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A solution ^{17}O-NMR approach for observing an oxidized cysteine residue in Cu,Zn-superoxide dismutase†

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Solution ^{17}O-NMR application to biological macromolecules is extremely limited. We describe here ^{17}O-NMR observation of the $^{17}O_2$-oxidized cysteine side chain of human Cu,Zn-superoxide dismutase in solution using selective $^{17}O_2$ oxidation. ^{17}O-NMR with the aid of ^{17}O-labeling has wide potential to probe the environment and dynamics of oxidizable functionalities in proteins.

^{17}O-NMR is a direct, non-degrading method for detecting oxygen-containing functionalities like hydroxyl (–OH), ether (–O–), carbonyl (C═O), phosphate (P═O), and sulfoxide (S═O) groups. It has found wide application in organic chemistry to identify structure and reaction mechanisms based on the wide chemical-shift range and distinguishable oxidation states.[1] In contrast, its use in biological applications for protein analysis has been hampered by low sensitivity because the natural abundance of NMR active ^{17}O is only 0.0037%. A clear signal usually requires stable-isotope labeling using a ^{17}O-donatable species. Another drawback is that the ^{17}O-NMR signal is broader than those obtained by ^{1}H- and ^{13}C-NMR because ^{17}O has a quadrupole moment (I = 5/2). For these reasons, solid-state ^{17}O-NMR is more applicable for biological samples.[2–4] In contrast, solution ^{17}O-NMR spectroscopy has been limited to protein–ligand interactions with ^{17}O-labeled low-molecular-weight ligands, such as ^{17}O-labeled carbon monoxide binding to heme proteins and ^{17}O-labeled palmitic acid, oxalate and biotin binding to model proteins.[5–9] As yet, no attempts have been made to observe solution ^{17}O-NMR signals directly derived from ^{17}O-labeled proteins.

The thiol group of cysteine (Cys) residues in proteins is susceptible to reactive oxygen species (ROS). Upon oxidation by ROS, the thiol group can form a disulfide by recruiting a surrounding thiol group or oxo-acid derivatives involving sulfenic acid (–SOH), sulfinic acid (–SO_2H) and sulfonic acid (–SO_3H). The produced oxo-acid at Cys potentially alters the local hydrogen-bond environment and electrostatic polarities and might induce structural and dynamic changes in the protein. Actually, such a modified Cys side chain often influences protein function.[10–14]

Human Cu,Zn-superoxide dismutase (SOD1) is a key enzyme for protecting cells against the highly reactive superoxide anion radical by converting it to hydrogen peroxide. SOD1 is a homodimer containing one copper ion and one zinc ion in each 16-kDa subunit (Fig. 1). Mutations in the SOD1 gene have been found in patients with familial amyotrophic lateral sclerosis (FALS). The FALS-linked mutant SOD1 proteins have a propensity to form aggregates that are

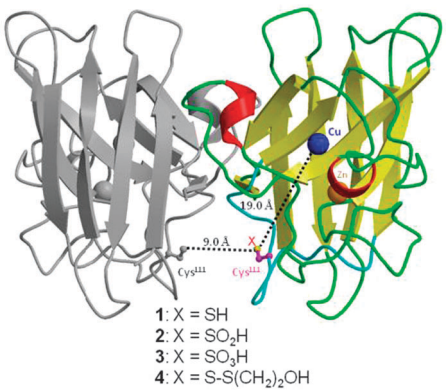

Fig. 1 Structure of the SOD1 dimer (pdb: 1SPD). Cys[111] is shown in magenta, Zn in orange, and Cu in blue. $^{17}O_2$-oxidized SOD1 contains three forms as in **1**, **2**, and **3**.

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implicated in neuronal toxicity. Even wild-type SOD1 aggregates after oxidation, and gains properties reminiscent of FALS mutant SOD1s. We previously reported that the free –SH group on Cys^{111} of human SOD1 (**1**) is selectively oxidized to $–SO_2H$ (**2**) and $–SO_3H$ (**3**) under atmospheric oxygen (Fig. 1).[15] Intriguingly, only a Cys^{111} at one of the subunits is asymmetrically air oxidized.[15–17] The oxidation of Cys^{111} affects aggregation and resulting cytotoxicity.[18,19] Immunohistochemical analysis using a specific antibody against Cys^{111}–SO_3H indicated labeling of Lewy-body-like hyaline inclusions and vacuole rims in the spinal cord of FALS model mice.[15]

A method for analysing the chemical environment and function of $R–SO_2H$/$R–SO_3H$ forms of Cys residues will therefore help understand the role of oxidized proteins in such conformational changes and associated disease processes. Previously ^{13}C-NMR has been used for the investigation of oxidized Cys, coupled with mass spectrometry.[20] The C_β chemical shift of Cys is known to reflect the oxidative state of the sulfhydryl group. In this context, we considered that ^{17}O-NMR might be an alternative and efficient technique to identify the $R–SO_nH$ group on Cys residues in a non-degrading manner. Furthermore, the direct observation technique includes the potential to analyse the structural property of the $R–SO_nH$ group because the chemical shifts and line-widths of the $R–SO_nH$ signal could be good reporters of the surrounding environment. Here we selectively label Cys^{111}–SH of human recombinant SOD1 with ^{17}O and successfully analyse it using solution ^{17}O-NMR.

Oxidation of Cys^{111}–SH SOD1 by $^{17}O_2$-gas was performed according to previously reported protocols with some modifications.[15] The oxidation state of ^{17}O–SOD1 was confirmed using peptide-mapping combined ESI-TOF MS (Fig. S1 and S2, ESI†). The molar ratio of **1** : **2** : **3** = 63 : 16 : 21 based on the HPLC profile of the peptides was identical to the previously reported pattern.[15] ^{1}H-NMR spectra of Cu^{2+}- and Cu^+-forms of $^{17}O_2$-oxidized SOD1 were measured to evaluate their structural identities (Fig. S3 and S4, ESI†). To monitor the oxidation state of the Cu cation and the structure of the catalytic site, NMR signals of imine protons on the His side chains chelating Zn^{2+} and the Cu cation were utilized as a probe.[21] The reduced SOD1 is of the Cu^+-form as evidenced by the presence of sharp His signals (Fig. S4(b) and (c), ESI†), while the original and re-oxidized SOD1 is of the Cu^{2+}-form, which was judged by the broadening of most His signals due to the paramagnetic effect of Cu^{2+} (Fig. S4(a) and (d), ESI†). Although the three forms, **1**, **2** and **3**, are contained in $^{17}O_2$-oxidized SOD1, prepared *via* the above-mentioned procedure, only a single pattern of ^{1}H-NMR signals was observed (Fig. S3 and S4, ESI†), showing that oxidation of Cys^{111} of SOD1 does not induce large structural differences in the protein. In contrast, reduction of Cu^{2+} to Cu^+ induces structural changes, as observed in ^{1}H-NMR and CD spectra (Fig. S3 and S5, ESI†).

Before performing ^{17}O-NMR on the ^{17}O-SOD1, the standard ^{17}O-chemical shifts of $R–SO_3H$ and $R–SO_2H$ were confirmed using AcNH–Cys–SO_3H **8** and AcNH–Cys–SO_2H **9** (Fig. 2). As to ^{17}O-NMR spectra of AcNH–Cys–SO_3H **8**, a signal was observed at 171 ppm (line-width: 720 Hz; Fig. 2a). In contrast, as shown in Fig. 2b, AcNH–Cys–SO_2H **9** provided a broader signal at 141 ppm (line-width: 1890 Hz), which would be due to the unsymmetrical oxygen nucleus. The results indicate that the ^{17}O-NMR technique apparently distinguishes between Cys–SO_2H and Cys–SO_3H in terms of chemical shifts and line-widths.

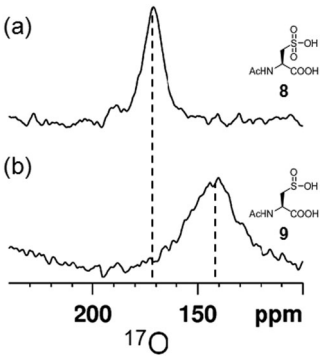

Fig. 2 ^{17}O-NMR spectra of *N*-acetylated cysteinesulfonic acid **8** (a; 1.4 M) and cysteinesulfinic acid **9** (b; 1.0 M) in 10 mM phosphate buffer (pH 5.0) at 20 °C. A ^{17}O-signal with natural abundance was detected at chemical shifts of 171 ppm (a) and 141 ppm (b), respectively.

Then, our interest shifted to observe Cys^{111}–SO_3H/–SO_2H signals in $^{17}O_2$-oxidized SOD1 by ^{17}O-NMR. We expected that it would be potentially possible because a quadrupole central transition signal would be observed at the slow motion limit (discussed later).[8,9] In fact, clear ^{17}O-NMR spectra of $^{17}O_2$-oxidized SOD1 in the Cu^{2+}-form, Cu^+-form, and peptide mixture-form were successfully obtained (Fig. 3). First, the spectrum of $^{17}O_2$-oxidized SOD1 (Cu^{2+}-form) showed a signal at 165 ppm (line-width: 470 Hz) (Fig. 3a). To examine the paramagnetic Cu^{2+} effect, Cu^{2+} at the catalytic centre was reduced with isoascorbic acid or 2-mercaptoethanol to form Cu^+.[22,23] As shown in Fig. 3b, the Cu^+-form prepared by addition of isoascorbic acid gave a sharp ^{17}O-NMR signal at 164 ppm (line-width: 130 Hz). Reduction with 2-mercaptoethanol also

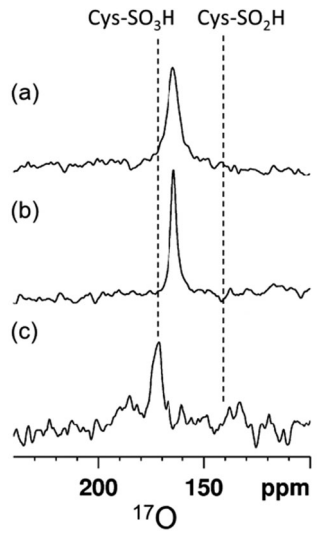

Fig. 3 ^{17}O-NMR spectra of $^{17}O_2$-oxidized SOD1. All spectra were measured in 10 mM phosphate buffer (pH 5.0) at 20 °C; (a) original Cu^{2+}-form, (b) Cu^+-form prepared by addition of isoascorbic acid (10 mM). (c) SOD1 peptide mixture produced by treatment with lysylendopeptidase.

provides the Cu^{+}-form, giving an identical signal (data not shown). Hence the signal-broadening effect observed in Fig. 3a is partially caused by a Cu^{2+} paramagnetic effect. The distance between the Cu cation at the active site and Cys^{111} is about 19 Å based on a previously reported SOD1 structure (PDB ID: 1SPD; Fig. 1).[24] Although the distance is rather far for such a paramagnetic effect from the active site Cu^{2+} to ^{1}H (usually sensitive up to 11 Å) and ^{13}C (up to 6 Å) nuclei, ^{17}O-nuclei at Cys^{111} still experience a paramagnetic effect.[22] The slight down-field shift by 1 ppm of the Cys^{111}–SO_3H signal from Cu^{+}-SOD1 indicates the subtle structural or environmental difference between Cu^{2+}-SOD1 and Cu^{+}-SOD1. A previous NMR study showed that the copper ion in the Cu^{+}-bound dimer form moves further inside the active cavity ($\sim$1.7 Å) and thus His63 loses the coordination to the Cu ion.[23] Since additional structural comparisons have not been reported, further experiments are necessary to establish the conformational differences between Cu^{2+}-SOD1 and Cu^{+}-SOD1. The ^{17}O-NMR line width is highly dependent on $\omega_0\tau_c$,[8] where ω_0 is the Larmor angular frequency of the ^{17}O nucleus under detection and τ_c is the molecular rotational correlation time. For biological macromolecules at high magnetic fields (slow motion limit, $\omega_0\tau_c \gg 1$), the ^{17}O signal is expected to show a relatively narrow spectral line. For the SOD1 dimer under the experimental conditions, $\omega_0\tau_c$ is estimated to be 9 ($\tau_c = 4\pi\eta r^3/3kT$, where η is the viscosity of water, k is the Boltzmann constant, T is the temperature and r is the effective hydrodynamic radius of the protein, 2.5 nm (ref. 25)). This condition is expected to provide a rather sharp line width, consistent with the observation of a sharp ^{17}O-NMR signal.

Although the sample includes both the Cys^{111}–SO_3H form and the Cys^{111}–SO_2H form in a 16 : 21 ratio (Fig. S1, ESI†), only one signal was observed (Fig. 3a and b). The reason is likely to be due to broadening out of a signal especially that from the Cys^{111}–SO_2H form. Actually, a broad signal is observed even in the free amino acid form, AcNH–Cys–SO_2H **9** (Fig. 2). Another possibility is due to overlapping of signals from Cys^{111}–SO_2H and –SO_3H. However, this seems unlikely because the chemical shifts of R–SO_2H and R–SO_3H are 30 ppm apart in the free amino acid form.

In order to examine the molecular size-dependent ^{17}O relaxation property, a $^{17}O_2$-oxidized SOD1 peptide mixture was prepared by guanidine–DTT treatment and lysylendopeptidase digestion. The molecular weight of the peptide (residues 92–122) containing Cys^{111}–SO_3H is 3300 (Fig. S2, ESI†).[15] Since the peptide mixture does not contain metal ions, the Cu^{2+} paramagnetic effect can be excluded. The ^{17}O-NMR spectra of the digest exhibited a sharp signal at 171 ppm with a line-width of 250 Hz, which is analogous to the signal obtained with AcNH–Cys–SO_3H **8** (Fig. 2a). The signal is therefore attributed to Cys^{111}–SO_3H in the peptide. However, no signals were observed at 141 ppm, corresponding to Cys^{111}–SO_2H. The selective observation of the Cys^{111}–SO_3H signal is likely attributable to an inherent property due to the symmetrical oxygen nucleus.

In conclusion, we have described here the first example of solution ^{17}O-NMR for Cys^{111}–SO_3H/SO_2H on SOD1, a homo-dimeric protein composed of 16 kDa subunits. The chemical shifts and line-widths of Cys^{111}–SO_3H on SOD1 were distinct in the Cu^{2+}- and Cu^{+}-forms as well as the peptide-digested form, which strongly suggests environmental, structural and dynamic differences. Our results suggest that observing Cys–SO_3H by ^{17}O labelling and ^{17}O-NMR could be very useful for investigating protein structure and dynamics, complementing the conventional ^{1}H-, ^{13}C- and ^{15}N-NMR studies. Further, the method can potentially be applied to the study of the functional modulation of proteins containing Met or catalytic Cys by oxidative stress. Our progress in solution ^{17}O-NMR when combined with an efficient ^{17}O-labeling strategy promises to change that and direct and precise analysis of such biological molecules becomes feasible.

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