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Biosynthetic ^{13}C Labeling of Aromatic Side Chains in Proteins for NMR Relaxation Measurements

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Biosynthetic enrichment of macromolecules with stable isotopes has been used to great advantage in biomolecular NMR spectroscopy.[1] In studies of side chain dynamics by spin relaxation methods, site-specific ^{13}C labeling offers a desirable means of eliminating unwanted relaxation pathways and coherent magnetization transfer. Here we present a straightforward and cost-effective method for biosynthetic incorporation of ^{13}C specifically at the δ-position of the aromatic rings of Phe and Tyr, together with positions $\delta2/\epsilon1$ in His and $\delta1/\epsilon2/\epsilon3$ in Trp. This labeling scheme greatly simplifies the measurements and interpretation of ^{13}C relaxation rates in aromatic side chains and is well suited for characterization of chemical exchange by CPMG or spin-lock relaxation methods. Aromatic residues occur frequently in the hydrophobic core and ligand binding sites of proteins. Thus, dynamic information obtained from aromatic ^{13}C relaxation is expected to complement and extend that generated by existing protocols for ^{13}C methyl relaxation.

In the context of relaxation experiments, specific labeling schemes must be employed whenever the spin system of interest cannot be decoupled from undesired interactions by means of pulse sequence design, as exemplified by band-selective refocusing pulses in the case of CPMG refocusing pulse trains,[2,3] or judicious choices of radio frequency field strengths and offsets in the case of continuous-wave spin-lock ($R_{1\rho}$) experiments.[4,5] In aromatic rings, the problems are particularly severe due to the sizable *J*-couplings, combined with the relatively small chemical shift differences between aromatic carbons, with an average of approximately 1.5 ppm in the case of Phe, as calculated from the BMRB database (http://www.bmrb.wisc.edu/). Consequently, measurements of transverse relaxation rates (R_2) for ^{13}C spins in aromatic ring systems of uniformly labeled proteins are hampered by highly efficient magnetization transfer among the spins, which renders the relaxation decays nonexponential.

A number of strategies for biosynthetic incorporation of isolated (i.e., non-neighboring) ^{13}C nuclei have been devised. Bacterial cultures grown on [2-^{13}C]-glycerol or [1,3-$^{13}C_2$]-glycerol produce amino acids with isolated ^{13}C nuclei throughout the aliphatic parts of most side chains.[6] The use of [2-^{13}C]-glycerol will also introduce isolated ^{13}C nuclei in the aromatic rings of Phe, Tyr, and Trp. Similarly, random and fractional labeling has been achieved using a mixture of acetate enriched in ^{13}C at positions 1 or 2, or both.[7] [3-^{13}C]-Pyruvate has been utilized to obtain isolated ^{13}C nuclei in the methyl groups of Ala, Val, and Leu, and the $\gamma2$ methyl of Ile.[8,9] Specific labeling of both ϵ-carbons in Phe has been achieved by growing *Escherichia coli* on [ϵ,ϵ-$^{13}C_2$]-Phe synthesized from commercially available [ϵ,ϵ-$^{13}C_2$]-Tyr.[10] A major drawback of the various labeling strategies mentioned above is the relatively high costs of the labeled metabolites. Here we present a favorable alternative, which apparently has been used rarely in the past, and for other purposes.[11] By using [1-^{13}C]-glucose as the sole carbon source in the growth media for protein overexpression in *E. coli*, partial labeling of isolated carbon positions in the aromatic side chains is achieved (Figure 1). Briefly, the carbon skeletons of the aromatic amino acids stem from phosphoenolpyruvate (PEP), erythrose-4-phosphate (E4P), and ribose-5-phosphate (R5P).[12] The three-carbon species, PEP, is generated directly from glucose during glycolysis, resulting in approximately 50% ^{13}C enrichment at position 3 when glucose is labeled specifically at position 1. Both E4P and R5P are produced in the pentose phosphate pathway. Due to the rapid equilibria of the transketolase and pentose-5-phosphate isomerization reactions,[13] E4P will be ^{13}C enriched at position 1 and R5P at positions 1 and 5. As a result, each of the carbons highlighted in Figure 1 will be partially (maximally 50%) enriched in ^{13}C. Thus, [1-^{13}C]-glucose yields the same labeling pattern as [3-^{13}C]-pyruvate, albeit with 50% lower incorporation levels. In the large majority of proteins at near-physiological temperatures, $^{13}C^{\delta1}$ and $^{13}C^{\delta2}$ in Phe and Tyr will be magnetically equivalent due to rapid ring flips; consequently, scalar coupling between the two spins will be ineffective.[14] In His and Trp, the labeled carbons are separated by two or three covalent bonds and exhibit chemical shift differences of 17 ± 4 ppm (His, $\delta2$-$\epsilon1$), 11 ± 3 ppm (Trp, $\delta1$-$\epsilon2$), 6 ± 3 ppm (Trp, $\delta1$-$\epsilon3$), and 17 ± 7 ppm (Trp, $\epsilon2$-$\epsilon3$), as calculated from the average shifts listed in the BMRB. Hence, accurate relaxation rates can be measured also for these carbons, provided that the necessary experimental precautions are taken.[4,5] The same is expected to hold for Tyr in uniformly labeled systems because the average shift difference between δ and ϵ is 15 ± 3 ppm. However, the large one-bond coupling constant between these spins restricts the range of viable radio frequency field strengths and offsets in this case.

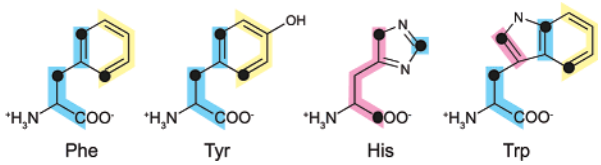

Figure 1. Labeling pattern of aromatic amino acids in *E. coli* cultures grown on media containing [1-^{13}C]-glucose as the sole carbon source, as predicted from the biosynthetic pathways of *E. coli*.[12] Carbon atoms marked with a black dot are partially enriched in ^{13}C. Colored shading indicates the origin of the different carbon atoms in these amino acids: cyan, PEP; magenta, R5P; and yellow, E4P. Continuous shading of two or more atoms indicates that the corresponding fragments are preserved from the precursors.

The labeling scheme utilizing [1-^{13}C]-glucose was applied to the E140Q mutant of the C-terminal domain of calmodulin (E140Q-Tr2C)[15] and bovine acyl-coenzyme A binding protein (ACBP).[16] Figure 2 verifies that the aromatic rings become ^{13}C labeled only at δ of Phe and Tyr, $\delta2/\epsilon1$ of His, and $\delta1/\epsilon3$ of Trp ($\epsilon2$ of Trp is not observable in a ^{13}C HSQC). Our results are consistent with what is expected based on the metabolic pathways of *E. coli*,[12] but disagree with a previous report, which suggested that ^{13}C is incorporated also at γ of Phe, ζ of Tyr, and $\delta2$ of His.[11]

The specific labeling pattern obtained using [1-^{13}C]-glucose permits straightforward measurements of relaxation rates for $^{13}C^{\delta}$

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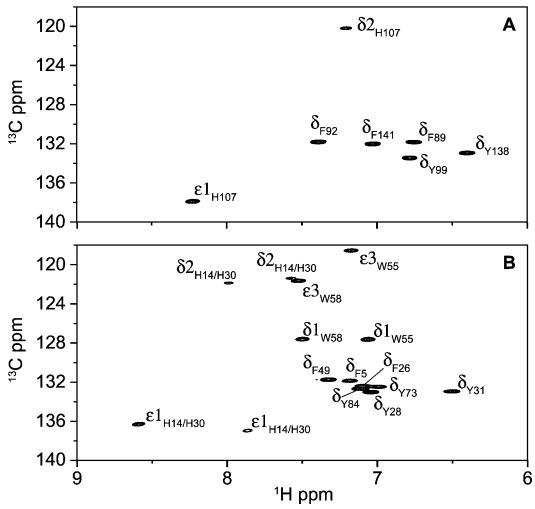

Figure 2. The aromatic region of gradient-enhanced $^{1}H-^{13}C$ HSQC spectra of (A) E140Q-Tr2C (2.9 mM in 10%/90% D_2O/H_2O, pH 6.0 at 301 K) and (B) ACBP (0.3 mM in 10%/90% D_2O/H_2O, pH 5.5 at 298 K), both expressed in *E. coli* cultures grown on [1-^{13}C]-glucose. Protein expression and purification followed published protocols.[15,17]

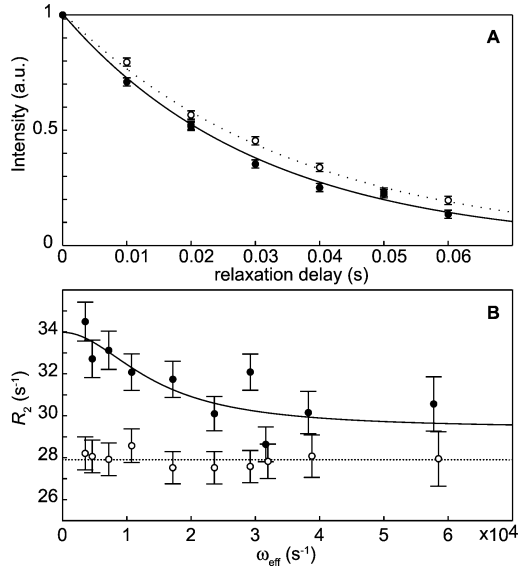

Figure 3. Representative ^{13}C $R_{1\rho}$ relaxation data for E140Q-Tr2C: Y138, filled symbols, solid line; F141, open symbols, dotted line. (A) Relaxation decays, obtained at a nominal tilt angle of $\theta = 90°$. The fitted curves are monoexponential functions. $R_{1\rho} = 33.0 \pm 0.9$ and 27.9 ± 0.8 s^{-1} for Y138 and F141, respectively. (B) Relaxation dispersion profiles of R_2 as a function of the effective field strength. The fitted curves correspond to $R_{1\rho} = R_1 \cos^2\theta + R_{2,0}\sin^2\theta + [k_{ex}\phi_{ex}/(k_{ex}^2+\omega_{eff}^2)]\sin^2\theta$. The exchange parameters extracted for Y138 were $k_{ex} = (1.4 \pm 0.9) \times 10^4$ s^{-1} and $\phi_{ex} = (6.4 \pm 4.3) \times 10^4$ s^{-2}, corresponding to a minimal shift difference of 0.6 ppm. F141 does not show significant exchange contributions to R_2. The pulse sequence, experimental details, and data analysis are described in the Supporting Information.

in aromatic side chains, as validated here by $R_{1\rho}$ experiments performed on E140Q-Tr2C. Figure 3a demonstrates that monoexponential relaxation decays are obtained. Figure 3b shows relaxation dispersion profiles, which identify conformational exchange dynamics for a subset of residues in E140Q-Tr2C. In general, the large intrinsic R_2 of aromatic carbons makes the experiment less sensitive than the corresponding one for ^{13}C methyls. The aromatic ring of Y138 exchanges with a rate of $k_{ex} = (1.4 \pm 0.9) \times 10^4$ s^{-1}, which is consistent with results obtained previously for backbone nuclei of E140Q-Tr2C.[4,18] Thus, our approach enables accurate measurement of microsecond to millisecond dynamics of aromatic side chains in Phe, Tyr, and His, as well as Trp (although the latter has not been demonstrated experimentally herein because E140Q-Tr2C does not contain any Trp). Furthermore, the approach is highly attractive because the cost of [1-^{13}C]-glucose is approximately the same as that of [U-$^{13}C_6$]-glucose, but only a fraction of that of [3-^{13}C]-pyruvate. In this context, it should also be noted that protein production yields are usually significantly higher with glucose compared to pyruvate.[8,19]

In summary, we have demonstrated a simple strategy for introducing isolated ^{13}C into aromatic amino acid side chains. The present method extends the available repertoire of spin relaxation experiments for characterizing side chain dynamics and serves as a useful complement to existing methods that measure ^{13}C methyl relaxation.[3,9]

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Supporting Information Available: Pulse sequence, experimental details, and data analysis; relaxation dispersion profiles for all aromatic side chains in E140Q-Tr2C. This material is available free of charge via the Internet at http://pubs.acs.org.

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