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Supporting Information:

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"Production and Incorporation of ^{15}N, ^{13}C, ^{2}H (^{1}H-δ1 methyl) Isoleucine into Proteins for Multidimensional NMR Studies"

Reaction Details

1. Production of (3-^{2}H), ^{13}C 2-ketobutyrate from ^{13}C threonine:

reaction conditions:

20 mM ^{15}N, ^{13}C threonine (Cambridge Isotope Labs; note that [^{15}N natural abundance], ^{13}C threonine will work as well)

50 mM potassium phosphate buffer, pH* 7.5 (uncorrected for deuterium isotope effects)

99.5% $^{2}H_2O$ (Cambridge Isotope Labs)

To this reaction, 10 μg *Escherichia coli* biosynthetic threonine deaminase was added (provided by Drs. Diana Chinchilla and Edward Eisenstein, Center for Advanced Research in Biotechnology, University of Maryland; additional enzyme was overexpressed in *E. coli* and purified (Eisenstein, 1991). The activity of the enzyme was assayed by monitoring the time-dependent increase of absorption at 230 nm (corresponding to the formation of 2-ketobutyrate) for a reaction at room temperature (~22°C) containing 50 mM threonine, 50 mM potassium phosphate buffer, pH* 7.5 (Eisenstein, 1991). The enzyme activity was found to be 128 (μmol Thr converted) (mg enzyme)$^{-1}$ min^{-1} in 99.5% D_2O as compared to 247 (μmol Thr converted) (mg enzyme)$^{-1}$ min^{-1} in H_2O.

The reaction was assembled in a total volume of 3.2 mL with 0.5 mL removed for real-time NMR assay (below). The reaction was run in the dark at 26°C until completion as judged by NMR (approximately 4 hr; shown in Supporting Figure 1).

Real-time NMR assay: 1D ^{1}H NMR spectra were recorded every five minutes on the 0.5 mL reaction sample; this assay was important as the enzyme V_{max} is affected by deuterium isotope effects (above) and threonine concentration (Eisenstein, 1991). These spectra were acquired with the following parameters: T=26°C, 4 scans acquired/FID, 15.2 s relaxation delay with ^{13}C decoupling during acquisition (2.2 kHz field, centered at 40 ppm). Peak integrals were monitored as a function of time to track the progress of the reaction by watching the disappearance of threonine peaks (3-CH (δ=4.20 ppm), 4-CH_3 (δ=1.28 ppm)) and the concomitant appearance of (3-^{2}H) 2-ketobutyrate (3-$CH^{2}H$ (δ=2.67 ppm), 4-CH_3 (δ=1.01 ppm)). These assignments are consistent with the chemical shifts and $^{3}J(^{1}H\text{-}^{1}H)$ splittings of natural abundance ^{13}C threonine and 2-ketobutyrate (data not shown).

2. Production of (3,3-$^{2}H_2$), ^{13}C 2-ketobutyrate from (3-^{2}H), ^{13}C 2-ketobutyrate:

Reaction conditions: to avoid aldol condensation resulting in dimerization of 2-ketobutyrate during the base-catalyzed exchange process, the reaction was diluted with 20 mL of 50 mM potassium phosphate buffer (99.5% $^{2}H_2O$, pH* 7.5). This led to a total volume of 23.2 mL, with a 2-ketobutyrate concentration of 2.7 mM.

The sample pH* was initially raised to 9.3 by the addition of 40% sodium deuteroxide (CIL) and incubated at 45°C for 2 hours; subsequently, the pH* was increased to 9.8 (incubated for 1 hour

at 45°C) and 10.2 (10 hours at 45°C). At each step, 0.5 mL of the reaction volume was removed for real-time NMR assay (as above, except T=45°C; shown in Supporting Figure 2). Along with the peaks identified above, spectra were monitored for the formation of aldol-condensed 2-ketobutyrate by the presence of a methyl peak from a CH_3 from the dimeric form (δ=0.82 ppm, verified by running this reaction on 25 mM natural abundance ^{13}C 2-ketobutyrate at pH* 12.5 to promote aldol condensation). Upon completion of the reaction, the pH* was decreased to 7.9, sterile filtered (0.22 μm cutoff filter) and stored at 4°C until used in the bacterial growth (below).

3. Incorporation of (3,3-$^2H_2$),^{13}C 2-ketobutyrate and ^{15}N,^{13}C valine by *E. coli*: BL21(DE3) *E. coli* cells were transformed with a plasmid carrying residues 663-759 (plus additional residues from cloning for a total of 105) of the C-terminal SH2 domain of bovine phospholipase Cγ1 (PLCC). As previously described (Rosen et al., 1996), bacteria were grown in stages to adapt them to 99.5% D_2O and pyruvate as their lone carbon source.

In the final stage of this protocol, bacteria were grown at 37°C in 150 mL minimal M9 media with 1.5 g/L ^{12}C-pyruvate, 1 g/L $^{15}NH_4Cl$ and 99.5% ^2H_2O to an O.D.(600 nm) = 0.2.

Protein expression was triggered by the addition of 0.25 mg/mL IPTG. Simultaneously, the following amino acid and biosynthetic precursor were added:

^{15}N, ^{13}C valine	50 mg/L
(3,3-$^2H_2$), ^{13}C 2-ketobutyrate	50 mg/L

An additional 1.5 g/L ^{12}C-pyruvate was added as well. Expression was allowed to proceed for 6.5 hr at 37°C before the cells were harvested. The PLCC SH2 domain was purified as previously described (Rosen et al., 1996).

Spectral parameters:

Figure 1: Spectral parameters: ^{13}C: 5000 Hz, t_1^{max}=32.0 ms (160 complex pts), final size: 512 pts; ^1H: 8000 Hz, t_2^{max}=64.0 ms (1024 complex pts), final size: 2048 pts (500 MHz).

Figure 2: Spectral parameters: ^{13}C: 6000 Hz, t_1^{max}=21.3 ms (128 complex pts), final size: 512 pts; ^1H: 9001 Hz, t_2^{max}=64.0 ms (1152 complex pts), final size: 2048 pts (600 MHz).

Quantitation of methyl labeling efficiency:

The efficiency of generating methyl-protonated valine, leucine and isoleucine in proteins that were otherwise highly deuterated at aliphatic positions was determined by NMR. Samples were prepared using the protocol listed above with D_2O-based minimal media supplemented with $^{15}NH_4Cl$, (^{15}N, ^{13}C, ^1H) valine, (^{12}C, ^1H) 2-ketobutyrate and (^{12}C, ^1H) glucose as a carbon source. Protein produced in this manner is uniformly ^{15}N labeled while only the valine backbone carbonyl groups are ^{13}C labeled. As such, the HNCO experiment (Muhandiram and Kay, 1994) can be suitably modified to record 2D ^{15}N-^1H correlation maps where the only crosspeaks that are observed arise from backbone ^{15}N-^1H pairs that are immediately adjacent to ^{13}C (not ^{12}C), ^{12}C (not ^{13}C) or either ^{13}C or ^{12}C labeled carbonyl groups. The efficiency was established as:

$$efficiency(i) = \frac{[^{15}N\text{–}^{1}H]^{i+1}_{^{13}C-only}}{[^{15}N\text{–}^{1}H]^{i+1}_{^{13}C-only} + [^{15}N\text{–}^{1}H]^{i+1}_{^{12}C-only}}$$

where $[^{15}N\text{–}^{1}H]^{i+1}_{^{13}C-only}$ is the crosspeak volume for the backbone ^{15}N-^{1}H correlation of residue *i+1* measured in spectra which record these correlations only for residues immediately following ^{13}C-labeled valine. As the carbonyl groups of leucine and isoleucine are ^{12}C labeled in these samples, these experiments cannot offer direct estimates of the labeling efficiencies of these amino acids. However, these efficiencies can be estimated by knowing the valine labeling efficiency (determined above) and comparing the relative intensities of Val, Leu and Ile(δ1) methyl crosspeak volumes in fully relaxed non-constant time ^{13}C-^{1}H HSQC experiments recorded on fully protonated and deuterated, [Val, Leu, Ile(δ1)]-protonated samples. These analyses indicate that the valine and leucine methyl groups are approximately 92±5% (^{13}C, ^{1}H) labeled, while the Ile(δ1) labeling efficiency is approximately 102±5%.

References:

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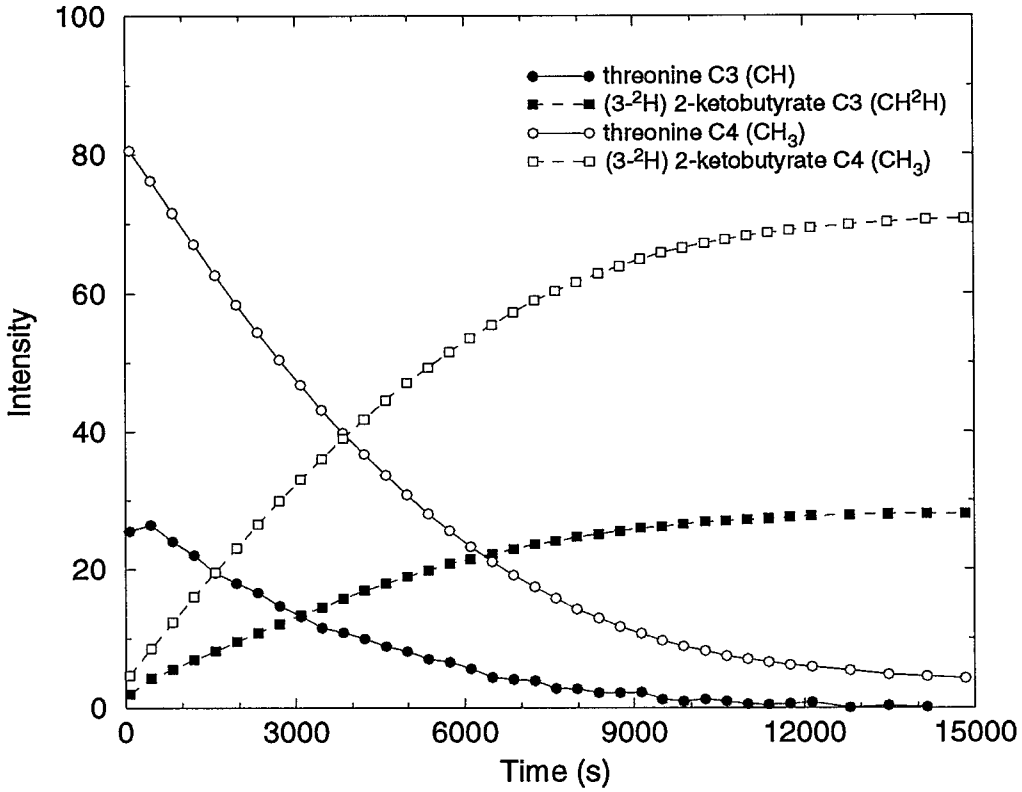
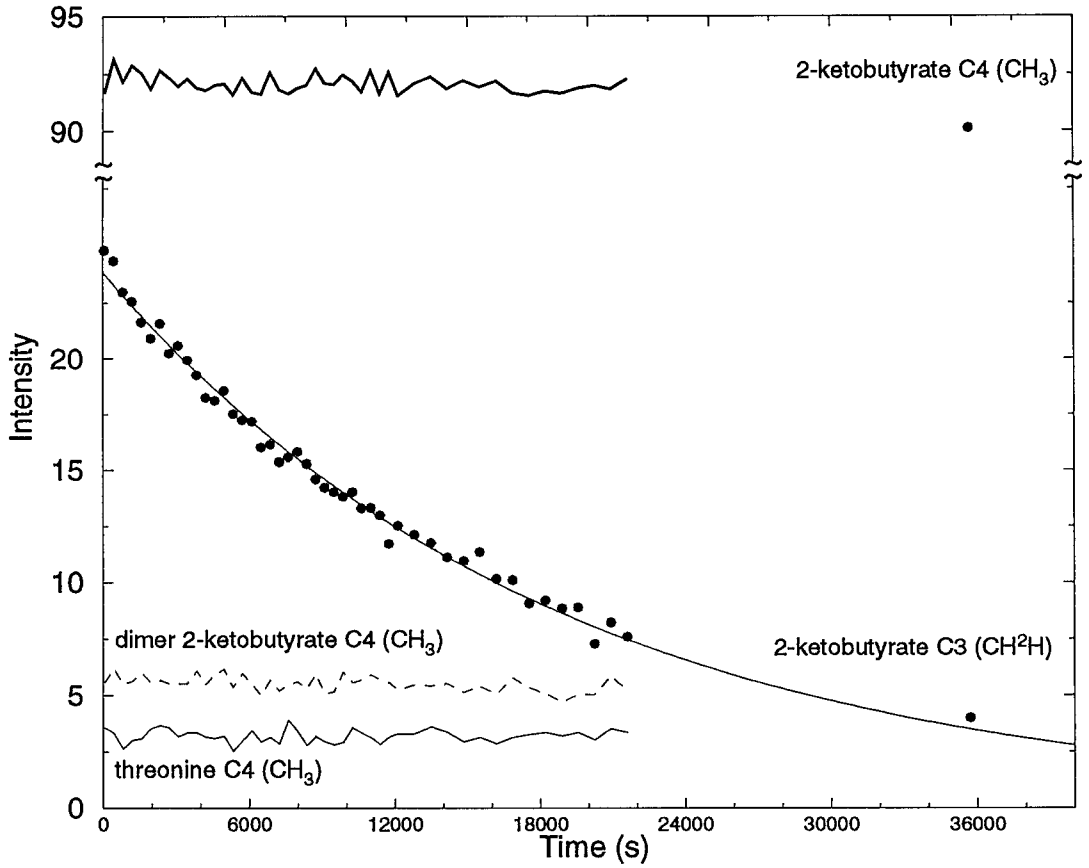


Supporting Figure 1: Progress of threonine deaminase catalyzed reaction. Peak integrals for each identified peak were recorded from each NMR spectrum.



Supporting Figure 2: Progress of base-catalyzed incorporation of deuterium at the 3-methylene group of 2-ketobutyrate at pH* 10.2. Peak integrals for each identified peak were recorded from each NMR spectrum.