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## Biomolecular NMR

### $\alpha$ -Ketoacids as Biosynthetic Precursors for Protein NMR

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The state-of-the-art NMR methodology for the studies of large proteins requires full deuteration of all non-exchangeable sites in the polypeptide chain. Unfortunately, full deuteration precludes the use of NOEs for structure determination. Since methyl groups give the most intense signals in NMR spectra of proteins due to their favorable relaxation properties and are frequently located in the hydrophobic cores of protein structures, it was suggested to protonate methyls selectively in otherwise fully deuterated proteins<sup>(1)</sup>. Selective protonation of methyl groups allows to retain protons in the key elements of protein structure and to measure methyl-methyl and methyl-HN NOEs with high sensitivity and resolution<sup>(2)</sup> for determination of global folds of larger proteins<sup>(1, 3-7)</sup>.

The development of strategies for selective methyl protonation of deuterated large proteins has a long history. It has been recognized that  $\alpha$ -ketoacids may serve as biosynthetic precursors for incorporation of several methyl moieties with desired isotopic labeling patterns into proteins overexpressed in minimal media. Initially, [U-<sup>13</sup>C] pyruvate was used as a primary carbon source for production of protonated Ala $\beta$ , Ile $\gamma$ , Val $\gamma$  and Leu $\delta$  methyls in highly deuterated protein molecules<sup>(1)</sup>. However, this approach leads to extensive generation of methyl isotopomers of other than <sup>13</sup>CH<sub>3</sub> variety (<sup>13</sup>CHD<sub>2</sub>, <sup>13</sup>CH<sub>2</sub>D and possibly <sup>13</sup>CD<sub>3</sub>) in protein samples. Due to isotope shifts, both <sup>13</sup>C and <sup>1</sup>H in each of these methyl variants resonate at different frequencies degrading resolution and sensitivity in NMR spectra. Although each of the isotopomers may be easily selected for spectroscopically, such losses in sensitivity are detrimental to NMR studies of high-molecular-weight (> ca. 30 kDa) proteins. Later, selective protonation of isoleucine  $\delta$ 1 methyls was suggested through addition of  $\alpha$ -ketobutyrate (2-ketobutyric acid) (obtained by enzymatic reaction from threonine) to the growth medium<sup>(8)</sup>. It was also shown that the addition of  $\alpha$ -ketoisovalerate (2-keto-3-methylbutyric acid) leads to efficient production of methyl-protonated Val and Leu residues<sup>(9)</sup>. Despite that the number of methyl-containing sites is restricted to Ile $\delta$ 1, Val $\gamma$ , and Leu $\delta$  in this case, the use of  $\alpha$ -ketobutyrate and  $\alpha$ -ketoisovalerate in the growth media does not lead to production of methyl isotopomers and is, generally, a robust and effective technique for selective protonation of methyl groups<sup>(9)</sup>. Sodium salts of  $\alpha$ -ketobutyric and  $\alpha$ -ketoisovaleric acids can be added to the D<sub>2</sub>O-based minimal growth medium in amounts of ~50 mg and ~80 mg/L, respectively, ca. 1 hour prior to induction of protein overexpression. These keto-acids can be obtained in the

protonated form at position 3 and quantitatively exchanged to 3-<sup>2</sup>H at high pH in D<sub>2</sub>O (pH 12.5, ~2-3 hrs. for  $\alpha$ -ketoisovalerate and pH 10.5, ~12-14 hrs. for  $\alpha$ -ketobutyrate) prior to use. In addition,  $\alpha$ -ketobutyrate (the source of  $\delta$ 1-methyl protonated Ile) and  $\alpha$ -ketoisovalerate (the source of methyl protonated Val and Leu) may be used separately or together without scrambling between Ile and Val, Leu labels in the course of protein production.

Building upon the efficiency and robustness of selective methyl protonation in deuterated protein structures through the use of  $\alpha$ -ketoacids, the Abbott group developed synthetic methods for production of  $\alpha$ -ketobutyric and  $\alpha$ -ketoisovaleric acids with <sup>13</sup>C enrichment exclusively at methyl positions<sup>(10)</sup>. The main purpose of this approach was to eliminate <sup>13</sup>C-<sup>13</sup>C scalar couplings that would allow recording very sensitive and highly resolved <sup>1</sup>H-<sup>13</sup>C methyl correlation maps for high throughput screening of ligands to larger proteins. This group demonstrated that the screening methods based on <sup>1</sup>H-<sup>13</sup>C spectroscopy of selectively [<sup>13</sup>C, <sup>1</sup>H]-labeled methyl groups of Ile, Leu and Val can be at least 3-5 times more sensitive than those based on <sup>1</sup>H-<sup>15</sup>N TROSY spectroscopy<sup>(10)</sup>. An alternative and more cost-effective synthetic strategy for production of these keto-acids was proposed later by Gross *et al.* and used for the studies of protein-ligand interactions<sup>(11)</sup>. Recently, Konrat and co-workers have reported a versatile synthetic procedure that allows to incorporate <sup>13</sup>CH<sub>3</sub>/<sup>12</sup>CD<sub>3</sub> (one methyl group - <sup>13</sup>CH<sub>3</sub>, the other - <sup>12</sup>CD<sub>3</sub>) isotope labels into isopropyl moieties of Val and Leu residues with high efficiency<sup>(12)</sup>. Indeed, the primary advantage of using  $\alpha$ -ketobutyric and  $\alpha$ -ketoisovaleric precursors is in the possibility of incorporation of practically any desired labeling pattern into the side-chains of Ile, Leu, Val residues as well as into methyl moieties themselves. For example, using appropriate precursors the side chains of these residues may be uniformly <sup>13</sup>C-labeled or, alternatively, the <sup>13</sup>C labels can be restricted to methyl groups. The isopropyl moieties of (<sup>13</sup>CH<sub>3</sub>/<sup>13</sup>CH<sub>3</sub>) or (<sup>13</sup>CH<sub>3</sub>/<sup>12</sup>CD<sub>3</sub>) type can be produced in Val/Leu side-chains. Likewise, methyl groups of <sup>13</sup>CH<sub>3</sub>, <sup>13</sup>CHD<sub>2</sub>, and <sup>13</sup>CH<sub>2</sub>D variety can be incorporated into Ile $\delta$ 1, Val $\gamma$ , and Leu $\delta$  sites. Such isotope labeling possibilities in methyl-bearing side-chains open new perspectives for NMR of large protein molecules.

Recently, Tugarinov & Kay showed that NMR assignments can be obtained for Leu and Val groups of very large proteins using 'linearization' of the corresponding spin-systems through the use

$\alpha$ -ketoisovalerate non-stereospecifically labeled with  $^{13}\text{C}$  in only one of its two methyl positions <sup>(13)</sup>. The same laboratory showed that factors of 2-to-3 in sensitivity can be achieved through the use of HMQC as opposed to HSQC experiments on protonated Ile  $\delta 1$  methyls (obtained using  $\alpha$ -ketobutyrate  $^{13}\text{C}$ -enriched only in the methyl position) in a highly deuterated protein environment <sup>(14)</sup>. The Methyl-TROSY methodology opened new perspectives for NMR studies of methyl groups attached to very large proteins and macromolecular complexes. However, as with  $^1\text{H}$ - $^{15}\text{N}$  TROSY, Methyl-TROSY requires extensive deuteration of the protein and a modification of standard methyl labeling strategies. The labeling strategy that maximizes the dipolar TROSY effect in Leu and Val side chains involves the  $^{13}\text{CH}_3/^{12}\text{CD}_3$  labeling of isopropyl groups <sup>(15)</sup>. Using the samples of a 723-residue monomeric protein Malate Synthase G (MSG, 82 kDa), selectively protonated at Ile  $\delta 1$  ( $^{13}\text{CH}_3$ ), and Val $\gamma$ /Leu $\delta$  sites ( $^{13}\text{CH}_3/^{12}\text{CD}_3$ ), the global fold of this protein could be derived very recently exclusively from solution NMR data <sup>(16-18)</sup>. Finally, the utility of  $^{13}\text{CHD}_2$  and  $^{13}\text{CH}_2\text{D}$  methyl isotopomers incorporated into the side chains of Ile/Val/Leu amino acids has been established recently for  $^2\text{H}$  and  $^{13}\text{C}$  relaxation studies in high-molecular-weight proteins in the 100 kDa range <sup>(19)</sup>.

Applications of the labeling methodology and the methyl-TROSY approach have been published in studies of a number of supra-molecular systems by the Kay group. Applications include the allosteric enzyme aspartate transcarbamoylase (306 kDa) <sup>(20)</sup>, the ClpP protease (300 kDa) <sup>(21)</sup> and the 20S core particle proteasome (670 kDa) <sup>(22)</sup>. A survey of the approaches used and some of the applications have recently been published as well <sup>(23)</sup>.

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