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A ^{13}C Labeling Strategy Reveals a Range of Aromatic Side Chain Motion in Calmodulin

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Supporting Information

ABSTRACT: NMR relaxation experiments often require site-specific isotopic enrichment schemes in order to allow for quantitative interpretation. Here we describe a new labeling scheme for site-specific $^{13}C-^{1}H$ enrichment of a single ortho position of aromatic amino acid side chains in an otherwise perdeuterated background by employing a combination of [4-^{13}C]erythrose and deuterated pyruvate during growth on deuterium oxide. This labeling scheme largely eliminates undesired contributions to ^{13}C relaxation and greatly simplifies the fitting of relaxation data using the Lipari−Szabo model-free formalism. This approach is illustrated with calcium-saturated vertebrate calmodulin and oxidized flavodoxin from *Cyanobacterium anabaena*. Analysis of ^{13}C relaxation in the aromatic groups of calcium-saturated calmodulin indicates a wide range of motion in the subnanosecond time regime.

Nuclear magnetic resonance (NMR) relaxation has proven to be a versatile probe of the link between fast internal protein motions and their relevance to function.[1−4] The motions of the polypeptide chain or of the amino acid side chains in proteins of significant size are most often studied using ^{15}N relaxation of amide nitrogen and deuterium or carbon relaxation in methyl groups.[2,3] This is generally due to restrictions arising from requirements of isotopic labeling and unfavorable relaxation properties of some sites within proteins. In other contexts, more specific and tailored enrichment schemes are often vital in order to eliminate unwanted dipolar and scalar interactions as well as to simplify data interpretation. Examples include the use of [3-^{13}C]pyruvate,[5] [2-^{13}C]glycerol, or [1,3-$^{13}C_2$]glycerol[6,7] or mixtures of singly ^{13}C-enriched acetates[8] as carbon precursors to generate isolated ^{13}C spins. Even more selective spin enrichment schemes are sometimes required in order to suppress unwanted spin interactions and often employ more complex biosynthetic precursors. Prominent examples include labeling schemes targeted for optimal relaxation in methyl groups.[5,9,10] Comprehensive chemical synthesis, though relatively expensive, has also proven viable.[11]

Here we focus on the use of NMR relaxation phenomena to characterize the fast subnanosecond motion of aromatic residues. Aromatic amino acid side chains have a rich structural role within proteins[12−15] and are often central to their biological function, particularly in the context of molecular recognition[16,17] and catalysis.[18] Thus, the motional character of aromatic residues would seem to be of central importance in a range of protein structure−function issues. In this context, NMR phenomena have long been used to characterize relatively slow motions that are manifested in line broadening or population exchange.[19]

For classical relaxation phenomena used to probe fast subnanosecond motions, aromatic residues present a difficult situation. In addition to the concern about the isolation of the spin interaction of interest from extraneous contributions, aromatic ring systems suffer from extensive homo- and heteronuclear scalar (J) interactions that can also significantly complicate the quality and information content of the obtained relaxation data. Previously, [1-^{13}C]- or [2-^{13}C]glucose has been used to create isolated ^{13}C sites in aromatic side chains and thereby eliminate one-bond $^{13}C-^{13}C$ interactions. However, this labeling scheme does not eliminate remote scalar or dipolar interactions with nonbonded ^{1}H spins.[20−22] In our hands, the [1-^{13}C]glucose labeling scheme, which is designed to place isolated ^{13}C at the δ position, results in minor scrambling of the label that confounds the subsequent analysis to some extent (see below). In addition, though anticipated to be less of an issue than for methyl ^{13}C relaxation studies,[9,23] the presence of remote ^{1}H spins does present a complication in the analysis of aromatic ^{13}C relaxation in proteins. Isolation of the $^{1}H-^{13}C$ pair in an otherwise perdeuterated background would eliminate potential complications from dipolar interactions with remote ^{1}H spins as well as scalar couplings with other ring hydrogens. Labeling strategies based on glucose are unable to provide this labeling scheme. To overcome this limitation to a large extent, we have developed a biosynthetic strategy that takes advantage of the flow of carbon from erythrose 4-phosphate to Tyr, Phe, and Trp through condensation with phosphoenolpyruvate during biosynthesis[24] (Figure 1). Consideration of this pathway suggested that the use of [4-^{13}C]erythrose as the sole source of ^{13}C in conjunction with deuterated (^{12}C)pyruvate should lead to the creation of an isolated bonded $^{1}H-^{13}C$ pair at a *single* δ position (the C2 position) within the aromatic ring. Protein expression during growth on [4-^{13}C]erythrose, deuterated (^{12}C)pyruvate, and 99% D_2O should allow for exchange of all other nonaromatic hydrogens while preserving that bonded to the target ^{13}C. In the case of phenylalanine and tryptophan, an additional ^{1}H spin is predicted to remain at the ζ (C4) position of the benzoid ring. Fortunately, this spin is greater than 3 Å away from the sole ^{13}C nucleus, rendering its contribution to relaxation negligible. All of the other aromatic carbons remain NMR-inactive ^{12}C nuclei. This labeling pattern should therefore largely eliminate the

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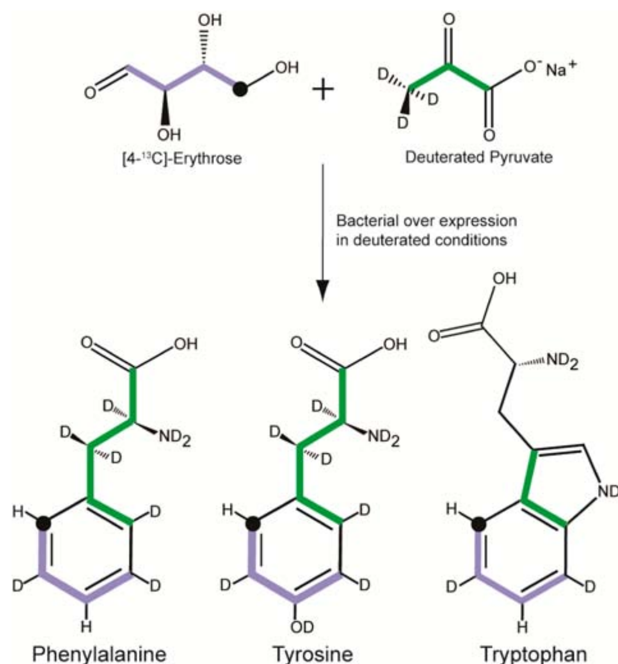

Figure 1. Carbon inflow into the aromatic pathway through the condensation reaction of erythrose 4-phosphate and phosphoenolpyruvate. Carbons originating from pyruvate and erythrose are shown in green and purple, respectively. The position of the ^{13}C label is indicated by the ● symbol.

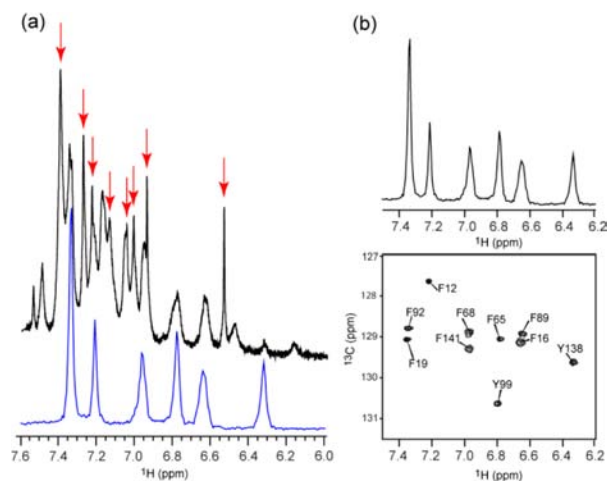

Figure 2. (a) Comparison of the aromatic regions of the ^{13}C-filtered and -decoupled 1D ^{1}H spectrum (lower, blue) and the unfiltered ^{1}H spectrum (upper, black) of calcium-saturated calmodulin expressed during growth on [4-^{13}C]erythrose, deuterated pyruvate, and D$_2$O, indicating the selective introduction of a ^{1}H−^{13}C pair at a single δ carbon of Phe and Tyr. Also evident are resonances arising from H bonded to ^{12}C at the ζ position of the aromatic ring of Phe (red arrows). These resonances have narrow line widths because of the absence of scalar coupling. A more detailed analysis of the ^{1}H spectrum is presented in Figures S1 and S2. (b) 2D ^{13}C HSQC spectrum of the aromatic region and the corresponding 1D ^{1}H spectrum (^{13}C-filtered). No significant ^{13}C labeling of other amino acids was observed.

potential complications of extraneous intraring scalar or dipolar interactions (^{1}H or ^{13}C) with the isolated ^{13}C spin. The low gyromagnetic ratio of the replacement deuterons should cause them to contribute insignificantly to relaxation of the isolated ^{13}C nucleus. Similarly, the contribution to the measured relaxation of the target ^{13}C nucleus from random ^{13}C at natural abundance should also be negligible.

Vertebrate calmodulin was expressed in *Escherichia coli*[25] to test this strategy. (^{12}C,^{2}H)pyruvate was used to suppress scrambling of ^{13}C to other amino acids and to aid in perdeuteration of the aromatic ring. One-dimensional (1D) ^{13}C-filtered and unfiltered ^{1}H spectra revealed essentially complete deuteration of the protein except at single δ positions in Tyr and Phe residues and the ζ position of Phe (Figure 2). No protonation at the ε (C3, C5) positions of the aromatic ring was observed. ^{13}C labeling was restricted to the δ positions of Tyr and Phe. The enrichment of ^{13}C−^{1}H pairs at the δ position was determined by comparison of the unfiltered and ^{13}C-filtered ^{1}H spectra with and without ^{13}C decoupling and was found to be uniformly 67% [Figure S1 in the Supporting Information (SI)]. Analysis of the 1D ^{13}C spectra with and without ^{1}H coupling confirmed that the isolated ^{13}C$_\delta$−^{1}H was not diluted with ^{2}H from the solvent (Figure S3). The underlabeling of ^{13}C is a consequence of using a (^{12}C,^{2}H)pyruvate/[4-^{13}C]erythrose ratio of 3:2, which was motivated by the need to suppress scrambling of ^{13}C to other amino acids or other positions in the aromatic rings. This is a reasonable price to pay to maintain the fidelity of the ^{13}C nucleus for relaxation studies.

Calmodulin does not contain tryptophan. To confirm that appropriate labeling of Trp was also achieved, flavodoxin C55A was similarly prepared.[26] Flavodoxin has four Trp, eight Tyr, and eight Phe residues along with one His residue. The 22 anticipated aromatic ^{1}H−^{13}C correlations were seen in the ^{13}C heteronuclear single-quantum coherence (HSQC) spectrum (Figure S4). No other significant ^{13}C labeling was observed. Thus, the desired labeling pattern was observed for all four aromatic amino acid side chains in the context of a perdeuterated background.

^{13}C R_1 and $R_{1\rho}$ relaxation in calcium-saturated calmodulin prepared using this labeling strategy was measured at three magnetic fields (11.7, 14.0, and 17.6 T) using standard two-dimensional (2D) sampling pulse sequences.[10] For comparison, similar measurements were done using calmodulin prepared with a labeling strategy based on [1-^{13}C]glucose[27,28] (Figure 3). The anisotropy of global macromolecular tumbling was characterized in the usual way[29] using the crystal structure of calcium-saturated calmodulin (PDB entry 3CLN) and assessing the two globular domains separately.[30]

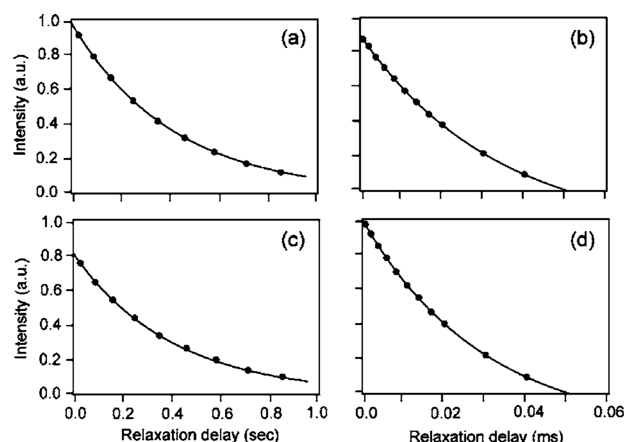

Figure 3. Aromatic ^{13}C R_1 and $R_{1\rho}$ relaxation in calcium-saturated calmodulin prepared using (a, b) the [4-^{13}C]erythrose/deuterated pyruvate/D$_2$O strategy and (c, d) a [1-^{13}C]glucose strategy. Relaxation at the δ2 position of F92 is shown.

Table 1. Lipari−Szabo Model-Free Parameters for Aromatic Ring Motion in Calcium-Saturated Calmodulin[a]

probe	O^2	τ_e (ps)	probe	O^2	τ_e (ps)
F12[b]	0.49 ± 0.03	236 ± 21	F89[c]	0.94 ± 0.02	900 ± 39
F16[b,d]	0.95 ± 0.01	127 ± 12	F92[c]	0.72 ± 0.03	624 ± 23
F19[b]	0.47 ± 0.01	232 ± 19	Y99[c]	0.79 ± 0.02	240 ± 21
F65[b]	0.70 ± 0.04	176 ± 23	Y138[c]	0.89 ± 0.02	604 ± 36
F68[b]	0.96 ± 0.01	292 ± 17	F141[c]	0.95 ± 0.01	101 ± 11

[a]Prepared using the [4-^{13}C]erythrose labeling scheme. Model-free squared generalized order parameters (O^2) and effective correlation times (τ_e) were determined using the simple model-free spectral density,[31] an effective C−H bond length of 1.09 Å, and the axially symmetric and fully anisotropic chemical shift anisotropy (CSA) tensors for $^{13}C_\delta$ of Phe and Tyr, respectively, as determined by Ye et al.[32] Macromolecular tumbling was characterized using ^{15}N relaxation with an effective N−H bond length[33] of 1.04 Å and a simple uniform ^{15}N CSA tensor breadth[34] of −170 ppm. The N- and C-terminal domains were treated separately. The precisions of O^2 and τ_e were estimated by Monte Carlo sampling. [b]Part of the N-terminal domain with an effective macromolecular tumbling time of 8.96 ± 0.14 ns. [c]Part of the C-terminal domain with an effective macromolecular tumbling time of 8.05 ± 0.10 ns. [d]Because of partial spectral overlap, only data obtained at 17.6 T were fitted.

Lipari−Szabo model-free squared generalized order parameters (O^2) and effective correlation times (τ_e) were determined using a grid search approach[35] employing an effective bond length of 1.09 Å and residue-specific chemical shift anisotropy (CSA) tensors[32] with axially symmetric and anisotropic[36] CSA values for Phe and Tyr, respectively. The analysis was carried out with an updated version (Relxn2A) of our in-house software.[23,35] Standard statistical F tests were used to determine whether R_{ex} terms were justified, and none were found. The contributions from ^{13}C−^{2}H remote dipolar coupling and ^{13}C−^{13}C dipolar interactions due to natural-abundance ^{13}C amounted to less than 0.05% of the ^{13}C−^{1}H direct bond dipolar interaction. Relaxation data obtained from the sample obtained using the [4-^{13}C]-erythrose labeling scheme fit well to the simple model-free spectral density (Table 1). The obtained O^2 values ranged from 0.47 to 0.96, indicating a rich spectrum of aromatic ring motion within calcium-saturated calmodulin on the subnanosecond time scale.

In contrast, though the primary R_1 and $R_{1\rho}$ relaxation time profiles derived from the calcium-saturated calmodulin obtained using the [1-^{13}C]glucose labeling scheme fitted reasonably well to single-exponential decays (Figure 3), the obtained relaxation rates largely gave relatively poor fits to the Lipari−Szabo model-free interpretation (5−10% vs <1% residual error) (Table S3 in the SI). Inclusion of remote ^{1}H spin dipolar interactions as well as dipolar interactions with $^{13}C_\varepsilon$ failed to recover the excellent statistics of the relaxation data derived from the more optimal [4-^{13}C]erythrose labeling scheme (Figure S5 and Table S3). It seems likely that unaccounted dipolar relaxation, intraring one-bond ^{13}C−^{13}C J coupling, and intraring two-bond $^{13}C_\delta$−$^1H_\varepsilon$ J coupling effects contaminated the measurement and interpretation of ^{13}C relaxation in structured proteins in the protonated background. This is consistent with the presence of additional peaks in the ^{13}C−^{1}H HSQC spectrum of calmodulin derived from the [1-^{13}C]glucose labeling scheme (Figure S6). These additional peaks arise from partial ^{13}C labeling (8−15%) at C_ε of the aromatic ring and are a consequence of the scrambling of the ^{13}C label when glucose was used as the carbon precursor. These considerations provide a plausible scenario where the relaxation profiles still fit reasonably well to single-exponential decays but fail to be fit reasonably by the Lipari−Szabo model-free formalism. Thus, the [4-^{13}C]erythrose labeling strategy described here would seem to be highly advantageous in providing high-fidelity relaxation for the study of fast aromatic ring dynamics.

In summary, we have demonstrated a strategy for producing perdeuterated proteins with isolated ^{1}H−^{13}C pairs in the aromatic ring systems. It has been shown that the aromatic side chains of calmodulin have a wide range of motion on the subnanosecond time scale, the observation of which has to date been restricted to detection at natural abundance using highly concentrated and relatively small proteins.[37] The ability to investigate the fast dynamics of aromatic side chains will provide a highly complementary perspective to that accessed by ^{13}C or ^{2}H relaxation in methyl groups.

■ ASSOCIATED CONTENT

Supporting Information

Description of the [4-^{13}C]erythrose labeling protocol; ^{13}C relaxation decay rates for aromatic side chains in calcium-saturated calmodulin collected at 500, 600, and 750 MHz (^{1}H); and the ^{13}C HSQC spectrum of flavodoxin prepared with the [4-^{13}C]erythrose labeling strategy. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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