ChemBioChem

Communications
doi.org/10.1002/cbic.202000659

Chemistry Europe
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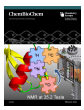

^{17}O NMR Studies of Yeast Ubiquitin in Aqueous Solution and in the Solid State

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We report a general method for amino acid-type specific ^{17}O-labeling of recombinant proteins in *Escherichia coli*. In particular, we have prepared several [1-^{13}C,^{17}O]-labeled yeast ubiquitin (Ub) samples including Ub-[1-^{13}C,^{17}O]Gly, Ub-[1-^{13}C,^{17}O]Tyr, and Ub-[1-^{13}C,^{17}O]Phe using the auxotrophic *E. coli* strain DL39 GlyA λDE3 (*asp*C$^-$ *tyr*B$^-$ *ilv*E$^-$ *gly*A$^-$ λDE3). We have also produced Ub-[η-^{17}O]Tyr, in which the phenolic group of Tyr59 is ^{17}O-labeled. We show for the first time that ^{17}O NMR signals from protein terminal residues and side chains can be readily detected in aqueous solution. We also reported solid-state ^{17}O NMR spectra for Ub-[1-^{13}C,^{17}O]Tyr and Ub-[1-^{13}C,^{17}O]Phe obtained at an ultrahigh magnetic field, 35.2 T (1.5 GHz for ^{1}H). This work represents a significant advance in the field of ^{17}O NMR studies of proteins.

Biomolecular NMR spectroscopy has become an indispensable tool in structural biology. Now, it is equally possible to determine the three-dimensional structure of biological macromolecules either in aqueous solution[1–5] or in the solid state.[6–9] While tremendous progress has been made over the past few decades in many aspects of biomolecular NMR, most NMR studies in this field have continued to rely on detection of "NMR friendly" spin-$^1/_2$ nuclei such as ^{1}H, ^{13}C, ^{15}N, and ^{31}P. Although the oxygen element is also ubiquitous in organic and biological molecules, its detection is severely hampered by the fact that the quadrupolar nature of the only NMR-active oxygen isotope, ^{17}O ($I = {}^5/_2$), often causes significant line broadening in NMR signals. In addition, because the natural abundance of ^{17}O is exceedingly low (0.037%), ^{17}O isotopic labeling is usually a prerequisite of any ^{17}O NMR study. In recent years, important advances have been made in the development of ^{17}O NMR spectroscopy as a useful tool in studying organic and biological molecules in the solid state.[10–12] In the context of biological macromolecules, ^{17}O NMR has been applied to study [^{17}O] ligand-protein complexes in the solid state[13–15] and in aqueous solution.[16,17] Solid-state ^{17}O NMR was also useful in probing membrane-bound proteins/peptides.[18–21] A particularly promising approach is to study large biomolecular systems with the ^{17}O quadrupole-central-transition (QCT) methodology.[22–24] However, one major obstacle is the lack of a general approach to incorporate ^{17}O isotopes into proteins. It is somewhat surprising that, although it is routine to produce selectively or uniformly ^{13}C- and ^{15}N-labeled recombinant proteins by heterologous expression in various host cell systems,[25–30] the same approach has not been reported in the literature for ^{17}O-labeling of proteins. In this work, we report successful amino acid-type specific ^{17}O-labeling of recombinant yeast ubiquitin by expression with an auxotrophic *Escherichia coli* strain and acquisition of ^{17}O NMR spectra for selectively ^{17}O-labeled yeast ubiquitin samples. The main goals of the present work are threefold: 1) to demonstrate the validity of this new ^{17}O-labeling approach, 2) to test the hypothesis that protein side-chain groups might be readily observable by ^{17}O NMR in aqueous solution, and 3) to explore the potential of solid-state ^{17}O NMR of proteins at an ultrahigh magnetic field, 35.2 T (1.5 GHz for ^{1}H).[31]

Ubiquitin is a small regulatory protein (76 amino acid residues) and one of the standard proteins for NMR methodology development. In this work, we utilized an auxotrophic *E. coli* strain DL39 GlyA λDE3 (*asp*C$^-$ *tyr*B$^-$ *ilv*E$^-$ *gly*A$^-$ λDE3)[32,33] to produce yeast ubiquitin (Ub) where glycine (Gly), tyrosine (Tyr), phenylalanine (Phe) residues were selectively ^{17}O-labeled. This general approach was first tested with ^{15}N-labeled amino acids and subsequently used to incorporate [1-^{13}C,^{17}O]-labeled Gly, Phe, Tyr as well as [η-^{17}O]Tyr into yeast ubiquitin for ^{17}O NMR studies. We chose this [1-^{13}C,^{17}O] double-labeling scheme for two reasons. First, the ^{13}C-labeling can be used to monitor for any potential isotope dilution and scrambling. Second, [1-^{13}C,^{17}O]-labeled protein samples may be useful for further development of heteronuclear ^{13}C/^{17}O correlation spectroscopy. All experimental details on chemical synthesis of ^{17}O-labeled amino acids, recombinant protein expression, purification, and characterization are provided in the Supporting Information.

Now let us first examine the hypothesis that, because protein side chains often experience substantial local motion in aqueous solution, their nuclear quadrupole relaxation characteristics may be similar to those of small molecules. As a result, it may be possible to detect ^{17}O NMR signals from protein side chains in aqueous solution. To this end, we prepared two

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Supporting information for this article is available on the WWW under https://doi.org/10.1002/cbic.202000659

samples: yeast ubiquitin (Ub)-[1-^{13}C,^{17}O]Gly and Ub-[η-^{17}O]Tyr. In yeast ubiquitin, there are six Gly residues, one of which, G76, is at the C-terminus. Thus, we may consider the COOH group of G76 as a "side chain". Since there is only one Tyr residue, Y59, in yeast ubiquitin, any ^{17}O NMR signal from Ub-[η-^{17}O]Tyr would be from the phenolic group of Y59. The ^{13}C solution NMR spectrum of Ub-[1-^{13}C,^{17}O]Gly confirmed that there was no substantial isotope scrambling and that all six Gly residues in yeast ubiquitin were [1-^{13}C,^{17}O]-labeled (Figure S6; our ^{1}H,^{15}N HSQC signals and ^{13}C chemical shifts are consistent with those reported in the literature).[34–36] Figure 1 shows the ^{17}O NMR spectra of Ub-[1-^{13}C,^{17}O]Gly and Ub-[η-^{17}O]Tyr in aqueous solution. Clearly, relatively sharp ^{17}O NMR signals are observed in both cases. The ^{17}O NMR signal from Ub-[1-^{13}C,^{17}O]Gly appears at 272 ppm, typical of carboxylic acids. This signal broadened as the temperature was lowered, but the signal position and line width were independent of the applied magnetic field, which suggests that G76 is under the so-called extreme narrowing condition (i.e., $\omega_0\tau_C \ll 1$; Figure S8). The ^{17}O NMR signals from other five Gly residues in yeast ubiquitin were too broad to be detected in aqueous solution at room temperature because they are in the slow motion regime, $\omega_0\tau_C > 1$. It is interesting to note that while G75 and G76 might have similar order parameters from the perspective of ^{13}C and ^{15}N NMR, the additional carboxylate 180°-flipping motion[37] must contribute to the very short correlation time of G76 from the ^{17}O NMR point of view. When the tumbling motion of yeast ubiquitin was slowed down by dissolving the protein in glycerol/H_2O (20%/80%), ^{17}O QCT NMR signals from G10, G35, G47, G53, and G75 residues were observed at 253 K, as shown in Figure S8.

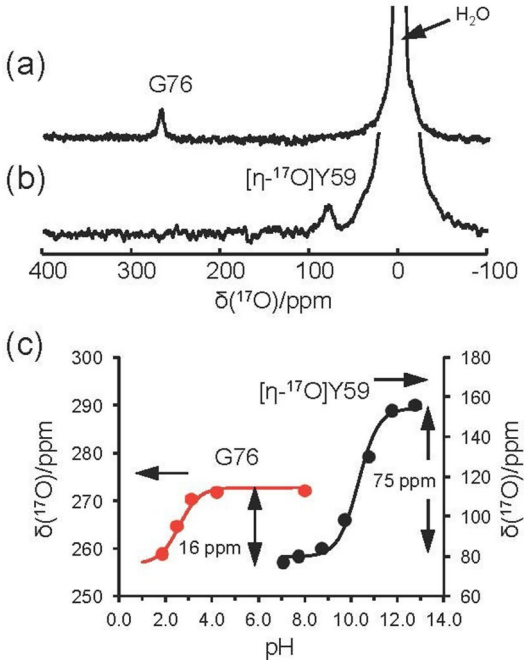

Figure 1. ^{17}O NMR spectra obtained at 16.4 T (700 MHz for ^{1}H) for a) Ub-[1-^{13}C,^{17}O]Gly and b) Ub-[η-^{17}O]Tyr in aqueous solution (1.2 mM protein, H_2O, 25 mM Tris buffer, 250 mM NaCl, pH 8, 25 °C). c) Titration results; for Ub-[1-^{13}C,^{17}O]Gly at low pH values, a 50-mM phosphate buffer was used.

This suggests that, under this condition, the protein is in the ultraslow motion regime (i.e., $\omega_0\tau_C \gg 1$).[12] However, we will not pursue this line of investigation further in the present study. As also seen in Figure 1, the ^{17}O NMR signal from Ub-[η-^{17}O]Tyr appears at 87 ppm, which is also consistent with that observed for the free amino acid L-Tyr.[38] Further, the ^{17}O NMR signal from the phenolic group in Y59 is broader than that for the carboxyl group in G76, because the phenolic group is known to have a larger C_Q(^{17}O) value.[39] This is the first time that ^{17}O NMR signals from protein side chains have been observed. One immediate application is to utilize ^{17}O NMR as a new probe for pK_a measurement. As seen in Figure 1c, the pK_a values for the carboxyl group of G76 and the phenolic group in Y59 were determined to be 2.32 ± 0.04 and 10.44 ± 0.04, respectively. It is interesting to note that, for the phenolic group of Y59, the ^{17}O chemical shifts in the two ionization states differ by 75 ppm. This is in agreement with the previous results for free amino acid L-Tyr both in aqueous solution[38] and in the solid state.[39] While the two pK_a values determined for yeast ubiquitin may not have any important implication for its biological functions per se, this proof-of-concept experiment provides a novel method for studying ionization state, hydrogen bonding or ion binding of oxygen-containing amino acid side chains (Asp, Glu, Ser, Thr, Asn, Gln) of proteins in aqueous solution.

To explore the potential of solid-state ^{17}O NMR in detecting ^{17}O signals from a protein backbone, we prepared two yeast ubiquitin samples: Ub-[1-^{13}C,^{17}O]Tyr and Ub-[1-^{13}C,^{17}O]Phe. Once again, because only one Tyr residue is present in yeast ubiquitin, Ub-[1-^{13}C,^{17}O]Tyr serves as an ideal sample to avoid any ambiguity in spectral analysis. Figure 2a shows an overlay of the 2D ^{1}H,^{15}N HSQC spectra of Ub-[^{15}N]Tyr and Ub-[^{15}N]Phe in aqueous solution. The observed ^{1}H and ^{15}N chemical shifts are consistent with the literature values.[34] This data confirmed that no substantial isotope scrambling occurred. Figures 2b and 2c show the ^{13}C CP/MAS NMR spectra of hydrated solid samples of Ub-[1-^{13}C,^{17}O]Tyr and Ub-[1-^{13}C,^{17}O]Phe, respectively. The observed line width of 0.4 ppm is similar to those observed for ubiquitin microcrystals.[40–42] The ^{13}C chemical shifts for Y59, F4, and F45 are also in agreement with those reported in previous solution[35,36] and solid-state NMR studies.[40,42] Figure 2d shows the ^{17}O MAS NMR spectrum of Ub-[1-^{13}C,^{17}O]Tyr obtained at 35.2 T. Since there is a single Tyr backbone carbonyl oxygen in this sample, the spectrum can be readily analyzed, which yielded the following ^{17}O NMR tensor parameters: $\delta_{iso} = 308$ ppm, $C_Q = 9.0$ MHz, $\eta_Q = 0.7$, $\Delta_{CS} = \delta_{33} - \delta_{iso} = -320$ ppm, $\eta_{CS} = (\delta_{22} - \delta_{11})/\Delta_{CS} = 0.2$. The ^{17}O MAS NMR spectrum of Ub-[1-^{13}C,^{17}O]Phe (Figure 2e) displays signals that are broader than those seen for Ub-[1-^{13}C,^{17}O]Tyr, suggesting the presence of two oxygen sites. This is also consistent with the ^{13}C and ^{15}N NMR data mentioned earlier. Once again, spectral analysis yielded a complete set of ^{17}O NMR tensor parameters for both sites: F45, $\delta_{iso} = 299$ ppm, $C_Q = 8.9$ MHz, $\eta_Q = 0.7$, $\Delta_{CS} = -320$ ppm, $\eta_{CS} = 0.2$; Y4, $\delta_{iso} = 281$ ppm, $C_Q = 8.2$ MHz, $\eta_Q = 0.4$, $\Delta_{CS} = -310$ ppm, $\eta_{CS} = 0.3$. This is the first time that complete ^{17}O NMR tensor parameters have been obtained for oxygen atoms on a protein backbone. All ^{17}O NMR tensor parameters obtained for the yeast ubiquitin samples are summarized in Table S1, together with some

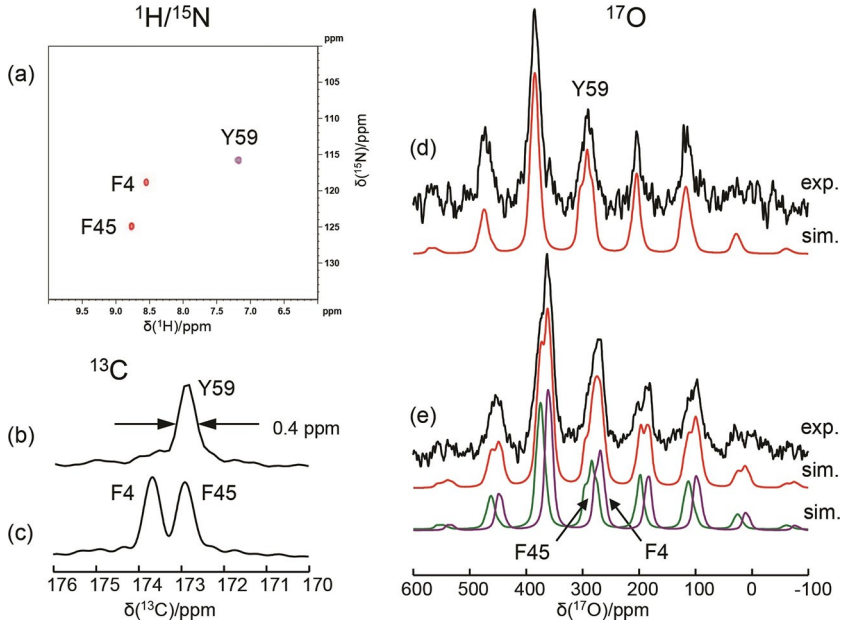

Figure 2. a) Overlay of the $^{1}H,^{15}N$ 2D HSQC NMR spectra obtained at 16.4 T (700 MHz for ^{1}H) for Ub-[^{15}N]Phe and Ub-[^{15}N]Tyr in aqueous solution (0.8 mM protein, 95 %H_2O/5 %D_2O, 25 mM Tris buffer, 300 mM NaCl, pH 8, 25 °C). Solid-state ^{13}C CP/MAS spectra obtained at 19.6 T (833 MHz for ^{1}H) for b) rehydrated Ub-[1-^{13}C,^{17}O]Tyr and c) Ub-[1-^{13}C,^{17}O]Phe. Solid-state ^{17}O 18-kHz MAS NMR spectra obtained at 35.2 T (1.5 GHz for ^{1}H) for d) Ub-[1-^{13}C,^{17}O]Tyr (665 600 transients, recycle delay 20 ms) and e) Ub-[1-^{13}C,^{17}O]Phe (313 856 transients, recycle delay 20 ms). Home-built 3.2-mm MAS probes were used at 19.6 and 35.2 T.

relevant literature data.[18–20,43–47] As seen from Table S1, the overall ^{17}O NMR parameters observed for yeast ubiquitin samples are similar to those reported previously for peptides.

The most striking observation is the sensitivity of $\delta_{iso}(^{17}O)$ and $C_Q(^{17}O)$ toward molecular structure. For example, the crystal structures of ubiquitin suggest that both F45 and Y59 are involved in loop regions, whereas F4 is within a β-sheet.[48,49] Thus, strong hydrogen bonding is expected around the backbone carbonyl oxygen of F4. Indeed, the values of $\delta_{iso}(^{17}O)$ and $C_Q(^{17}O)$ found for F45 and Y59 are similar, but are significantly larger than the corresponding values observed for F4. It is interesting to point out that the difference in $\delta_{iso}(^{13}C)$ values between F4 and F45 is approximately 1 ppm, as seen from Figure 2c, whereas the corresponding $\delta_{iso}(^{17}O)$ values differ by 18 ppm. These observations are entirely consistent with the previously known trends in the dependence of ^{17}O NMR parameters on hydrogen bonding (i.e., strong hydrogen bonding leads to reduction in both $\delta_{iso}(^{17}O)$ and $C_Q(^{17}O)$ values for carbonyl compounds).[10] The sensitivity offered by the 35.2-T magnet is also impressive. The levels of ^{17}O enrichment in the Ub-[1-^{13}C,^{17}O]Tyr and Ub-[1-^{13}C,^{17}O]Phe samples were about 30–40 %. Approximately 20 mg of lyophilized protein was packed in a 3.2-mm MAS rotor. The total experimental times to record the ^{17}O MAS spectra shown in Figures 2d and 2e were 4–5 h. With higher ^{17}O enrichment levels, it may be possible to apply techniques such as MQMAS[50] or STMAS[51] for proteins to obtain even higher spectral resolution. In principle, the solid-state ^{17}O NMR approach is not impacted by the size of the protein under study.

In summary, we have demonstrated that it is feasible to achieve amino acid-type specific ^{17}O-labeling of proteins via recombinant expression in an auxotrophic *E. coli* strain. This approach allows incorporation of ^{17}O isotopes into both the protein backbone and side chains. We have shown that since protein side chains often exhibit significant local motion they behave like small molecules from the perspective of nuclear quadrupole relaxation, thus making the detection of their ^{17}O NMR signals straightforward. Such an approach offers a new method for probing protein side-chain structure, interactions and dynamics. For example, it would be interesting to apply this ^{17}O NMR approach to study ion binding to protein side chains or hydrogen bonding. We have also explored the use of an ultrahigh magnetic field, 35.2 T, to record high-quality solid-state ^{17}O NMR spectra for proteins. The information offered by the complete ^{17}O NMR tensor characterization will be complementary to that obtainable from more conventional NMR probes, such as ^{1}H, ^{13}C and ^{15}N. The results reported in this study are encouraging. One can envision that the sensitivity and resolution in solid-state ^{17}O NMR for proteins can be further improved by $^{13}C/^{17}O$ heteronuclear correlation spectroscopy perhaps in combination with nuclear dynamic polarization. It is highly desirable to make ^{17}O accessible by NMR in studies of biological macromolecules in aqueous solution and in the solid state, so that one would be able to utilize all magnetic nuclei available in proteins, nucleic acids, and carbohydrates (^{1}H, ^{2}H, ^{13}C, ^{14}N, ^{15}N, ^{17}O, ^{31}P) to gain information about molecular structure, chemical bonding, and dynamics. The present work represents a key step towards this ultimate goal. Research to

further expand the current work is under way in our laboratories.

Acknowledgements

S.P.S. and G.W. thank the Natural Sciences and Engineering Research Council (NSERC) of Canada for Discovery Grants to support this work (grants no. 298508 and 23308-11). This work was also supported by the National High Magnetic Field Laboratory through NSF DMR-1644779 and the State of Florida and by a grant from NIH (P41 GM122698). We thank Professor Gary Shaw (University of Western Ontario) for providing the yeast ubiquitin encoding plasmid and Professor Lawrence P. McIntosh (University of British Columbia) for supplying the auxotrophic bacterial strain.

Conflict of Interest

The authors declare no conflict of interest.

Keywords: auxotrophic strains · isotope labeling · ^{17}O NMR · recombinant protein · ubiquitin

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Manuscript received: September 21, 2020
Revised manuscript received: October 14, 2020
Accepted manuscript online: October 15, 2020
Version of record online: November 6, 2020