Electronic Supplementary Material (ESI) for Chemical Communications

Supporting Information

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

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Contents

1. Experimental procedures

^{17}O-labeling with $^{17}O_2$-gas

Recombinant human SOD1 (residues 1-153) chemically modified with 2-mercaptoethanol (2-ME) at Cys^{111} (Cys^{111}-S-S-CH_2CH_2OH), designated as 2-ME-SOD1 **4**, was kindly provided by Ube Industries Ltd. Preparation of Wild-type(WT)-SOD1 **1** and $^{16}O_2$-oxidized SOD1 were performed according to previously reported protocols.[1] WT-SOD1 (Cys^{111}-SH form) **1** was initially prepared from 2-ME-SOD1 **4** by incubation with excess 2-ME for 1 h on ice and then the excess 2-ME was removed using a PD-10 column (GE Healthcare). For labeling with $^{17}O_2$-gas, the WT-SOD1 in milliQ water was degassed and then stirred under $^{17}O_2$ (70% isotope enrichment, ISOTEC) for 24 h at room temperature. The vacuum level was monitored with a vacuum gauge, and the loading of $^{17}O_2$-gas was repeated three times. This oxidation process produced a SOD1 mixture of Cys^{111}-SH **1**, Cys^{111}-SO_2H **2** and Cys^{111}-SO_3H **3**.

Peptide mapping and mass spectrometry

The ^{17}O-labeled position and labeling ratio at Cys^{111} of SOD1 were confirmed by peptide mapping according to previously reported protocols.[1, 2] Briefly, 0.05 mM SOD1 (calculated as monomer) was incubated in 50 mM Tris-HCl, pH 8.5, including 10 mM EDTA and 7 M guanidinium hydrochloride and 50 mM dithiothreitol (DTT) at 37 °C for 2 h. The liberated sulfhydryl groups were alkylated with 90 mM iodoacetamide in the dark for 30 min at room temperature. After the reaction, the mixture was desalted by dialysis against 50 mM Tris-HCl buffer, pH 8.5. Then, the protein was digested with 0.25% (w/w) lysylendopeptidase (Wako Pure Chemicals) at 37 °C for 16 h. The resultant peptide mixture was fractionated by high performance liquid chromatography (HPLC) with a reverse phase octadecylsilyl modified column (Inertsil ODS3, 4.6 mm×250 mm, particle size: 5 μm; GL Science) at a flow rate of 1.0 mL/min. Two linear gradients of 0–30% acetonitrile for 4 column volumes and 30–40% acetonitrile for 8 column volumes containing 0.05% (v/v) trifluoroacetic acid were employed for separation and the peptides were detected by absorbance at 215 nm. The fractions corresponding to Asp92-Lys122 were subjected to ESI-TOF-MS analysis for estimation of the ^{17}O enrichment ratio on Cys^{111}. High resolution ESI-TOF-MS measurements were performed using maXis (Bruker Daltonics). Each peptide sample was dissolved in 0.08% (v/v) formic acid / 20% (v/v) acetonitrile solution. MS spectra were collected with positive mode.

^{1}H- and ^{17}O-NMR spectroscopy

All NMR spectra were collected with Bruker DRX-600 spectrometer equipped with triple resonance inverse (TXI; for ^{1}H detection) or broad band observation (BBO; for ^{17}O detection) probes. Water signals in ^{1}H-NMR spectra were suppressed using a watergate pulse sequence. Chemical shifts of ^{1}H were referenced to the outer standard 4,4-dimethyl-4-silapentane-1-sulfonic acid (DSS) signal at 0 ppm. ^{17}O-NMR spectra were measured with aring pulse sequence to cancel acoustic ringing.[3] The chemical shifts of ^{17}O were referenced to the naturally abundant $H_2{}^{17}O$ signal at 0 ppm. The ^{17}O-NMR spectra were accumulated from 500 k to 1,000 k scans, and FID data were treated upon multiplication by an exponential function with a 150 Hz line broadening factor. The protein or peptides was dissolved in 20 mM sodium phosphate buffer (pH 5.0) containing 10% (v/v) D_2O, and probe temperature was set at 20 °C during the NMR experiments. The Cu^{2+}-form of $^{17}O_2$-oxidized SOD1 was reduced to produce the Cu^{+}-form using 10 mM 2-ME or 10 mM isoascorbic acid. Under the reducing conditions, the blue color of the SOD1 solution gradually faded, and finally became colorless. The time course of the reduction by 2-ME was followed by observing the fingerprint His-region of ^{1}H-NMR spectra. A digested peptide form was prepared according to the above-mentioned peptide mapping procedures. ^{1}H- and ^{17}O-NMR spectra of the peptide form were collected before HPLC fractionation.

CD spectroscopy

$^{16}O_2$-oxidized SOD1 (116 μM) in 20 mM phosphate buffer (pH 5.0) was incubated alone or in the presence of 10 mM 2-ME for 16 h at room temperature and each sample was diluted with the corresponding buffer to 40 μM, as Cu^{2+},Zn^{2+}- and Cu^{+},Zn^{2+}-SOD1, respectively. CD spectra were collected at 25 °C using a 1 mm path length cuvette on a JASCO J-725 spectropolarimeter.

2. Peptide mapping and MS analysis of $^{17}O_2$-oxidized SOD1

After oxidation with $^{17}O_2$ gas, the oxidation state and ^{17}O-labeling ratio of $^{17}O_2$-oxidized SOD1 were confirmed by a previously reported procedure, using HPLC peptide-mapping analysis combined with ESI-TOF-MS analysis (Figure S1 and S2).[1] The $^{17}O_2$-oxidized SOD1 contains oxidized forms with Cys^{111}-SO_2H **2** and Cys^{111}-SO_3H **3**, as well as Cys^{111}-SH **1**. As described in experimental procedures section, the denatured $^{17}O_2$-oxidized SOD1 treated with iodoacetamide was submitted to limited proteolysis using lysylendopeptidase and was analyzed by HPLC (Figure S1b). The cocktail of ^{17}O-oxidized peptide fragments contained peptides **5** (Cys^{111}-SH), **6**

(Cys^{111}-SO_2H), and **7** (Cys^{111}-SO_3H). As a control experiment, WT-SOD1 **1** was also digested by the same procedure, and the HPLC profile is shown in Figure S1a. The HPLC profile was almost identical to a previous report.[1] Two new peaks assigned as peptide **6** and **7** were identified, and the molar ratio of peptides **5**, **6**, and **7**, estimated by the corresponding peak areas, were 63:16:21. The two new peaks were collected and analyzed by high-resolution ESI-TOF-MS (Figure S2). As shown in Figure S2-1, the MS spectrum of peptide **5** provided a *m/z* of 830.9206, which corresponds to the tetravalent molecular ion peak $[M+4H]^{4+}$ of peptide **5** (calcd 830.9252). Next, as shown in Figure S2-2, the MS profile of SO_2H-peptide **6** revealed *m/z* of 824.6656, which corresponds to the tetravalent molecular ion peak ($[M+4H]^{4+}$ (calcd 824.6673). The peak-distribution was simulated and the ^{17}O enrichment of peptide **6** was around 50%. Figure S2-3 shows the MS spectrum of RSO_3H-peptide **7**, giving a *m/z* of 828.6749. The value is in good agreement with the $[M+4H]^{4+}$ ion peak of peptide **7** (calcd 828.6661). The ^{17}O enrichment of peptide **7** was estimated to be 64%.

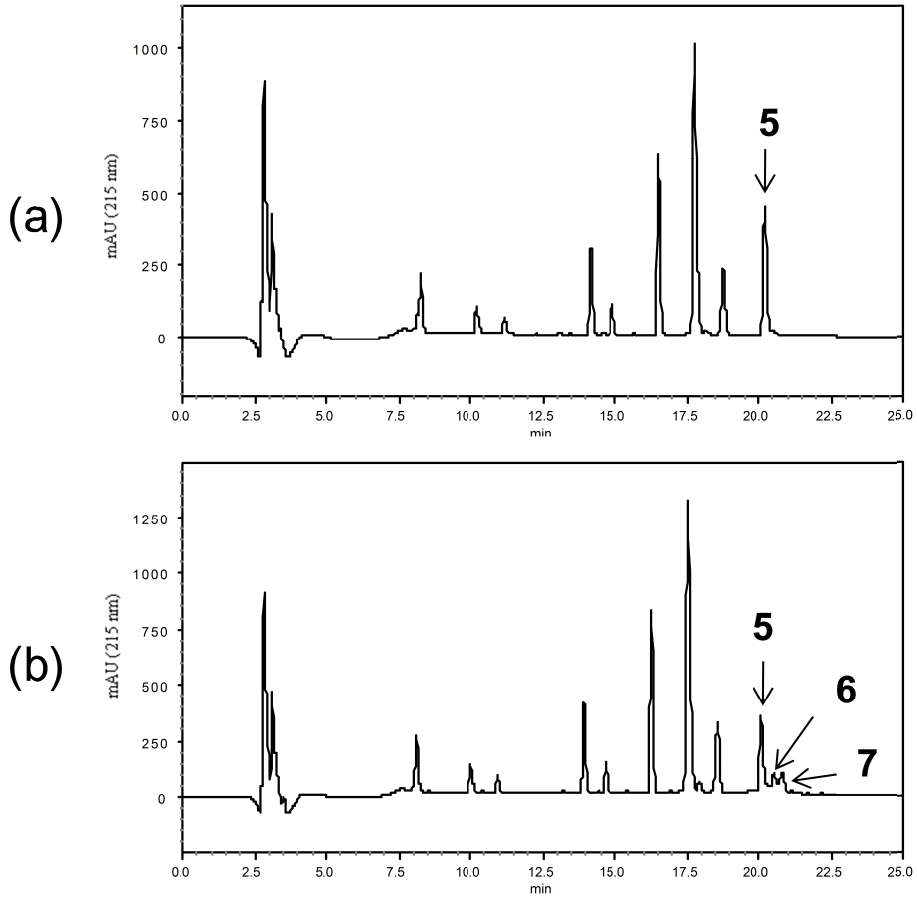
As described in the Experimental Section, 70% $^{17}O_2$ was employed for ^{17}O-labeling. However, the labeling rate of **6** and **7** did not reach 70%. The oxidation mechanism of SOD1 to form Cys^{111}-SO_2H and -SO_3H is not completely clear, but $H_2{}^{16}O$ in the buffer might be involved in the oxidative event by forming H-^{17}O-^{16}O-H at the superoxide degradation stage by SOD1 itself. Additionally, the ^{17}O-labeling ratios of SO_2H-peptide **6** and SO_3H-peptide **7** showed a significant difference (50% and 64%). The different oxidation ratio may suggest that some active species with varying oxidation potentials are playing a role.

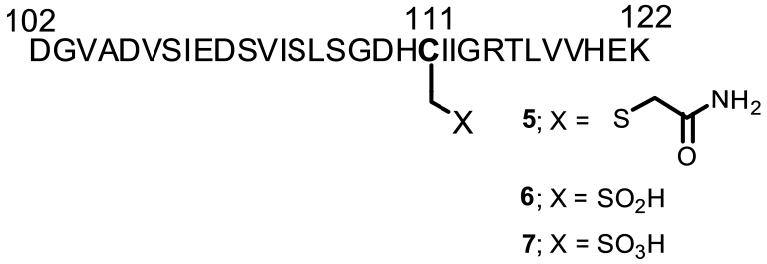
3. ^{1}H-NMR and CD spectra of $^{17}O_2$-oxidized SOD1 with Cu^{2+},Zn^{2+}- and Cu^{+},Zn^{2+}-states

^{1}H-NMR spectra as well as CD spectra of $^{17}O_2$-oxidized SOD1 with Cu^{2+} and Cu^{+} forms were measured (Figures S3, S4, and S5). In the superoxide degradation pathway of SOD1, reversible reduction/oxidation at the catalytic site Cu cation is essential. Overall protein structure was estimated by the patterns of the ^{1}H-NMR and CD spectra (Figure S3, S4 and S5). Local catalytic site structure in each Cu oxidation state was confirmed by the signal pattern on the imidazole NH protons of fingerprint His chelating the Zn and Cu cations (Figure S4).[4] The ^{1}H-NMR signal, especially at 8 to 9 ppm originating from amide protons, showed significant differences between the Cu^{2+}- and Cu^{+}-forms (Figure S3). The CD spectra also support the significant difference in secondary structure (Figure S5).

The Cu^{2+}-form provided few signals in the reporter His region (11-16 ppm) because of the potent paramagnetic effect of the divalent Cu cation (Figure S4a). The observable signals shown in Figure S4a, are assigned as His43 ε2[4], which is about 11 Å from the Cu^{2+} center, a distance usually long enough to eliminate paramagnetic effects.[5] The Cu^{2+} paramagnetic effect was diminished under reductive conditions by addition of either 10 mM isoascorbic acid (Fig S4b) or 2-ME (Fig. S4c). The time course of the reduction was monitored by ^{1}H-NMR focusing on the reporter His region in each case. The Cu^{2+} in SOD1 was gradually reduced at 5 °C to give complete conversion to the Cu^{+} form within 4 h (data not shown). ^{1}H-NMR of the Cu^{+} state is shown in Figure S4b, providing a set of sharp metal ion-coordinating His signals (His71 ε2; 15.4 ppm, His43 ε2; 14.0 ppm, His46 ε2; 13.4 ppm, His 43 δ1, 12.8 ppm, His80 ε2; 12.7 ppm, His63 ε2; 12.5 ppm, His48 δ1; 12.3 ppm). The assignment of the His signals was based on a previous report.[4] Each Cys^{111}-oxidation form of **2** and **3** were not distinguishable from the SH form **1** in the ^{1}H-NMR spectral data. It suggests that the local conformation of the catalytic-site is not significantly different in the three oxidation states. This data is compatible with the finding that $^{16}O_2$-oxidized SOD1 is as active as wild-type SOD1.[1]

4. Supporting figures





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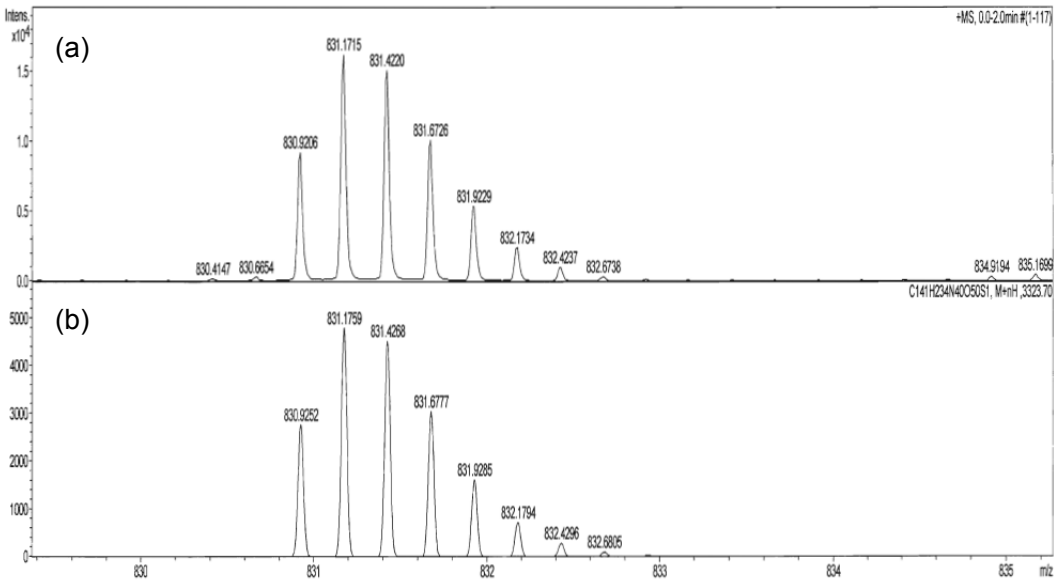

Figure S2-1.

ESI-TOF MS profile of peptide **5** having amidomethyl-S-modification. $[M+4H]^{4+}$ region was expanded. (a) The spectrum of peptide **5**; (b) MS profile simulation of **5**.

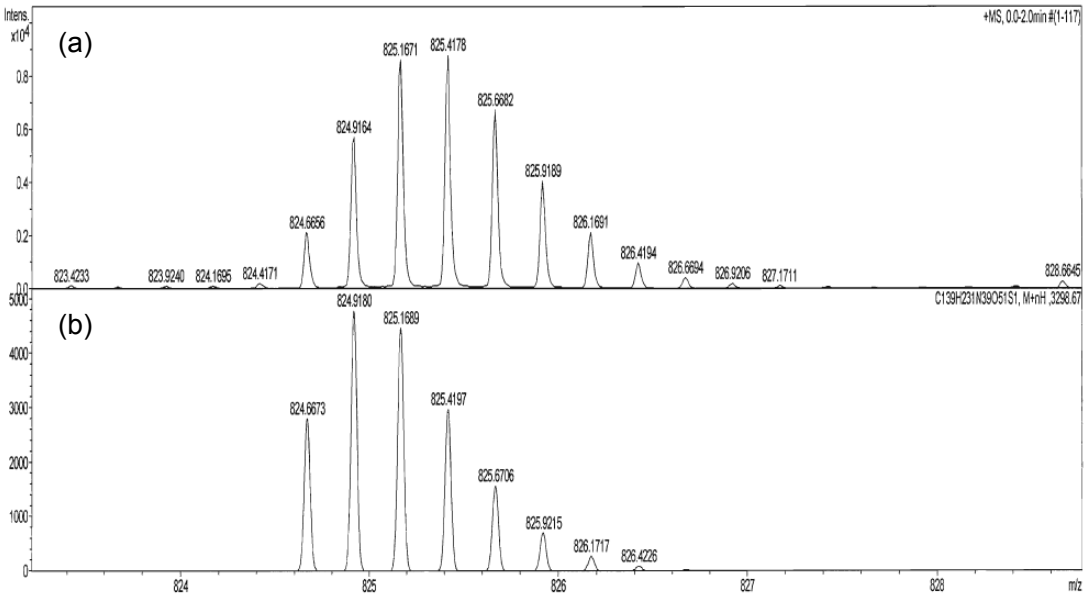

Figure S2-2.

ESI-TOF MS profile of peptide **6**. $[M+4H]^{4+}$ region was expanded. (a) The spectrum of peptide **6**; (b) MS profile simulation of **6**. The differential MS distribution pattern indicates the ^{17}O-labeling.

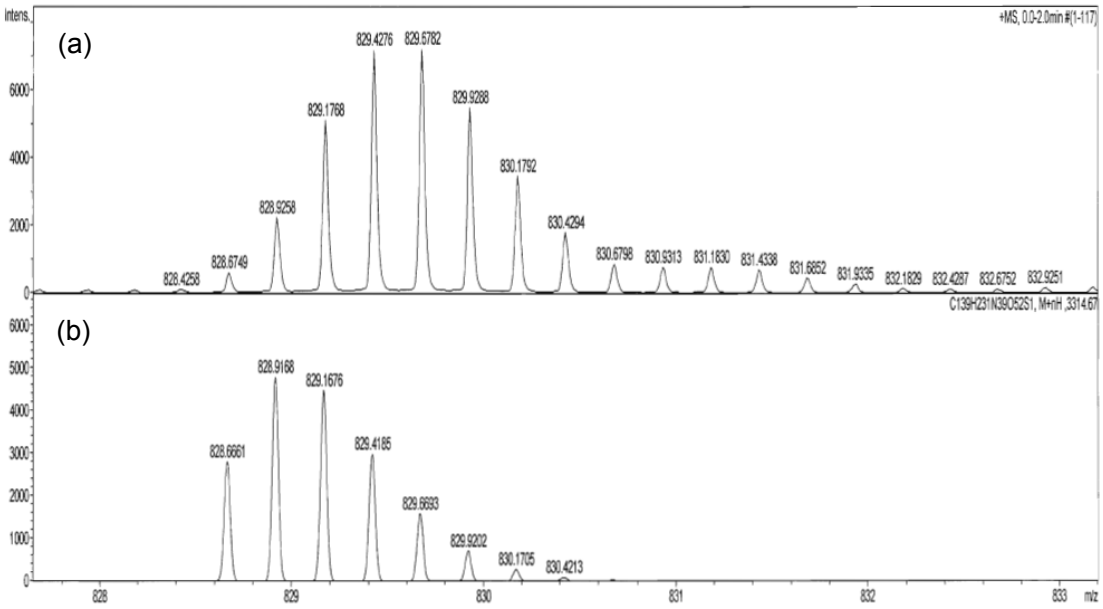

Figure S2-3.

ESI-TOF MS profile of peptide **7**. The $[M+4H]^{4+}$ region is expanded. (a) The spectrum of peptide **7**; (b) MS profile simulation of **7**. The differential MS distribution pattern indicates the ^{17}O-labeling. A contaminant in the S-alkylated form **5** was also found at *m/z* of 830.93.

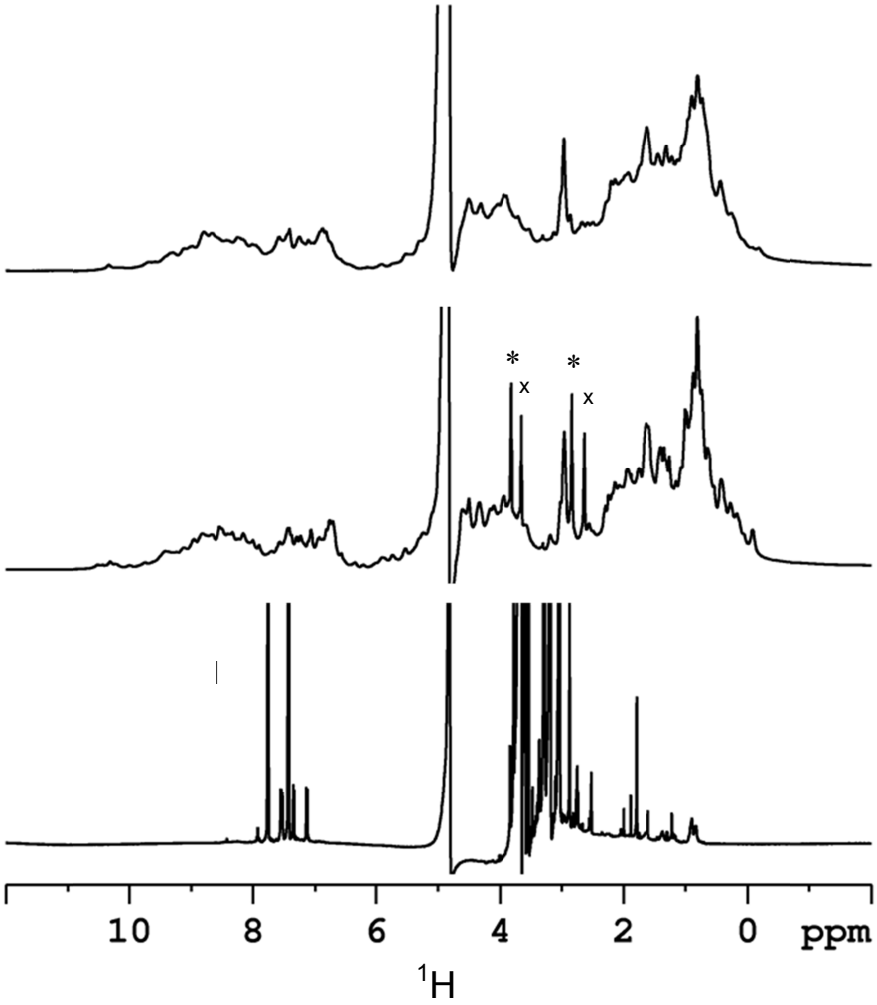

Figure S3.

^{1}H-NMR spectra of $^{17}O_2$-oxidized SOD1 in 20 mM phosphate buffer composed with 10%D_2O (v/v) with pH of 5.0. (a) Cu^{2+}-form, (b) Cu^{+}-form prepared by an addition of 10 mM 2-ME, (c) digested peptide form. *; originating from (HO-CH_2-CH_2-S)$_2$. ^{x}; originating from HO-CH_2-CH_2-SH.

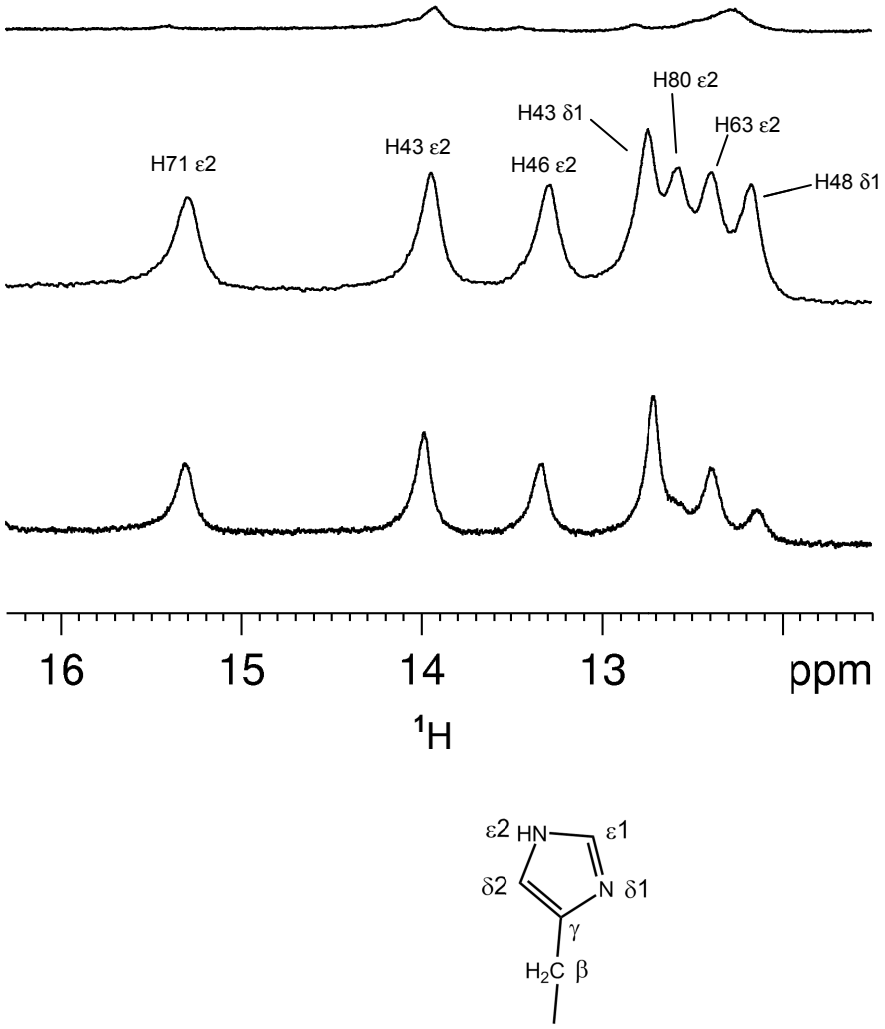

Figure S4.

^{1}H-NMR spectra of the fingerprint His regions of $^{17}O_2$-oxidized SOD1. (a) Cu^{2+}-form, (b) Cu^{+}-form prepared by addition of 10 mM isoascorbic acid, (c) Cu^{+}-form prepared by addition of 10 mM 2-ME (10 mM).

Figure S5.

CD spectra of Cu^{2+}-form (blue) and Cu^{+}-form (red), prepared by addition of 2-ME (10 mM), of $^{16}O_2$-oxidized SOD1

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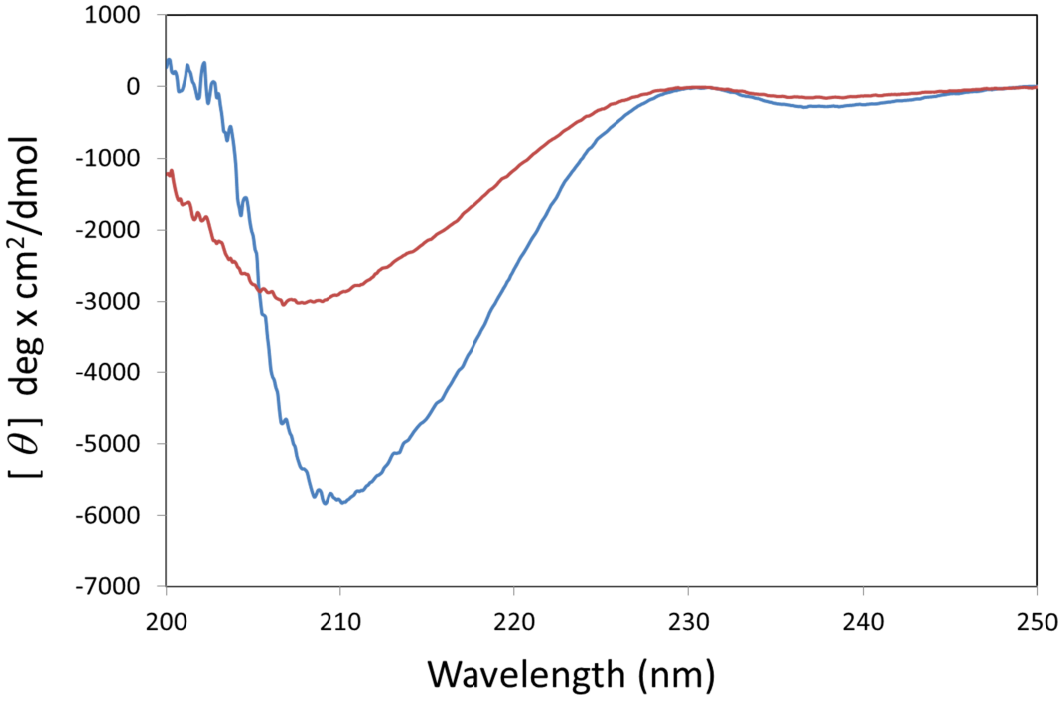

5. References

1. N. Fujiwara, M. Nakano, S. Kato, D. Yoshihara, T. Ookawara, H. Eguchi, N. Taniguchi and K. Suzuki, *J. Biol. Chem.*, 2007, **282**, 35933.
2. N. Fujiwara, Y. Miyamoto, K. Ogasahara, M. Takahashi, T. Ikegami, R. Takamiya, K. Suzuki and N. Taniguchi, *J. Biol. Chem.*, 2005, **280**, 5061.
3. S. L. Patt, *J. Magn. Reson.*, 1982, **49**, 161.
4. L. Banci, I. Bertini, F. Cantini, N. D'Amelio and E. Gaggelli, *J. Biol. Chem.*, 2006, **281**, 2333.
5. H. X. Deng, A. Hentati, J. A. Tainer, Z. Iqbal, A. Cayabyab, W. Y. Hung, E. D. Getzoff, P. Hu, B. Herzfeldt, R. P. Roos, C. Warner, G. Deng, E. Soriano, C. Smyth, H. E. Parge, A. Ahmed, A. D. Roses, R. A. Hallewell, M. A. Pericak-Vance and T. Siddique, *Science*, 1993, **261**, 1047.