# Site-Specific Amide Hydrogen/Deuterium Exchange in *E. coli* Thioredoxins Measured by Electrospray Ionization Mass Spectrometry

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**Abstract:** Mass spectrometry as an analytical tool to study protein folding and structure by hydrogen/deuterium exchange is a relatively new approach. In this study, site-specific amide deuterium content was measured in oxidized and reduced *E. coli* thioredoxins by using the $b_n$ ions in electrospray ionization CID MS/MS experiments after 20-s incubation in $D_2O$ phosphate-buffered solution (pH 5.7). The deuterium levels correlated well with reported NMR-determined H/D exchange rate constants. The deuterium measured by $y_n$ ions, however, showed much less reliable correlation with rate exchange data. In general, residues in α helices and $\beta$ sheets, when measured by $b_n$ ions, showed low incorporation of deuterium while loops and turns had high deuterium levels. Most amide sites in the two protein forms showed similar deuterium levels consistent with the expected similarity of their structures, but there were some differences. The turn consisting of residues 18−22 in particular showed more variability in deuterium content consistent with reported structural differences in the two forms. The deuterium uptake by thioredoxins alkylated at Cys-32 by *S*-(2-chloroethyl)glutathione and *S*-(2-chloroethyl)-cysteine, in peptides 1−24 and 45−58, was similar to that observed for oxidized and reduced thioredoxins, but several residues, particularly Leu-53 and Thr-54, showed slightly elevated deuterium levels, suggesting that structural changes had occurred from alkylation of the protein at Cys-32. It is concluded that $b_n$ ions are reliable for determining the extent of site-specific amide hydrogen isotope exchange and that mass spectrometry is useful as a complementary technique to NMR and other analytical methods for probing regional structural characteristics of proteins.

## Introduction

The application of mass spectrometry (MS) to monitor H/D exchange in peptides and proteins was first introduced by Katta and Chait in 1991.[1] Mass spectrometric methods when used for probing protein conformations by hydrogen/deuterium exchange offer certain advantages in comparison to NMR methods. These include the ability to (a) study proteins in the micromolar as opposed to the millimolar range, (b) access proteins that precipitate or aggregate at high concentrations, (c) study protein samples that are larger than 20 kDa, (d) observe coexisting conformations simultaneously, (e) work with mixtures or nonpurified samples, and (f) determine the exact hydrogen isotope content and the location by analyzing peptic peptides. The chief advantage enjoyed by NMR until recently was the ability to determine deuterium levels in H/D exchange experiments at the amide nitrogen of individual peptide linkages. A reluctance to relying too heavily on mass spectrometry to measure the isotopic content on individual amide linkages after hydrogen isotope exchange stems from studies that showed scrambling of the label during the collision process necessary to distinguish the amide linkages and their hydrogen isotope composition.[2] Despite these potential problems with scrambling, recent pulse-labeling studies with cytochrome *c* suggested that it is, in fact, possible to obtain reliable H/D exchange information on backbone amide groups of peptides by tandem MS/MS.[3] These studies have shown that the level of deuteration of amide groups in the $b_n$ ions and even in the $y_n$ ions following exchange-in from $D_2O$ for 10 s can be qualitatively correlated with H/D exchange rate constants obtained by NMR. Fast exchange rates are usually associated with high levels of deuterium at amide sites, while slow exchange rates correlate with low deuterium levels.

MS methods were applied in the current studies to measure the degree of H/D exchange in oxidized, reduced, and alkylated *E. coli* thioredoxin so that differences in deuterium uptake could be related to differences in their structures. *E. coli* thioredoxin (TRX) is a small 108 amino acid residue protein (MW = 11 673.40) with a redox-active site sequence $Cys_{32}$-Gly-Pro-$Cys_{35}$.[4] TRXs are ubiquitous and are highly conserved in both prokaryotic and eukaryotic species and mammalian TRXs have about 25% sequence homology with *E. coli* TRX.[5] Although TRXs have many cellular functions, their most important activity is the reduction of protein disulfide bonds.

The $Cys_{32}-Cys_{35}$ linkage in oxidized thioredoxin (Oxi-TRX) is reduced by NADPH through the action of a flavoprotein enzyme, thioredoxin reductase.[6] With the exception of the active-site region there is little structural difference between Oxi-TRX and its reduced form (Red-TRX) and indeed, hydrogen

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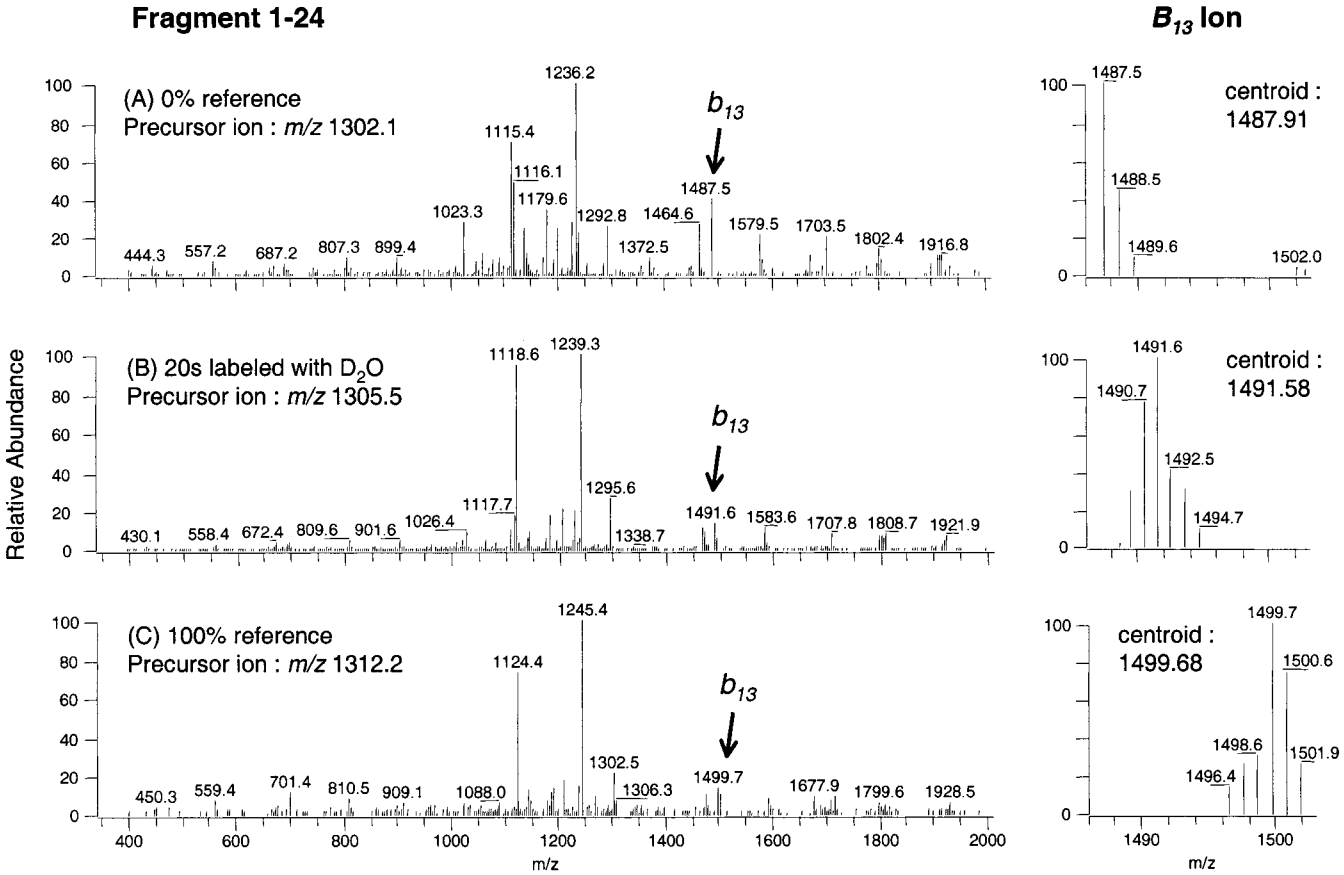


**Figure 1.** CID MS/MS spectra of Oxi-TRX peptide 1−24 of nondeuterated (A, 0% reference), 20 s labeled (B), and fully deuterated (C, 100% reference) protein. Right panels represent isotope patterns of the CID fragment, $b_{13}$.

isotope exchange studies by NMR have shown that only 3−5 more backbone amide hydrogens are exchanged in Red-TRX compared to Oxi-TRX[7] even after extended incubation in the exchange medium.[8] In addition, the exchange rates for most protons were essentially identical between the two forms.[9] However, certain protons in or near the active site appeared to exchange with slightly faster rates in Red-TRX. We show here that amide H/D exchange of *E. coli* TRXs measured by electrospray mass spectrometry complements the information obtained by the NMR method.

## Results and Discussion

The equilibration and exchange-in experiments were performed in phosphate-buffered solution (10 mM, pH 5.7) so that the results for Oxi- and Red-TRX could be compared to previously reported NMR hydrogen exchange rate constants.[9] Cys-32-modified ethylglutathionyl (GS-ethyl-TRX) and ethylcysteinyl (Cys-ethyl-TRX) structures prepared from the reaction of Red-TRX with *S*-(2-chloroethyl)glutathione and *S*-(2-chloroethyl)cysteine[10] were also studied to determine if possible structural changes could be detected by H/D exchange. The equilibrated TRXs were exposed to $D_2O$-buffer solution for 20 s at room temperature. After incubation, the TRXs were digested and analyzed by collision-induced dissociation tandem mass spectrometry (CID MS/MS) to determine the H/D content at individual amide linkages of the peptic peptides. Almost all CID MS/MS experiments of peptic peptides of TRXs were carried out on the doubly charged precursor ions as they showed greater peak intensities than did the singly charged ions.

Doubly charged ions of a peptide 1−24 of nondeuterated (0% reference), 20 s deuterated, and fully deuterated (100% reference) Oxi-TRX were used as precursor ions for CID MS/MS (Figure 1). Fully deuterated proteins were obtained by incubating TRXs in 1% AcOD/$D_2O$ at 80 °C for 1 h. Reference samples, 0% and 100%, were used to adjust for deuterium loss during analysis. The amide deuterium content remaining on Oxi-TRX peptide 1−24 after analysis under minimal exchange conditions was 85%, i.e., ((D content of fully deuterated peptide)/(D content of theoretically 100% deuterated peptide)) × 100 = (19.51/23) × 100 = 85%. Isotope patterns for the CID fragment ion, $b_{13}$, derived from the MS/MS spectrum of fragment 1−24 for the 0% reference, 20 s deuterated, and 100% reference Oxi-TRX samples are shown in the right side panels (Figure 1). The MS/MS spectra of the peptides after H/D exchange normally show a broad distribution of peaks contributed to by the naturally occurring isotopes, $^{13}C$ and $^{15}N$. The centroidal mass values were obtained by using published software.[11] Some isotopic distributions of CID fragments showed overlapped isotopic multiplets as, for example the $b_{12}$ and $y_{13}$ ions (Figure 2), whose protonated masses were 1372.67 and 1377.73, respectively after 20 s labeling. The deconvoluted multiplets indicated by dashed lines were obtained by a nonlinear deconvolution algorithm (PeakFit) and used to determine the centroid mass values of each component. In some cases, the overlapped multiplets could not be resolved because they were too close.

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**Table 1.** Deuterium Levels Found at Individual Amide Linkages in Peptide 1−24 of Oxi-TRX after 20 s Incubation in $D_2O$ (phosphate buffer, 10 mM, pD 5.7) at 25 °C. Average values from 3-6 scans

| ions (charge) | | protonated mass (calcd) | 20 s deut. mass (obsd) | no. of D on ions | adjusted no. of D[a] | no. of D on residue | residue | NMR[c] log $k_{ex}$ ($s^{-1}$) | |
|---|---|---|---|---|---|---|---|---|---|
| (1+) | $b_7$ | 807.47 | 809.3 | 1.83 | 2.18 | | 7Leu | −4 | S |
| | $b_8$ | 908.52 | 910.41 | 1.89 | 2.25 | 0.1 | 8Thr | −5.01 | S |
| | $b_9$ | 1023.55 | 1025.6 | 2.05 | 2.44 | 0.2 | 9Asp | −3.11 | S |
| | $b_{10}$ | 1138.57 | 1141.51 | 2.94 | 3.50 | 1.1 | 10Asp | −0.21 | F |
| | $b_{11}$ | 1225.61 | | | | (0.0)[b] | 11Ser | −3.09 | S |
| | $b_{12}$ | 1372.67 | 1375.68 | 3.01 | 3.58 | (0.0)[b] | 12Phe | −4.18 | S |
| | $b_{13}$ | 1487.7 | 1491.58 | 3.88 | 4.62 | 1.0 | 13Asp | *d* | M |
| | $b_{14}$ | 1588.75 | | | | (0.4)[b] | 14Thr | −3.24 | S |
| | $b_{15}$ | 1703.78 | 1708.33 | 4.55 | 5.41 | (0.4)[b] | 15Asp | −3.51 | S |
| | $b_{16}$ | 1802.84 | 1807.5 | 4.66 | 5.50 | 0.1 | 16Val | −5.4 | S |
| | $b_{17}$ | 1915.93 | 1920.69 | 4.76 | 5.66 | 0.1 | 17Leu | −5.93 | S |
| (2+) | $b_{18}$ | 1022.52 | 1025 | 4.96 | 5.90 | 0.2 | 18Lys | −4.41 | S |
| | $b_{19}$ | 1058.03 | 1060.63 | 5.2 | 6.19 | 0.3 | 19Ala | −2.75 | S |
| | $b_{20}$ | 1115.55 | 1118.49 | 5.88 | 7.00 | 0.8 | 20Asp | *d* | M |
| | $b_{21}$ | 1144.06 | | | | (0.4)[b] | 21Gly | −0.21 | F |
| | $b_{22}$ | 1179.58 | 1182.83 | 6.5 | 7.74 | (0.4)[b] | 22Ala | *d* | M |
| | $b_{23}$ | 1236.12 | 1239.37 | 6.5 | 7.74 | 0.0 | 23Ile | −7.7 | S |
| | $b_{24}$ | 1292.66 | 1295.78 | 6.24 | 7.43 | −0.3 | 24Leu | −7.8 | S |
| (2+) | $y_{22}$ | 1200.64 | 1203.85 | 6.42 | 7.64 | 2.8 | 3Lys | −0.02 | F |
| | $y_{21}$ | 1136.59 | 1138.62 | 4.06 | 4.83 | −0.2 | 4Ile | −2.71 | S |
| | $y_{20}$ | 1080.05 | 1082.18 | 4.26 | 5.07 | −1.6 | 5Ile | −4.06 | S |
| | $y_{19}$ | 1023.51 | 1026.3 | 5.58 | 6.64 | 1.5 | 6His | −2.6 | S |
| (1+) | $y_{18}$ | 1908.94 | 1913.22 | 4.28 | 5.09 | −0.5 | 7Leu | −4 | S |
| | $y_{17}$ | 1795.86 | 1800.59 | 4.73 | 5.63 | 1.0 | 8Thr | −5.01 | S |
| | $y_{16}$ | 1694.81 | 1698.66 | 3.85 | 4.58 | 0.0 | 9Asp | −3.11 | S |
| | $y_{15}$ | 1579.79 | 1583.62 | 3.83 | 4.56 | −0.1 | 10Asp | −0.21 | F |
| | $y_{14}$ | 1464.76 | 1468.64 | 3.88 | 4.62 | 1.0 | 11Ser | −3.09 | S |
| | $y_{13}$ | 1377.73 | 1380.8 | 3.07 | 3.65 | (0.0)[b] | 12Phe | −4.18 | S |
| | $y_{12}$ | 1230.66 | | | | (0.0)[b] | 13Asp | *d* | M |
| | $y_{11}$ | 1115.63 | 1118.65 | 3.02 | 3.59 | 0.1 | 14Thr | −3.24 | S |
| | $y_{10}$ | 1014.58 | 1017.52 | 2.94 | 3.50 | 0.1 | 15Asp | −3.51 | S |
| | $y_9$ | 899.56 | 902.4 | 2.84 | 3.38 | −0.1 | 16Val | −5.4 | S |
| | $y_8$ | 800.49 | 803.38 | 2.89 | 3.41 | −0.1 | 17Leu | −5.93 | S |
| | $y_7$ | 687.4 | 690.4 | 3 | 3.57 | | 18Lys | −4.41 | S |

[a] From $A = D\times(Q/P)$, $D$ is measured deuteriums, $Q$ is the number of amide hydrogens in the fragment ion, $P$ is the number of deuteriums on the fully deuterated sample, i.e., 100% reference. [b] For missing fragment ions, the difference in the number of deuteriums is equally divided. [c] NMR data were taken from published results.[9] [d] The intermediate exchange rates are designated as M, which is in the range 0.0025−0.25 $s^{-1}$ (log value −2.6 to −0.6).

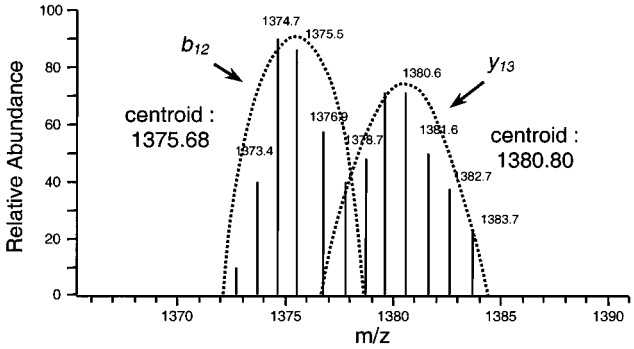


**Figure 2.** Averaged isotopic multiplets of $b_{12}$ and $y_{13}$ ions derived from CID MS/MS spectra of peptic peptide 1−24 of 20 s labeled Oxi-TRX.

The mass values of the $b_n$ and $y_n$ ions of peptides were used to determine the deuterium levels at the amide linkages (Table 1). The number of deuteriums in a CID fragment ion was calculated by subtracting the protonated mass from the deuterated mass, which was then adjusted for deuterium loss.[12] The deuterium content on individual amide sites was determined by subtracting the adjusted content of the $b_{n-1}$ ions from the $b_n$ ions. Incomplete fragmentation resulted in missing ions such as the $b_{11}$, $b_{14}$, $b_{21}$, and $y_{12}$ ions. In these cases, the difference in deuterium content of the $b_n$ and $b_{n+2}$ ions was assigned equally to the intervening amino acid residues. In some regions, for example in the active site of Oxi-TRX, fragmentation failed to occur over a sequence of several amino acids and no site specific deuterium label information was available.

The NMR rate constants for Oxi-TRX reported by Dyson and co-workers[9] are designated as F when log $k_{ex} > -0.6$, and S when log $k_{ex} < -2.6$. These investigators found some amide protons exchanged too slowly to be measured by saturation transfer, and too rapidly to be measured on the NMR time scale. The exchange rate constants for such protons fall in the range log $k_{ex}$ −2.6 to −0.6 and are here designated as M.

The amide deuterium content at each site should range between 0 and 1.0. Amide hydrogens in the $b_n$ ions with small exchange rate constants generally showed low incorporation of deuterium (Table 1). The ranges were mostly between −0.1 and 0.2. Occasionally, there were larger values such as 0.4 for Thr-14 and Asp-15 or −0.3 for Leu-24. The fast exchanging amide hydrogen on Asp-10 resulted in a deuterium level of 1.1. Asp-13 and Asp-20 residues have amide hydrogens with intermediate exchange rates and the deuterium content at these sites was also high with a value of 1.0 for the former. The exchange rates for Gly-21 and Ala-22 are fast and intermediate, respectively. Since cleavage by CID failed to occur between the two residues, a deuterium content of 0.4 was assigned to each but more deuterium could have been located on Gly-21 and, thereby, better match the NMR data. Thr-14 and Asp-15 are reported to have small rate constants, but showed deuterium

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**Table 2.** Comparison of Two Possible Values for $b_{11}-b_{13}$ Ions in the Assignment of Deuterium Levels by Using Two Differenet Vlues for the $b_{12}$ Ion Isotopic Clusters.[c] The $b_{12}$ ions represent a single scan. All other $b_n$ ions are averages from 3-6 scans

| cases | ions | protonated mass (calced) | 20 s deut. mass (obsd) | no. of D on ions | adjusted no. of D | no. of D on residue | residue | NMR log $k_{ex}$ ($s^{-1}$) | |
|---|---|---|---|---|---|---|---|---|---|
| (**A**) | $b_{10}$ | 1138.57 | 1141.51 | 2.94 | 3.50 | 1.1 | 10Asp | −0.21 | F |
| | $b_{11}$ | 1225.61 | | | | **(0.1)**[a] | 11Ser | −3.09 | S |
| | $b_{12}$ | 1372.67 | 1375.73 | 3.06 | 3.64 | **(0.1)**[a] | 12Phe | −4.18 | S |
| | $b_{13}$ | 1487.7 | 1491.58 | 3.88 | 4.62 | 1.0 | 13Asp | *b* | M |
| (**B**) | $b_{10}$ | 1138.57 | 1141.51 | 2.94 | 3.50 | 1.1 | 10Asp | −0.21 | F |
| | $b_{11}$ | 1225.61 | | | | **(−0.1)**[a] | 11Ser | −3.09 | S |
| | $b_{12}$ | 1372.67 | 1375.39 | 2.72 | 3.24 | **(−0.1)**[a] | 12Phe | −4.18 | S |
| | $b_{13}$ | 1487.7 | 1491.58 | 3.88 | 4.62 | 1.4 | 13Asp | *b* | M |

[a] For missing fragment ions, the difference in the number of deuteriums is equally divided. [b] The intermediate exchange rates are designated as M, which is in the range 0.0025−0.25 $s^{-1}$ (log value −2.6 to −0.6). [c] The calculation method is the same as that used in Table 1.

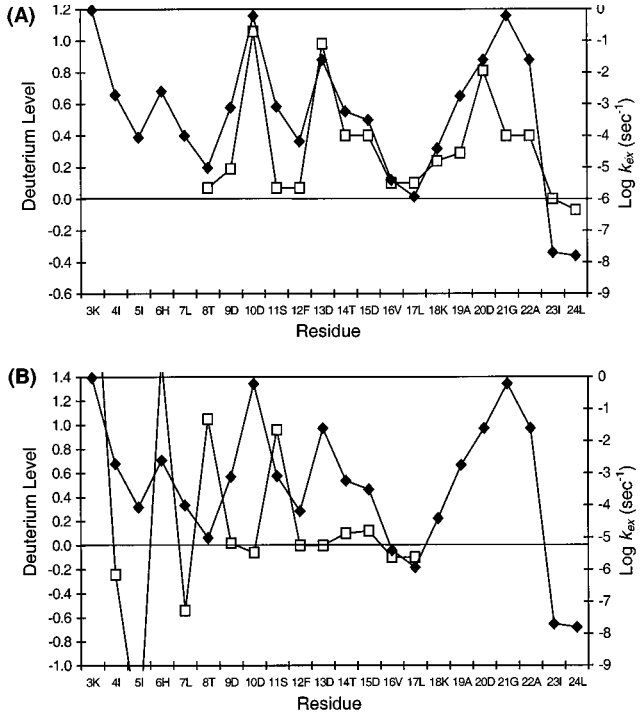


**Figure 3.** Correlation of deuterium levels (□) on peptide amide linkages in peptide 1−24 of Oxi-TRX with hydrogen exchange rate constants (◆, NMR data): (A) $b_n$ ions and (B) $y_n$ ions. The midpoint value of log $k_{ex}$ −1.6 was used for intermediate range rate constants.

levels of about 0.4. Overall, the correlation between the NMR exchange rates for the amide hydrogens and the level of deuterium incorporated at amide sites is excellent (Figure 3A). The deuterium content on the amides within peptide 1−24 as measured by the $y_n$ series showed essentially no correlation with the NMR rate data (Table 1, Figure 3B). The high deuterium level at Lys-3 and the very large negative values of deuterium on the immediately following residues in both Oxi- and Red-TRX may suggest a mobile proton phenomenon.[2a] However, rigorous conclusions concerning the mechanism of a possible transfer cannot be drawn from these data because the deuterium content for the first eight residues in Oxi- and Red-TRX is so different (Table 3). In the present studies, the $y_n$ ions were not used further for H/D exchange information.

In some cases the deuterium levels were observed to be outside the expected limits, i.e., 1 and 0. One possible explanation for these values is that some low-level scrambling occurs during the collision process. There is also some uncertainty in the deuterium levels when they are calculated from weak $b_n$ ion isotopic clusters. For instance, the $b_{12}$ ion of Oxi-TRX showed a weak signal, i.e., less than 3% of the base

**Table 3.** Deuterium Levels Found at Individual Amide Linkages in Peptide Fragment 1−24 of Oxi-, Red-, and GS-TRX Based on the Analysis of $b_n$ Ions and $y_n$ Ions (in italics) Produced by CID MS/MS

| | no. of D on residue[a] | | | | | log $k_{ex}$ ($s^{-1}$)[b,c] | | | |
|---|---|---|---|---|---|---|---|---|---|
| residue | Oxi-TRX | | Red-TRX | | GS-TRX | Oxi-TRX | | Red-TRX | |
| 3Lys | | *2.8* | | *3.9* | | −0.02 | F | −0.02 | F |
| 4Ile | | *−0.2* | | *−0.5* | | −2.71 | S | −2.81 | S |
| 5Ile | | *−1.6* | | *−0.7* | | −4.06 | S | −4.13 | S |
| 6His | | *1.5* | 0.0 | *−0.6* | | −2.6 | S | −2.6 | S |
| 7Leu | | *−0.5* | 0.1 | *−0.6* | (0.2) | −4 | S | −4.17 | S |
| 8Thr | 0.1 | *1.0* | 0.0 | *0.5* | (0.2) | −5.01 | S | −5.02 | S |
| 9Asp | 0.2 | *0.0* | 0.0 | *0.0* | −0.1 | −3.11 | S | −3.28 | S |
| 10Asp | 1.1 | *−0.1* | 0.9 | *0.8* | 0.8 | −0.21 | F | 0.12 | F |
| 11Ser | (0.1)[d] | *1.0* | (0.2) | (*0.2*) | 0.0 | −3.09 | S | −3.22 | S |
| 12Phe | (0.1)[d] | (*0.0*) | (0.2) | (*0.2*) | 0.0 | −4.18 | S | −4.18 | S |
| 13Asp | 1.0[d] | (*0.0*) | 0.9 | (*0.1*) | 0.7 | *c* | M | −0.4 | F |
| 14Thr | (0.4) | *0.1* | 0.2 | (*0.1*) | 0.1 | −3.24 | S | −3.31 | S |
| 15Asp | (0.4) | *0.1* | 0.2 | *0.6* | 0.2 | −3.51 | S | −3.49 | S |
| 16Val | (0.1) | (*−0.1*) | (0.1) | (*0.2*) | (0.1) | −5.4 | S | −5.44 | S |
| 17Leu | (0.1) | (*−0.1*) | (0.1) | (*0.2*) | (0.1) | −5.93 | S | −5.97 | S |
| 18Lys | 0.2 | | 0.0 | | (0.3) | −4.41 | S | −4.43 | S |
| 19Ala | 0.3 | | 0.1 | | (0.3) | −2.75 | S | −2.8 | S |
| 20Asp | 0.8 | | 0.6 | | 0.8 | c | M | −0.19 | F |
| 21Gly | (0.4) | | 0.7 | | (0.9) | −0.21 | F | −0.12 | F |
| 22Ala | (0.4) | | 0.8 | | (0.9) | c | M | −0.34 | F |
| 23Ile | 0.0 | | 0.2 | | 0.2 | −7.7 | S | −7.6 | S |
| 24Leu | −0.1 | | 0.1 | | 0.0 | −7.8 | S | −7.8 | S |

[a] In cases of missing fragment ions, the difference in the number of deuteriums incorporated was equally divided among the residues and these are shown in parentheses. [b] The rate constants were classified as S (slow), M (medium), and F (fast). [c] The intermediate exchange rates are designated as M, which are in the range 0.0025−0.25 $s^{-1}$ (log value −2.6 to −0.6). [d] See Figure 4, Table 2.

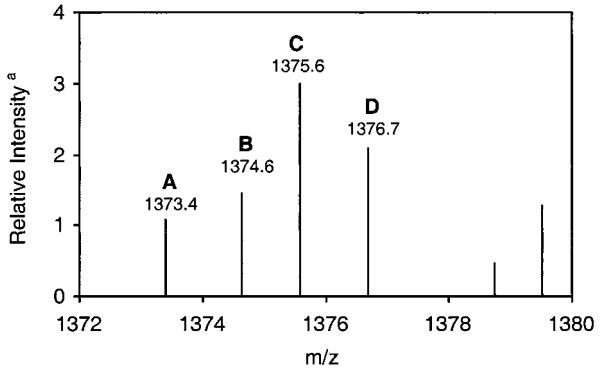


**Figure 4.** Isotopic clusters of $b_{12}$ ion of Oxi-TRX. Footnote a: Relative intensities were set according to the assumption in which the value of base peak is 100. Values from a single scan.

peak (Figure 4). If the centroid value is calculated by using three isotopic peaks (B−D), the mass obtained is 1375.73. However, if it is calculated from four isotopic peaks (A−D), the mass is 1375.39. Although this difference seems small, the

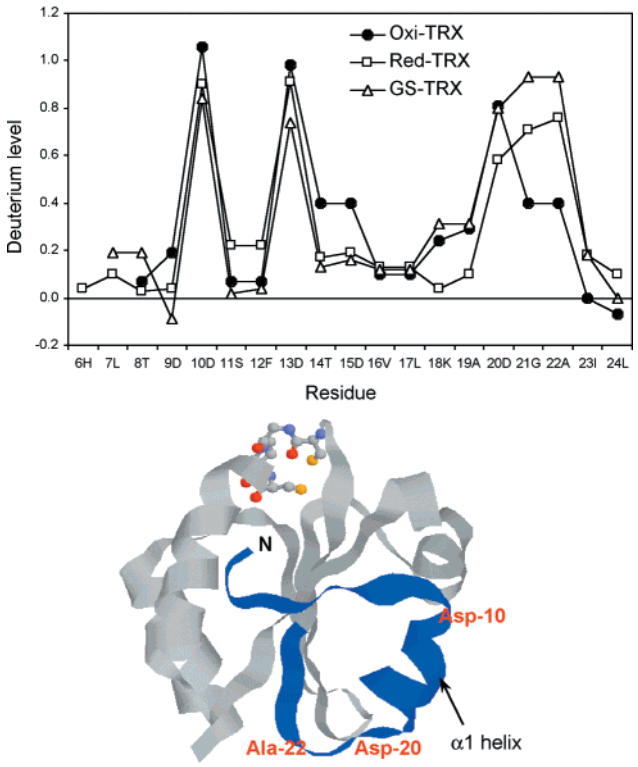


**Figure 5.** (A, top) Deuterium levels found at peptide amide linkages in peptic peptide 1−24 of Oxi-, Red-, and GS-TRX. (B, bottom) NMR solution structure of Oxi-TRX.[13] The sequence 1−24 is highlighted in blue. The active site is represented in the ball-and-stick format.

results for the isotope content are dramatically different. In the former case reasonable deuterium levels are observed for Ser-11 through Asp-13 and these values are in excellent agreement with that expected from NMR exchange rate data (Table 2, case A). In the latter case, negative values are obtained for Ser-11 and Phe-12 and excess deuterium (1.4) is found on Asp-13 (Table 2, case B). A closer examination of the isotopic envelope suggests that peak A may be too large in comparison to the peaks that follow. From this example, it is clear that weak isotope clusters may not yield accurate results.

A comparison of deuterium levels observed on the amides of peptic peptide 1−24 of Oxi-TRX, Red-TRX, and GS-ethyl-TRX by using the $b_n$ ions suggests that the structures among the three protein forms are similar (Table 3, Figure 5A). The amide residue of Asp-9 of GS-ethyl-TRX had a slightly negative value, otherwise there were no other negative values, and none greater than 0.9 in the Red-TRX and GS-ethyl-TRX. There was also better agreement between the deuterium content and the NMR exchange rate data for amides in the Red-TRX peptide. In particular, Gly-21 showed greater incorporation of deuterium, which was consistent with the large rate constant. Residues 14−19 showed very low incorporation, which was consistent with the small exchange rate constants. The differences in the deuterium uptake between Oxi- and Red-TRX in the turn region consisting of residues 18−22 appear to be significant. The crossover in deuterium levels observed between Asp-20 and Gly-21, or Gly-21 and Ala-22, in the two forms may be a reflection of the variability in the dihedral angles and hydrogen bonding patterns in this region.[13] It is significant that, in peptide 1−24 of the alkylated form, GS-ethyl-TRX (Figure 5A), there

**Table 4.** Deuterium Levels Found at Individual Amide Linkages in Peptide Fragment 48−58 of Labeled TRXs after 20 s Exposure to $D_2O$ (phosphate buffer, 10 mM, pD 5.7) at 25 °C

| | no. of D on TRX residues | | | | log $k_{ex}$ ($s^{-1}$) | | | |
|---|---|---|---|---|---|---|---|---|
| residue | Oxi-[a] | Red- | GS- | Cys- | Oxi-TRX | | Red-TRX | |
| 48Glu | | 0.3 | 0.1 | 0.1 | −4.31 | S | −4.36 | S |
| 49Tyr | | 0 | −0.2 | −0.1 | −4.04 | S | −4.03 | S |
| 50Gln | | −0.1 | 0.2 | −0.1 | −2.85 | S | −2.86 | S |
| 51Gly | 0.8 | (0.7)[b] | (0.8)[b] | (0.7)[b] | 0.13 | F | 0.28 | F |
| 52Lys | 0.5 | (0.7)[b] | (0.8)[b] | (0.7)[b] | −0.51 | F | −0.19 | F |
| 53Leu | 0 | −0.1 | 0.4 | 0.4 | −4.62 | S | −4.23 | S |
| 54Thr | 0.1 | 0 | 0.3 | 0.4 | −6.54 | S | −6.37 | S |
| 55Val | −0.1 | −0.1 | 0.2 | 0 | −5.1 | S | −5.09 | S |
| 56Ala | 0 | 0 | 0.2 | 0.1 | −8.4 | S | −7 | S |
| 57Lys | 0.2 | 0.1 | 0.2 | 0.2 | −5.6 | S | −5.66 | S |
| 58Leu | 0.1 | 0.2 | 0.1 | 0.1 | −8.2 | S | −7.5 | S |

[a] Data for Oxi-TRX were taken from a part of the exchange data for peptide 48−80. [b] In cases of missing fragment ions, the difference of deuterium numbers was equally divided between the two sites in the sequence possible sites shown in parentheses.

was closer agreement in the pattern of deuterium uptake with the peptide of Red-TRX than with that of Oxi-TRX. These results are consistent with the expectation that there should be less difference in backbone structure and solvent accessibility between the reduced and alkylated forms than between the oxidized and alkylated forms.

A general analysis of the deuterium content of various peptides for the entire sequence of both Oxi-TRX and Red-TRX showed excellent correlation with the hydrogen exchange rate constants throughout the proteins, with the agreement being somewhat better for Red-TRX (Figure 6). As expected, deuterium incorporation was low in α-helices and $\beta$-sheets where smaller exchange rate constants prevail. In turns for which CID-derived sequence information is available, i.e., between α2 and $\beta$3, α3 and $\beta$4, and $\beta$5 and α4, the deuterium levels in Red- and Oxi-TRXs track each other closely, as expected from structural data that show the dihedral angles are the same in these turns.

The deuterium levels at Gly-51 and Lys-52 in the turn region were high in Oxi- and Red-TRX as expected from the high exchange rates (Table 4 and Figure 7). High deuterium levels were also observed at these sites in GS-ethyl-TRX and Cys-ethyl-TRX, but in addition there were slightly higher levels of deuterium on residues 53−57 in GS-ethyl-TRX and on residues 53−54 in Cys-ethyl-TRX, suggesting that the ethylglutathionyl and ethylcysteinyl groups caused some structural changes in the protein. Additional changes in the deuterium incorporation were observed nearer to the active site of GS-ethyl-TRX and Cys-ethyl-TRX.[14]

The limitations of NMR to provide definitive amide H/D exchange information when rates are in the intermediate range can be addressed by mass spectrometry. The difference between intermediate and fast or intermediate and slow rates measured by NMR may not be very significant. Mass spectrometry can give an indication at which end of the exchange rate scale the rates lie. Dyson and co-workers[9] reported that several fast exchanging amide protons in loops and turns of Red-TRX belong to the intermediate rate group in Oxi-TRX (Table 5). Others fall into the intermediate range in both protein forms. To the extent that small differences in deuterium content can be measured accurately, the mass spectrometry data generally show little difference in the uptake of deuterium at these sites in the two forms. Intermediate levels of deuterium (mostly 0.6)

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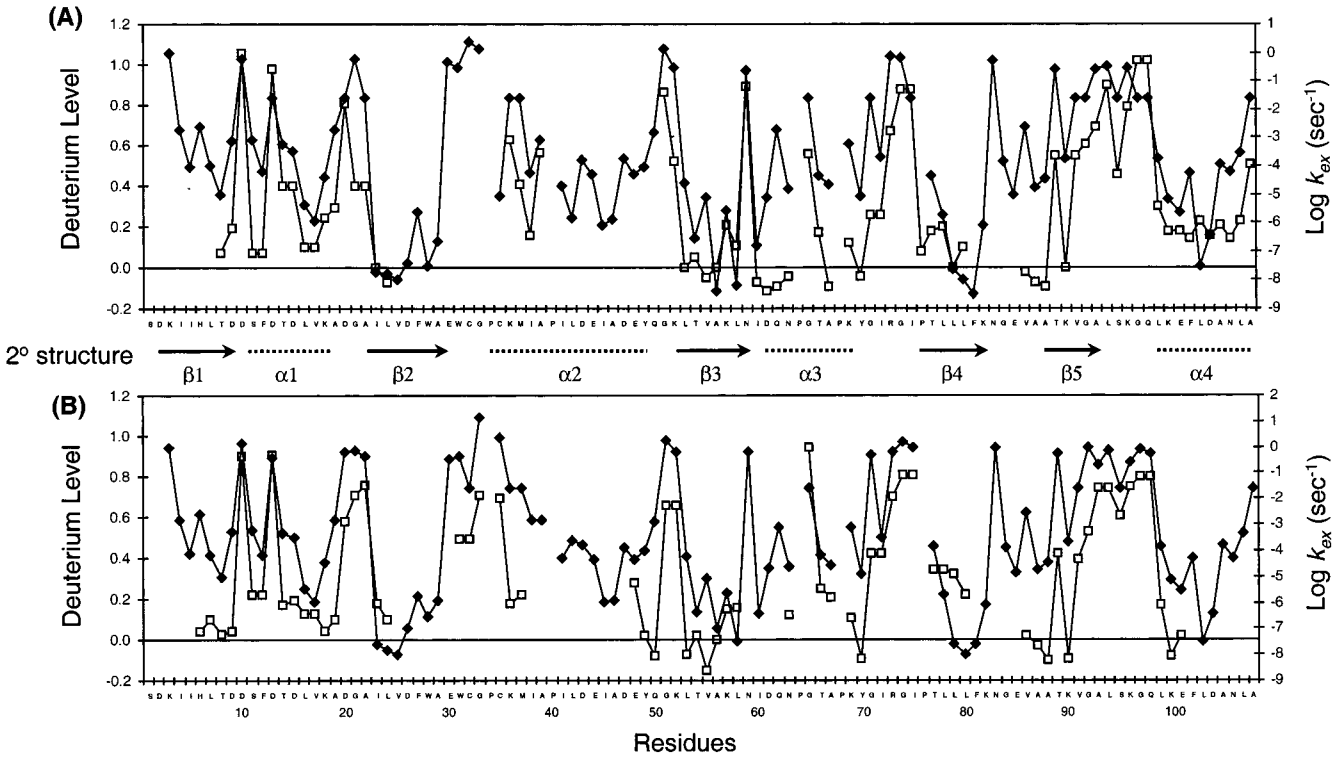


**Figure 6.** Correlation of H/D exchange rate constants (◆) from NMR studies[9] with deuterium levels (□) measured by *b* ions from CID MS/MS experiments on peptide amide linkages. (A) Oxi-TRX and (B) Red-TRX after 20 s incubation in $D_2O$. The midpoint value log $k_{ex}$ −1.6 was used for intermediate exchange rate constants. Secondary structures are indicated; *β*-sheets are designated with arrows and α-helices are shown with dotted lines.

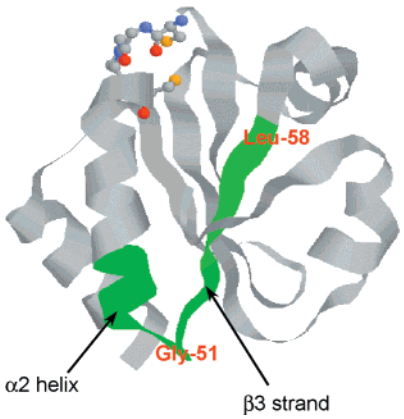


**Figure 7.** NMR solution structure of Oxi-TRX.[13] The sequence 45−58 is highlighted in green. The active site region is represented by the ball-and-stick format.

were observed in the α2 and α3 helices and the *β*5 sheet of Oxi-TRX. In these structural regions of Red-TRX the levels were found to cover a broader range (0.2−0.9) of deuterium content. The lower level of deuterium found at Cys-32 in Red-TRX agrees well with the intermediate exchange rate data obtained by NMR. Dyson and co-workers,[9] however, questioned their results in this case as they expected the amide hydrogen on Cys-32 to be in the fast exchange rate group.

The structures of the two forms of the protein are similar, but Oxi-TRX has a melting temperature about 10 K higher than Red-TRX.[15] In bacteriophage systems Red-TRX is active while Oxi-TRX is not. But, the reactivity of the reduced form seems to have less to do with the presence of the sulfhydryl groups and more with the structure, since mutants in which the cysteine residues have been replaced are also active.[16] The difference between the two protein forms has been suggested to be due to greater backbone flexibility within the active site region of Red-TRX and to solvation effects.[9] Complementary studies by mass spectrometry and NMR confirm that there are subtle differences between the two forms in the amide proton exchange rates.

**Table 5.** Deuterium Levels Found at Amide Linkages in Oxi- and Red-TRX Based on an Analysis of $b_n$ Ions in CID MS/MS Experiments

| | | no. of D on residue | | log $k_{ex}$ (s$^{-1}$) | | | |
|---|---|---|---|---|---|---|---|
| residue | region | Oxi-TRX | Red-TRX | Oxi-TRX | | Red-TRX | |
| 13Asp | α1 | 1.0 | 0.9 | M | | −0.4 | F |
| 20Asp | turn | 0.8 | 0.6 | M | | −0.19 | F |
| 22Ala | turn | 0.4 | 0.8 | M | | −0.34 | F |
| 31Trp | turn | | (0.5)[a] | −0.55 | F | −0.34 | M |
| 32Cys | turn | | (0.5)[a] | 0.37 | F | M | |
| 36Lys | α2 | 0.6 | 0.2 | M | | M | |
| 37Met | α2 | 0.3 | 0.2 | M | | M | |
| 65Gly | α3 | 0.6 | 0.9 | M | | M | |
| 71Gly | α3 | (0.3)[a] | (0.4)[a] | M | | −0.31 | F |
| 72Ile | turn | (0.3)[a] | (0.4)[a] | −3.69 | S | −3.52 | S |
| 75Ile | turn | (0.9)[a] | 0.8 | M | | −0.01 | F |
| 91Val | *β*5 | 0.6 | 0.4 | M | | M | |
| 92Gly | *β*5 | 0.6 | 0.5 | M | | −0.02 | F |
| 95Ser | turn | 0.5 | 0.6 | M | | M | |
| 97Gly | α4 | (1.0)[a] | (0.8)[a] | M | | −0.08 | F |
| 98Gln | α4 | (1.0)[a] | (0.8)[a] | M | | −0.25 | F |

[a] In cases of missing fragment ions, the difference of deuterium numbers was divided equally between the two sites in the sequence possible sites shown in parentheses.

## Conclusions

Studies with Oxi- and Red-TRX for which NMR exchange rate data are available generally showed excellent qualitative

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correlations between the hydrogen rates of exchange and the amount of deuterium label at the amide sites in the $b_n$ ions (Figure 3A and 6). Known structural differences in the loop region (residues 18−20) of Oxi- and Red-TRX were detected and a lower abundance of deuterium on Cys-32 in Red-TRX agreed with the NMR exchange rate data. The adduction of Cys-32 by ethylglutathionyl and ethylcysteinyl groups resulted in slightly greater deuterium uptake in Leu-53 and Thr-54, but for the most part, peptides 1−24 and 48−58 showed levels of deuterium that were about the same as in Red-TRX. Some negative values of deuterium content in the $b_n$ ions and others greater than one may be due to low-level hydrogen scrambling. The mechanism of amide hydrogen scrambling in the CID process still is not known with certainty. Although Smith and co-workers did not report observing negative deuterium values in their exchange-in experiments in peptides of cytochrome *c*, several amide sites from the $b_n$ series had deuterium values of 1.1. To determine accurate deuterium levels on the $b_n$ ions, it is critical to accurately measure the centroids of the isotopic peak clusters, a task that can be difficult when clusters overlap or when the ion signals are weak. A computer algorithm to simulate isotopic clusters for comparison with the experimental results, which is based on either experimental NMR rate exchange data[17] or the mass spectrometrically determined deuterium levels, should add confidence to the measured H/D ratios.

These studies have shown that a comparison of mass spectrometry and NMR H/D exchange data can yield complementary information. In particular, mass spectrometry can give an indication whether amide protons with intermediate exchange rates that cannot be accurately measured by NMR fall closer to the fast or slow exchange category or somewhere between these limits. Data obtained by NMR also are not completely free of errors particularly for measuring fast exchanging hydrogens by saturation transfer,[9] and mass spectrometry can provide additional information that either supports or raises questions about the NMR results. Most importantly, mass spectrometry can be used when the experiments are inaccessible by NMR as, for example, when proteins are large or not very soluble. The major limitation with mass spectrometry at the present time is the incomplete cleavage between certain residues in the CID experiments. Part of the difficulty here is that smaller $b_n$ ions from the N-terminal region of the peptides are very weak or are not produced at all by CID, when using instruments such as the LCQ mass spectrometer. Multiple MS/MS experiments on peptic peptides[18] or tandem mass spectrometry of the entire protein[19] may be solutions to this problem.

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## Experimental Section

**Protein Preparation.** Oxi-TRX, GS-ethyl-TRX, and Cys-ethyl-TRX were equilibrated (0.85 mM) in $H_2O$ phosphate buffer (10 mM, pH 5.7) at room temperature (25 °C) for 1 h. Red-TRX was obtained by dissolving Oxi-TRX (0.85 mM) in 8 mM TCEP/phosphate buffer (pH 5.7).

**Deuterium Exchange and Peptic Digestion.** The equilibrated protein solution was diluted 20-fold into $D_2O$ phosphate buffer (10 mM, pD 5.7) and incubated for 20 s. The samples were chilled immediately in an ice−water bath (0 °C) and acidified with 0.1 M HCl (pH 2.5). This quench step takes roughly 30 s. Samples were then digested with pre-cooled pepsin solution (Sigma, 3 mg/mL in 5% formic acid/$H_2O$) for 5 min at 0 °C (Enz/Sub = $^1/_2$). In the case of Oxi-TRX, urea (8 M, 0.5 M TRIS, 5 mM EDTA, pH 2.5) was added during the quench step for efficient digestion. The final urea concentration was 1.85 M.

**HPLC.** The digested samples were injected with a pre-chilled syringe into an HPLC injector port, which was immersed in an ice bath. All components from the injector to the capillary line just before the mass spectrometer were maintained at low temperature in the ice bath. The peptides were separated by a microbore reverse-phase HPLC on a C18 column (20 cm × 0.32 mm, 15 $\mu$m, LUNA). The elution gradient was linear from 30% B to 60% B in 20−30 min; the flow rate was 10−15 $\mu$L/min. Solvent A was 0.05% TFA/$H_2O$, and solvent B was 0.05% TFA/$CH_3CN$. All parameters were adjusted to maintain retention times of 5 to 10 min.

**Mass Spectrometry.** All mass spectra were acquired with a Finnigan LCQ ion-trap mass spectrometer (ThermoQuest, San Jose). Mass spectrometer conditions were set with an ion spray voltage of 4 kV, a capillary voltage of 46 V, and a tube lens offset of 30 V. Data-dependent MS/MS conditions were set with a default collision energy of 30 V, a default charge state of 2, and an isolation width of 2 ($m/z$). Scans were taken over the mass range $m/z$ 100 to 2000.

**Data Treatment.** MS and MS/MS spectra of deuterated peptides normally showed a broad distribution of isotopic peaks contributed to by the naturally occurring isotopes. The exact mass value of the isotopic cluster was determined by measuring the centroid mass value. Mass spectra were obtained by the operating software "Xcalibur" and the centroid values were calculated by "Magtran" software.[11]

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