Journal of Biomolecular NMR, **13:** 369–374, 1999.
KLUWER/ESCOM

A robust and cost-effective method for the production of Val, Leu, Ile (δ1) methyl-protonated ^{15}N-, ^{13}C-, ^{2}H-labeled proteins

Natalie K. Goto[a,b], Kevin H. Gardner[c,*], Geoffrey A. Mueller[b,c], Randall C. Willis[b] & Lewis E. Kay[c]

[a]*Department of Biochemistry, University of Toronto, Toronto, ON, Canada M5S 1A8*
[b]*Department of Biochemistry and Structural Biology, Hospital for Sick Children, 555 University Avenue, Toronto, ON, Canada M5S 1X8*
[c]*Protein Engineering Network Centres of Excellence and Departments of Medical Genetics and Microbiology, Biochemistry and Chemistry, University of Toronto, Toronto, ON, Canada M5S 1A8*

Received 8 December 1998; Accepted 15 January 1999

Key words: deuteration, α-ketobutyrate, α-ketoisovalerate, methyl protonation

Abstract

A selective protonation strategy is described that uses [3-^{2}H] ^{13}C α-ketoisovalerate to introduce (^{1}H-δ methyl)-leucine and (^{1}H-γ methyl)-valine into ^{15}N-, ^{13}C-, ^{2}H-labeled proteins. A minimum level of 90% incorporation of label into both leucine and valine methyl groups is obtained by inclusion of ≈100 mg/L α-ketoisovalerate in the bacterial growth medium. Addition of [3,3-$^{2}H_2$] α-ketobutyrate to the expression media (D_2O solvent) results in the production of proteins with (^{1}H-δ1 methyl)-isoleucine (>90% incorporation). ^{1}H-^{13}C HSQC correlation spectroscopy establishes that CH_2D and CHD_2 isotopomers are not produced with this method. This approach offers enhanced labeling of Leu methyl groups over previous methods that utilize Val as the labeling agent and is more cost effective.

The application of multidimensional NMR spectroscopy to study the structure and dynamics of macromolecules requires techniques that maximize both spectral resolution and sensitivity. To this end, uniform labeling of molecules with NMR-active isotopes, combined with the development of triple-resonance experiments has permitted detailed NMR analyses of systems up to 20 kDa in molecular mass (Bax, 1994). Beyond this limit, spectra become increasingly complicated by peak overlap, while shorter transverse relaxation times lead to decreases in both sensitivity and resolution. In this regard deuteration of aliphatic carbons is increasingly used as a means of spectral

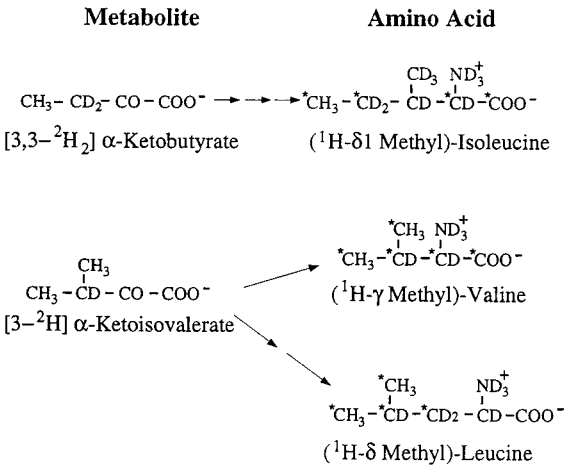
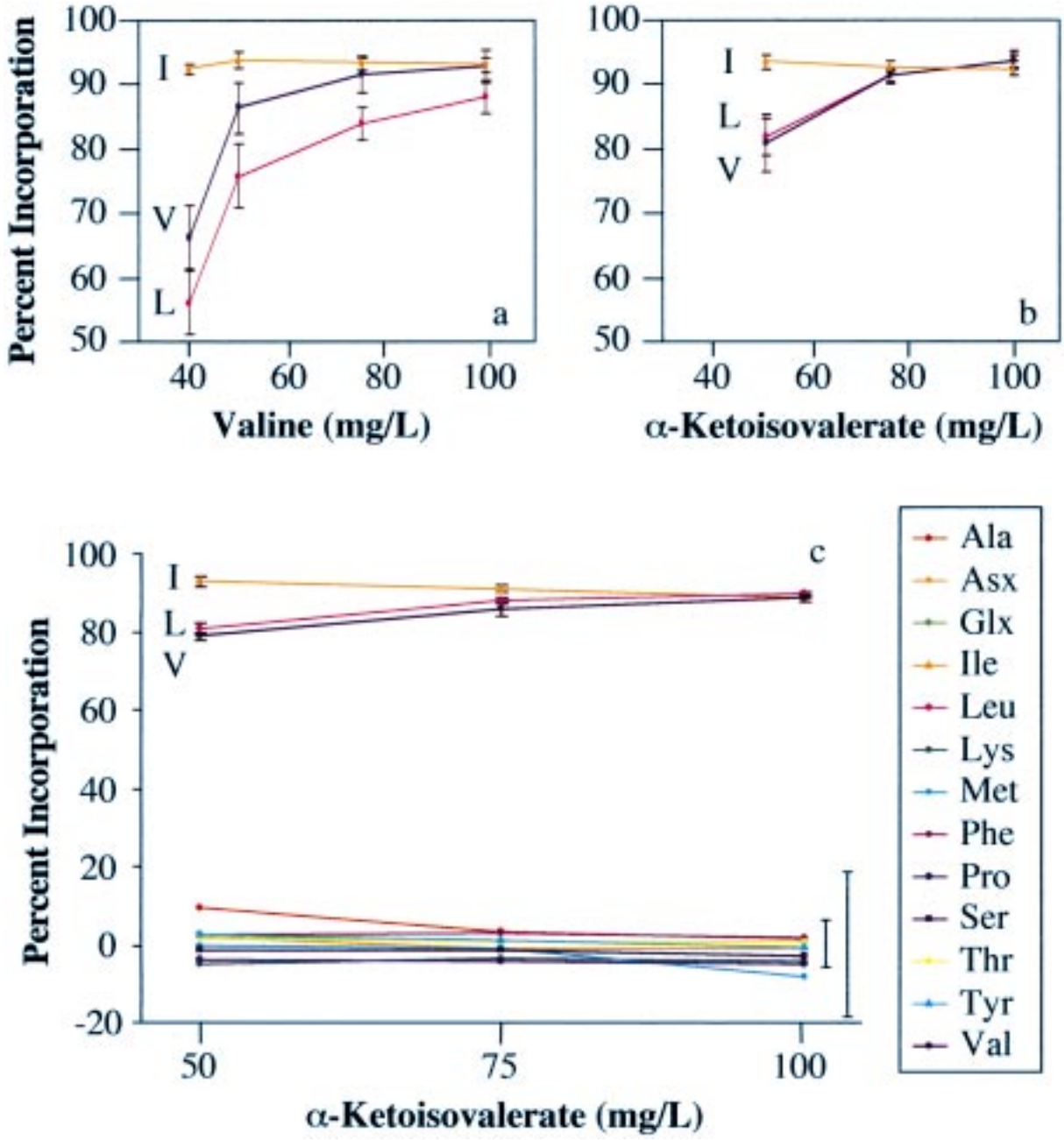


Figure 1. Chemical structures of metabolites supplementing the ^{13}C,^{2}H-glucose *E. coli* growth media (D_2O) used in the selective protonation strategy described in the text and the resulting amino acid products produced (Gottschalk, 1986). Carbons derived from each of the metabolite precursors are indicated by *.

*Present address: Department of Biochemistry, University of Texas Southwestern Medical Center at Dallas, 5323 Harry Hines Blvd., Dallas, TX 75235-9038, U.S.A.

Supporting information available: One table showing percent incorporation versus α-ketoisovalerate added obtained from GC-MS (with errors at each value of added α-ketoisovalerate). This material can be obtained by e-mailing N.K.G. (gnat@pound.med.utoronto.ca).



simplification and sensitivity enhancement (reviewed by Gardner and Kay, 1998).

Although many of the amide-detected triple-resonance correlation experiments for chemical shift assignment are best performed on samples with high levels of deuteration (Grzesiek et al., 1993; Yamazaki et al., 1994; Shan et al., 1996; Constantine et al., 1997), the concomitant reduction in the number of NOE correlations that can be observed in NOESY spectra presents a serious challenge for structure calculations. Strategies have therefore been developed to selectively reintroduce ^{1}H spins into non-exchangeable sites of highly perdeuterated proteins (Metzler et al., 1996; Rosen et al., 1996; Smith et al., 1996; Gardner and Kay, 1997). Initial work in our laboratory focused on selective methyl protonation

Figure 2. Percent ^{12}C incorporation (from ^{12}C precursors, see below) into the methyl positions of Leu (L), Val (V) and Ile (I, δ1 only) in MBP produced from $^{15}NH_4Cl$ and ^{13}C,^{1}H-glucose upon addition of (a) ^{12}C, ^{14}N valine and (b) ^{12}C α-ketoisovalerate, with 50 mg/L ^{12}C α-ketobutyrate included in all cases. For each methyl type, the ratios of methyl peak volumes in ^{1}H-^{13}C CT-HSQC spectra of samples prepared with ^{12}C-metabolite relative to volumes of corresponding peaks in the fully ^{13}C-labeled control (prepared in the absence of any ^{12}C precursors) were calculated. Normalization factors which account for differences in protein concentrations between each sample and the ^{13}C-labeled control were calculated analogously from Ala, Met, Thr and Ile γ1 methyl peak volumes (which are essentially independent of metabolite addition). The percent incorporation of ^{12}C indicated on the graph is defined as $100\% \times (1 - I_{methyl,i}/I_{methyl,c})$ where $I_{methyl,i}$ and $I_{methyl,c}$ are the normalized methyl intensities in spectrum i, where ^{12}C-metabolite is added, and the control spectrum (no ^{12}C-metabolite), respectively. Error bars indicate standard deviations of ratios over the population of peaks used in the calculation, where the minimum numbers of peaks used in each calculation were 21, 12 and 12 for Leu, Val and Ile, respectively. All spectra were recorded on a Varian Inova 600 MHz spectrometer at 37 °C, with acquisition times of 28.7 and 64 ms in (t_1, t_2), and measuring times ranging from 7 to 14 h. Data analysis and processing were performed with NMRPipe (Delaglio et al., 1995). MBP samples were purified as described (Gardner et al., 1998) and final solutions were 0.2–1.0 mM MBP, containing 20 mM sodium phosphate buffer (pH 7.2), 3 mM NaN_3, 100 μM EDTA and 10% D_2O with 3 mM β-cyclodextrin. (c) Percent ^{12}C incorporation as a function of the amount of ^{12}C α-ketoisovalerate added to the growth media (with 50 mg/L ^{12}C α-ketobutyrate added to all samples), measured by GC-MS of TAB-derivatized protein hydrolysates. Values represent the average of duplicate runs of hydrolysis, derivatization and measurement (excluding Met, where sufficient signal was observed in only one trial). Percent incorporation calculations utilized amplitude-weighted averages of the mass distribution for *N*-trifluoroacetyl-iminium (TFA) ions, where the ion peaks were chosen to represent side-chain labeling patterns (and exclude the carbonyl group). For non-ILV amino acids, percent incorporation was taken as the difference between the average mass of the fully ^{13}C-, ^{15}N-labeled species (control) and that of the sample, divided by the number of carbons derived form the amino acid in the TFA ion. Error bars indicated on the graph on the right-hand side denote the average error (6%) and maximum error (19%) observed for the non-ILV series of amino acids. Percent incorporation calculations for ILV assume addition of the metabolite as a complete unit to the amino acid in question. Values were calculated according to the following equation: $\%\ \text{Incorporation} = 100\% \times (M^{control} - M^{sample})/n$, where $M^{control}$ and M^{sample} are the molecular masses of the samples produced in the absence and presence of ^{12}C α-ketoisovalerate, respectively, and $n = 3$ and 4 for α-ketobutyrate and α-ketoisovalerate, respectively. (Note that the ion peaks used to calculate masses do not include backbone carbonyl groups, which in the case of Ile and Val are derived from the metabolite, see Figure 1.) Error bars for each ILV point are indicated on the graph. Amino acid hydrolysis and derivatizations were all performed as described previously (Rosen et al., 1996). Any non-ILV amino acids not appearing were either destroyed or deaminated during hydrolysis (Trp, Gln and Asn), not observed in the GC spectrum (Arg, His, Cys), or had mass differences between labeled and unlabeled species that were too small for accurate detection at this resolution of mass spectrometry (Gly). GC-mass spectra were recorded on a Fisons Trio 1000 spectrometer interfaced to a Hewlett-Packard 5890 Series II gas chromatograph, using a 30 m × 0.25 mm (i.d.) DB-1 column with 0.25 μm film thickness. Samples were injected (∼0.5 μL) at 50 °C and separated under a 10 °C/min gradient to 240 °C. The spectrometer voltage was 70 eV and mass spectra were acquired at a rate of 4/s. Interpretation of mass spectra was done according to the published spectra of Leimer et al. (1977).

of highly deuterated proteins using a bacterial protein expression system with ^{13}C, ^{1}H pyruvate as the sole carbon source in D_2O media (Rosen et al., 1996). Although highly deuterated proteins with 40–80% protonation levels at the methyl positions of Ala, Val, Leu and Ile (γ2 only) were obtained, the production of significant levels of isotopomers (CH_2D and CHD_2) complicates spectral analysis. A strategy which does not produce these isotopomers is one in which [2,3-$^{2}H_2$] ^{15}N, ^{13}C Val is added to the growth media containing deuterated ^{13}C-glucose as the sole carbon source to generate proteins with methyl-protonated Val and Leu (Gardner and Kay, 1997). Addition of [3,3-$^{2}H_2$] ^{13}C α-ketobutyrate results in high levels (> 90%) of protonation at the δ1 methyl position of Ile. Chemical shift assignments of the 370-residue maltose binding protein (MBP) prepared using this procedure have recently been reported (Gardner et al., 1998), and experiments recording methyl-methyl (Zwahlen et al., 1998a, b), HN-HN (Grzesiek et al., 1995; Venters et al., 1995) and methyl-HN (Muhandiram et al., 1993) NOEs have been used to provide restraints in subsequent structure calculations.

Although supplementation of the D_2O growth media with [2,3-$^{2}H_2$]-Val results in high levels of incorporation of (^{1}H-γ methyl)-Val, the level of methyl protonation for Leu is somewhat lower. For example, supplementation with 50 mg/L Val resulted in Val methyl-protonation levels on the order of 85% in deuterated MBP, while Leu methyl protonation is on the order of 75% (see below). These difficulties with uniform incorporation of both (^{1}H-γ methyl)-Val and (^{1}H-δ methyl)-Leu have prompted efforts to optimize labeling conditions. Here we describe the development of a new strategy using α-ketoisovalerate, a metabolite that is common to both Val and Leu biosynthesis as shown in Figure 1 (Gottschalk, 1986).

To optimize the labeling protocol, prototrophic *E. coli* containing the expression plasmid for MBP were grown in $^{1}H_2O$/^{13}C, ^{1}H glucose/$^{15}NH_4Cl$ media, supplemented with varying amounts of either unlabeled Val or unlabeled α-ketoisovalerate (^{12}C) 1 h prior to induction. A comparison of peak intensities in

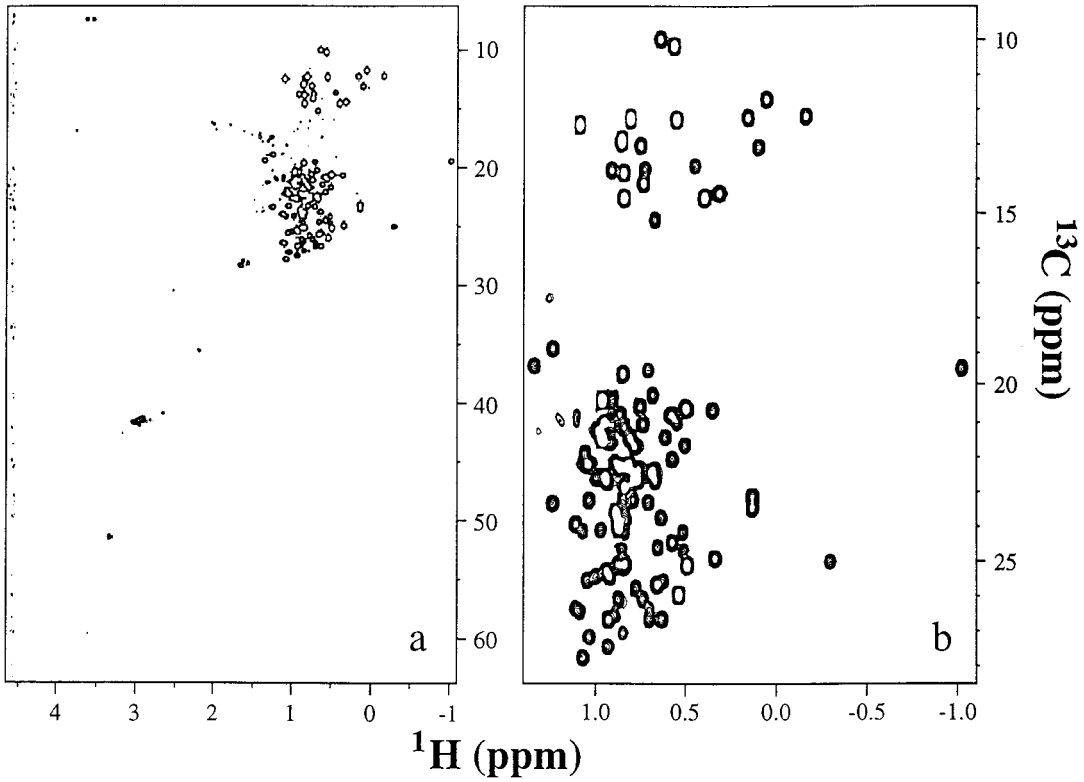


Figure 3. ^{1}H-^{13}C CT HSQC spectrum of a sample of 1.5 mM Val, Leu, Ile (δ1) methyl-protonated MBP, 2 mM β-cyclodextrin, 20 mM sodium phosphate (pH 7.2), 3 mM NaN_3, 200 μM EDTA, 0.1 mg/mL Pefabloc, 1 μg/μL pepstatin and 10% D_2O recorded at 37 °C on a Varian Unity+ 500 MHz spectrometer. Acquisition times of 28 and 64 ms were employed in (t_1,t_2) along with a relaxation delay of 1.5 s, for a total measuring time of 3 h. (a) Aliphatic region of the ^{1}H-^{13}C correlation map of MBP illustrating the selectivity of labeling. Small amounts of residual protonation are observed at the Cγ positions of a number of Pro/Arg residues, the Cβ positions of Asp and Ser (aliased) residues and the Cγ2 methyl positions of Ile. In all cases intensities of these cross peaks are less than 10% of the methyl peaks. (b) Methyl region of the ^{1}H-^{13}C HSQC.

^{1}H-^{13}C CT-HSQC spectra (Santoro and King, 1992; Vuister and Bax, 1992) of proteins produced in this manner relative to proteins generated in the same way but without addition of amino acid precursors allows quantitation of the extent of incorporation of unlabeled Val or α-ketoisovalerate. For example, the absence of a given methyl peak would indicate complete (100%) incorporation of the ^{12}C precursor. Noting that branched-chain amino acid biosynthetic pathways are linked by common enzymes and feedback mechanisms and because samples produced for NMR study will also be protonated at the Ile δ1-position [via α-ketobutyrate (Gardner and Kay, 1997)], we also included 50 mg/L unlabeled α-ketobutyrate in the media 1 h prior to induction.

NMR results demonstrate that incorporation of precursor Val into Leu can be significantly enhanced by addition of larger amounts of Val to the growth media. As shown in Figure 2a, when 100 mg/L Val is added, incorporation is >90% for Val and >85% for Leu. However, even at these high levels of media supplementation, Leu methyl group labeling does not approach the efficiency of Val methyl labeling. In contrast, Figure 2b demonstrates that when α-ketoisovalerate is used as a precursor, Val and Leu methyl groups are 'labeled' in virtually equal amounts with approximately 90% incorporation when 75 mg/L of α-ketoisovalerate is employed. A small enhancement in labeling can additionally be obtained by raising the amount of α-ketoisovalerate added to 100 mg/L. NMR results show that α-ketoisovalerate is thus more effective than Val at simultaneously labeling Val and Leu methyl groups.

An important consideration in selective labeling protocols is the diversion of metabolites away from desired pathways, especially when high levels are

present in the growth media. For example, excess α-ketoisovalerate can be converted into acetyl-CoA (Massey et al., 1976) and reincorporated into other amino acids via the TCA cycle. To investigate this possibility, GC-MS was performed on *N*-trifluoroacetyl *n*-butyl-ester (TAB) derivatives of protein hydrolysates prepared from the α-ketoisovalerate-, α-ketobutyrate-supplemented series of growths (see above). Figure 2c demonstrates that incorporation of ^{12}C into the non-ILV (Ile, Leu, Val) amino acid side chains was, to within experimental error, not detectable, even at 100 mg/L α-ketoisovalerate. Hence the catabolism of either α-ketoisovalerate or α-ketobutyrate by alternative pathways is not significant under these conditions. In contrast, mass spectra of Val and Leu trifluoroiminium ions provide evidence for incorporation of intact units of α-ketoisovalerate (see Figure 1). For example, the average mass of the trifluoroiminium species of Leu obtained from hydrolysis of MBP produced with ^{13}C-glucose and $^{15}NH_4Cl$ as the carbon and nitrogen sources is 188.04 ± 0.02, while an average mass of 184.44 ± 0.03 is obtained for Leu from MBP produced when 50 mg/L α-ketobutyrate and 100 mg/L α-ketoisovalerate are added to the growth media.

As shown in Figure 2, the level of label incorporation of the precursor α-ketobutyrate into Ile is high, between 90–95%. This is in accord with previous results where the amount of α-ketobutyrate added to each sample in these experiments (50 mg/L) was shown to give high labeling levels at the Ile δ1 position (Gardner and Kay, 1997). It is interesting to note that the GC-MS data does show a small but significant decrease in α-ketobutyrate incorporation into Ile at higher α-ketoisovalerate concentrations. This is likely due to the interconnected metabolic regulation of the biosynthetic pathways involving branched-chain amino acids. One important site of regulation is the inter-conversion of threonine and α-ketobutyrate mediated by threonine deaminase (Eisenstein, 1991). This enzyme is inhibited by high levels of Ile, which would be present upon metabolic conversion of exogenously supplied α-ketobutyrate into Ile. In the presence of high levels of Val however, this enzyme is activated, potentially leading to the production of α-ketobutyrate from Thr. Since the Thr is derived from glucose it would not possess the desired selective labeling pattern (methyl protonation) and subsequent incorporation into Ile would lower the level of δ1 methyl Ile protonation. Hence, the use of excess amounts of α-ketoisovalerate should be avoided as its facile conversion to Val can decrease (slightly) the incorporation of added α-ketobutyrate into Ile.

In practice, highly deuterated ^{15}N, ^{13}C, ^{2}H, (Val, Leu, Ile δ1)-methyl-protonated protein is produced using $D_2O/^{13}C$, ^{2}H glucose media with selective protonation achieved by addition of the precursors [3-^{2}H] ^{13}C α-ketoisovalerate and [3,3-$^{2}H_2$] ^{13}C α-ketobutyrate, as described in Figure 1 of Gardner et al. (1998). We have previously described the production of [3,3-$^{2}H_2$] ^{13}C α-ketobutyrate from ^{13}C-Thr (Gardner and Kay, 1997), although recently Cambride Isotope Laboratories (CIL, Andover, MA) has begun to supply this compound. Fully protonated ^{13}C α-ketoisovalerate is available commercially (CIL, US$ 2/mg) and the substitution of ^{2}H for ^{1}H at the 3 position is readily achieved by incubating the compound in D_2O (25 mM) at pH 12.5, 45 °C for 2–3 h. The reaction is easily monitored by NMR and goes to completion with no detectable production of other products.

As a demonstration of the utility of the method ^{15}N, ^{13}C, (Val, Leu, Ile δ1)-methyl-protonated MBP was grown in $D_2O/^{13}C$, ^{2}H glucose/$^{15}NH_4Cl$ with 85 mg/L [3-^{2}H] ^{13}C α-ketoisovalerate and 50 mg/L [3,3-$^{2}H_2$] ^{13}C α-ketobutyrate added 1 h prior to induction. MBP was produced as described by Gardner et al. (1998) with the exception that 85 mg/L α-ketoisovalerate was added (in place of Val) with 50 mg/L α-ketobutyrate and the protein was harvested 4 h after induction to minimize label scrambling and ensure that methyl isotopomers are not produced. The ^{1}H-^{13}C CT-HSQC spectrum (Santoro and King, 1992; Vuister and Bax, 1992) in Figure 3 demonstrates that there is a high degree of protonation of Leu δ, Val γ and Ile δ1 methyl groups and only a very small amount of residual protonation at other sites. Notably, $^{13}CH_2D$ and $^{13}CHD_2$ isotopomers are not observed.

In summary, a selective methyl protonation strategy for the production of highly deuterated proteins is described that uses α-ketoisovalerate as the labeling agent for Val and Leu. Approximately 90% incorporation of (^{1}H-δ methyl)-Leu, (^{1}H-γ methyl)-Val and (^{1}H-δ1 methyl)-Ile can be obtained by supplementing the growth media with 100 mg/L [3-^{2}H] α-ketoisovalerate and 50 mg/L [3,3-$^{2}H_2$] α-ketobutyrate. The simple conversion of commercially available ^{1}H, ^{13}C α-ketoisovalerate into the [3-^{2}H]-form translates into a more cost-effective method for selective methyl protonation than the previously described approach based on using ^{15}N, ^{13}C-[2,3-$^{2}H_2$]-Val as the precursor for Val and Leu.

Acknowledgements

This research was supported by a grant from the Medical Research Council of Canada. We would like to thank Grace DeSantis (Univiversity of Toronto) for her guidance in the amino acid derivatization reactions and Dr. Mike Rosen (Sloan-Kettering, New York) for his helpful advice. We also gratefully acknowledge the technical assistance provided by Dr. Alex Young (Univiversity of Toronto) with the GC-MS analyses and Rey Interior (Hospital for Sick Children Biotechnology Center) for performing the protein hydrolysis. The authors are grateful to Cambridge Isotope Laboratories (Andover, MA) for supplying α-ketoisovalerate free of charge. K.H.G. gratefully acknowledges a postdoctoral fellowship from the Helen Hay Whitney Foundation and N.K.G. is a recipient of an NSERC postgraduate scholarship. L.E.K. is an International Howard Hughes Research Scholar.

References

Bax, A. (1994) *Curr. Opin. Struct. Biol.*, **4**, 738–744.

Constantine, K.L., Mueller, L., Goldfarb, V., Wittekind, M., Metzler, W.J., Yanchunas, J., Jr., Robertson, J.G., Malley, M.F., Friedrichs, M.S. and Farmer, B.T.I. (1997) *J. Mol. Biol.*, **267**, 1223–1246.

Delaglio, F., Grzesiek, S., Vuister, G.W., Zhu, G., Pfeifer, J. and Bax, A. (1995) *J. Biomol. NMR*, **6**, 277–293.

Eisenstein, E. (1991) *J. Biol. Chem.*, **266**, 5801–5807.

Gardner, K.H. and Kay, L.E. (1997) *J. Am. Chem. Soc.*, **119**, 7599–7600.

Gardner, K.H. and Kay, L.E. (1998) *Annu. Rev. Biophys. Biomol. Struct.*, **27**, 357–406.

Gardner, K.H., Zhang, X., Gehring, K. and Kay, L.E. (1998) *J. Am. Chem. Soc.*, **120**, 11738–11748.

Gottschalk, G. (1986) In: *Bacterial Metabolism*, 2nd ed., Springer-Verlag, New York, NY, p. 359.

Grzesiek, S., Anglister, J., Ren, H. and Bax, A. (1993) *J. Am. Chem. Soc.*, **115**, 4369–4370.

Grzesiek, S., Wingfield, P., Stahl, S., Kaufman, J. and Bax, A. (1995) *J. Am. Chem. Soc.*, **117**, 9594–9595.

Leimer, K.R., Rice, R.H. and Gehrke, C.W. (1977) *J. Chromatogr.*, **141**, 121–144.

Massey, L.K., Sokatch, J.R. and Conrad, R.S. (1976) *Bacteriol. Rev.*, **40**, 42–54.

Metzler, W.J., Wittekind, M., Goldfarb, V., Mueller, L. and Farmer, B.T. (1996) *J. Am. Chem. Soc.*, **118**, 6800–6801.

Muhandiram, D.R., Xu, G.Y. and Kay, L.E. (1993) *J. Biomol. NMR*, **3**, 463–470.

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.

Santoro, J. and King, G.C. (1992) *J. Magn. Reson.*, **97**, 202–207.

Shan, X., Gardner, K.H., Muhandiram, D.R., Rao, N.S., Arrowsmith, C.H. and Kay, L.E. (1996) *J. Am. Chem. Soc.*, **118**, 6570–6579.

Smith, B.O., Ito, Y., Raine, A., Teichmann, S., Ben-Tovim, L., Nietlispach, D., Broadhurst, R.W., Terada, T., Kelly, M., Oschkinat, K., Shibata, T., Yokoyama, S. and Laue, E.D. (1996) *J. Biomol. NMR*, **8**, 360–368.

Venters, R.A., Metzler, W.J., Spicer, L.D., Mueller, L. and Farmer, B.T. (1995) *J. Am. Chem. Soc.*, **117**, 9592–9593.

Vuister, G.W. and Bax, A. (1992) *J. Magn. Reson.*, **98**, 428–435.

Yamazaki, T., Lee, W., Arrowsmith, C.H., Muhandiram, D.R. and Kay, L.E. (1994) *J. Am. Chem. Soc.*, **116**, 11655–11666.

Zwahlen, C., Gardner, K.H., Sarma, S.P., Horita, D.A., Byrd, R.A. and Kay, L.E. (1998a) *J. Am. Chem. Soc.*, **120**, 7617–7625.

Zwahlen, C., Vincent, S.J.F., Gardner, K.H. and Kay, L.E. (1998b) *J. Am. Chem. Soc.*, **120**, 4825–4831.