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Improved labeling strategy for ^{13}C relaxation measurements of methyl groups in proteins

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Summary

Selective incorporation of ^{13}C into the methyl groups of protein side chains is described as a means for simplifying the measurement and interpretation of ^{13}C relaxation parameters. High incorporation (>90%) is accomplished by using pyruvate (3-^{13}C, 99%) as the sole carbon source in the growth media for protein overexpression in *E. coli*. This improved labeling scheme increases the sensitivity of the relaxation experiments by approximately fivefold when compared to randomly fractionally ^{13}C-labeled protein, allowing high-quality measurements on relatively dilute (<1 mM) protein samples at a relatively low cost.

The selective isotopic labeling of proteins has proven to be useful in many NMR applications, most recently in the quantification of protein internal dynamics and in the estimation of related physical parameters. The measurement of ^{13}C, ^{15}N and ^{2}H relaxation parameters is of particular interest given their potential as a rich source of information about protein side-chain motions (Daragan and Mayo, 1996; LeMaster and Kushlan, 1996; Li et al., 1996; Palmer et al., 1996; Yang and Kay, 1996). As NMR relaxation is inherently complex (Werbelow and Grant, 1977), it is often advantageous to examine relaxation in the simplest possible context. For this reason, selective ^{13}C labeling strategies are of obvious utility. Specifically, it is advantageous to avoid the potentially contaminating effects arising from ^{13}C-^{13}C and extraneous ^{1}H-^{13}C interactions when examining the ^{1}H-mediated relaxation of a particular ^{13}C nucleus. In previous ^{13}C relaxation studies of proteins and large peptides in solution, isolated ^{13}C nuclei have been introduced through various methods: detection at natural abundance ^{13}C (Richarz et al., 1980; Dellwo and Wand, 1989); in situ generation or incorporation of specifically labeled amino acids (Nicholson et al., 1992); random fractional ^{13}C incorporation in combination with low-pass ^{13}C-^{13}C J-filtration (Wand et al., 1995); and metabolically directed alternate carbon labeling (LeMaster and Kushlan, 1996). Nevertheless, the difficulty in generating enriched, isolated ^{13}C methyl sites has remained an obstacle to obtaining high-quality data on relatively dilute (<1 mM) protein samples. In this communication, we show that an adaptation of a recently reported method for the selective incorporation of ^{1}H can be used to selectively introduce isolated ^{13}C sites into side-chain methyl groups of bacterially expressed proteins.

Kay and co-workers have recently reported the selective incorporation of methyl protons into perdeuterated proteins (Rosen et al., 1996). This was achieved by overexpression of the protein in *E. coli* grown on media containing 100% D_2O and protonated pyruvate as a carbon source. There the emphasis was on the development of a method for obtaining selective ^{1}H-^{1}H NOEs in large and otherwise extensively deuterated proteins. Consideration of the underlying metabolic scheme reveals that straightforward modification of this protocol allows for the incorporation of isolated ^{13}C methyl sites for the purpose of ^{13}C relaxation studies. To illustrate this, we have expressed human ubiquitin in *E. coli* (strain BL21-DE3) during

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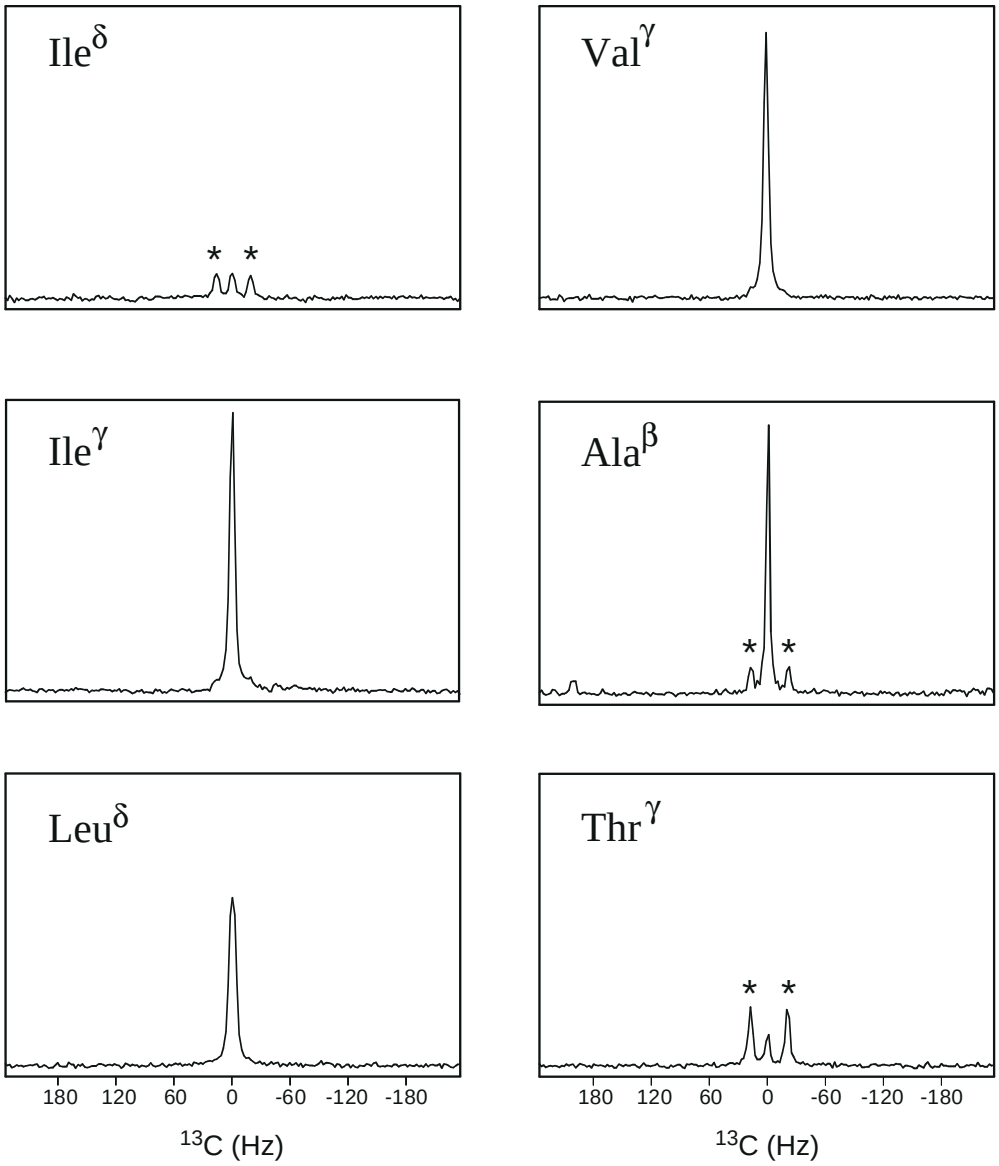

Fig. 1. One-dimensional vectors taken along the ^{13}C dimension of a ^{13}C/^{1}H HSQC spectrum through the six different methyl types in ubiquitin. Peaks centered at the 0 Hz position arise from isolated ^{13}C sites. Doublets with an ~40 Hz splitting arise from that fraction of the sample population that has adjacent ^{13}C nuclei and are further identified with asterisks. No apodization was used along the ^{13}C dimension. The spectrum was acquired at 45 °C on a Varian Unity Inova 600 MHz spectrometer.

growth on M9 minimal medium with 0.3–0.4% (w/v) sodium pyruvate (3-^{13}C, 99%) added as the sole carbon source instead of glucose. No adaptation of the bacteria to this medium was required, and the cells grew at a rate comparable to growths in glucose-containing media. Overexpression was induced with 1 mM IPTG at an OD_{600} of 0.6, and the cells were harvested after 4 h of shaking at 37 °C. The purification was carried out as described previously (Wand et al., 1996). The protein yield was approximately 70% of that normally obtained on glucose-containing M9 media.

The incorporation of ^{13}C into Leu$^{\delta}$, Val$^{\gamma}$, Ile$^{\gamma}$, and Ala$^{\beta}$ methyl groups was >90% as judged from a ^{13}C-edited spin-echo experiment and relative peak intensities from ^{13}C HSQC spectra. These methyl groups are biosynthetically derived from the methyl carbon of pyruvate. As expected, Thr$^{\gamma}$ and Ile$^{\delta}$ showed a low incorporation of ^{13}C since these methyl carbons are derived from aspartate, which in turn is derived from the citric acid cycle (Bender, 1985). Scrambling of ^{13}C into the natural abundance pool is essentially complete for these methyl carbons. There is also one methionine in ubiquitin, but methionine methyl carbons are expected to have incorporation levels similar to Thr$^{\gamma}$ and Ile$^{\delta}$ since methionine synthesis branches from the same biosynthetic pathway. For the purpose of ^{13}C relaxation experiments, it is important that individual ^{13}C nuclei are not directly bonded to other ^{13}C nuclei. The presence of any ^{13}C-^{13}C pairs will be manifested as an ~40

Hz scalar coupling. Figure 1 illustrates the frequency of ^{13}C-^{13}C pairs in all methyl groups (except methionine) by analysis of the ~40 Hz splitting. The 1D vectors shown are taken along the ^{13}C dimension of a ^{13}C HSQC spectrum for each methyl type in ubiquitin. Ile$^{\gamma}$, Leu$^{\delta}$, and Val$^{\gamma}$ methyls have <5% of ^{13}C-^{13}C pairs and should therefore be useful for measuring the ^{13}C-^{1}H dipolar interaction. In contrast, Ile$^{\delta}$ and Thr$^{\gamma}$ methyls have a high (>50%) frequency of ^{13}C-^{13}C pairs, in addition to low ^{13}C incorporation levels. Ala$^{\beta}$ methyls have a ^{13}C-^{13}C frequency of ~20%.

The minimal occurrence of ^{13}C-^{13}C pairs in Ile$^{\gamma}$, Leu$^{\delta}$, and Val$^{\gamma}$ methyl groups will have a negligible effect on measured methyl ^{13}C T_1 values. Even the ~20% ^{13}C-^{13}C pair frequency for alanine is expected to have a minor albeit complicating contribution to the relaxation behavior. This, however, can in principle be removed by low-pass filtration (Wand et al., 1995) or by the acquisition of coupled spectra with high digital resolution. Figure 2 shows sample ^{13}C T_1 decays measured from selected ubiquitin methyl groups. All curves exhibit single-exponential behavior, confirming the lack of significant ^{13}C-^{13}C coupling effects. Finally, it is important to note that this ^{13}C enrichment strategy improves the sensitivity over the random fractional approach (Wand et al., 1995) by approximately fivefold for Ile$^{\gamma}$, Leu$^{\delta}$, Ala$^{\beta}$, and Val$^{\gamma}$ methyl groups. This makes it possible to carry out relaxation studies on samples of low effective concentration. For example, the data in Fig. 2 were acquired on a 1.8 mM ubiquitin sample in a sapphire tube specially designed for use in high-pressure NMR studies (Urbauer et al., 1996). This sample cell has an inner diameter of 1 mm and holds an active volume of 80 µl. Even though the overall sensitivity using this tube is ~8 times lower than a standard 5 mm tube, the data indicate that high-quality relaxation data may be obtained.

In conclusion, the use of pyruvate (3-^{13}C, 99%) as the sole carbon source for overexpressing proteins in *E. coli* generates isolated ^{13}C nuclei in a large number of methyl sites. Such sites are preferred for ^{13}C relaxation studies, and we have shown that high-quality data can be obtained from samples of submillimolar effective concentration. The dynamics information in ^{13}C relaxation parameters is, in principle, complementary to ^{2}H relaxation (Muhandiram et al., 1995) since these nuclei sample different frequencies of the spectral density. The labeling strategy

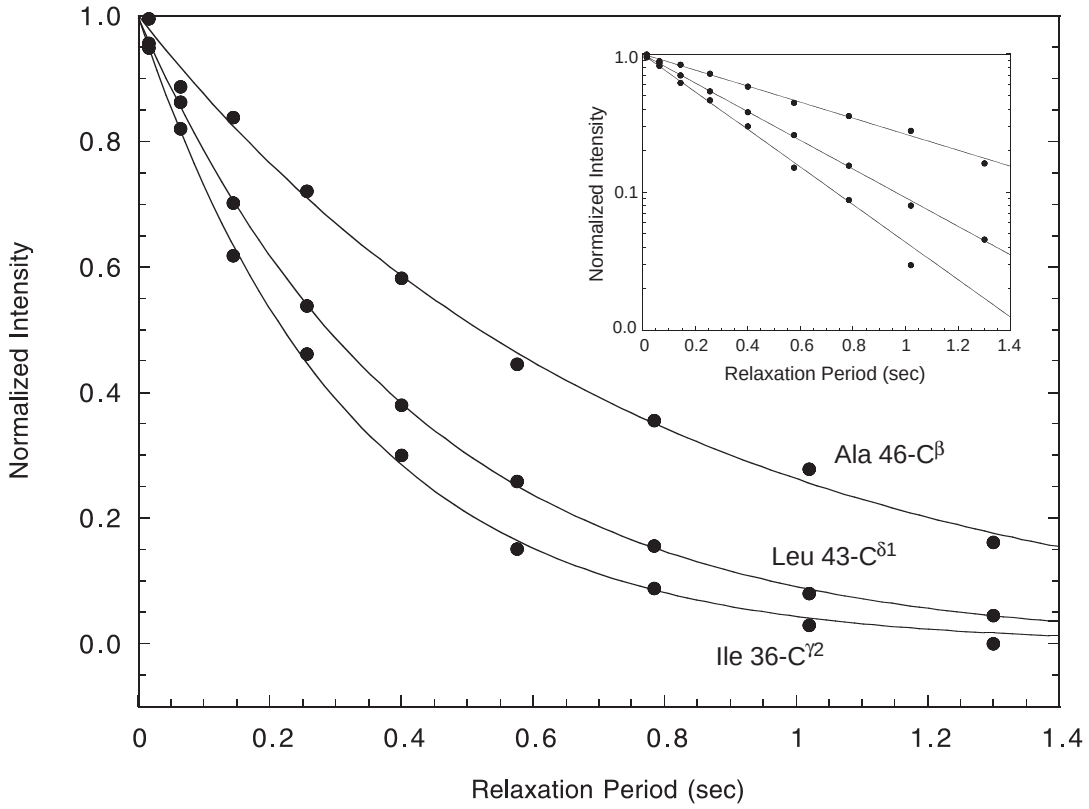

Fig. 2. Representative methyl ^{13}C T_1 decays from ubiquitin obtained at pH 4.6 (uncorrected for the isotope effect) and 30 °C at 14 T. A series of 2D $^{13}C/^{1}H$ correlation spectra were acquired as a function of an increasing ^{13}C T_1 relaxation period. The data were acquired over a period of 32 h in a sapphire tube assembly suitable for high-pressure NMR applications (see Urbauer et al. (1996)). The effective sample concentration was <250 µM (see text). Data points correspond to normalized intensities from the 2D cross peaks. The inversion-recovery pulse sequence used for measuring T_1 is essentially that given previously (Nicholson et al., 1992), except that ^{1}H decoupling was extended into the ^{13}C t_1 evolution period, and pulsed-field gradients were added. Sensitivity enhancement was not employed.

reported here is also potentially useful for the introduction of deuterium at isolated ^{13}C-labeled methyl sites.

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References

Bender, D.A. (1985) *Amino Acid Metabolism*, 2nd ed., Wiley, Chichester, U.K.

Daragan, V.A. and Mayo, K.H. (1996) *J. Phys. Chem.*, **100**, 8378–8388.

Dellwo, M.J. and Wand, A.J. (1989) *J. Am. Chem. Soc.*, **111**, 4571–4578.

LeMaster, D.M. and Kushlan, D.M. (1996) *J. Am. Chem. Soc.*, **118**, 9255–9264.

Li, Z., Raychaudhuri, S. and Wand, A.J. (1996) *Protein Sci.*, **5**, 2647–2650.

Muhandiram, D.R., Yamazaki, T., Sykes, B.D. and Kay, L.E. (1995) *J. Am. Chem. Soc.*, **117**, 11536–11544.

Nicholson, L.K., Kay, L.E., Baldisseri, D.M., Arango, J., Young, P.E., Bax, A. and Torchia, D.A. (1992) *Biochemistry*, **31**, 5253–5263.

Palmer, A.G., Williams, J. and McDermott, A. (1996) *J. Phys. Chem.*, **100**, 13293–13310.

Richarz, R., Nagayama, K. and Wüthrich, K. (1980) *Biochemistry*, **19**, 5189–5196.

Rosen, M.K., Gardner, K.H., Willis, R.C., Parris, W.E., Pawson, T. and Kay, L.E. (1996) *J. Mol. Biol.*, **263**, 627–636.

Urbauer, J.L., Ehrhardt, M.R., Bieber, R.J., Flynn, P.F. and Wand, A.J. (1996) *J. Am. Chem. Soc.*, **118**, 11329–11330.

Wand, A.J., Bieber, R.J., Urbauer, J.L., McEvoy, R.P. and Gan, Z. (1995) *J. Magn. Reson.*, **B108**, 173–175.

Wand, A.J., Urbauer, J.L., McEvoy, R.P. and Bieber, R.J. (1996) *Biochemistry*, **35**, 6116–6125.

Werbelow, L.G. and Grant, D.M. (1977) *Adv. Magn. Reson.*, **9**, 189–299.

Yang, D. and Kay, L.E. (1996) *J. Mol. Biol.*, **263**, 369–382.