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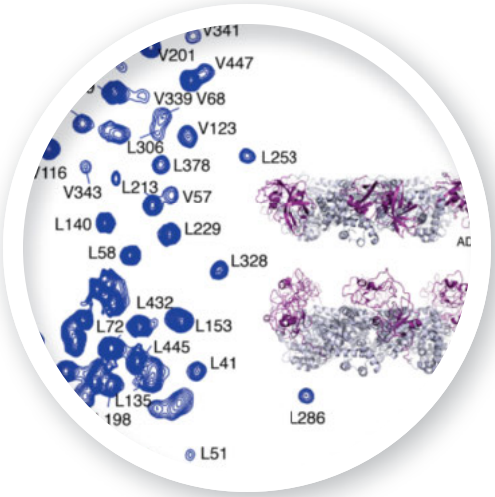

Stereospecific Leu/Val Methyl Labeling: An Important Technology for NMR Studies of High-Molecular-Weight Complexes

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The development of modern biomolecular solution NMR spectroscopy has paralleled innovations in labeling technologies. For example, early advances in heteronuclear double- and triple-resonance NMR spectroscopy were closely coupled to the emergence of methodologies for the effective uniform labeling of biomolecules with ^{15}N and/or ^{13}C.[1,2] Despite arguments that uniform ^{13}C (U-^{13}C) labeling would be prohibitively expensive, the high demand for U-^{13}C-labeled precursors has significantly lowered costs, and the preparation of biomolecules with ^{13}C incorporation is most often the approach of choice for NMR studies.[3] As the size of the system studied increases, both the sensitivity and the resolution of the resulting spectra suffer, and this has led to the development of amide-TROSY-based experiments[4] that are significantly improved through the use of perdeuteration.[5] A powerful labeling scheme for studies of proteins over a wide range of molecular masses, but, in particular, for systems with aggregate masses in excess of 100 kDa involves $^{13}CH_3$-labeling in a highly deuterated background.[6] Initial schemes focused on Ile (δ1), Leu and Val methyl groups, where for Leu and Val only one of the isopropyl methyls is $^{13}CH_3$ (the other is $^{12}CD_3$), but more recently precursors for the other methyl positions have become commercially available[7-11] so that it is possible to label the methyl group of choice. Once again, the availability of precursors stimulated the development of new NMR experiments, in this case those exploiting a methyl-TROSY effect,[12] which has resulted in applications involving protein complexes in excess of 1 MDa.[13]

The continued success of NMR studies of molecular machines over the past years has led to the study of increasingly complex targets. For example, initial studies by our laboratory focused on the 670 kDa proteasome core particle,[14] that is comprised of 14 copies of α and β-subunits, each with a molecular mass of approximately 25 kDa. The relatively small size of each protomer, albeit within the context of a large complex, meant that resolution was not limiting and that experiments could be performed using samples where methyl groups of Leu and Val were not stereospecifically $^{13}CH_3$ labeled, with one isopropyl methyl NMR-active in each Leu and Val. As the size of the individual protomers of the complex increases, spectral resolution is challenged as a pair of signals is measured for each isopropyl group. Boisbouvier and coworkers have developed an elegant approach that significantly addresses this limitation by introducing a synthetic route for the production of specifically methyl-labeled acetolactate, subsequently converted into either *proR*- or *proS*-$^{13}CH_3$ α-ketoisovalerate *in vivo* that then produces Leu and Val with $^{13}CH_3$ labeling confined to a single prochiral methyl[15] (**Figure 1A**). In addition, to simplifying the resulting NMR spectrum, the sensitivity of cross-peaks is increased two-fold relative to samples prepared with the racemic *pro-R/pro-S* $^{13}CH_3$,$^{12}CD_3$-isovalerate mixture as all of the label is focused on a single position rather than a 50%-50% split. This is an important consideration in applications involving samples that are concentration limited, and, in general in studies of very high-molecular-weight complexes.

Our recent work on the proteostasis protein p97[16] provides an example of the utility of the stereospecific biosynthetic labeling scheme of Boisbouvier.[15] Here we focused on a 320 kDa hexameric fragment of p97, with each of the protomers comprising residues 1-480 of the 540 kDa intact protein. A series of p97 disease mutants had been characterized biochemically prior to our study, but little structural information was available that provided a firm understanding of the relation between the mutants and p97 malfunction; indeed X-ray-derived structures of wild-type and mutated proteins were very similar. **Figure 1B** shows the Leu/Val region of the ^{13}C-^{1}H HMQC spectrum recorded on a sample of highly deuterated, Ile $C^{\delta1}$-$^{13}CH_3$, *proR* Leu,Val C^{δ},C^{γ}-$^{13}CH_3$,

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Met C^{ε}-$^{13}CH_3$ p97, highlighting the excellent sensitivity and resolution of the data set, along with cartoon representations of the structures in both ADP and ATP states, as established by X-ray studies. Note that one of the domains (colored purple) changes position significantly between the different nucleotide states. We have been able to show through NMR studies of a series of disease mutants that this domain changes orientation in response to disease severity, establishing a connection between structural-dynamics, and disease that was missing from X-ray analyses.

Molecular machines are ubiquitous in the cell, and they involve moving parts that, in principle, can now be characterized in quantitative detail by solution NMR methods. The development of stereospecific methyl labeling has been an important advance in achieving this goal.

Products of Interest

Catalog No.	Description	Package Size	Media Volume*
CDLM-10508-0.25	Ethyl 2-hydroxy-2-methyl-3-oxobutanoate (4-^{13}C, 99%; 2-methyl-D_3, 98%) (*proR*)	0.25 g	1 L
CDLM-10508-1	Ethyl 2-hydroxy-2-methyl-3-oxobutanoate (4-^{13}C, 99%; 2-methyl-D_3, 98%) (*proR*)	1 g	4 L
CLM-9935-0.25	Ethyl 2-hydroxy-2-methyl-3-oxobutanoate (methyl-^{13}C, 99%) (*proS*)	0.25 g	1 L
CLM-9935-1	Ethyl 2-hydroxy-2-methyl-3-oxobutanoate (methyl-^{13}C, 99%) (*proS*)	1 g	4 L

Note: The product must be hydrolyzed prior to use according to the instructions included with the product.

*Final volume of minimal media that can be prepared. Larger package sizes are available; please inquire. Minimal media reagents are not included.

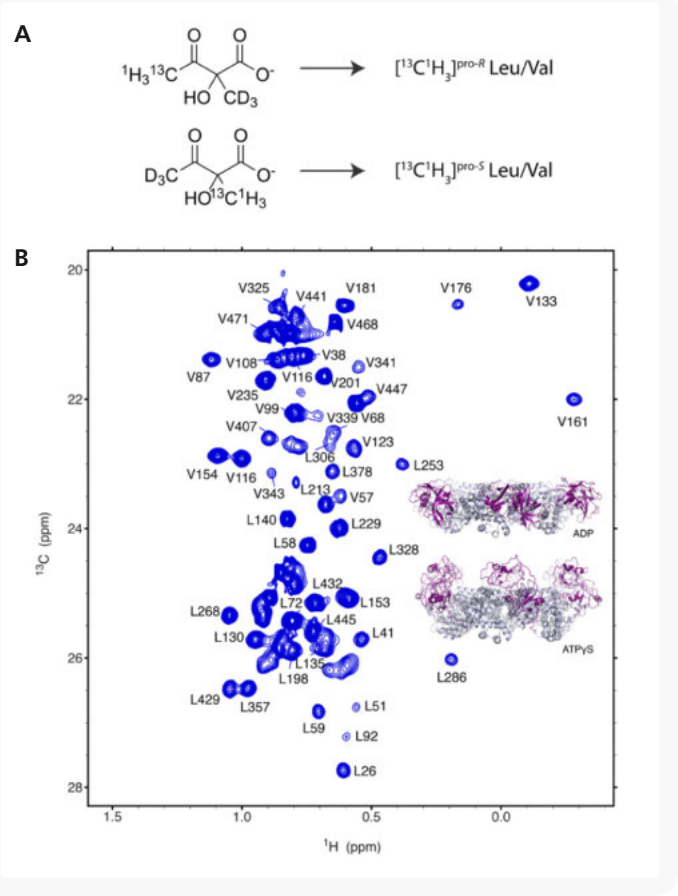

Figure 1. (A) Precursors used to produce stereospecific methyl labeling at *proR* (top) and *proS* (bottom) positions of Leu/Val residues. The *proR* precursor is prepared by dissolving the commercially available ethyl acetolactate compound in sodium phosphate buffer (9 g/L dibasic in H_2O) at a volume-to-volume ratio of 16.7% (acetolactate to buffer), followed by hydrolysis at pH 12 for 5-10 minutes. The formation of acetolactate is monitored by 1D NMR. After the completion of the reaction the solution is neutralized to pH 7.4, aliquoted for further usage and stored at -80°C. The *proS* precursor is prepared in the same fashion except that the initial hydrolysis reaction is carried out in D_2O buffer, followed by an incubation period of ~0.5 h during which exchange of the CH_3 moiety at position 4 to CD_3 is monitored by 1D NMR. NMR samples are all highly deuterated by expression in M9 minimal media, 99% D_2O, with d_7-glucose as the sole carbon source. Stereospecific methyl labeling was achieved by adding 230 mg L-1 of the prepared precursor 1 h prior to the induction of protein overexpression using 1 mM IPTG (OD600 ~ 0.8, expression at 18°C overnight). **(B)** Leu/Val region of ^{13}C-^1H HMQC spectrum of a hexameric 320 kDa construct comprising the N terminal domains (purple), the D1 domains (gray), and linker regions of p97. The spectrum was recorded on an 800 MHz spectrometer, 50°C. Figure modified from Schuetz and Kay.[16]

References

1. Ikura, M.; Kay, L.E.; Bax, A. **1990**. A novel approach for sequential assignment of ^{1}H, ^{13}C, and ^{15}N spectra of proteins: heteronuclear triple-resonance three-dimensional NMR spectroscopy. Application to calmodulin. *Biochemistry, 29(19),* 4659-4667.
2. Clore, G.M.; Gronenborn, A.M. **1991**. Structures of larger proteins in solution: three- and four-dimensional heteronuclear NMR spectroscopy. *Science, 252(5011),* 1390-1399.
3. Goto, N.K.; Kay, L.E. **2000**. New developments in isotope labeling strategies for protein solution NMR spectroscopy. *Curr Opin Struct Biol, 10(5),* 585-592.
4. Pervushin, K.; Riek, R.; Wider, G.; Wüthrich, K. **1997**. Attenuated T2 relaxation by mutual cancellation of dipole-dipole coupling and chemical shift anisotropy indicates an avenue to NMR structures of very large biological macromolecules in solution. *Proc Natl Acad Sci USA, 94,* 12366-12371.
5. Salzmann, M.; Pervushin, K.; Wider, G.; Senn, H.; Wuthrich, K. **1998**. TROSY in triple-resonance experiments: new perspectives for sequential NMR assignment of large proteins. *Proc Natl Acad Sci USA, 95(23),* 13585-13590.
6. Tugarinov, V.; Kay, L.E. **2004**. An isotope labeling strategy for methyl TROSY spectroscopy. *J Biomol NMR, 28(2),* 165-172.
7. Ayala, I.; Sounier, R.; Use, N.; Gans, P.; Boisbouvier, J. **2009**. An efficient protocol for the complete incorporation of methyl-protonated alanine in perdeuterated protein. *J Biomol NMR, 43(2),* 111-119.
8. Velyvis, A.; Ruschak, A.M.; Kay, L.E. **2012**. An economical method for production of (2)H, (13)CH_3-threonine for solution NMR studies of large protein complexes: application to the 670 kDa proteasome. *PLoS One, 7(9),* e43725.
9. Ruschak, A.M.; Velyvis, A.; Kay, L.E. **2010**. A simple strategy for (1)(3)C, (1)H labeling at the Ile-gamma2 methyl position in highly deuterated proteins. *J Biomol NMR, 48(3),* 129-35.
10. Ayala, I.; Hamelin, O.; Amero, C.; Pessey, O.; Plevin, M.J.; Gans, P.; Boisbouvier, J. **2012**. An optimized isotopic labelling strategy of isoleucine-gamma2 methyl groups for solution NMR studies of high molecular weight proteins. *Chem Commun (Camb), 48(10),* 1434-1436.
11. Isaacson, R.L.; Simpson, P.J.; Liu, M.; Cota, E.; Zhang, X.; Freemont, P.; Matthews, S. **2007**. A new labeling method for methyl transverse relaxation-optimized spectroscopy NMR spectra of alanine residues. *J Am Chem Soc, 129(50),* 15428-15429.
12. Tugarinov, V.; Hwang, P.; Ollerenshaw, J.; Kay, L.E. **2003**. Cross-correlated relaxation enhanced ^{1}H-^{13}C NMR spectroscopy of methyl groups in very high molecular weight proteins and protein complexes. *J Am Chem Soc, 125,* 10420-10428.
13. Ruschak, A.M.; Kay, L.E. **2012**. Proteasome allostery as a population shift between interchanging conformers. *Proc Natl Acad Sci USA, 109(50),* E3454-3462.
14. Sprangers, R.; Kay, L.E. **2007**. Quantitative dynamics and binding studies of the 20S proteasome by NMR. *Nature, 445(7128),* 618-622.
15. Gans, P.; Hamelin, O.; Sounier, R.; Ayala, I.; Dura, M.A.; Amero, C.; Noirclerc-Savoye, M.; Franzetti, B.; Plevin, M.J.; Boisbouvier, J. **2010**. Stereospecific isotopic labeling of methyl groups for NMR spectroscopic studies of high molecular weight proteins. *Angew Chem Int Ed, 49,* 1958-1962.
16. Schuetz, A.K.; Kay, L.E. **2016**. A dynamic molecular basis for malfunction in disease mutants of p97/VCP. *eLife, 5.*

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