saturation of oxaloacetate C2 spins. In our investigation, the pseudo-first-order rate constant ($k_{Asp \rightarrow Oxa}$) and the corresponding unidirectional aspartate $\rightarrow$ oxaloacetate flux ($V_{AAT(Asp \rightarrow Oxa)}$) were calculated to be $0.17 \pm 0.01 \text{ s}^{-1}$ and $29 \pm 4 \mu\text{mol/g/min}$ (mean $\pm$ SD) [26]. The unidirectional oxaloacetate $\rightarrow$ aspartate flux rate is also $\sim 29 \mu\text{mol/g/min}$ because of the near equilibrium nature of the AAT-catalyzed reactions.

Fig. 5 shows the spectra for the half-reaction glutamate $\leftrightarrow \alpha$ -ketoglutarate using ^{13}C saturation transfer with ^1H detection method infused with $[1,6\text{-}^{13}\text{C}_2]\text{glucose}$. The spectra were measured on the same spectrometer and performed under the same anesthetic conditions as used in Fig. 4, but with a different set of surface coils. The effect of saturating α -ketoglutarate C2 became apparent on the glutamate H2 resonance at 3.75 ppm with an overlapped glutamine H2 resonance at 3.76 ppm (Glx H2 = glutamate H2 + glutamine H2). With the additional information regarding the known pool sizes of total glutamate and glutamine, the pseudo-first-order rate constant glutamate $\rightarrow \alpha$ -ketoglutarate was calculated to be $0.12 \pm 0.01 \text{ s}^{-1}$ (mean $\pm$

SD) [28], in quantitative agreement with the determination based on ^{13}C saturation transfer using the ^{13}C detection method [24].

In addition to AAT, alanine aminotransferase, GABA transaminase, and ornithine aminotransferase also use α -ketoglutarate as the primary amino group receptor. In the brain, the aminotransferase activities using the α -ketoglutarate $\leftrightarrow$ glutamate half-reaction are overwhelmingly dominated by that of AAT, which represents > 97% of glutamate-related aminotransferase activities [92]. Glutamate dehydrogenase also involves the α -ketoglutarate $\leftrightarrow$ glutamate exchange, but in brain homogenate, its overall activity measured in the direction of reductive amination is less than the activity of AAT (70–130 $\mu\text{mol/g/min}$ measured in both directions) by a factor of 10–20 [92]. Furthermore, the activity of glutamate dehydrogenase in the direction of oxidative deamination glutamate $\rightarrow$ α -ketoglutarate was approximately 1/10 of its activity in the opposite direction [99]. The unidirectional glutamate $\rightarrow$ α -ketoglutarate flux rate determined in our study was close to the reported Michaelis-Menten parameter V_{max} of AAT-catalyzed full transamination reaction. It is most likely that the measured glutamate $\rightarrow$ α -ketoglutarate flux rate is dominated by the α -ketoglutarate $\leftrightarrow$ glutamate exchange reaction while AAT is far from saturated in vivo for the full transamination reaction in the glutamate + oxaloacetate $\rightarrow$ α -ketoglutarate + aspartate direction [92].

5. Malate Dehydrogenase (MDH)

MDH catalyzes the reversible near equilibrium interconversion between malate and oxaloacetate using nicotinamide adenine dinucleotide (oxidized form: NAD, and reduced form: NADH) as coenzymes:

The C2 carbons of malate and oxaloacetate are marked with a "*". The enzyme is located in both the mitochondria and cytoplasm. At equilibrium, the reaction energetically favors the reduction of oxaloacetate into malate. MDH is the final enzyme of the tricarboxylic acid cycle. Both mitochondrial and cytoplasmic MDH, as well as AAT (see above), participate in the malate-aspartate shuttle for translocating electrons produced during the tricarboxylic acid cycle across the impermeable inner membrane of the mitochondrion. In cytoplasm, MDH plays an important role in generating the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH) needed for reductive biosynthesis. The carbon skeleton of malate transported out of mitochondria is also used for gluconeogenesis.

Oxaloacetate is a substrate for both MDH and AAT catalyzed reactions in vivo. Although both oxaloacetate and malate are below the detection threshold of in vivo MRS, studying the chemical exchange process catalyzed by MDH can be conducted by relaying the perturbation of magnetization between malate and the MRS-detectable aspartate via the rapid turnover of oxaloacetate, which is shared by the catalyzed reactions of both AAT and MDH.

The modified Bloch-McConnell equations for the longitudinal magnetization of malate C2 (M_{Mal}), oxaloacetate C2 (M_{Oxa}), and aspartate C2 (M_{Asp}) are as follows:

$$\frac{dM_{Mal}}{dt} = \frac{(M_{0Mal}M_{Mal})}{T_{1Mal}} + k_{Oxa \rightarrow Mal}M_{Oxa} - k_{Mal \rightarrow Oxa}M_{Mal} \quad (13)$$

$$\frac{dM_{Oxa}}{dt} = \frac{(M_{0Oxa}M_{Oxa})}{T_{1Oxa}} + k_{Mal \rightarrow Oxa}M_{Mal} + k_{Asp \rightarrow Oxa}M_{Asp} - (k_{Oxa \rightarrow Mal} + k_{Oxa \rightarrow Asp})M_{Oxa} \quad (14)$$

$$\frac{dM_{Asp}}{dt} = \frac{(M_{0Asp}M_{Asp})}{T_{1Asp}} + k_{Oxa \rightarrow Asp}M_{Oxa} - k_{Asp \rightarrow Oxa}M_{Asp} \quad (15)$$

Where $k_{Oxa \rightarrow Mal}$, $k_{Mal \rightarrow Oxa}$, $k_{Asp \rightarrow Oxa}$ and $k_{Oxa \rightarrow Asp}$ are the unidirectional pseudo-first-order rate constants for oxaloacetate $\rightarrow$ malate, malate $\rightarrow$ oxaloacetate, aspartate $\rightarrow$ oxaloacetate, and oxaloacetate $\rightarrow$ aspartate, respectively. T_{1Mal} , T_{1Oxa} , and T_{1Asp} are the longitudinal relaxation times of malate, oxaloacetate, and aspartate, respectively, in the absence of any chemical exchange. M_{0Mal} , M_{0Oxa} , and M_{0Asp} are the thermal equilibrium values of M_{Mal} , M_{Oxa} , and M_{Asp} , respectively.

Defining $V_{AAT} = k_{Asp \rightarrow Oxa}M_{0Asp} - k_{Oxa \rightarrow Asp}M_{0Oxa}$ for AAT ($Asp \leftrightarrow Oxa$) and $V_{MDH} = k_{Mal \rightarrow Oxa}M_{0Mal} - k_{Oxa \rightarrow Mal}M_{0Oxa}$ for MDH, Eq. (14) and Eq. (15) can be solved for the steady-

state condition $\frac{dM_{Oxa}}{dt} = \frac{dM_{Asp}}{dt} = 0$ and $M_{Mal} = 0$ resulting from radio frequency saturation of the malate C2 resonance at 71.2 ppm. Assuming $k_{Asp \rightarrow Oxa}M_{0Asp} = k_{Oxa \rightarrow Asp}M_{0Oxa}$, then

$$M_{Oxa}^{ss} = \frac{\frac{M_{0Oxa}}{T_{1Oxa}} + k_{Asp \rightarrow Oxa}M_{Asp}}{T_{1Oxa}^{-1} + k_{Oxa \rightarrow Mal} + k_{Oxa \rightarrow Asp}} \quad (16)$$

$$\frac{M_{Asp}^{ss}}{M_{0Asp}} = \frac{T_{1Asp}^{-1} + \alpha}{T_{1Asp}^{-1} + k_{Asp \rightarrow Oxa} + \beta} \quad (17)$$

Where M_{Oxa}^{ss} and M_{Asp}^{ss} are the steady state magnetization of oxaloacetate C2 and aspartate C2 resonances and

$$\alpha = \frac{k_{Asp \rightarrow Oxa}}{1 + T_{1Oxa}(k_{Oxa \rightarrow Mal} + k_{Oxa \rightarrow Asp})} \quad (18)$$

$$\beta = \frac{k_{Oxa \rightarrow Asp} k_{Asp \rightarrow Oxa}}{T_{1Oxa}^{-1} + k_{Oxa \rightarrow Mal} + k_{Oxa \rightarrow Asp}} \quad (19)$$

When $\alpha = \beta = 0$, Eq. (17) reduces to the well-known saturation transfer equation for two-site

$$\text{exchange } \frac{M_{Asp}^{ss}}{M_{0Asp}} = \frac{T_{1Asp}^{-1}}{T_{1Asp}^{-1} + k_{Asp \rightarrow Oxa}}, \text{ which is the same as Eq. (7) (see [14]).}$$

Because $M_{0Oxa} \ll M_{0Mal}$ and M_{0Asp} , the turnover rate of oxaloacetate is expected to be several orders of magnitude greater than the longitudinal relaxation rate of its unprotonated carbonyl carbon. We can assume $k_{Oxa \rightarrow Asp} T_{1Oxa}^{-1}$. Also, assuming $k_{Oxa \rightarrow Asp}/k_{Oxa \rightarrow Mal} = V_{AAT}/V_{MDH}$, Eq. (17) can thus be rewritten as:

$$\frac{\Delta M_{Asp}}{M_{0Asp}} = \frac{k_{Asp \rightarrow Oxa}}{k_{Asp \rightarrow Oxa} + T_{1Asp}^{-1} \left(1 + \frac{V_{AAT}}{V_{MDH}}\right)} \quad (20)$$

where $\Delta M_{Asp} \equiv M_{0Asp} - M_{Asp}^{ss}$. Because $k_{Asp \rightarrow Oxa} M_{Asp} \frac{M_{0Oxa}}{T_{1Oxa}}$ and $k_{Oxa \rightarrow Mal} + k_{Oxa \rightarrow Asp} T_{1Oxa}^{-1}$, Eq. (15) becomes:

$$\frac{M_{Oxa}^{ss}}{M_{0Oxa}} = \frac{\left(\frac{M_{Asp}^{ss}}{M_{0Asp}}\right)}{1 + \frac{V_{MDH}}{V_{AAT}}} \quad (21)$$

The ^{13}C saturation transfer with ^{13}C detection method was used to determine V_{MDH} . The setup for the experiment was similar to that used to study the AAT reaction. When the relayed ^{13}C saturation transfer spectrum was acquired, the malate C2 at 71.2 ppm (see Table 1) was saturated using a train of spectrally selective 2ms Gaussian pulses with a nominal flip angle of 180° spaced 12 ms apart. When the control spectrum was acquired, the saturating pulse train was placed at an equal spectral distance from aspartate C2 at 53.2 ppm (see Table 1) but on the opposite side of Mal C2. According to the measurement of $k_{Asp \rightarrow Oxa}$ ($0.17 \pm 0.02 \text{ s}^{-1}$, mean $\pm$ SD) and V_{AAT} ($29 \pm 4 \text{ } \mu\text{mol/g/min}$, mean $\pm$ SD) for the oxaloacetate $\leftrightarrow$ aspartate half reaction (Section 4), and the T_{1Asp} ($2.2 \pm 0.1 \text{ s}$, mean $\pm$ SD) measured from the inversion-recovery null experiment, V_{MDH} was calculated using Eq. (20) to be $9 \pm 2 \text{ } \mu\text{mol/g/min}$ (mean $\pm$ SD) [26].

If oxaloacetate participates in reactions catalyzed by other enzymes, it can cause V_{MDH} to be underestimated, because of the leakage of perturbed magnetization of oxaloacetate or influx of unperturbed magnetization into oxaloacetate during the relayed magnetization transfer experiment. In brain, pyruvate carboxylase and citrate synthetase are the two main enzymes that use oxaloacetate as the substrate for the reactions. The overall activity of pyruvate carboxylase is negligibly low compared to that of AAT or MDH [100–102]. The citrate synthetase reaction is essentially irreversible in the forward direction [70]. The upper limit of the leakage of perturbed oxaloacetate magnetization due to the citrate synthetase reaction is, therefore, set by the tricarboxylic acid cycle rate (V_{TCA}). Under similar experimental conditions, our previous work [103] found that $V_{TCA}/V_{MDH} \approx 5\%$, which is markedly smaller than the coefficient of variance of the measured V_{MDH} . Therefore, the possibility of significant

interference from the two enzymes can be ruled out. As described earlier in this review, the transamination activity in brain is overwhelmingly dominated by that of AAT. In the brain, the other transamination reactions that use oxaloacetate as an amino group acceptor are not distinguishable from the AAT reaction in magnetization transfer between oxaloacetate and aspartate. Because the dominant keto form of oxaloacetate [104,105] acts as the substrate for MDH and AAT [104,106], the chemical exchange between the keto form of oxaloacetate with its minor enol and gem-diol forms causes no significant leakage of perturbed magnetization.

In vitro investigations of MDH in several species suggest that this enzyme operates at well below its Michaelis constant (K_m) values for oxaloacetate and NADH [107,108] in vivo. A study of the homogenate of adult rat cerebral cortex showed that the maximum reaction rate (V_{max}) of the Michaelis-Menten kinetics for MDH was 69 $\mu\text{mol/g}$ wet weight/min [109]. In our study [26], in vivo V_{MDH} was $\sim 13\%$ of its V_{max} , providing evidence for the low availability of substrates for this enzyme in vivo.

The relatively large frequency separation between aspartate, oxaloacetate, and malate C2 resonances has made it possible to completely eliminate any radio frequency spill-over effect by using a spectrally selective Gaussian pulse train for saturating malate C2 at 11.7 Tesla field strength. Because malate C2 is coupled to malate H2 with $^1J_{CH} = 146$ Hz, the bandwidth of the Gaussian pulse has to be significantly greater than $^1J_{CH}$. The very selective radio frequency pulses commonly employed in ^{31}P magnetization transfer MRS studies should not be used here.

6. Exchange between Mitochondrial α -Ketoglutarate/Oxaloacetate and Cytosolic Glutamate/Aspartate (V_x)

Most of the tricarboxylic acid intermediates are transported into and/or out of mitochondria through a host of exchanger and co-transporter mechanisms [70,110]. Carriers such as the aspartate/glutamate carrier (AGC) and oxaloglutarate carrier (OGC) couple the exchange of aspartate between mitochondria and cytosol on the four-carbon carbon side of the tricarboxylic acid cycle to that of glutamate on the five/six side (Fig.6a). In addition to the unidirectional aspartate/glutamate carrier, a reversible glutamate/hydroxyl carrier also functions on brain mitochondria [111]. Likewise, there are other mitochondria-cytosol transport mechanisms involving four-carbon dicarboxylates in brain [110,112,113]; for example, the dicarboxylate carrier mediates an exchange between malate and phosphate ions across the mitochondrial inner membrane [110]. Reactions of the TCA cycle that occur in the cytosol undoubtedly also contribute to the exchange between cytosolic and mitochondrial pools observed by in vivo ^{13}C MRS during infusion of ^{13}C -labeled substrates.

Many enzymes from the tricarboxylic acid cycle, such as MDH, aconitase, and isocitrate dehydrogenase, are also found in the cytosol of neurons and astroglia [70,114,115]. In contrast, several others (e.g., fumarase, pyruvate dehydrogenase, citrate synthase) are exclusively localized to mitochondria in brain [70,114,116,117]. In vivo ^{13}C MRS has been used to follow the flow of ^{13}C -enriched substrates, via the tricarboxylic acid cycle in mitochondria, to many metabolites including GABA, glutamate and aspartate in the brain. Using in vivo ^{13}C MRS to measure the brain tricarboxylic acid cycle rate, it was noted that glutamate C4 turnover time-course data alone was not sufficient to separate the TCA cycle flux rate (V_{TCA}) from the rate of exchange between mitochondrial α -ketoglutarate/oxaloacetate and cytosolic glutamate/aspartate (V_x) [118,119]. This is because MRS detects the total, and therefore predominantly cytosolic, glutamate signal while the mitochondrial glutamate pool size is negligibly small. Time-course data of the labeling of glutamate C2 and C3 also depend on V_{TCA} and V_x . In principle, using metabolic models to fit the combined time-course data of glutamate C4 and other positions may allow one to extract both V_{TCA} and V_x simultaneously. Whether V_x is slow

($V_x \approx V_{TCA}$) or rapid ($V_x \gg V_{TCA}$) in brain is a matter of considerable controversy in the field of cerebral ^{13}C MRS and has been vigorously debated [111,118–124]. The main focus of this debate has been on metabolic modeling of ^{13}C label incorporation into glutamate and aspartate for extraction of V_x .

As noted above, α -ketoglutarate $\leftrightarrow$ glutamate and oxaloacetate $\leftrightarrow$ aspartate exchange reactions catalyzed by AAT were found to be very rapid and cause a relatively large magnetization transfer effect on glutamate and aspartate when α -ketoglutarate and oxaloacetate C2 resonances are irradiated using radiofrequency pulses [24]. When malate is irradiated using radiofrequency pulses, a relayed magnetization transfer effect was also detected on aspartate because of the rapid and linear malate $\leftrightarrow$ oxaloacetate $\leftrightarrow$ aspartate exchange reactions [26]. The enzymes AAT, MDH, and their substrates are located in both cytosolic and inner mitochondrial compartments [70]. Because of the extremely small fraction of contributions from mitochondrial pools [125,126], the magnetization transfer effects catalyzed by AAT and MDH described in Sections 4 and 5 are predominantly of cytosolic origin and cannot be used to extract V_x . Fortunately, brain fumarate is exclusively localized to mitochondria. Together with MDH and AAT, these three enzymes catalyze a linear chain of rapid exchange reactions in mitochondria. None of these three enzymes are involved in the control of the TCA cycle [127]. The mitochondrial and cytosolic exchange systems are connected via V_x . Therefore, we hypothesized that when fumarate is irradiated using radiofrequency pulses a rapid V_x ($V_x \gg V_{TCA}$) between mitochondrial matrix and cytosol would lead to a detectable relayed ^{13}C magnetization transfer effect on cytosolic aspartate. If V_x is slow ($V_x \approx V_{TCA}$), no detectable relayed ^{13}C magnetization transfer effect between mitochondrial fumarate and cytosolic aspartate should be expected.

Fig 6a depicts the exchange between mitochondrial and cytosolic pools on the four-carbon side of the TCA cycle. Cytosolic malate enters mitochondria via the OGC. Aspartate leaves mitochondria via the AGC [70]. The rapid exchanges between malate and oxaloacetate and between oxaloacetate and aspartate are catalyzed by MDH and AAT, respectively. In the mitochondrial compartment, malate is also in rapid exchange with fumarate catalyzed by the action of fumarase. Among all these metabolites, only cytosolic aspartate is detectable by MRS in vivo while the rest are below the detection threshold of in vivo MRS due to their low concentration. To extract V_x using ^{13}C saturation transfer, the rapidly exchanging small mitochondrial fumarate, malate, oxaloacetate, and aspartate pools were lumped into a single mitochondrial site, which is saturated by the radio frequency irradiation of fumarate C2 at 136.1 ppm (Fig 6b). This simplification attributes the loss of saturation of magnetization inside mitochondria to V_x . The value of V_x calculated via this simplified model therefore represents the lower limit of V_x . Using our four-site exchange model (Fig. 6b) analysis [29], the Bloch-McConnell equations at steady state are

$$\frac{M_{0Asp}(1 - f_{Asp})}{T_{1Asp}} + f_{Oxa} V_{AAT} - f_{Asp} V_{AAT} = 0 \quad (22)$$

$$\frac{M_{0Oxa}(1 - f_{Oxa})}{T_{1Oxa}} + f_{Mal} V_{MDH} + f_{Asp} V_{AAT} - f_{Oxa} V_{AAT} - f_{Oxa} V_{MDH} = 0 \quad (23)$$

$$\frac{M_{0Mal}(1 - f_{Mal})}{T_{1Mal}} - f_{Mal} V_x - f_{Mal} V_{MDH} + f_{Oxa} V_{MDH} = 0 \quad (24)$$

where M_{0Asp} , M_{0Mal} , and M_{0Oxa} are the equilibrium magnetizations of cytosolic aspartate C2, malate C2, and oxaloacetate C2 carbons, respectively. T_{1Asp} , T_{1Mal} , and T_{1Oxa} are the longitudinal relaxation times of cytosolic aspartate C2, malate C2, and oxaloacetate C2 carbons, respectively, in the absence of any chemical exchange. Defining

$f_{Asp} = M_{Asp}^{ss} / M_{0Asp}$, $f_{Mal} = M_{Mal}^{ss} / M_{0Mal}$, and $f_{Oxa} = M_{Oxa}^{ss} / M_{0Oxa}$, V_{AAT} and V_{MDH} are the exchange flux rates catalyzed by cytosolic AAT (for aspartate $\leftrightarrow$ oxaloacetate) and MDH, respectively; V_x is the rate of isotopic exchange between cytosol and mitochondria.

Because $M_{0Oxa}/T_{1Oxa} \ll V_{AAT}$ and V_{MDH} , the term $M_{0Oxa}(1 - f_{Oxa})/T_{1Oxa}$ in Eq. (23), can be omitted. A similar treatment was used in Section 5. V_x can then be estimated from Eqs. (22)–(24):

$$V_x = \left(\frac{f_{Oxa}}{f_{Mal}} - 1 \right) V_{MDH} + \left(\frac{1}{f_{Mal}} - 1 \right) M_{0Mal} / T_{1Mal}, \quad (25)$$

where $f_{Oxa} = (f_{Asp} V_{AAT} - M_{0Asp}(1 - f_{Asp})/T_{1Asp})/V_{AAT}$ and

$$f_{Mal} = (f_{Oxa}(V_{AAT} + V_{MDH}) - f_{Asp} V_{AAT})/V_{MDH}.$$

A value for f_{Asp} is calculated using $f_{Asp} = 1 - \Delta M_{Asp}/M_{0Asp}$, and $\Delta M_{Asp}/M_{0Asp}$ is obtained from the relayed ^{13}C saturation transfer experiment with complete radiofrequency saturation of fumarate C2 resonance at 136.1 ppm.

Fig. 7 shows in vivo relayed ^{13}C magnetization transfer results from both a single rat (a), and summed from eight rats (b). In the control spectrum, the Gaussian saturation pulse train was placed at -29.7 ppm . The pulse train was placed at fumarate C2 resonance 136.1 ppm in the fumarate-saturated spectrum. The control frequency and the fumarate saturation frequency are symmetrical with respect to the resonance frequency of aspartate C2 at 53.2 ppm. In the difference spectrum, a small but well-defined peak at the resonance frequency of aspartate C2 (53.2 ppm) was clearly detected. In comparison, the nearby and much more intense α -glucose C6 (61.7 ppm), β -glucose C6 (61.8 ppm), glutamate C2 (55.2 ppm), glutamine C2 (55.1 ppm), and N -acetylaspartate C2 (54.0 ppm) resonances were completely cancelled. The ratio $\Delta M_{Asp}/M_{0Asp}$ was determined to be 4.2% with a relative standard deviation of 15%. The relative standard deviation was calculated by integrating the aspartate C2 signal and its neighboring spectral regions in the difference spectrum using the same interval length. For using Eq. (25) to estimate V_x only T_{1Mal} was unavailable because malate is below the detection threshold of in vivo MRS while cytosolic aspartate concentration ($[\text{Asp}] = 2.8 \mu\text{mol/g}$, malate concentration ($[\text{Mal}] = 0.3 \mu\text{mol/g}$, $V_{AAT(Asp \leftrightarrow Oxa)} = 29 \mu\text{mol/g/min}$, $V_{MDH} = 9 \mu\text{mol/g/min}$, and $T_{1Asp} = 2.2 \text{ s}$ (mean $\pm$ SD)) (see [24,26,70]). We estimated V_x by assuming $T_{1Mal} = 0.5 \sim 2.0 \times T_{1Asp}$ because both malate and aspartate C2 carbons are singly protonated and the two molecules are small and similar in size [128]. Using Eq. (25), the estimated V_x is in the range 11–23 $\mu\text{mol/g/min}$ in isoflurane-anesthesia rat brain [29]. Under the same experimental conditions (1.5% isoflurane anesthesia), V_{TCA} was found to be in the range of 0.40–0.48 $\mu\text{mol/g/min}$ [129]. These results clearly demonstrate that $V_x \gg V_{TCA}$. Note that in Fig. 6, it was the unidirectional cytosolic aspartate $\rightarrow$ cytosolic oxaloacetate $\rightarrow$ cytosolic malate $\rightarrow$ mitochondrial fumarate relay pathway that was actually measured therefore we have f_{Asp} (95.8%) $> f_{Oxa}$ (84.7%) $> f_{Mal}$ (49.1%) $> f_{Fum}$ (0%). The values of f_{Oxa} and f_{Mal} were calculated using $f_{Oxa} = (f_{Asp} V_{AAT} - M_{0Asp}(1 - f_{Asp})/T_{1Asp})/V_{AAT}$ and $f_{Mal} = (f_{Oxa}(V_{AAT} + V_{MDH}) - f_{Asp} V_{AAT})/V_{MDH}$, respectively.

Because the mitochondrial pools of malate, oxaloacetate, and aspartate are very small [118, 130], the linear exchanges among fumarate, malate, oxaloacetate and aspartate in mitochondria are very rapid. Previous brain metabolic models considered these fast-exchanging mitochondrial pools as a single kinetic pool [118,119,131]. When the turnover time constant due to exchange is much shorter than T_I , loss of saturation of magnetization is negligible [26]. This line of reasoning has allowed us to directly measure V_x using relayed ^{13}C magnetization transfer spectroscopy and to simplify the analysis of the relayed ^{13}C magnetization transfer results by gathering the mitochondrial fumarate, malate, oxaloacetate, and aspartate pools into a single pool as shown in Fig. 6b [29]. Any loss of saturation inside mitochondria would underestimate V_x . Thus, using the simplified four-site exchange model in Fig. 6b, the lower limit of V_x was obtained. Quantitatively, if we also ignore T_I relaxation of the fast-exchanging malate and oxaloacetate extant in the cytosolic compartment, the four-site exchange model described in Fig. 6b is reduced to the simpler two-site exchange model commonly used in metabolic modeling of ^{13}C turnover kinetics for glutamate and aspartate [76]. In this further simplified two-site exchange model, cytosolic aspartate exchanges directly with tricarboxylic acid cycle intermediates with a flux rate of V'_x . The pool size of cytosolic malate (0.3 $\mu\text{mol/g}$, [70]) is not negligibly small, nor are cytosolic V_{AAT} and V_{MDH} fluxes infinitely fast [24,26]. As a result, V'_x is smaller than that given by Eq. (25) because loss of magnetization saturation through longitudinal relaxation in the cytosol is lumped into V'_x . Using the two-site exchange model as well as Eq. (7), we find that $V'_x \approx \Delta M_{\text{Asp}}/M_{0\text{Asp}} \times [\text{Asp}]_0/T_{I\text{Asp}} = 0.042 \times 2.8 \mu\text{mol/g}/(2.2/60 \text{ min}) = 3.2 \mu\text{mol/g/min}$, while the actual V_x is greater than V'_x .

If $V_x/V_{\text{TCA}} \approx 1$, as argued by Gruetter and colleagues [121], the observed $\Delta M_{\text{Asp}}/M_{0\text{Asp}}$ would have to be approximately 0.002 for $T_{I\text{Mal}} = 0.5 \times T_{I\text{Asp}}$, and approximately 0.003 for $T_{I\text{Mal}} = 2 \times T_{I\text{Asp}}$ [29]. Under either situation, we would not be able to detect any ^{13}C magnetization transfer effect on aspartate when mitochondrial fumarate is saturated, because of slow V_x . The results from our relayed ^{13}C magnetization transfer study clearly demonstrate that $V_x \gg V_{\text{TCA}}$ regardless of the value of $T_{I\text{Mal}}$. Viewed another way, our fundamental conclusion that the exchange between mitochondrial tricarboxylic acid cycle intermediates and cytosolic metabolites is rapid is also independent of specific exchange models used for analyzing the relayed ^{13}C magnetization transfer data; this is because V_x has to be fast enough on the time scale of ^{13}C T_I relaxation to be detectable using magnetization transfer spectroscopy. Therefore, for an exchange process to be detectable using this method, the exchange rate multiplied by T_I of the observed signal has to be a significant fraction of the pool size of the observed signal. If $V_x \approx V_{\text{TCA}}$, $V_x \times T_{I\text{Asp}} \approx 0.44 \mu\text{mol/g/min} \times 2.2/60 \text{ min} = 0.016 \mu\text{mol/g}$, or less than 0.6 % of the pool size of aspartate.

7. Lactate Dehydrogenase (LDH)

LDH catalyzes a rapid near equilibrium interconversion between pyruvate and lactate in the presence of coenzymes NAD^+ and NADH :

via an ordered ternary complex mechanism. The C2 carbons of pyruvate and lactate are marked with a ^{13}C . The LDH enzyme plays an important role in cellular energy production. It converts pyruvate, the final product of glycolysis, to lactic acid when oxygen is absent or in short supply. It performs the reverse reaction during the Cori cycle. The conversion of pyruvate to lactate by LDH regenerates NAD^+ to allow glycolysis to proceed in active skeletal muscle and erythrocytes. At high concentrations of lactate, the enzyme exhibits feedback inhibition and the rate of conversion of pyruvate to lactate is reduced. Organs that are relatively rich in LDH include heart, kidney, liver, and muscle. Because the plasma membrane of most cells is highly permeable to both lactate and pyruvate, the two substances can diffuse into the bloodstream fairly easily. In brain it has been hypothesized that lactate derived from aerobic glycolysis in astrocytes is transported as an additional energy substrate to neurons in conjunction with glutamatergic activity [132,133].

LDH has five isoforms: LDH-1 (H_4), LDH-2 (H_3M_1), LDH-3 (H_2M_2), LDH-4 (H_1M_3), and LDH-5 (M_4). Each contains four 35-kD subunits (the M type and the homologous H type) that are encoded by similar genes (LDH-A and LDH-B, respectively). LDH-A is thought to play a key role in tumor growth. Recently, it was shown that blocking LDH-A by RNA interference reduces the growth rate of cancer cells approximately 100-fold [134]. LDH-1 is predominantly found in the heart, erythrocytes, brain, and kidney; LDH-3 is predominantly found in leukocytes; and LDH-5 is predominantly found in skeletal muscle and liver. In the cerebral cortex of adult rat brain (relevant to our investigation of brain LDH), the H type subunit dominates, with the LDH-1 isozyme accounting for 58% of total LDH activity [135].

Lactate is well-known to be significantly elevated in a variety of physiological and pathological conditions (e.g., in the brain of individuals with panic disorder, in exercised muscle, and in various cancerous tissues). Nearly every type of cancer may cause elevated blood LDH levels, as well as dramatically altered total activity and isozyme composition of LDH [136]. In keeping with the dependence of astrocytoma on aerobic glycolysis, a large shift occurs in the production of the M subunit in malignant astrocytoma, with LDH-5 approximately five times higher than in the normal brain [137]. Blood LDH levels have been used to monitor treatment of some cancers, including testicular cancer, Ewing's sarcoma, non-Hodgkin's lymphoma, and some types of leukemia. However, elevated LDH activity in the bloodstream generally lacks the specificity for tissue locations. It would be useful to develop a method that would allow in vivo assessment of the LDH reaction in cancerous tissues themselves. In vivo assessment of the LDH reaction may also be useful for studying other diseases with significantly elevated lactate concentrations, such as panic disorder [138], metabolic acidosis [139], and Huntington's disease [140].

We first reported the observation of rapid in vivo exchange between pyruvate and lactate catalyzed by LDH at the 22nd Annual Meeting of the European Society for Magnetic Resonance in Medicine and Biology [23]. Using both ^{13}C saturation transfer with ^{13}C detection [25] and ^{13}C saturation transfer with ^1H detection methods [28], the in vivo kinetics of cerebral LDH-catalyzed unidirectional lactate $\rightarrow$ pyruvate flux were quantified [25,28]. We demonstrated the feasibility of detecting the ^{13}C saturation transfer effect catalyzed by LDH after raising lactate levels by intravenous infusion of the GABA_A receptor antagonist bicuculline, which generates sustained elevation of lactate concentration. Our investigation also showed that the magnetization transfer effect of LDH can be detected in intracranial glioma rat models [25]. Therefore, the magnetization transfer effect of the LDH reaction can be used as a valuable marker for cancer accessible to noninvasive MRS techniques.

In our study [25], the experimental procedure was similar to those used in the AAT reaction study [24]. Fig. 8a shows the spectra for the exchange between lactate and pyruvate using ^{13}C saturation transfer with the ^{13}C detection method in the bicuculline-treated rat brains

infused with [2-¹³C]glucose. The unidirectional $k_{Lac \rightarrow Pyr}$ rate constant was found to be $0.08 \pm 0.01 \text{ s}^{-1}$ (mean $\pm$ SD) in the bicuculline-treated rat brain. The corresponding unidirectional lactate pyruvate flux rate was determined to be $25 \pm 13 \text{ } \mu\text{mol/g/min}$ (mean $\pm$ SD). The saturation transfer experiment was repeated postmortem, where no magnetization effect was detected because of the deletion of pyruvate (see Fig. 8b). The difference spectra obtained showed significant intensity changes in lactate C2 at 69.33 ppm (see Table 1) in live rats. The possible contribution of the non-specific off-resonance magnetization transfer effect, which is presumably due to a small and immobilized lactate pool in which there is a dipolar coupling between lactate C2 and nearby protons, was investigated by shifting the frequency of the saturation pulse by 75 kHz while keeping the frequency of the continuous irradiation pulse in the control scans unchanged. No significant non-specific off-resonance ¹³C magnetization transfer effect was detected for either glucose or lactate in the difference spectra (Fig. 9). A result obtained from the 6C glioma model is shown in Fig. 10. In Fig. 10a, as shown in the coronal and horizontal anatomical images, the 6C tumor had approximately homogeneous tissue contrast. The signal intensity change of lactate C2 ($\Delta M_{Lac}/M_{0Lac}$) was found to be -19% due to saturation of pyruvate. The second 6C glioma rat (Fig. 10b.), which showed significant tissue heterogeneity in its images, had a much higher lactate signal intensity and a much smaller percent change of lactate C2 (-10%) upon saturation of pyruvate C2 at 207.9 ppm (see Table 1).

The large chemical shift dispersion (>18000 Hz at 11.7 T) between the α -carbons of the amino acids and the carbonyl carbons of their cognate α -keto acids provides an ideal situation for ¹³C saturation transfer experiments without any significant interference from the radio frequency spillover effects between the saturated spins and the observed spins (Section 3.1). For example, in the AAT-catalyzed reaction, the chemical shift difference between α -ketoglutarate C2 and glutamate C2 is approximately 150 ppm. It is therefore very convenient to saturate α -ketoglutarate C2 and observe its exchange partner glutamate C2 in both the ¹³C and ¹H detection experiments. Because the chemical shift difference between lactate C2 and pyruvate C2 is approximately 134 ppm, it is ideal to measure the ¹³C magnetization transfer effect using [2-¹³C]glucose or [2,5-¹³C₂]glucose infusion, saturation of pyruvate C2 resonance and direct ¹³C detection. However, it is practically impossible to use the pyruvate C2 $\rightarrow$ lactate C2 $\rightarrow$ lactate H2 pathway for ¹³C saturation transfer with ¹H detection measurement because the signal of lactate H2 is distributed among four resonance lines and is very close to that of water. Instead, pyruvate C3 can be used for the saturation, and the methyl protons of lactate H3 can function as the reporter signal. Pyruvate and lactate C3 carbons can be labeled using [1-¹³C]glucose or [1,6-¹³C₂]glucose infusion. The chemical shift separation between pyruvate C3 and lactate C3 is only 8.3 ppm. The pyruvate C3 signal is even less convenient, because of its quartet structure arising from the one-bond scalar coupling to the three methyl protons of pyruvate, which makes it difficult to attain spectrally selective radio frequency saturation.

Although bicuculline administration significantly increases tissue [NADH]/[NAD⁺], [H⁺], [pyruvate], and [lactate]/[pyruvate] [141], the LDH activity per se is not modified by bicuculline [142]. The altered concentration of substrates undoubtedly affects the unidirectional lactate $\rightarrow$ pyruvate flux rate [143] and its pseudo first-order rate constant measured using the ¹³C saturation transfer experiment. Despite the metabolic changes associated with bicuculline treatment, our study [25,28] clearly demonstrates the feasibility of quantifying the in vivo ¹³C saturation transfer effect catalyzed by LDH. Given the low lactate concentrations in the normal brain [144], we made no attempt to measure the ¹³C saturation transfer effect under normal physiological conditions using direct ¹³C MRS. It is suggested that the magnetization transfer effect described here may be used to quantify the exchange rate between pyruvate and lactate after administration of hyperpolarized [1-¹³C]pyruvate [145]. The in vivo unidirectional lactate $\rightarrow$ pyruvate flux determined in this study can be compared to the LDH activity determined in samples of rat brain homogenate obtained from normal rats.

In the pyruvate $\rightarrow$ lactate direction, the activity of LDH was determined to be 107–119 $\mu\text{mol/g/min}$ [146,147]. In the lactate $\rightarrow$ pyruvate direction, the whole-brain LDH activity was determined to be 32 $\mu\text{mol/g/min}$ using 19 mM lactate, 1 mM NAD^+ at pH = 8.5 and 25°C [148], which is comparable to the above-mentioned $\sim 25 \mu\text{mol/g/min}$ unidirectional lactate pyruvate flux measured in bicuculline-treated rat brain. However, it should be noted that many factors regulate the rate of LDH reaction. Thus, the enzyme activity of LDH determined under in vitro conditions should not be considered an accurate representation of its V_{max} in vivo.

The partial participation of extra-cellular lactate in saturation transfer of the LDH reaction can cause an underestimation of the pseudo first-order rate constant k due to the use of total lactate magnetization, because lactate distributes both in the intra- (in) and extra-cellular (ex) space. Extracellular fluid comprises approximately 15% of brain volume [70]. Lactate is transported with H^+ across plasma membranes, predominantly via facilitated diffusion mediated by a family of proton-linked monocarboxylate carriers, which are reversible transporters that operate under near-equilibrium conditions to allow equilibration of lactate across the plasma membrane even under conditions of rapid metabolism [149]. That is, $[\text{lactate}]_{\text{ex}}[\text{H}^+]_{\text{ex}} = [\text{lactate}]_{\text{in}}[\text{H}^+]_{\text{in}}$. Cultured astrocytes express powerful, low-affinity monocarboxylate carriers. Studies of lactate uptake kinetics have shown low-affinity uptake into astrocytes, with V_{max} values of approximately 174–250 nmol/min/mg protein ($\approx 23\text{--}33 \mu\text{mol/min/g}$ wet weight [143]), and a K_m value of $\geq 7.7 \text{ mM}$ [150]. Both high-affinity ($K_m = 0.68 \text{ mM}$, $V_{\text{max}} = 3.4 \text{ nmol/min/mg protein}$) and low-affinity ($K_m = 8.1 \text{ mM}$, $V_{\text{max}} = 29 \text{ nmol/min/mg protein}$) lactate uptake into cultured cerebral inter-neurons have been found [150]. The time scale of lactate transport across the plasma membrane of cultured cells overlaps with that of saturation transfer measured in our studies [25,28]. Considering the small extra-cellular volume fraction, the error in k due to partial participation of extra-cellular lactate in ^{13}C saturation transfer should be quite small. However, the unidirectional lactate $\rightarrow$ pyruvate flux depends on ΔM_{Lac} instead. Therefore, any error in the unidirectional flux rate due to partial participation of extra-cellular lactate in the ^{13}C saturation transfer process is expected to be insignificant.

8. Carbonic Anhydrase (CA)

CAs form a family of enzymes that, in mammals, are present in at least 14 different isoforms or carbonic anhydrase-related proteins [151–154]. The active site of most CAs contains a zinc ion and is classified as a metalloenzyme. CA catalyzes the interconversion between carbon dioxide and bicarbonate anion, a reaction that occurs rather slowly in the absence of a catalyst,

and permits near equilibrium even at low substrate concentrations [151]. The carbon atoms of carbon dioxide and bicarbonate are marked with a "*". CA isozymes have different kinetic properties and are distributed in various tissues and cell compartments. CA I, II, the sulfonamide-resistant III, and VII are cytoplasmic, CA V is mitochondrial, and CA VI is present in salivary secretions. CA IV, IX, XII, and XIV are membrane proteins with CA IV being a glycosyl-phosphatidylinositol-anchored protein and CA IX, XII, and XIV are transmembrane proteins. Several of the isozymes, including II, IV, and V, are present in the brain.

The fundamental change catalyzed by CA is from the non-polar linear carbon dioxide gas to the polar triangular carbonic acid or its conjugate base bicarbonate ion. The interconversion between carbonic acid and bicarbonate is physiologically instantaneous. Carbon dioxide plays a vital role in life. It is produced by the metabolism of all cells, and is removed from the body by red blood cells that convert most of it to bicarbonate via carbonic anhydrase for transport, and then back to carbon dioxide to be exhaled from the lungs. CA isozymes catalyze the important reactions during respiration and transport of carbon dioxide/bicarbonate between metabolizing tissues and lung, electrolyte secretion, pH and carbon dioxide homeostasis, lipogenesis, ureagenesis, gluconeogenesis, tumorigenicity, bone resorption, calcification, signal transduction, and many other important physiological and pathophysiological processes [151,152,155–158]. In brain tissues, CA is primarily expressed in glial and choroid cells [159,160], not in neurons. If CA were very active in neurons, the enzyme would impede the rapid removal of carbon dioxide by free diffusion through cell membranes. The compartmentation of CA in the brain leads to the processing of carbon dioxide primarily in glial cells, and renders glial cells as sinks of carbon dioxide. Deitmer [161,162] recently proposed that glial processing of carbon dioxide and energy transfer is coupled with its high-affinity glutamate uptake and other transport processes at the glial and neuronal cell membranes under conditions of high neuronal activities.

The aromatic and heterocyclic sulfonamides can strongly inhibit the catalytic function of the CA isozymes (but not the sulfonamide-resistant CA III). These CA inhibitors possess important clinical applications (e.g., in the treatment of epilepsy, glaucoma [154], and other neurological disorders [163,164]). Many potent CA inhibitors also inhibit the growth of certain tumor cell lines in vitro and in vivo, thus making them interesting leads for developing novel antitumor therapies [165]. On the other hand, CA activators have been used to manage conditions in which learning and memory are impaired, such as in Alzheimer's disease and aging [166], and for treating genetically inherited CA deficiencies [152,158,167]. Considering the ubiquitousness and importance of CA, and ongoing active research endeavors to design drugs for its inhibition or activation, a noninvasive method capable of directly measuring the rate of carbonic anhydrase-catalyzed exchange reaction between carbon dioxide and bicarbonate in vivo is clearly of significant value.

^{31}P MRS has been employed to indirectly investigate CA functions in vivo, in order to assess intracellular pH and high-energy phosphates in skeletal muscle of CA III knockout mice [168]. In our study [27], we measured the in vivo ^{13}C magnetization transfer effect of the CA-catalyzed carbon dioxide-bicarbonate reaction, quantified the pseudo-first-order unidirectional bicarbonate $\rightarrow$ carbon dioxide rate constant, and demonstrated the effect of acetazolamide treatment on ^{13}C magnetization transfer between carbon dioxide and bicarbonate.

Because the labile proton of HCO_3^- is not observable in ^1H MRS, the magnetization transfer effect catalyzed by CA cannot be detected using the ^1H detection method described in Section 3.2.2; we thus saturated the ^{13}C spin of carbon dioxide at 125.0 ppm and measured the ^{13}C signal changes of HCO_3^- at 160.7 ppm using direct ^{13}C detection. Also, the small two-bond ^1H - ^{13}C scalar coupling in $\text{H}^{13}\text{CO}_3^-$ is not observable, even in the absence of heteronuclear decoupling because of the rapid exchange between carbon dioxide and HCO_3^- [79]. Therefore, heteronuclear decoupling and NOE enhancement are not necessary for measuring the exchange rate between carbon dioxide and bicarbonate. Fig. 11 shows the ^{13}C magnetization transfer effect due to the rapid exchange between carbon dioxide and bicarbonate in the brains of a control rat (Fig. 11a) and an acetazolamide-treated rat (Fig. 11b) infused with $[\text{U-}^{13}\text{C}_6]\text{glucose}$. The bicarbonate resonance was the only significant signal in

the difference spectrum with $\frac{\Delta M_{\text{HCO}_3^-}}{M_{\text{OHCO}_3^-}}$ at 0.85. A much smaller intensity change in the bicarbonate signal was seen in the acetazolamide-treated rat brain than that of the control,

because of the CA inhibition effect of acetazolamide. The in vivo ^{13}C T_1 of bicarbonate was determined to be $11.8 \pm 0.4 \text{ s}^{-1}$ (mean $\pm$ SD). The pseudo-first-order unidirectional bicarbonate $\rightarrow$ carbon dioxide rate constants $k_{\text{HCO}_3^- \rightarrow \text{CO}_2}$ were calculated to be $0.47 \pm 0.05 \text{ s}^{-1}$ (mean $\pm$ SD) for the control brain, and $0.073 \pm 0.007 \text{ s}^{-1}$ (mean $\pm$ SD) for the acetazolamide-treated brain [27].

The carbon dioxide $\leftrightarrow$ bicarbonate exchange catalyzed by CA in vitro has been extensively studied using various enzymatic methods, including NMR spectroscopy [54–61]. In the presence of micromolar amounts of CA in solution, the carbon dioxide $\leftrightarrow$ bicarbonate exchange can cause a large line broadening of the bicarbonate signal, which correlates to the carbon dioxide $\leftrightarrow$ bicarbonate exchange rate [54,56,57]. The carbon dioxide hydration activities of the Mn(II) human CA B and I were studied using $\text{NaH}^{13}\text{CO}_3$ and an inversion transfer NMR approach [55,58]. A study of carbon dioxide hydration activity found that a derivative of the native bovine CA III modified with methyl methanethiosulfonate could increase carbon dioxide hydration rate three times [60]. The carbon dioxide $\leftrightarrow$ bicarbonate exchange was also studied using two-dimensional NOE/exchange NMR spectroscopy [61]. The exchange of bicarbonate across the membrane of packed erythrocytes was investigated using radiofrequency saturation of carbon dioxide and inclusion of relevant transmembrane transport terms into the Bloch-McConnell equations [59]. We found that the combination of relatively long ^{13}C T_1 of bicarbonate and fast exchange between carbon dioxide and bicarbonate catalyzed by CA causes a large and quantifiable ^{13}C magnetization effect in vivo [27]. Although only the cerebral carbon dioxide $\leftrightarrow$ bicarbonate exchange catalyzed by CA was investigated, we expect that this technique can also be used to study carbonic anhydrase-catalyzed carbon dioxide $\leftrightarrow$ bicarbonate exchange in other organs or tissues because of the wide distribution of carbonic anhydrase, carbon dioxide, and bicarbonate [151].

Previous enzyme kinetics studies have shown that the transportation of proton ions which are produced in the hydration of carbon dioxide from the active site or to the active site in bicarbonate dehydration is the rate-limiting step in the turnover of carbon dioxide or bicarbonate catalyzed by CAs [169]. Although addition of buffer to the reactant solution helps to bring about some of this proton transfer by external transport and increases the turnover rate, there remains an internal proton transfer step that ultimately limits the turnover rate of carbon dioxide or bicarbonate catalyzed by the CA. As a result, the turnover rate of carbon dioxide or bicarbonate catalyzed by CA is not limited by classic Michaelis-Menten kinetics, but by proton transfer [169]. In an earlier study, Simonsson and colleagues [54] had shown by NMR that the reversible exchange between ^{13}C -labeled carbon dioxide and bicarbonate is significantly more rapid than the turnover rate of carbon dioxide or bicarbonate. This process causes a significant amplification effect in favor of the in vivo detection of the ^{13}C magnetization transfer effect catalyzed by CA. The advantage of this significant amplification effect is that a large signal change at the bicarbonate resonance can be observed. Furthermore, this amplification effect makes the ^{13}C magnetization transfer effect very sensitive to alterations in the catalytic environment; these, for instance, can be affected by ion substitution [58], by mutation of CA residues that are important for stabilizing the zinc-bound hydroxide ion and for maintaining it in a highly reactive state [55,170], and by the action of sulfonamide inhibitors as shown in Fig. 11. On the other hand, the amplification effect also makes the ^{13}C saturation transfer effect insensitive to the proton transfer step, and therefore to the turnover rate of bicarbonate from carbon dioxide or to that of carbon dioxide from bicarbonate catalyzed by CA.

The hydration and dehydration of carbon dioxide can also proceed without CA catalysis. The hydration reaction of carbon dioxide is generally very slow in the absence of a catalyst. The true first-order rate constant of the uncatalyzed hydration reaction is 0.04 s^{-1} at 37°C [151]. The apparent ionization constant of carbonic acid ($K_a' = [\text{H}^+][\text{HCO}_3^-]/[\text{CO}_2]$) equals approximately 6.8×10^{-7} . The predicted uncatalyzed pseudo first-order dehydration constant

$k_{\text{HCO}_3^- \rightarrow \text{CO}_2}$ is 0.005 s^{-1} at $\text{pH} = 7.0$. This predicted $k_{\text{HCO}_3^- \rightarrow \text{CO}_2}$ of the uncatalyzed carbon dioxide $\leftrightarrow$ bicarbonate exchange is in exact agreement with our experimentally determined $\sim 0.005 \text{ s}^{-1}$ using ^{13}C saturation transfer and the 50 mM $\text{NaH}^{13}\text{CO}_3$ phantom [27]. In comparison, a $k_{\text{HCO}_3^- \rightarrow \text{CO}_2}$ of 0.47 s^{-1} was determined using ^{13}C magnetization transfer spectroscopy in isoflurane-anesthetized adult rat brain in vivo [27]. The acceleration of the nonenzymatic carbon dioxide $\leftrightarrow$ bicarbonate exchange rate in the in vivo brain due to CA catalysis was found to be highly significant in our study [27]. Acetazolamide is a highly specific sulfonamide inhibitor of CA [171]. As demonstrated in Fig. 11b, intralateral ventricular administration of acetazolamide causes a large reduction in the ^{13}C magnetization transfer effect in vivo, consistent with its expected strong inhibitory effect on CA. The $k_{\text{HCO}_3^- \rightarrow \text{CO}_2}$ values determined from phantom, control rats, and rats treated with acetazolamide are plotted in Fig. 12.

9. Conclusions

Approximately thirty years after the discovery of in vivo magnetization transfer effects exhibited by the few enzymes catalyzing transfer of phosphate groups, it has been surprising to uncover hidden rapid exchanges underneath commonplace MRS signals such as glutamate, aspartate, and lactate considering that magnetization transfer effect on the proton MRS spectra has been thoroughly investigated [e.g., 172,173]. Two factors may explain why enzyme-specific magnetization transfer effects were missed in previous investigations of off-resonance magnetization transfer effect on proton MRS signals: i) The chemical shift differences between proton resonant signals of amino/hydroxy acids and corresponding keto acids are relatively small. Among the glutamate/ α -ketoglutarate, aspartate/oxaloacetate and lactate/pyruvate exchange pairs, the 1.05 ppm chemical shift difference between pyruvate and lactate methyl protons is the largest; ii) The longitudinal relaxation times of the relevant proton signals are much shorter than the carbon signals. In the case of LDH-catalyzed exchange reaction between pyruvate and lactate the maximum change in proton signals due to saturation transfer is estimated to be $\sim 11\%$ based on the pseudo first order rate constants determined in Section 7 and the known lactate methyl proton T_1 ($\sim 1.5 \text{ s}$ at 11.7 Tesla). The dramatically increased crowdedness of the proton spectra (leading to RF spillover described in Section 3.1) and shortened longitudinal relaxation times make detection of magnetization transfer in the proton channel much more difficult. In comparison, the relatively longer ^{13}C T_1 values and the distinctly different chemical environments among the α -carbons of amino, hydroxy, and keto acids have allowed maximum transfer of saturation and clean subtraction to reveal the hidden rapid exchange reactions in the difference spectra.

The measurements of in vivo kinetic parameters such as chemical exchange rates and fluxes can provide important information about enzymatic activities. In this review, we showed that the in vivo ^{13}C magnetization transfer effects of certain enzymes in rat brain can be detected [24–27]. We also showed that the sensitivity of the ^{13}C magnetization transfer technique can be improved by a ^{13}C saturation transfer with ^1H detection method (e.g. inverse ^{13}C -to- ^1H INEPT-based polarization transfer technique) [28].

One of the main advantage of a relayed ^{13}C magnetization transfer method is that it offers an opportunity to measure chemical exchanges generally considered undetectable [26,29]. One of the examples discussed in this review is the study of MDH. Instead of trying to measure resonance signals from malate or oxaloacetate, which are both undetectable by in vivo NMR due to their low concentration, we measured the ^{13}C signal from aspartate, a substrate that is well above the detection threshold of in vivo MRS and connected to malate via the small and rapidly turning over oxaloacetate pool [26]. A similar design was also used to study the exchange between mitochondrial α -ketoglutarate/oxaloacetate and cytosolic glutamate/aspartate pools [29].

The valuable in vivo ^{13}C magnetization transfer technique provides a new way to both study the kinetics of biochemical systems, and diagnostically characterize pathological states. Application of ^{13}C magnetization transfer spectroscopy to studying rapid enzyme reactions or exchange processes in isolated cells and organelles, prepared cell cultures, as well as perfused tissues or organs are readily conceivable. Like the magnetization transfer effect of the creatine kinase reaction the newfound enzyme-specific magnetization transfer effects may also be exploited as possible magnetic resonance reporters of gene expression.

Finally, all ^{13}C magnetization transfer effects have been discovered in brain. The demonstration of the existence of these enzyme-specific magnetic resonance effects open the possibility of using similar strategies to study enzymes in other organs in vivo. For example, the exchange between acetaldehyde and alcohol catalyzed by alcohol dehydrogenase is known to be very rapid in liver and alcohol dehydrogenase is a key marker for liver function. ^{13}C magnetization transfer may offer a noninvasive in vivo method to characterize alcohol dehydrogenase as well as many other enzymes which catalyze rapid chemical exchange reactions.

Glossary

α -KG	α -ketoglutarate
AAT	aspartate aminotransferase
AGC	aspartate/glutamate carrier
AHP	half-passage pulse
ATP	adenosine triphosphate
Asp	aspartate
CA	carbonic anhydrase
CSF	cerebrospinal fluid
CK	creatine kinase
CW	continuous wave
FA	fumarase
Fum	fumarate
GABA	γ -aminobutyric acid
Glu	glutamate
INEPT	insensitive nuclei enhanced by polarization transfer
Lac	lactate
LDH	lactate dehydrogenase
Mal	malate
MDH	malate dehydrogenase
MRS	magnetic resonance spectroscopy
NAD(H)	nicotinamide adenine dinucleotide oxidized (reduced)
NADP(H)	nicotinamide adenine dinucleotide phosphate (reduced)
NOE	nuclear Overhauser enhancement
OGC	oxaloglutarate carrier

Oxa	oxaloacetate
PCr	phosphocreatine
Pyr	pyruvate
RARE	rapid acquisition with relaxation enhancement
TCA	tricarboxylic acid
WALTZ	wideband alternating phase low-power technique for zero-residue splitting

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Fig. 1.

The two-site exchange model for substrate A $\leftrightarrow$ substrate B. M_{0A} and M_{0B} represent the thermal equilibrium magnetization of substrate A and B, respectively. T_{1A} , T_{1B} and T_{2A} , T_{2B} represent the longitudinal, transverse relaxation time in the absence of exchange between substrate A and B. k_{AB} and k_{BA} are the pseudo-first-order rate constants of the unidirectional A $\rightarrow$ B and B $\rightarrow$ A fluxes, respectively.

Fig. 2.

A surface coil pulse sequence for the ^{13}C saturation transfer with ^{13}C detection experiments. Broadband $^1\text{H} \rightarrow ^{13}\text{C}$ NOE is generated using a train of non-selective hard pulses with a nominal flip angle of 180° spaced at $\Delta = 100$ ms apart. A simultaneous CW pulse or a train of spectrally selective Gaussian pulses is executed for radiofrequency saturation at substrate A resonance or at the control frequency during NOE generation. An adiabatic AHP and WALTZ-4 pulses were used for excitation and subsequent proton decoupling, respectively. Additional spatial localization may be added prior to ^{13}C excitation.

Fig. 3.

Pulse sequence for the ^{13}C saturation transfer with ^1H detection experiments (depiction of gradient pulses is omitted for clarity). Substrate A is irradiated using a continuous train of 2 ms nominally 180° Gaussian pulses and a simultaneous train of nominally 180° 200 μs rectangular pulses spaced 200 ms apart. At the end of the saturation pulse trains, an optional 500 μs AHP pulse and crusher gradients is used for further water suppression. A nonadiabatic 90°_x pulse is then used for excitation of ^{13}C equilibrium signals. A pair of identical hyperbolic secant pulses (sech, 2.5 ms, $\mu = 3$, 1% truncation) are used to generate an adiabatic double spin echo with an ^{13}C echo time (TE1) of 8.3 ms. A 1.5-ms sech pulse is also applied to the proton channel to reintroduce heteronuclear J evolution. A nonadiabatic 200 μs $90^\circ_{\pm y}$ pulse is used to convert the antiphase terms into longitudinal two-spin orders. Then, proton outer volume suppression using six nominal 90° sech pulses (2 ms, $\mu = 5$, 1% truncation) along the x (10 mm slab), -x (10 mm slab), y (3 mm slab), -y (5 mm slab), z (10 mm slab), -z (10 mm slab) directions plus crusher gradients, and CHESS water suppression (15 ms, Gaussian 90° pulse plus gradient crushers) are executed. Finally, the single-shot, fully adiabatic proton localization sequence is used to convert longitudinal two-spin orders into antiphase transverse proton coherence and to achieve transverse spatial localization for selection of a 5 (x) $\times$ 2.9 (y) $\times$ 5 (z) mm^3 (72.5 μL) voxel. A hyperbolic secant pulse is applied to the ^{13}C channel to refocus heteronuclear J evolution. After a nominal delay of $1/2^1J_{\text{CH}}$, the in-phase transferred proton coherence is detected with simultaneous ^{13}C decoupling using WALTZ4. Reprinted from [28], with permission from Elsevier Inc.

Fig. 4.

(a) Comparison of a 500 MHz ^1H saturation transfer spectrum with the corresponding symmetric control spectrum for α -ketoglutarate $\leftrightarrow$ glutamate. The relaxation delay was $\text{TR} = 10$ s. The strength of the saturation pulse was $B_{1\text{sat}} = 158$ Hz. A line broadening of $\text{lb} = 30$ Hz was applied, and the total number of scans was $NS = 512$. (b) Comparison of saturation transfer spectrum with the corresponding symmetric control spectrum for oxaloacetate $\leftrightarrow$ aspartate. $\text{TR} = 10$ s. $B_{1\text{sat}} = 790$ Hz. $LB = 30$ Hz. Total $NS = 512$. Upper traces: control spectra; middle traces: saturation transfer spectra; lower traces: difference spectra. The saturation transfer spectra were acquired with CW saturation of carbonyl carbons of the keto acids. The control spectra were acquired with the saturation pulse placed at an equal distance from amino acid C2, but on the opposite site of its cognate keto acid C2. Asp: aspartate; GABA: γ -aminobutyric acid; Glc: glucose; Glu: glutamate; NAA: *N*-acetylaspartate. Fig.4a was reprinted from [76], with permission from John Wiley & Sons, Ltd.

Fig. 5. 125 MHz ^{13}C spectra from a $[1,6\text{-}^{13}\text{C}_2]$ glucose infusion experiment showing the saturation transfer effect caused by rapid exchange between α -KG and Glu using ^{13}C saturation transfer with ^1H detection method. A 72.5 μl voxel was localized in the rat brain. The relaxation delay was $\text{TR} = 7.1$ s with a total number of scans of $\text{NS} = 512$. A Lorentz-Gaussian apodisation was applied with $\text{lb} = -20$ Hz and $\text{gb} = 0.02$. In the upper and middle traces, resonances from Glx H2 (glutamate H2 + glutamine H2) (3.75–3.76 ppm), glutamate H4 (2.3–2.4 ppm), and residual water (4.65 ppm) were observed. The difference spectrum (bottom trace) shows the expected magnetization transfer effect at 3.75 ppm. Reprinted from [28], with permission from Elsevier Inc.

Fig. 6.

(a) The exchange of four-carbon molecules between cytosol and mitochondria (adapted from Fig. 46 [70]). Cytosolic malate enters mitochondria through the oxoglutarate carrier (OGC). Mitochondrial aspartate enters cytosol through the aspartate/glutamate carrier (AGC). The rapid exchange reactions between malate and oxaloacetate and between oxaloacetate and aspartate are catalyzed by malate dehydrogenase (MDH) and aspartate aminotransferase (AAT), respectively. In mitochondria, malate is also in rapid exchange with fumarate catalyzed by fumarase (FA). (b) Simplified four-site exchange model for estimating V_x using relayed ^{13}C magnetization transfer spectroscopy and the Bloch–McConnell equations. The small mitochondrial fumarate, malate, oxaloacetate, and aspartate pools are lumped into one

site denoted by fumarate. This simplification attributes loss of saturation inside the mitochondrial matrix to V_x . The V_x value calculated through this model therefore represents its lower limit. The large, bold font for cytosolic aspartate indicates that its pool size is overwhelmingly larger than all other pools in the diagram. Asp, cytosolic aspartate; Fum, mitochondrial fumarate; Mal, cytosolic malate; Oxa, cytosolic oxaloacetate. Reprinted from [29], with permission from Blackwell Publishing.

Fig. 7.

(a) In vivo relayed 125 MHz ^{13}C magnetization transfer results from one rat. Top trace: control spectrum with the Gaussian saturation pulse train placed at -29.7 ppm (NS=1280); middle trace: fumarate-saturated spectrum with the Gaussian saturation pulse train placed at 136.1 ppm; bottom trace and inset: difference spectrum. (b) In vivo relayed ^{13}C magnetization transfer results summed from eight rats. Top trace: control spectrum summed from eight rats; middle trace: fumarate-saturated spectrum summed from eight rats (NS=10,240); bottom trace and inset: difference spectrum summed from eight rats. Glc C6, α - and β -glucose C6; Glu C2, glutamate C2; Gln C2, glutamine C2; Asp C2, aspartate C2. Reprinted from [29], with permission from Blackwell Publishing.

Fig. 8. 125 MHz ^{13}C spectra from a $[2\text{-}^{13}\text{C}]$ glucose infusion experiment showing the saturation transfer effect caused by rapid exchange between pyruvate and lactate. A relaxation delay of $\text{TR} = 9\text{ s}$ was used, and a total number of scans $\text{NS} = 128 \times 4$ were acquired. A Gaussian window function was used with $\text{lb} = -10\text{ Hz}$, $\text{gb} = 0.03$. The saturating field used was $\gamma B_{1\text{sat}} = 315\text{ Hz}$. (a) Bicuculline-treated rat brain, (b) Postmortem. Top traces: symmetric control spectra. Middle traces: with saturation of pyruvate C2 at 207.9 ppm. Bottom trace: difference spectra. Spectra from each stack were summed from four rats. Reprinted from [25], with permission from John Wiley & Sons, Ltd.

Fig. 9.

Investigation of nonspecific off-resonance magnetization transfer effect. 125 MHz ^{13}C Spectra acquired using the same parameters as in Fig. 8 except that the saturation pulse placed at the resonance frequency of pyruvate C2 was shifted by 75 kHz. A significant, nonspecific, off-resonance magnetization transfer effect, which could arise from exchange between a putative small pool of immobilized lactate and free lactate could not be detected. Spectra were summed from four rats. Reprinted from [25], with permission from John Wiley & Sons, Ltd.

Fig. 10.

Examples of 125 MHz ^{13}C saturation transfer effect observed in rat brain with C6 glioma. (a) From a rat after 16 days of inoculation. ^{13}C MRS parameters: A relaxation delay of $\text{TR} = 9$ sec was used, and a total number of scans $\text{NS} = 192$ were acquired. A Gaussian window function was used with $\text{lb} = -10$ Hz, $\text{gb} = 0.03$. Anatomical imaging parameters: A relaxation delay of $\text{TR} = 3050$ ms and an echo time of $\text{TE} = 30$ ms were used. The slice thickness used was $\text{st} = 2$ mm with a field of view $\text{FOV} = 2.56$ cm. The matrix size of the image was 128×128 . The rat was infused with $[2\text{-}^{13}\text{C}]\text{glucose}$. $\Delta M_{\text{Lac}}/M_{0\text{Lac}} = 0.19$. (b) From a second rat after 15 days of inoculation. $\Delta M_{\text{Lac}}/M_{0\text{Lac}} = 0.10$. All NMR parameters were the same as in (a). Reprinted from [25], with permission from John Wiley & Sons, Ltd.

a

b

Fig. 11.

125 MHz ^{13}C spectra from a $[\text{U-}^{13}\text{C}]$ glucose infusion experiment showing the saturation transfer effect due to rapid exchange between CO_2 and HCO_3^- in rat brains. A relaxation delay of $\text{TR} = 53$ s was used, and a total number of scans $\text{NS} = 256$ were acquired. A line broadening of, $\text{lb} = 15$ Hz was applied. (a) Control; (b) acetazolamide-treated. Top traces: symmetric control spectra; Middle traces: with saturation of CO_2 at 125.0 ppm. Bottom trace: difference spectra. Reprinted from [27], with permission from John Wiley & Sons, Ltd.

Fig. 12.

Pseudo first-order unidirectional dehydration rate constant $k_{HCO_3^- \rightarrow CO_2}$ determined from phantom, control rats, and rats treated with acetazolamide. Reprinted from [27], with permission from John Wiley & Sons, Ltd.

Table 1¹³C and ¹H chemical shifts of molecules involved in enzyme-specific ¹³C magnetization transfer effects

Resonances	¹³ C Chemical Shift (ppm)	¹ H Chemical Shift (ppm)
α-ketoglutarate C2	206.2	/
aspartate C2	53.2	3.89
bicarbonate	160.7	/
carbon dioxide	125.0	/
fumarate C2	136.1	6.52
glutamate C2	55.7	3.75
lactate C2	69.4	4.11
lactate C3	20.8	1.32
malate C2	71.2	4.28
oxaloacetate C2	201.3	/
pyruvate C2	207.9	/
pyruvate C3	29.1	2.37