Extensive Investigations on Oxidized Amino Acid Residues in H_2O_2-Treated Cu,Zn-SOD Protein with LC-ESI-Q-TOF-MS, MS/MS for the Determination of the Copper-Binding Site

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Abstract: The ESI (electrospray ionization)-Q-TOF (tandem quadrupole/orthogonal-acceleration time-of-flight) mass spectrometer combined with the nano-HPLC (high performance liquid chromatography) system was utilized to pinpoint the Cu-binding site in Cu,Zn-SOD (superoxide dismutase) protein. Cu,Zn-SOD was treated with hydrogen peroxide, intended to specifically oxidize histidine residues coordinated to the copper ion as a mass spectrometric probe. The oxidized Cu,Zn-SOD was then fragmented with the successive treatment of endoproteinase Asp-N and DTT (dithiothreitol). Separation of the peptide mixture with the nano-HPLC and the on-line ESI-Q-TOF MS analysis revealed that only two peptide fragments were oxidized to a significant extent. Further analyses of oxidized peptide fragments with LC-ESI-Q-TOF-MS/MS disclosed that three out of four Cu-coordinated histidine residues were specifically oxidized by action of a redox-active copper ion and hydrogen peroxide, demonstrating the copper-catalyzed oxidation of amino acid ligands could be a versatile tool for the mass spectrometric determination of the copper-binding site. In addition, proline and valine residues in the proximity of the Cu ion were found to be oxidized upon H_2O_2 treatment.

An ATPase called TIME-EA4 which was isolated from the diapausing eggs of the silkworm, *Bombyx mori*, as a key to the termination of embryonic diapause, has a closely similar amino acid sequence to that of Cu,Zn-SOD (superoxide dismutase).[1] Notably, amino acid residues constituting the Cu,Zn-binding site in Cu,Zn-SOD are exactly identical to those in TIME-EA4. Thus, a three-dimensional model of TIME-EA4 was constructed, with the Cu,Zn-binding site as a core, on the basis of the known crystallographic structure of Cu,Zn-SOD. Despite the similarity in the amino acid sequence, these two proteins have quite different functions. Cu,Zn-SOD catalyzes a dismutation of superoxide to dioxgen and hydrogen peroxide through an alternate reduction and oxidation of the active-site copper ion. TIME-EA4, on the other hand, is seemingly not involved in such redox reactions. Instead, TIME-EA4 was reported to exhibit a transitory ATPase activity in coincidence with the completion of the diapause development, probably because of a continuous conformational change.[2] Considering the completely different function of these two proteins, the homology-based tertiary structure of TIME-EA4 seems insecure and requires further experimental supports.

To give an experimental foundation to the proposed three-dimensional structure of TIME-EA4, we have initiated the project to pinpoint the metal-binding site in TIME-EA4. A possible dynamic nature of its metal-binding site renders it difficult to utilize a common methodology such as X-ray crystallography. We have introduced the ESI (electrospray ionization)-Q-TOF (tandem quadrupole/orthogonal-acceleration time-of-flight)[3] mass spectrometer combined with the appropriately adjusted nano-HPLC system equipped with a column (internal diameter; 0.3 mm × 150 mm) to accurately determine the position of introduced probes in proteins. The hybrid type mass spectrometer with quadrupole/time-of-flight having orthogonal acceleration after the collision chamber can perform with high resolution (<0.1 Da) and high sensitivity (<10 pmol) compared with the traditional MS/MS equipped with the EB-configuration (including electric and magnetic sectors) conducted with many slits. The nonsplitting nano-HPLC system, which was carefully constructed to minimize dead volume to the nL level, purifies and concentrates a small amount of a crude sample in a buffered solution to the optimum conditions for direct introduction into the ESI source. For determining the Cu-binding site, a probe could be attached by "specific" oxidation of histidine residues coordinated to a redox-active copper ion. However, it is not clear at present whether amino acid residues near the Cu-binding site are specifically oxidized and the other amino acids are not affected to any significant extent under certain oxidation conditions. To establish the methodology to pinpoint oxidized amino acids and optimize oxidation conditions for the specific oxidation of amino acid residues in the close proximity to the Cu-binding site, we utilized Cu,Zn-SOD as a structurally well-defined model protein instead of a target TIME-EA4 protein. It was already reported that, in the case of Cu,-Zn-SOD, the Cu-coordinated His118 is oxidized to oxo-histidine upon treatment with hydrogen peroxide, but it was not clarified whether the other Cu-coordinated histidine residues are oxidized in the same manner.[4a]

Identification of oxidized amino acids in oxidatively damaged proteins usually begins with chromatographic analyses of tryptic

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maps of native and oxidized proteins.[4] New peaks which become visible after oxidative treatment are isolated and analyzed with an amino acid analyzer or a mass spectrometer. Experiments to determine the oxidized position by such a method were time- and sample-consuming, and the obtained results were sometimes not conclusive, partly because of several unknown oxidative reactions. Recently, tandem mass spectrometry and MALDI (matrix-assisted laser desorption ionization)-PSD (post source decay) spectrometry have been applied to identify a chemically modified amino acid in proteins labeled with an exogenous chemical group.[5] Protein oxidation has also been investigated with MS/MS spectrometry.[6] An oxidatively damaged protein is surely one of the most challenging targets for analysis at the amino acid level, because quite a few amino acids such as methionine, cysteine, histidine, and tryptophan are susceptible to oxidation, and then oxidized amino acid residues could be distributed in various forms all over the oxidized protein.

We described herein the use of LC-ESI-Q-TOF-MS, MS/MS for analysis of H_2O_2-treated Cu,Zn-SOD. The on-line mass spectrometric analysis has made it possible to minimize sample loss and evaluate the oxidation yield for each digested peptide fragment in a semiquantitative manner, assuming similar ionization efficiencies of intact and oxygen-attached peptides. Accuracy of Q-TOF has enabled detailed discussion on structures of oxidized peptides. We found out that (1) oxidative damage of amino acid residues was restricted within the proximity of the Cu-binding site well enough to determine the metal center, although oxidation yield for four Cu-coordinated histidine residues was distinctively different depending on the coordination environment, (2) proline and valine residues near the Cu-binding site were unexpectedly oxidized upon H_2O_2-treatment, and (3) a dioxygenized histidine which was supposed to be a histidine hydroperoxide was formed selectively at position 46. Full details were disclosed in the following sections.

Results

Analysis of H_2O_2-Treated Cu,Zn-SOD. To investigate all the possible chemical changes in Cu,Zn-SOD upon H_2O_2 treatment, oxidized Cu,Zn-SOD was analyzed without enzymatic digestion. Then, Cu,Zn-SOD was incubated in phosphate buffer at pH 7.2 at 37 °C for 30 min at various concentration of H_2O_2 (0.05, 0.5, 5, and 50 mM). The solution was frozen just after 30 min and then lyophilized. After removing a trace of hydrogen peroxide by repeated lyophilization, the obtained reaction mixture (~60 pmol of the starting Cu,Zn-SOD) was analyzed with LC-ESI-Q-TOF-MS without any off-line purification operation. Shown in Figure 1 are HPLC profiles of native and oxidized Cu,Zn-SODs. As the concentration of hydrogen peroxide was increased, the peak corresponding to Cu,Zn-SOD became broadened, suggesting that several oxidative products were formed.

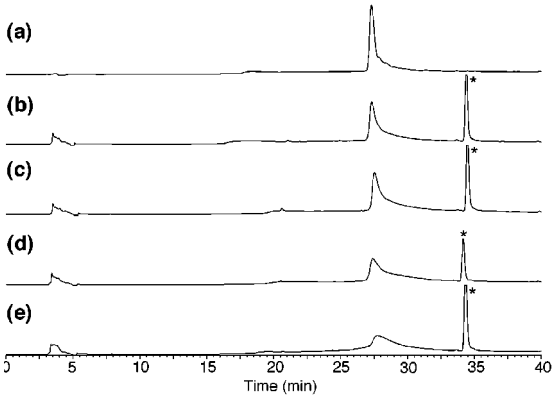

Figure 1. Develosil ODS-HG-5 chromatographic analyses of SOD: (a) native, (b) oxidized at 0.05 mM H_2O_2, (c) oxidized at 0.5 mM H_2O_2, (d) oxidized at 5 mM H_2O_2, and (e) oxidized at 50 mM H_2O_2, for 30 min. The column effluent was monitored at 210 nm; * indicates an unidentified peak.

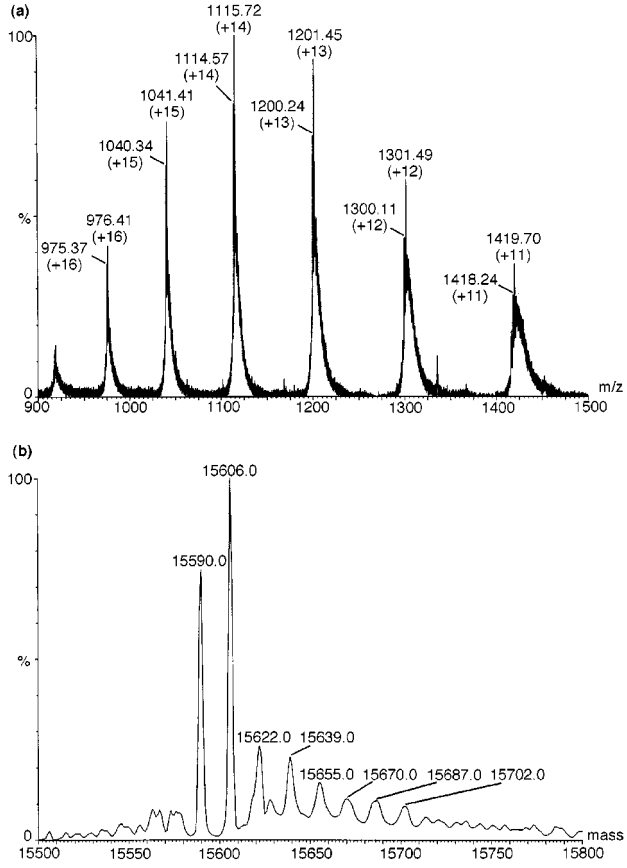

Figure 2. (a) Raw electrospray data of the H_2O_2-treated (5 mM) SOD which was eluted around 28 min as shown in Figure 1d. Shown in parentheses is the charge state of the ion peak. (b) Mass spectrum produced by MaxEnt1 processing of the raw electrospray data.

A representative on-line electrospray mass spectrum of the broadened peak (oxidized Cu,Zn-SOD with 5 mM of H_2O_2) is shown in Figure 2a. Typical molecular ion envelopes consisting of various charge states were observed. MaxEnt1 processing[7] of the raw electrospray data produced the mass of the species

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(6) Schey, K. L.; Finley, E. L. *Acc. Chem. Res.* **2000**, *33*, 299−306 and references therein.

(7) MaxEnt1, supplied from Micromass (Manchester, UK), is a computer algorithm to produce true molecular mass spectra from multiply charged electrospray spectra.

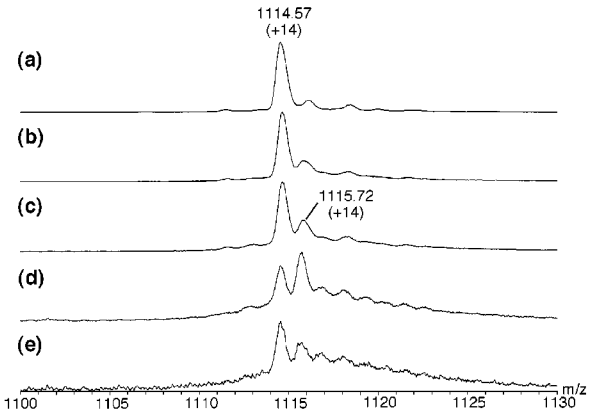

Figure 3. Electrospray mass spectrometry of SOD: (a) native, (b) oxidized at 0.05 mM H_2O_2, (c) oxidized at 0.5 mM H_2O_2, (d) oxidized at 5 mM H_2O_2, and (e) oxidized at 50 mM H_2O_2, for 30 min. Shown in parentheses is the charge state of the ion peak.

contained in the broadened peak (Figure 2b). The major species in the oxidized sample had masses of 15 590 and 15 606, corresponding to the apo-SOD and the mono-oxygen adduct of apo-SOD, respectively (theoretical average mass of apo-SOD is 15 591). The double- and triple-oxygen adducts seemed to be formed as minor products. It was noteworthy that formation of other degradation products was not significant.

Although a major oxidized product after H_2O_2 treatment was a simple mono-oxygen adduct of Cu,Zn-SOD, it seemed to contain various forms of oxidized proteins as deduced from the broadened chromatographic trace.

To find out the optimum oxidation conditions, H_2O_2-concentration-dependence and time-dependence were monitored for the formation of oxygen adducts of SOD. Shown in Figure 3 are electrospray mass spectra of native and oxidized Cu,Zn-SOD treated at different concentration of H_2O_2. As the concentration of H_2O_2 was increased from 0.05 to 5 mM, the ratio of the oxidized SOD was gradually increased. When Cu,-Zn-SOD was oxidized at 50 mM of H_2O_2, the ratio of the oxidized SOD was rather decreased, suggesting that some undesirable oxidative fragmentation reaction occurred. On the other hand, oxidation of Cu,Zn-SOD did not depend on the incubation time, because the ratio of the oxidized to the intact SOD was unchanged when Cu,Zn-SOD was incubated at 5 mM of H_2O_2 for 15 min to 6 h (data not shown). To obtain specifically oxidized Cu,Zn-SOD in the highest yield without undesirable secondary oxidation, the oxidation reaction was done by incubating Cu,Zn-SOD at 5 mM of H_2O_2 for 30 min in the following experiments.

Identification of Oxidized Peptide Fragments. The oxidized Cu,Zn-SOD was fragmented both enzymatically and chemically to a mixture of peptides of an appropriate size. Usual trypsin digestion was not suitable for LC-ESI-Q-TOF-MS analysis, because not all the fragments were observed. In the case of Cu,-Zn-SOD, endoproteinase Asp-N turned out to be an enzyme of choice. Endoproteinase Asp-N digestion of Cu,Zn-SOD and subsequent reductive cleavage of the disulfide bond with DTT (dithiothreitol) yielded all the expected peptide fragments (Figure 4). To avoid loss of any peptide, the obtained peptide mixture was subjected to LC-ESI-Q-TOF-MS analysis without any off-line purification. Shown in Figure 5 are chromatographic analyses of peptide mixtures from native and oxidized SOD, as well as the on-line ESI-Q-TOF-MS assignments of 12 peptide fragments.[8] The assignment of each peptide fragment was further confirmed with LC-ESI-Q-TOF-MS/MS. Now that all the peptide fragments from Cu,Zn-SOD were characterized with

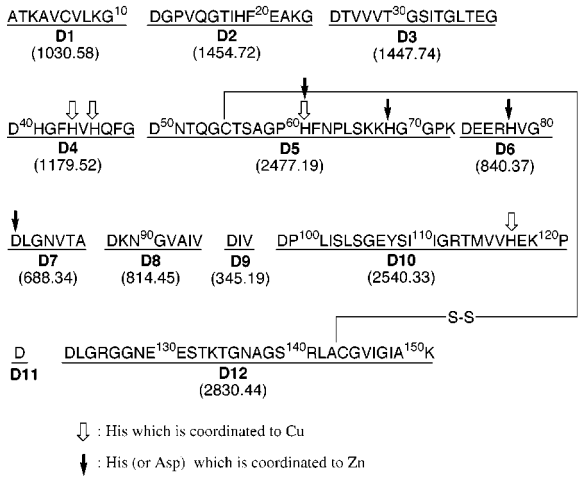

Figure 4. Primary structure of Cu,Zn-SOD and assignments of endoproteinase Asp-N-digested fragments. Shown in parentheses is the monoisotopic mass of each fragment. Monoisotopic mass of D5 and D12 was calculated after reductive cleavage of the Cys55-Cys144 disulfide bond.

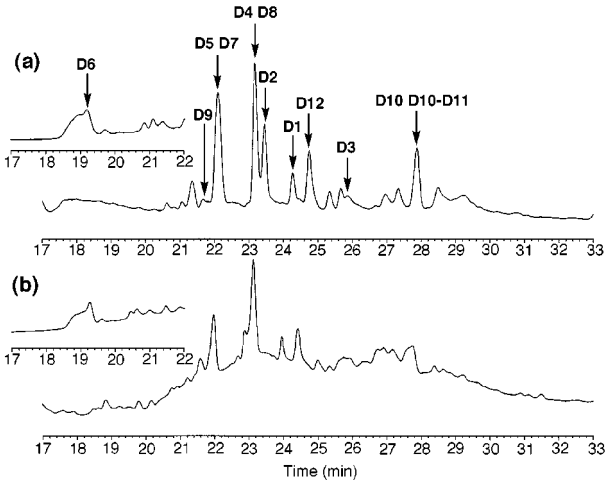

Figure 5. Develosil ODS-HG-5 chromatographic analyses of the peptide mixture from (a) Cu,Zn-SOD and (b) oxidized SOD successively treated with endoproteinase Asp-N and DTT. Insets: Develosil C30-UG-5 chromatographic analysis of the peptide mixture from (a) Cu,Zn-SOD and (b) oxidized Cu,Zn-SOD successively treated with endoproteinase Asp-N. The column effluent was monitored at 210 nm.

accuracies less than 0.1 Da using LC-ESI-Q-TOF-MS/MS, any modification in any position could be pinpointed.

As already mentioned, the mono-oxygen adduct was found to be a major product, as shown in Figure 2. The next thing to do was to identify all the singly oxidized peptide fragments and evaluate their formation in a semiquantitative manner. Then, we surveyed all the possible oxidized peptides on the total ion chromatogram. Shown in Figure 6 are ion chromatograms for molecular ions of native and oxidized peptides, which contain Cu- or Zn-coordinated histidine residues (for other peptide fragments, see Supporting Information). The oxidized peptides, in which one oxygen atom was added to the native peptide, were observed only for D4, D5, and D10. Almost no mono-oxygen adduct was detected for other peptide fragments, including those peptides containing the Zn-coordinated histidine residues, although they are in the proximity of the active site. The D10 + 16 fragment consisted of at least two components

(8) The hydrophilic D6 fragment eluted with the solvent front when analyzed with the Develosil ODS-HG-5 column. This fragment was detected using the Develosil C30-UG-5 column.

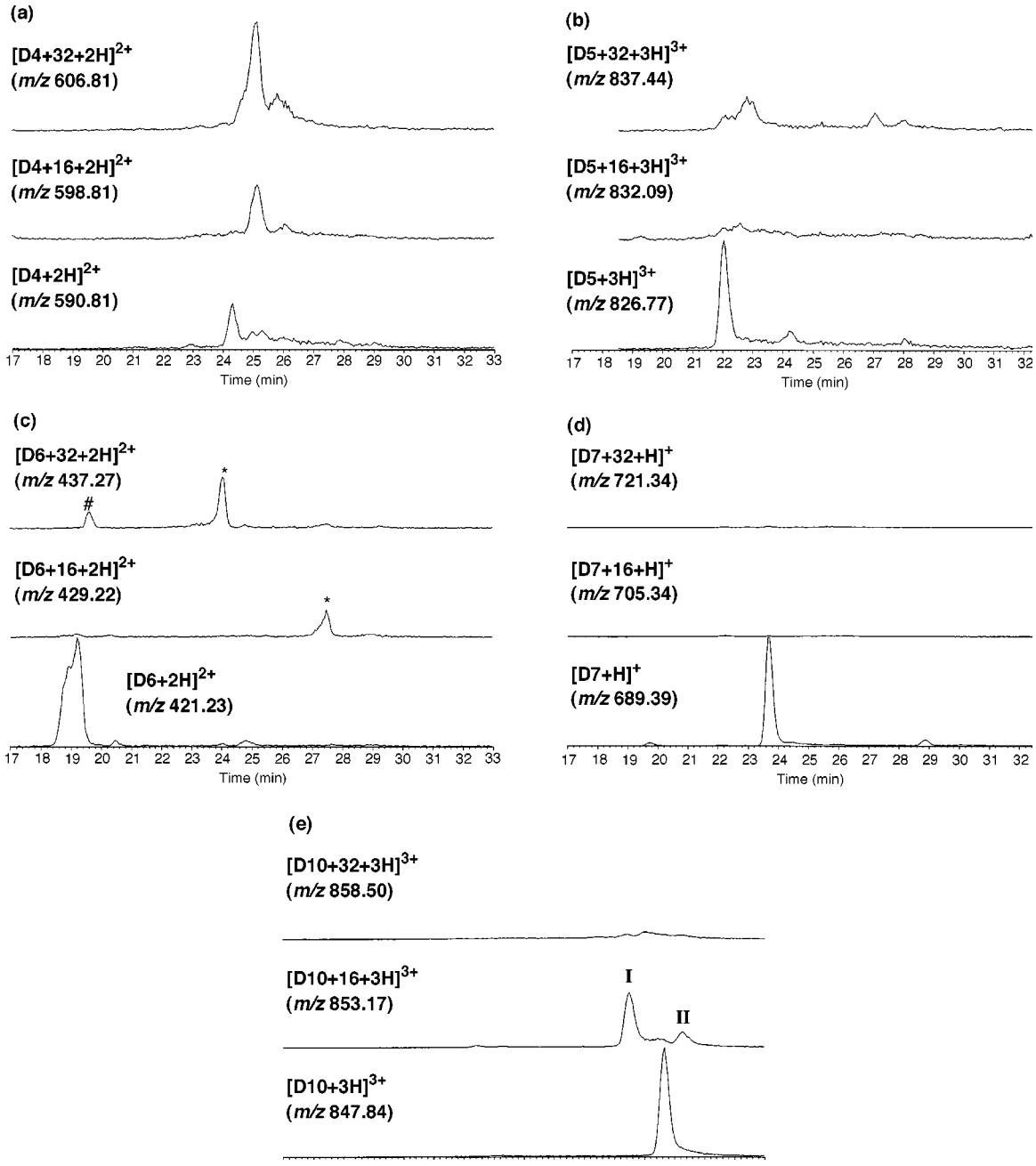

Figure 6. Ion chromatograms of (a) D4, D4 + 16, D4 + 32; (b) D5, D5 + 16, D5 + 32; (c) D6, D6 + 16, D6 + 32; (d) D7, D7 + 16, D7 + 32; and (e) D10, D10 + 16, D10 + 32. All of the ion chromatograms are plotted on the same scale for each peptide and its products; * indicates an unidentified peptide of which monoisotopic mass differed more than 1 Da from the expected mass, and # indicates an unidentified peptide of which monoisotopic mass was exactly as expected, but the ESI-Q-TOF-MS/MS data suggested that this peptide was utterly unrelated to the D6 fragment (data not shown).

(peaks I and II). The D5 + 16 fragment and peak I of the D10 + 16 fragment were observed without H_2O_2 treatment, probably because of air oxidation during the enzymatic digestion. The doubly oxidized peptides, in which two oxygen atoms were attached to the native peptide, were substantially formed for the D1, D4, D5, and D12 fragments, and in the case of the D1 fragment, a triply oxidized peptide was also detected, although doubly and triply oxidized peptides were rather minor products, as shown in Figure 2. The D4 + 32 and D5 + 32 fragments seemed to contain more than one product. The first peak of the D5 + 32 and the D12 + 32 peptide was observed without H_2O_2 treatment. It should be noted that the peptide fragments susceptible to air oxidation (D5, D10, and D12) contain cysteine or methionine residues, but the other peptides do not.[9]

Identification of Oxidized Amino Acids. Utilizing LC-ESI-Q-TOF-MS/MS, we attempted to pinpoint specifically oxidized amino acids in the oxidized peptide fragments. First, the D4 and the D4 + 16 fragments were analyzed with LC-ESI-Q-TOF-MS/MS, as shown in Figure 7a and b, respectively. The b fragment ion series was observed relatively well, compared to the y series. The b_2 (*m*/*z* 253.09), b_3 (*m*/*z* 310.12), and b_4 (*m*/*z* 457.18) ions appeared in both D4 and D4 + 16, and no fragment ions shifted by 15.99 Da above the predicted masses were observed for D4 + 16. A fragment ion observed at *m*/*z* 473.77,

(9) The D5 and the D12 fragments were analyzed after reductive cleavage of the disulfide bond with DTT. A possible oxidized D5 and D12 fragment would be stable to the reaction condition, because treatment of the peptide mixture from oxidized Cu,Zn-SOD with DTT did not affect the D10 + 16, D4 + 16, and D4 + 32 fragments at all.

D4

116.03	**253.09**	**310.12**	**457.18**	**594.24**	**693.31**	**830.37**	**958.43**	**553.25**	-
b_1	b_2	b_3	b_4	b_5	b_6	b_7	b_8	b_9	-
Asp	His	Gly	Phe	His	Val	His	Gln	Phe	Gly
-	y''_9	y''_8	y''_7	y''_6	y''_5	y''_4	y''_3	y''_2	y''_1
-	**533.25**	**928.44**	**871.42**	**724.35**	**587.29**	**488.23**	**351.17**	**223.11**	76.04

D4+16

				610.24	**709.31**	**846.36**	**974.42**	**561.25**	-
116.03	**253.09**	**310.12**	**457.18**	**594.24**	**693.31**				
b_1	b_2	b_3	b_4	b_5	b_6	b_7	b_8	b_9	-
Asp	His	Gly	Phe	**His+16**	Val	**His+16**	Gln	Phe	Gly
-	y''_9	y''_8	y''_7	y''_6	y''_5	y''_4	y''_3	y''_2	y''_1
					587.29	488.23	**351.17**	**223.11**	76.04
-	**541.25**	**944.44**	**887.42**	**740.35**	603.29	**504.22**			

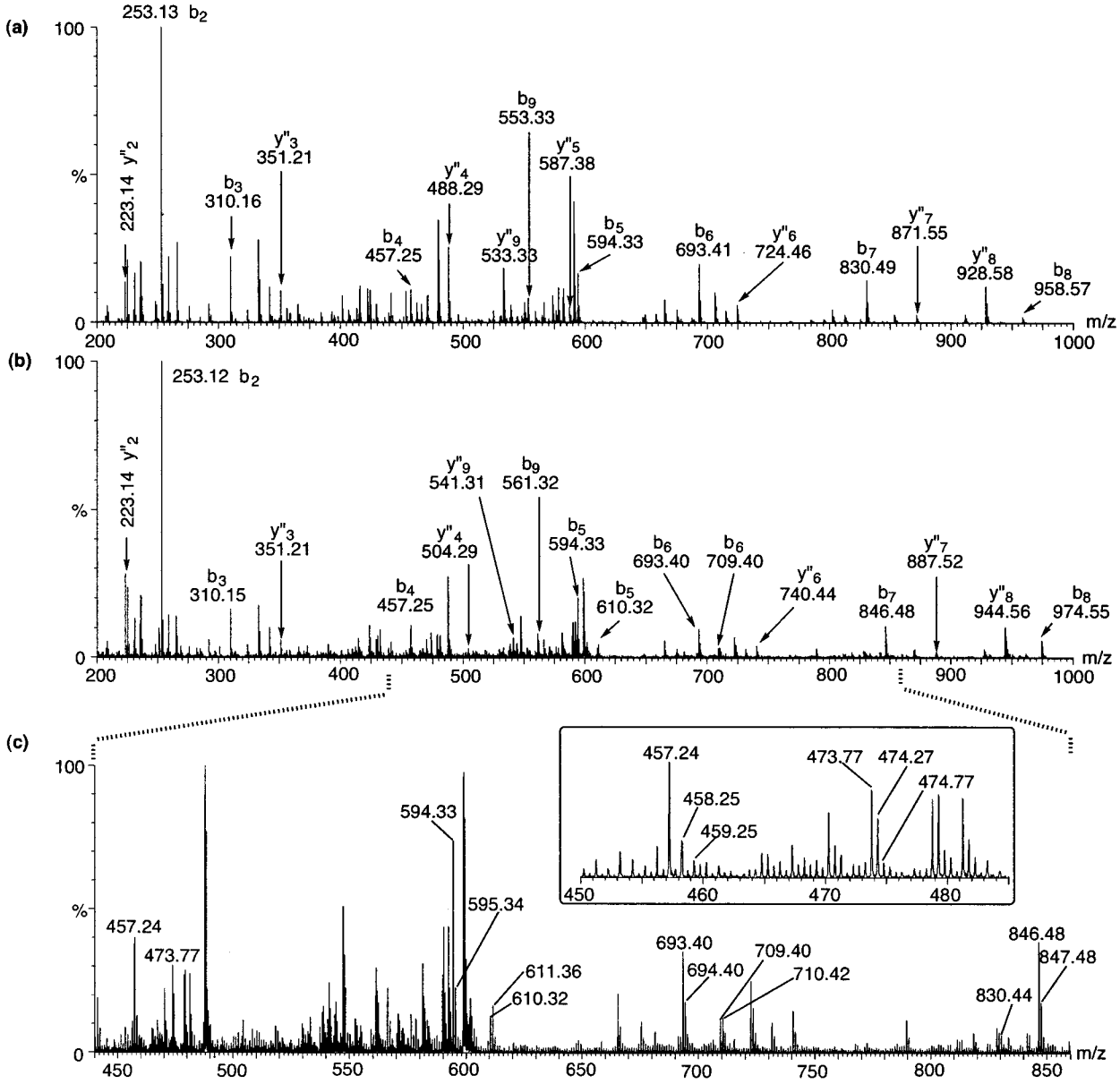

Figure 7. ESI-Q-TOF-MS/MS spectra of (a) the D4 fragment (*m*/*z* 590.80, in the doubly charged state) and (b) the D4 + 16 fragment (*m*/*z* 598.83, in the doubly charged state). (c) Expansion of the designated region in (b). Inset: Expansion of the region from *m*/*z* 450 to 485 in (c). Observed b- and y″-fragments are shown in the spectra. In the peptide sequences are shown the theoretical values of b- and y″-fragments. Observed ions are shown in bold. Doubly charged ions are assigned as b_9 and y''_9.

roughly corresponding to $b_4 + 16$, might be misleading without a Q-TOF-MS/MS. However, as clearly seen in the expansion of the region from *m*/*z* 450 to 485 (Figure 7c, inset), this fragment ion was in the doubly charged state and therefore had

D4+32

116.03	**253.09**	**310.12**	**457.18**	**594.24**	**693.31**	**862.36**	**990.42**	**569.25**	-
b_1	b_2	b_3	b_4	b_5	b_6	b_7	b_8	b_9	-
Asp	His	Gly	Phe	His	Val	**His+32**	Gln	Phe	Gly
-	y''_9	y''_8	y''_7	y''_6	y''_5	y''_4	y''_3	y''_2	y''_1
-	**549.25**	**960.43**	903.41	756.34	619.28	520.22	**351.17**	**223.11**	76.04

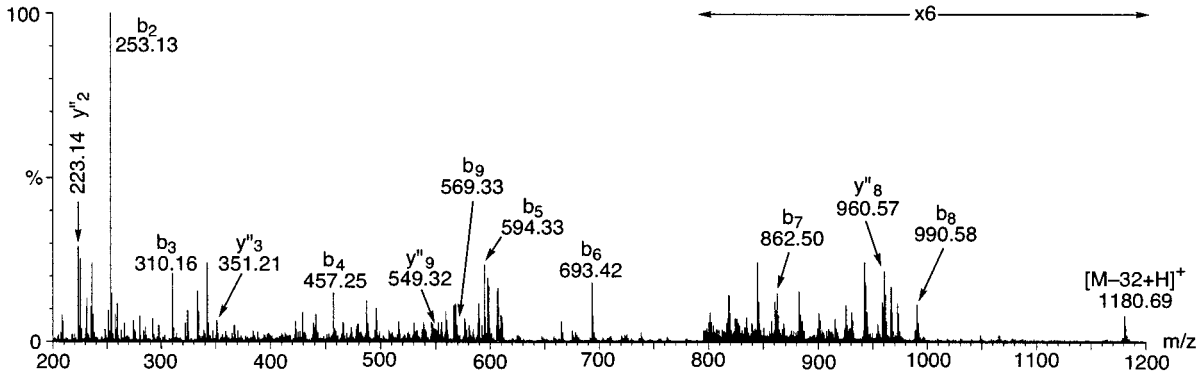

Figure 8. ESI-Q-TOF-MS/MS spectrum of the D4 + 32 fragment (*m*/*z* 606.82, in the doubly charged state). Observed b- and y″-fragments are shown in the spectrum. Shown in the peptide sequence are the theoretical values of b- and y″-fragments. Observed ions are shown in bold. Doubly charged ions are assigned as b_9 and y''_9.

nothing to do with b_4 + 16. The b_5 ion (fragmentation on the C-terminal side of His44), on the other hand, split into the native b_5 (*m*/*z* 594.33) and the b_5 + 16 fragments (*m*/*z* 610.32), as shown in Figure 7c. The following b_6 fragment ion exhibited the same heterogeneity (*m*/*z* 693.40 and 709.40), but the b_7 fragment (fragmentation on the C-terminal side of His46) was observed exclusively as a fragment ion shifted by 15.99 Da (*m*/*z* 846.48). The subsequent b_8 and b_9 ions were also shifted by 15.99 Da above the predicted masses. Analysis of the b fragment ion series clearly indicated that the D4 + 16 peptide was a mixture of mono-oxygen adducts in which His44 or His46 was specifically oxidized.

The D4 fragment was the only peptide to which two oxygen atoms were significantly attached upon H_2O_2 treatment, although the double-oxygen adduct was a rather minor product (Figure 2). As seen in Figure 6a, D4 + 32 was eluted as two peaks. However, the structure of the minor D4 + 32 could not be analyzed unambiguously because of incomplete separation. We then investigated the major D4 + 32 peptide with LC-ESI-Q-TOF-MS/MS (Figure 8). The b fragment ion series was analyzed in the same manner. In the case of the D4 + 32 fragment, not only the b_2, b_3, and b_4 ions but also the b_5 and b_6 ions were observed exclusively at the predicted *m*/*z* values. In contrast to the D4 + 16 fragment, neither b_5 + 16 nor b_6 + 16 ions were observed at all. Instead, the b_7 ion was found to be shifted by 31.99 Da (*m*/*z* 862.50), indicating double oxidation of His46. The following b_8 and b_9 ions were also observed at *m*/*z* values shifted by 31.99 Da. Interestingly, a fragment ion corresponding to the D4 + 32 minus 31.99 was observed at *m*/*z* 1180.69 in the singly charged state, indicating the oxygen−oxygen bond in the oxidized D4 peptide. Although a 4- or 5-hydroxy-2-imidazolone product was already proposed as a double-oxygen adduct of histidine,[6,10] a histidine hydroperoxide product seemed more likely in this case because of a fragment ion at *m*/*z* 1180.69. The formation of amino acid and protein peroxides has been studied using an iodometric assay so far. The fragment ion shifted by 31.99 Da below the parent ion could be another diagnosis for the formation of peroxides. It is interesting to note that histidine was reported to be not susceptible to peroxidation.[11]

$$\underbrace{DP^{100}LISLSGEYSI^{110}IGR}_{\textbf{D10T1}\ (1618.86)} \quad \underbrace{TMVVHEK^{120}P}_{\textbf{D10T2}\ (939.48)}$$

Figure 9. Assignments of tryptic D10. Shown in parentheses is the monoisotopic mass of each fragment.

The D10 + 16 fragment was subjected to the LC-ESI-Q-TOF-MS/MS analysis likewise, but the length of the D10 fragment consisting of 23 amino acids prevented us from obtaining a clear-cut MS/MS spectrum. Thus, the D10 peptide was further digested into two peptide fragments D10T1 and D10T2 at the C-terminal side of Arg113 by trypsin (Figure 9). To minimize sample loss, the D10 peptide was digested without isolation as a peptide mixture from the endoproteinase Asp-N digestion. The obtained peptide mixture was directly analyzed with LC-ESI-Q-TOF-MS without any off-line purification. As shown in Figure 10, both D10T1 and D10T2 were clearly detected in the doubly charged state. Amino acid sequences of D10T1 and D10T2 were confirmed with LC-ESI-Q-TOF-MS/MS. We would like to point out that exactly the same amount of the starting Cu,Zn-SOD was required for analyses of D10T1 and D10T2, despite the additional trypsin digestion. Ion chromatograms in Figure 10 showed that D10T1 was intact upon H_2O_2 treatment, indicating that oxidation occurred specifically at the D10T2 part. In fact, two peaks, I′ and II′, corresponding to the molecular ion of D10T2 + 16 were detected on the total ion chromatogram. Considering the intensity and the retention time, peaks I′ and II′ should correspond to peaks I and II observed for D10 + 16, respectively.

Peaks I′ and II′ were analyzed with LC-ESI-Q-TOF-MS/MS and were extensively compared with the MS/MS spectrum of the intact D10T2 fragment (Figure 11). Shown in Figure 11a and b are the ESI-Q-TOF-MS/MS spectra of the intact D10T2 fragment and major peak I′, respectively. The N-terminus-containing b_2 − b_7 were all shifted by 15.99 Da above the intact

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fragment ions. The C-terminus-containing $y''_1 - y''_6$ ions, on the other hand, remained intact. The y''_7 ion coming from fragmentation between Thr114 and Met115 was found to be shifted by 15.99 Da. Then, methionine was identified to be oxidized to methionine sulfoxide in the major D10T2 + 16 fragment (peak I′), namely the major D10 + 16 fragment (peak I). The formation of methionine sulfoxide at position 115 was further confirmed by the fragment ion peaks at *m*/*z* 185.09, 284.15, 383.21, 520.27, 649.31, 777.41, and 791.41, which were 64.00 Da lower than those of $b_2 - b_7$ and y''_7 ions, respectively. It was reported that the mass difference of 64.00 Da is due to the loss of sulfinic acid (CH_3SOH) from methionine sulfoxide and is diagnostic for the formation of methionine sulfoxide.[12] It is interesting to note that among the y″ fragment ion series, only the y''_7 ion accompanied the fragment ion coming from loss of sulfinic acid, which is in complete agreement with Met115 oxidation.

D10T2

102.06	233.10	332.16	431.23	568.29	697.33	825.43	-
b_1	b_2	b_3	b_4	b_5	b_6	b_7	-
Thr	Met	Val	Val	His	Glu	Lys	Pro
-	y''_7	y''_6	y''_5	y''_4	y''_3	y''_2	y''_1
-	839.44	708.40	609.34	510.27	373.21	244.17	116.07

D10T2+16 (peak I')

102.06	249.09	348.16	447.23	584.29	713.33	841.42	-
b_1	b_2	b_3	b_4	b_5	b_6	b_7	-
Thr	Met+16	Val	Val	His	Glu	Lys	Pro
-	y''_7	y''_6	y''_5	y''_4	y''_3	y''_2	y''_1
-	855.44	708.40	609.34	510.27	373.21	244.17	116.07

D10T2+16 (peak II')

		348.16	447.23	584.29	713.33	841.42	-
102.06	233.10	332.16	431.23				
b_1	b_2	b_3	b_4	b_5	b_6	b_7	-
Thr	Met	Val+16	Val+16	His+16	Glu	Lys	Pro
-	y''_7	y''_6	y''_5	y''_4	y''_3	y''_2	y''_1
			609.34	510.27	373.21	244.17	116.07
-	855.44	724.40	625.33	526.26			

Minor peak II′ was analyzed with LC-ESI-Q-TOF-MS/MS (Figure 11c). In the case of peptides from peak II′, the y″ fragment ion series was observed more clearly. The y''_1, y''_2, and y''_3 ions appeared at the predicted *m*/*z* values. The y''_4 ion (fragmentation on the N-terminal side of His118) was shifted by 15.99 Da, indicating specific oxidation at His118, as already reported.[4a] Unexpectedly, the y''_4 ion also remained intact at *m*/*z* 510.25, suggesting that peak II′ did not contain only the His118-oxidized peptide. The following y''_5 ion was also observed as both intact and oxidized (*m*/*z* 609.33 and 625.32, respectively). As clearly seen from Figure 11d, the ratio of the oxidized to the intact ion was larger for the y''_5 than the y''_4 ion. The y''_6 ion, corresponding to the fragmentation between Met115 and Val116, was observed exclusively as a fragment ion shifted by 15.99 Da (*m*/*z* 724.38). The most probable explanation for this is that an oxygen atom was attached to Val116 and Val117 by action of a Cu ion and hydrogen peroxide. It is noteworthy that valine residues, which are considered to be inert for such oxidation conditions, were found

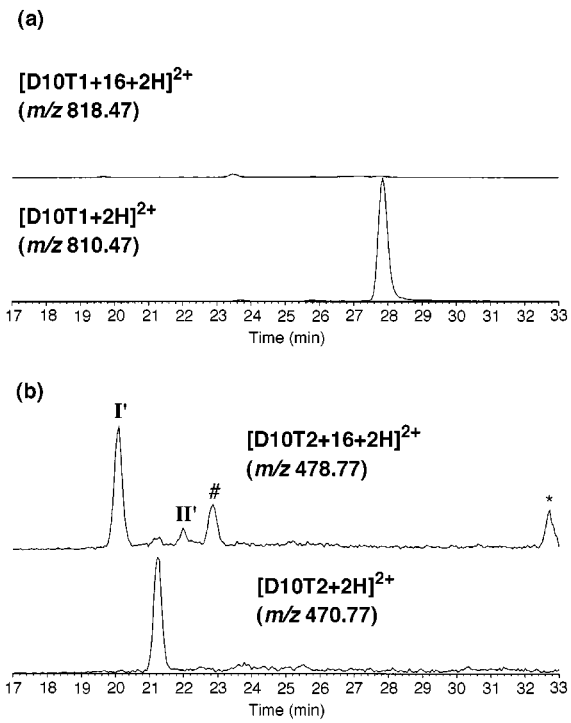

Figure 10. Ion chromatograms of (a) D10T1, D10T1 + 16 and (b) D10T2, D10T2 + 16. All of the ion chromatograms are plotted on the same scale for each peptide and its products; * indicates an unidentified peptide of which monoisotopic mass differed more than 1 Da from the expected mass, and # indicates an unidentified peptide of which monoisotopic mass was exactly as expected, but the ESI-Q-TOF-MS/MS data suggested that this peptide was utterly unrelated to the D10T2 fragment (data not shown).

to be oxidized,[13] even if these valine residues are positioned in the proximity of the active site.

The D5 fragment was also too large to be analyzed directly with LC-ESI-Q-TOF-MS/MS. Then, the D5 peptide was further subjected to trypsin digestion in the same manner. Among the three peptide fragments (Figure 12), D5T1 was the only fragment that could be detected with LC-ESI-Q-TOF-MS. LC-ESI-Q-TOF-MS/MS analyses of the D5 + 16 and the D5 + 32 fragments indicated that oxidation occurred from Cys55 to Pro64, although the oxidized amino acid could not be determined (data not shown). Indeed, the D5T1 + 32 fragment was found to exhibit a similar elution profile to the D5 + 32 fragment, as shown in Figure 13. The D5T1 + 16 fragment, on the other hand, was scarcely observed, probably because an oxidized amino acid in D5 + 16 was further oxidized or hydrolyzed during the additional trypsin digestion. On the basis of the MS/MS analysis of the D5T1 + 32 peptide described below, the most probable was mono-oxidation of Cys55, which might be converted to cysteine sulfinic acid to give the D5T1 + 32 fragment.

LC-ESI-Q-TOF-MS/MS analysis of the first peak of the D5T1 + 32 fragment suggested the oxidation of Cys55, because the b_6 ion corresponding to the fragmentation on the C-terminal side of Cys55 appeared at *m*/*z* 651.38 (data not shown). Shown in Figure 14 are the MS/MS spectra of the D5T1 fragment and the second peak of the D5T1 + 32 fragment. The y''_8 and y''_9 ions of the D5T1 peptide were found to be shifted by 32.02 Da, while the $y''_1 - y''_6$ ions were observed at the predicted *m*/*z*

(12) Jiang, X.; Smith, J. B.; Abraham, E. C. *J. Mass. Spectrom.* **1996**, *31*, 1309−1310.

(13) The formation of valine hydroperoxides upon gamma radiolysis of valine was reported: Fu, S.; Hick, L. A.; Sheil, M. M.; Dean, R. T. *Free Radical Biol. Med.* **1995**, *19*, 281−292.

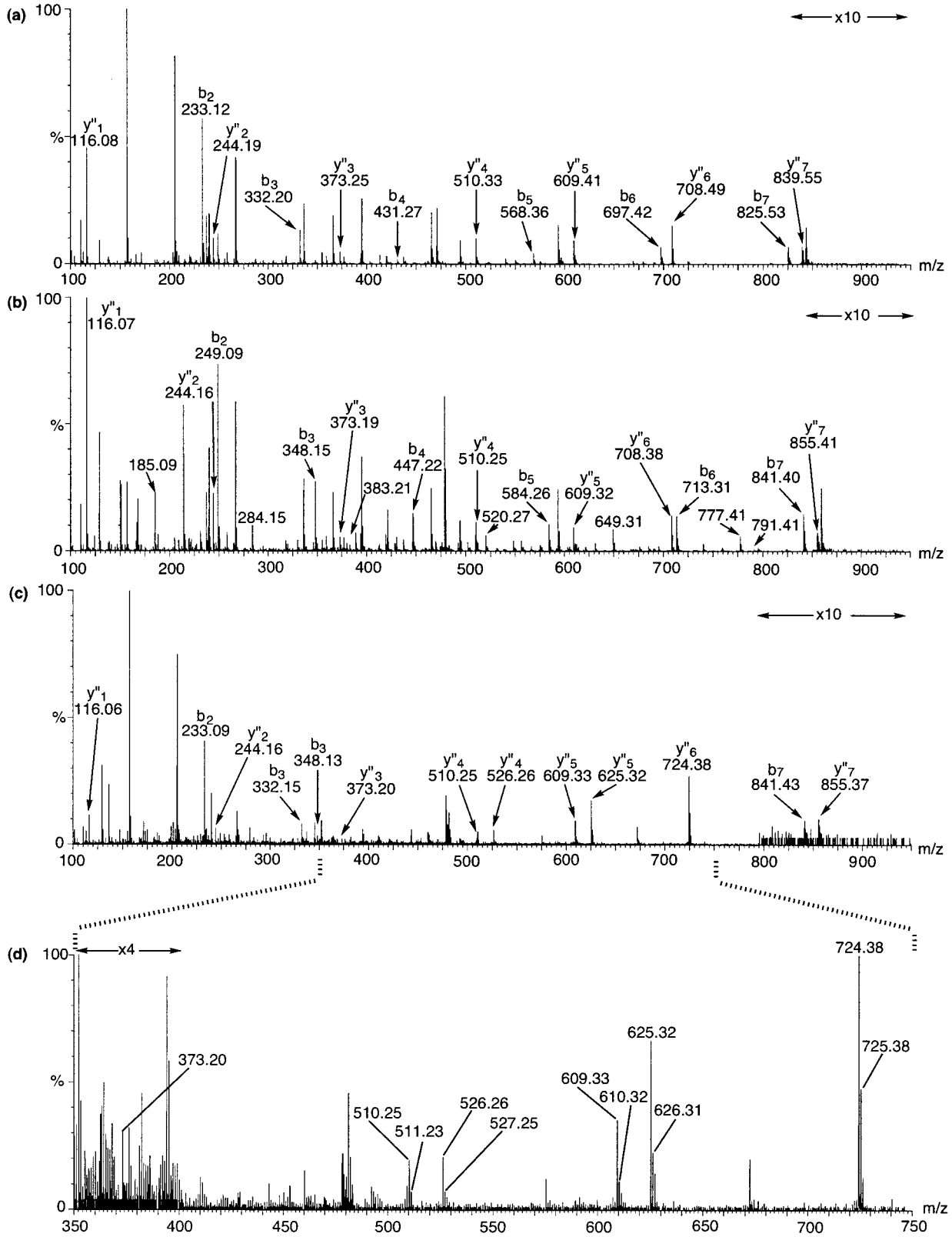

Figure 11. ESI-Q-TOF-MS/MS spectra of (a) the D10T2 fragment (*m*/*z* 470.78, in the doubly charged state), (b) the D10T2 + 16 fragment I′ (*m*/*z* 478.70, in the doubly charged state), and (c) the D10T2 + 16 fragment II′ (*m*/*z* 478.70, in the doubly charged state). (d) Expansion of the designated region in (c). Observed b- and y″-fragments are shown in the spectra. Shown in the peptide sequences are the theoretical values of b- and y″-fragments. Observed ions are shown in bold.

values. Analysis of the y″ fragment ion series thus indicated that Pro60 or His61 was oxidized. Absence of the y''_7 ion corresponding to the fragmentation on the N-terminal side of the His61 prevented the unambiguous determination of the oxidized amino acid. However, conversion of Pro60 to glutamic acid seemed most probable, because the amino acid analysis indicated the considerable loss of proline and the increase of glutamic acid,[4a] and the present LC-ESI-Q-TOF-MS/MS analyses revealed that the other four proline residues were intact upon H_2O_2 treatment.

$$\underbrace{D^{50}NTQGCTSAGP^{60}HFNPLSK}_{\text{D5T1}\ (1872.84)}\ \underbrace{K}_{\text{D5T2}}\ \underbrace{HG^{70}GPK}_{\text{D5T3}\ (494.26)}$$

Figure 12. Assignments of tryptic D5. Shown in parentheses is the monoisotopic mass of each fragment.

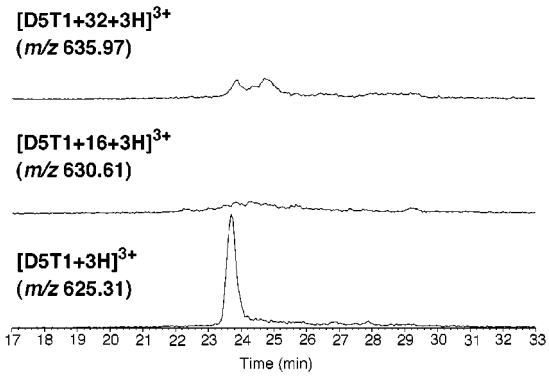

Figure 13. Ion chromatograms of D5T1, D5T1 + 16, and D5T1 + 32. All of the ion chromatograms are plotted on the same scale for each peptide and its products.

Finally, D1 + 32, D1 + 48, and D12T4 + 32 (one of the tryptic peptides of the D12 + 32 peptide) were analyzed with LC-ESI-Q-TOF-MS/MS. The D1 + 32 and D1 + 48 fragment became apparent after H_2O_2 treatment, while the D12 + 32 fragment was observed without H_2O_2 treatment. In both cases, cysteine residues (Cys6 and Cys144) were identified to be oxidized to cysteine sulfinic acid or cysteine sulfonic acid (see Supporting Information). It is interesting to note that the thiol functionality of cysteine was resistant to air oxidation, but the sulfide and the disulfide functionality of methionine and the oxidized form of cysteine was susceptible to air oxidation.

Discussion

To utilize an oxygen atom as a mass spectrometric probe for metal-binding ligands in metalloproteins, a metal-catalyzed oxidation of ligands must be demonstrated to be specific to a considerable extent. Although protein oxidation has been actively studied so far, it has been extremely difficult to prove the specific oxidation of amino acid residues around the Cu-binding site. Our methodology relied on the nano-HPLC separation/on-line ESI-Q-TOF-MS, MS/MS analysis combination which has enabled a direct survey for a certain species on the basis of its mass with less than 0.1 Da accuracy. All the peptides in chemically and enzymatically treated samples could be surveyed by molecular weight. In fact, all the peptide fragments from Cu,Zn-SOD were identified as described in this paper. Furthermore, the D10T1 and D10T2 fragments were successfully identified with ESI-Q-TOF-MS, MS/MS from the complete mixture obtained by H_2O_2-oxidation, endoproteinase Asp-N digestion, and trypsin digestion. Therefore, it could be reliably concluded that all the possible oxidized fragments are detectable with our methodology. It should also be stressed that use of ESI-Q-TOF-MS/MS allows more accurate determination of the position of oxidized amino acids, while ambiguity often remains in the interpretation of traditional MS/MS spectra with poor resolution.

Utilizing nano-LC-ESI-Q-TOF-MS and MS/MS, we have conducted extensive investigations on oxidized Cu,Zn-SOD and have obtained the following findings. The thiol, sulfide, and disulfide functionality of cysteine and methionine was oxidized to some extent in a nonspecific manner. In the case of oxidation of these sulfur-containing amino acids, the noncatalytic oxidation with H_2O_2 seemed to proceed substantially under the present oxidation conditions. The distances between the sulfur atoms of Met115, Cys6, Cys55, Cys144, and the central copper ion is 9.25, 12.68, 9.96, and 10.32 Å, respectively. Therefore, it seems generally difficult to determine whether these sulfur-containing amino acid residues come close to the Cu-binding site from the oxidation experiment. However, other amino acid residues which were oxidized upon H_2O_2 treatment were strictly restricted within the proximity of the Cu-binding site. As anticipated, the present methodology could be readily applied to the determination of Cu-coordinated histidine residues in metalloproteins. In the case of Cu,Zn-SOD, three out of four Cu-coordinated histidine residues were identified as oxo-histidine having 2-imidazolone upon H_2O_2 treatment. Another oxidized form of histidine having dehydro-2-imidazolone has been proposed,[6] but this oxidative product was not formed to any significant extent. The stereochemical arrangement of ligating amino acid residues and other oxidized amino acid residues are shown in Figure 15. Oxidation of His44 and His46 was especially remarkable. The oxidation yield of His118 was far lower but still well above those of other free histidines. The higher oxidation yield for His44 and His46 could probably come from the bidentate nature of the His44-Val45-His46 sequence. On the other hand, His61 coordinated to both Cu and Zn was relatively not susceptible to H_2O_2 oxidation, probably because of deactivation by a Lewis acidic Zn atom. Other Zn-binding histidine residues (His69 and His78) were not affected at all, although they are in the proximity of the Cu-binding site. Pro60, Val116, and Val117, which are not coordinated to Cu but are positioned in the close proximity to Cu, were found to be oxidized to a considerable extent. It is interesting to note that Val116 is the only amino acid residue that comes within 5 Å around the copper ion, except for the Cu-coordinated histidine residues. This result suggests that the metal-catalyzed oxidation of metalloproteins could also be utilized to elucidate noncoordinated amino acid residues in the close proximity of the metal center with the exception of sulfur containing amino acids.

Conclusions

We described in this paper the general methodology to determine oxidized amino acids in oxidatively damaged metalloproteins. In the case of Cu,Zn-SOD, three out of four histidine residues coordinated to the copper ion were identified as oxo-histidines well above the noise level, demonstrating that the metal-catalyzed oxidation of amino acid ligands could be a versatile tool for pinpointing the metal-binding site. The advantage of the present methodology was highlighted by several new findings: (1) a dioxygenized histidine which was supposed to be a histidine hydroperoxide was found to be formed selectively at position 46, and (2) proline (Pro60) and valine residues (Val116 and Val117) in the close proximity of the copper ion were found to be oxidized upon H_2O_2 treatment. The present highly sensitive mass spectrometric method combined with the nano-HPLC system will prove to be especially powerful for the analyses of metalloproteins of low purity and limited availability. The determination of the metal-binding site in TIME-EA4 from *Bombyx* diapause eggs will be reported as a first example in near future. Dynamic aspects around a metal-binding site, such as metal-insertion and displacement, in the context of biological events will be the next challenge.

Experimental Section

Instrumentation. MS and MS/MS spectra were measured utilizing a Q-TOF mass spectrometer (Micromass, Manchester, UK) equipped

D5T1

116.03	**230.08**	**331.13**	**459.18**	516.21	**619.21**	720.26	807.29	878.33	935.35	516.71	585.24	658.77	715.79	764.32	820.86	864.38	-
b_1	b_2	b_3	b_4	b_5	b_6	b_7	b_8	b_9	b_{10}	b_{11}	b_{12}	b_{13}	b_{14}	b_{15}	b_{16}	b_{17}	-
Asp	Asn	Thr	Gln	Gly	Cys	Thr	Ser	Ala	Gly	Pro	His	Phe	Asn	Pro	Leu	Ser	Lys
-	y''_{17}	y''_{16}	y''_{15}	y''_{14}	y''_{13}	y''_{12}	y''_{11}	y''_{10}	y''_9	y''_8	y''_7	y''_6	y''_5	y''_4	y''_3	y''_2	y''_1
-	879.92	822.89	**772.37**	**708.34**	**679.83**	**628.33**	**577.80**	**534.29**	**996.53**	**939.51**	**842.45**	**705.39**	**558.33**	**444.28**	347.23	**234.15**	**147.11**

D5T1+32

116.03	**230.08**	**331.13**	**459.18**	516.21	**619.21**	720.26	807.29	878.33	935.33	532.70	601.23	674.77	731.79	780.31	836.86	880.37	-
b_1	b_2	b_3	b_4	b_5	b_6	b_7	b_8	b_9	b_{10}	b_{11}	b_{12}	b_{13}	b_{14}	b_{15}	b_{16}	b_{17}	-
Asp	Asn	Thr	Gln	Gly	Cys	Thr	Ser	Ala	Gly	**Pro+32**	His	Phe	Asn	Pro	Leu	Ser	Lys
-	y''_{17}	y''_{16}	y''_{15}	y''_{14}	y''_{13}	y''_{12}	y''_{11}	y''_{10}	y''_9	y''_8	y''_7	y''_6	y''_5	y''_4	y''_3	y''_2	y''_1
-	895.91	838.89	788.37	724.34	695.83	644.32	593.80	550.28	**1028.52**	**971.49**	842.45	**705.39**	**558.33**	**444.28**	347.23	**234.15**	**147.11**

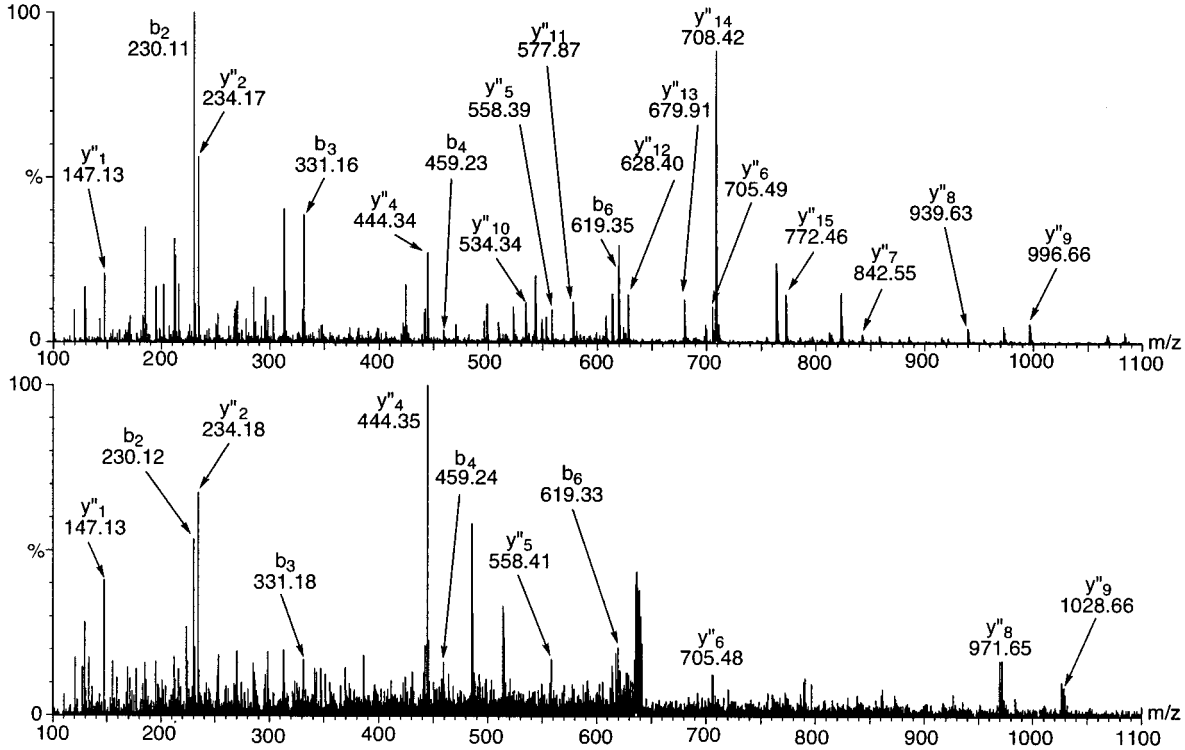

Figure 14. ESI-Q-TOF-MS/MS spectra of (a) the D5T1 fragment (*m*/*z* 625.32, in the triply charged state) and (b) the D5T1 + 32 fragment (*m*/*z* 635.97, in the triply charged state). Shown in the peptide sequences are the theoretical values of b- and y″-fragments. Observed ions are shown in bold. Doubly charged ions are assigned as $b_{11} - b_{17}$ and $y''_{10} - y''_{17}$.

with a Z-spray type ESI source. All experiments were performed in the positive ion mode. Data were acquired and processed using MassLynx version 3.4. All samples were desalted and separated by the appropriately adjusted nano-HPLC system (JASCO, Tokyo, Japan) before on-line ESI-MS and MS/MS analysis. Columns used were Develosil C30-UG-5 (Nomura, Seto, Japan, 15 cm × 0.3 mm i.d.) for the analysis of the hydrophilic D6 fragment and Develosil ODS-HG-5 (Nomura, 15 cm × 0.3 mm i.d.) for the analyses of the others. The columns were equilibrated with 260 μL of water containing 0.025% trifluoroacetic acid at a flow rate of 10 μL/min and then developed using a linear gradient from 0% to 100% of acetonitrile containing 0.025% trifluoroacetic acid for 40 min at a flow rate of 5 μL/min. The column effluent was monitored at 210 nm and then introduced into the electrospray nebulizer without splitting.

Materials. Superoxide dismutase from bovine erythrocytes, hydrogen peroxide (30%), disodium dihydrogen ethylenediaminetetraacetate dihydrate, DTT, acetonitrile (HPLC grade), and trifluoroacetic acid were purchased from Nacalai tesque (Kyoto, Japan). Endoproteinase Asp-N and trypsin (sequence grade) were purchased from Roche Diagnostics (Mannheim, Germany).

General Procedure for H_2O_2-Oxidation of Cu,Zn-SOD. To a solution of Cu,Zn-SOD (16.5 μg, 1050 pmol) in phosphate buffer (24 μL, 62.5 mM, pH 7.2) was added a solution of hydrogen peroxide (6 μL, 25 mM). The solution was incubated at 37 °C for 30 min. Then, the oxidized sample was frozen in dry ice−acetone and lyophilized for 3 h. To remove a trace of hydrogen peroxide, the lyophilization was repeated twice by adding water (20 μL) to the reaction tube. The obtained sample together with the phosphate salt was dissolved in water (28 μL) and subjected to enzymatic digestion as described in the following section. When oxidized Cu,Zn-SOD was analyzed without enzymatic digestion, the sample was dissolved in water (30 μL), from which 2 μL was injected for the LC-ESI-Q-TOF-MS analysis.

General Procedure for Enzymatic and Chemical Fragmentation of Intact and Oxidized Cu,Zn-SOD. The solution of H_2O_2-treated Cu,Zn-SOD in phosphate buffer or a solution of Cu,Zn-SOD (16.5 μg, 1050 pmol) in phosphate buffer (28 μL, 54 mM, pH 7.2) was heated at about 90 °C for 5 min to denature the protein. After being cooled in an ice−water bath, a solution of endoproteinase Asp-N (0.08 μg, 3.0 pmol) in water (2 μL) was added. The solution was incubated at 37 °C for 18 h. The enzyme was deactivated by heating the solution at about 90 °C for 5 min. The obtained sample was stored at −30 °C before analysis. For LC-MS or LC-MS/MS analysis, 1 μL or 2 μL of the sample was utilized, respectively. For LC-MS/MS analyses of oxidized peptide fragments, 10 μL of the sample was injected.

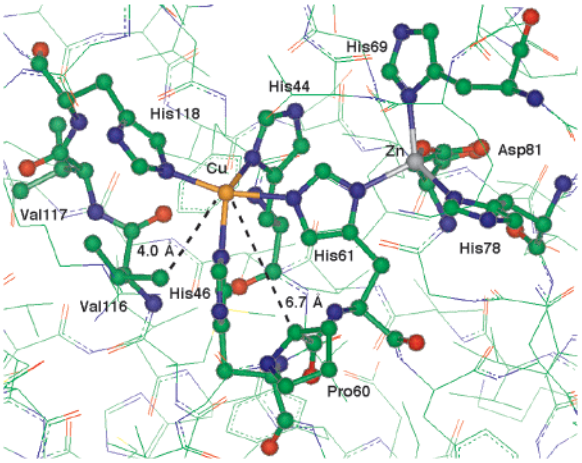

Figure 15. Detail of the Cu- and Zn-binding site in Cu,Zn-SOD. Carbon atoms are in yellowish green, oxygen atoms are in red, and nitrogen atoms are in blue. Hydrogen atoms of amino acid residues are omitted. The illustration was created using Insight II (Molecular Simulations, Inc.) with coordinates obtained from PDB file 2SOD.

The D5 and D12 peptide fragments, connected via the disulfide bond, were analyzed after reductive cleavage of the disulfide bond. Thus, to an aliquot (15 μL) from the solution of the peptide mixture were added a solution of disodium dihydrogen ethylenediaminetetraacetate dihydrate (3 μL, 14 mM) and a solution of DTT (3 μL, 35 mM) successively. The solution was incubated at 37 °C for 2 h; 1 μL of the obtained solution was utilized for LC-MS, and 2 μL for LC-MS/MS without any off-line purification. Note that the reductive operation must be done just before analysis and that the sample thus obtained cannot be used any more after storage.

Trypsin digestion of the D5 and the D10 fragment was conducted as follows. To the solution of endoproteinase Asp-N digested SOD (30 μL) was added a solution of trypsin (0.83 μg, 35 pmol) in water (3 μL). After incubation at 37 °C for 18 h, trypsin was deactivated by heating the solution at about 90 °C for 5 min. The amount of the sample required for analysis was exactly the same: 1 μL for LC-MS, 2 μL for LC-MS/MS, and 10 μL for LC-MS/MS analysis of the oxidized fragment.

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Supporting Information Available: Ion chromatograms of D1, D2, D3, D8, D9, and D12 after H_2O_2 treatment and D1, D5, D10, and D12 without H_2O_2 treatment. ESI-Q-TOF-MS/MS spectra of D1, D2, D3, D5, D6, D7, D8, D9, D10, D10−D11, D12, D10T1, D1 + 32, D1 + 48, D12T4, and D12T4 + 32 (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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