Rapid Characterization of Amyloid-β Side-Chain Oxidation by Tandem Mass Spectrometry and the Scoring Algorithm for Spectral Analysis

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Purpose. Amyloid-β (Aβ) is a self-aggregating protein found in senile plaques in Alzheimer's disease (AD) brain and is thought to play a major role in the disease process. Oxidative stress may be a predominant cause of the formation of these Aβ aggregates. This study aims at identifying possible sites of copper-catalyzed oxidation of Aβ1-40 using liquid chromatography tandem mass spectrometry (LC/MS/MS) and scoring algorithm for spectral analysis (SALSA). Traditionally, identification of post-translational modifications by tandem mass spectrometric analysis requires users to inspect manually thousands of MS/MS spectra, which can be a tedious and time-consuming process. With the use of SALSA, users can automatically search for post-translational modifications based on the spacing of the m/z values associated with the ion series of an amino acid sequence.

Methods. Aβ1-40 was subjected to copper-catalyzed oxidative stress. LC/MS/MS and SALSA analyses were used to determine the sites of post-translational modification within the tryptic fragments.

Results. Oxidation was found to occur preferentially at the histidine residues His13 and His14 and at the methionine residue (Met35) of Aβ1-40.

Conclusions. The combination of LC/MS/MS and SALSA searches could dramatically improve the efficiency and accuracy of determining the specific sites of oxidation of *in vitro*, copper-oxidized Aβ1-40 as well as other oxidized proteins.

KEY WORDS: amyloid-β; LC/MS/MS; oxidation; SALSA.

INTRODUCTION

Senile plaques in Alzheimer's disease (AD) brain are primarily composed of the 40-42 amino acid amyloid-β (Aβ) peptide, which is derived from the amyloid precursor protein (APP) (1). The deposition of Aβ plays a primary role in the pathology of the disease (1). Aβ1-40 is the soluble form of the amyloid protein found in the brain; however, the longer isoform of the peptide, Aβ1-42, has been shown to be the main component of the neuritic plaques found in AD brain (2–4).

Oxidative stress in the brain has been suggested to be a factor in Aβ aggregate formation (5–7). In addition, oxidative stress has also been shown to play a role in not only AD (5–9), but also in the pathogenesis of many other neurodegenerative diseases (10–13). Reactive oxygen species (ROS), generated by oxidative stress, can cause abundant damage to proteins, lipids, and DNA (8,9,14,15). The role of transition metals in the brain and their involvement in the generation of ROS has gained increased attention. An imbalance of these metal ions (Fe, Al, Cu, Zn) in the brain can cause oxidative damage through the generation of ROS via the reduction of the metal ion (16). A study by Lovell *et al.* found elevated levels of copper, iron, and zinc in AD senile plaques (16). Also, *in vitro*, Aβ1-40 and Aβ1-42, in the presence of these metals, have been shown to reduce Fe(III) and Cu(II), to produce hydrogen peroxide, and to form hydroxyl radicals (17,18). In addition, Cu(II) has been demonstrated to cause aggregation of the Aβ peptide in a slightly acidic environment (pH 6.8) (19), which is comparable to the pH (6.6) of AD brain tissue (20).

In an attempt to use highly sensitive methods to determine the effects of copper-oxidation on the Aβ peptide, liquid chromatography tandem mass spectrometry (LC/MS/MS) studies have previously been used (21,22). Schoneich *et al.* suggested a conversion of His13 and His14 to 2-oxo-histidine residues in copper-oxidized Aβ1-28 and Aβ1-40 (21), and Lim *et al.* also found copper-oxidized His6, His13, and His14 residues of the peptide Aβ1-16 (22). The current study aims at using a novel approach for the detection of sites of oxidation within the Aβ1-40. The approach consists of LC/MS/MS analysis of digested, copper-oxidized Aβ1-40 and interpretation of the data via the scoring algorithm for spectral analysis (SALSA) algorithm.

SALSA (23,24) is a pattern-recognition algorithm in which MS/MS spectra can be analyzed with user-specified search parameters. Specific fragment ion masses or a complete ion sequence can be entered into the algorithm based on the user's criteria. The search parameters entered will then generate a "theoretical ruler" with which to match and score the MS/MS spectra. This study has shown that SALSA can provide a useful tool in determining sites of post-translational modifications such as oxidation. This method allows for quick and accurate searching of thousands of MS/MS spectra from a given LC/MS/MS acquisition for site-specific post-translational modifications based on a researcher's criteria.

MATERIALS AND METHODS

Synthesis of Aβ1-40

Aβ1-40 was synthesized by fluoren-9-ylmethoxy carbonyl chemistry using a continuous flow semiautomatic instrument as previously described (25).

Copper-Oxidation of Aβ1-40

One milligram of lyophilized Aβ1-40 was dissolved in 250 μl 1,1,1,3,3,3-hexafluoro-2-propanol, 99+% (HFIP), and incubated for 0.5 h in a 1.5-ml Eppendorf tube. The HFIP was

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ABBREVIATIONS: Aβ, amyloid-β; AD, Alzheimer's disease; CID, collision-induced dissociation; LC/MS/MS, liquid chromatography tandem mass spectrometry; ROS, reactive oxygen species; RP, reverse phase; SALSA, scoring algorithm for spectral analysis; SCX, strong cation exchange; σ, standard deviation.

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evaporated-off under a flow of nitrogen gas. One milliliter of double-distilled water was added to the tube and sonicated for 30 s. Fifty microliters of 0.2 M phosphate buffer, pH 7.4, 5.7 μl 0.3% H_2O_2, and 5 μl 0.25 mg/ml freshly made $CuSO_4$ were added to 50 μl of the Aβ1-40 solution. The mixture was incubated for 4 h at 37°C to oxidize the Aβ1-40 peptide. The oxidation reaction was stopped by the addition of 20 μl of 5 mM ethylenediamine-tetraacetic acid (EDTA). Reagents were from Sigma, Aldrich (St. Louis, MO, USA), or Mallinckrodt (St. Louis, MO, USA).

HPLC Purification

The copper-oxidized sample was purified by high-performance liquid chromatography (HPLC) using a Vydac 259VHP Polymer Reverse Phase Column (Western Analytical Products, Murietta, CA, USA) at 65°C. Sample was loaded onto the column with 95% HPLC solvent A (5 mM NH_4HCO_3, 5% acetonitrile) and 5% HPLC solvent B (5 mM NH_4HCO_3, 90% acetonitrile). Peptide was eluted-off the column with a gradient of 5–20% HPLC solvent B from 0 to 5 min, and 20–80% HPLC solvent B from 5 to 50 min. Fractions containing peptide were collected and lyophilized according to absorbances detected at 280 nm and 215 nm.

Tryptic Digest

The copper-oxidized Aβ1-40 was digested with 1% (w/v) trypsin (Sigma or Promega) in 0.001 N HCl and 0.1 mM $CaCl_2$. Digestion reactions were carried out twice for 2 h at 37°C and once in an overnight incubation at 37°C. The reaction was stopped with the addition of 5% acetic acid, and the digested samples were frozen and lyophilized.

LC/MS/MS

All samples were analyzed using a Finnigan LCQ™ Classic ion trap mass spectrometer (Thermo Finnigan, San Jose, CA, USA). Liquid chromatography was conducted with an Ultra Plus II Proteomic System (Micro-Tech Scientific, Inc., Vista, CA, USA) equipped with a 10 cm × 300 μm (ID) Partisphere SCX (strong cation exchange) capillary column and a 15 cm × 75 μm (ID) reverse phase (RP) capillary column (in-tube end frits packed with 5 μm C18, 300 Å particles) from Micro-Tech Scientific. Samples were loaded onto the SCX column with 99% mobile phase solvent A (5% acetonitrile, 1% formic acid) and 1% solvent B (95% acetonitrile, 0.8% formic acid). Peptides were eluted from the SCX with 100% ammonium acetate (0.5 M) onto the RP column and subsequently eluted with a gradient of 1–60% solvent B for 0–50 min and 60–80% solvent B from 50 to 55 min. MS and MS/MS data were acquired on both salt and organic elution steps as Aβ1-5 and Aβ6-16 do not readily adhere to the C18 RP column and are primarily detected in the salt elution step. The mass spectrometer was equipped with an LCQ™ nanospray ion source (Thermo Finnigan) and 10-μm (ID) noncoated SilicaTip PicoTip nanospray emitters (New Objective, Woburn, MA, USA). The electrical contact was made through a liquid junction at the polyetheretherketone (PEEK) union. The spray voltage of the mass spectrometer was set to 1.5 kV and the heated capillary temperature to 160°C.

Bioinformatic Programs

To interpret MS/MS data, the SALSA search algorithm was used using user-defined parameters as discussed in the "Results" section. The Sequest analyses were performed through the TurboSequest search option in the Bioworks Browser rev. 3.1 Beta 7 software (Thermo Finnigan). All Sequest analyses were run against an Aβ1-42 Fasta database file with His, Tyr, and Met oxidation entered as differential amino acid modifications. Sequest data was sorted and filtered using the X-correlation score vs. charge state filter (+1 ions: 1.5; +2 ions: 2.0; +3 ions: 2.5). Mascot analyses of the MS/MS spectra were performed through the Web-based version of the program (www.matrixscience.com). Mascot analyses were run against a MSDB human database with histidine or methionine oxidation entered as variable modifications. Quantitation was performed using the Qual Browser feature found in the Xcalibur 1.3 software (Thermo Finnigan).

RESULTS

Characterization of Aβ1-40 Tryptic Fragments by LC/MS/MS

In order to identify the specific amino acid sites of oxidation due to copper metal ions, oxidized Aβ1-40 was purified by HPLC, digested with trypsin, and analyzed by LC/MS/MS as described in "Materials and Methods." Four possible tryptic fragments of Aβ1-40 can be generated based on the enzyme's preferential cleavage of a peptide after arginine and lysine residues (Fig. 1). The Aβ1-40 tryptic fragments include Aβ1-5 (DAEFR), Aβ6-16 (HDSGYEVHHQK), Aβ17-28 (LVFFAEDVGSNK), and Aβ29-40 (GAIIGLMVGGVV). Our study focuses on the second and fourth tryptic fragments, Aβ6-16 and Aβ29-40, because these fragments contain amino acids that are susceptible to oxidative attack by copper ions (His6, Tyr10, His13, His14, and Met35) (26).

MS/MS spectra were acquired by the direct elution of the digested peptide mixture, after LC separation, into the ion source of the LCQ ion trap mass spectrometer. In contrast to a matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometer, a quadrupole ion trap mass spectrometer is able to select and isolate specific mass ions for fragmentation in order to determine ultimately the exact positions of the modifications.

The MS data showed the presence of 3 tryptic fragments in the copper-oxidized sample: Aβ6-16, Aβ17-28, and Aβ29-40, which correspond to +1 ion mass-to-charge ratios (m/z) of 1337.2, 1326.5, and 1086.4, respectively. Aβ6-16 was predominately found in the +2 charge state (m/z = 669.2) (Fig. 2A), and Aβ17-28 displayed relatively equal abundances between the +1 and +2 (m/z +2 ion = 663.7) charge states (data not shown). An extremely low abundance of unmodified Aβ29-40 was found in the +1 or +2 charge states (Fig. 2B).

The MS data also revealed that the tryptic fragments, Aβ6-16 and Aβ29-40, were also present in oxidized forms (Fig. 2). The parent peptide masses of the mono-oxidized fragments correspond to a 16-Da increase (equivalent to the addition of an oxygen molecule), yielding m/z values of 1353.2 for Aβ6-16 (+1 ion) and 1102.4 for Aβ29-40 (+1 ion). The mono-oxidized and di-oxidized forms of Aβ6-16 were found predominantly in the +2 charge state (m/z = 677.2 and m/z =

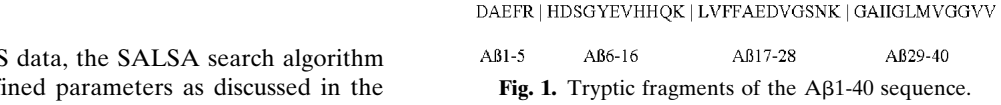

Fig. 1. Tryptic fragments of the Aβ1-40 sequence.

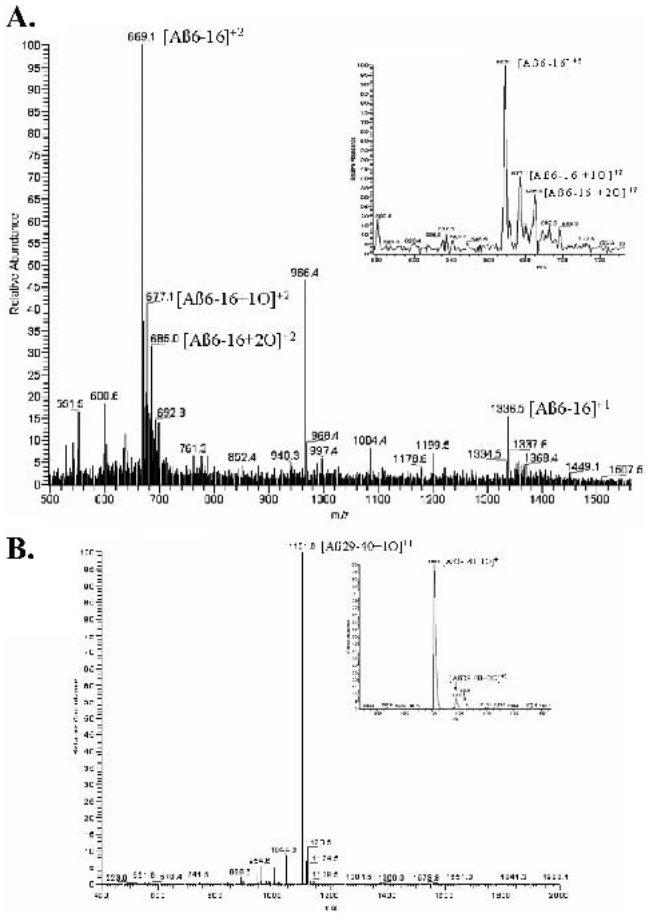

Fig. 2. MS spectra of copper-oxidized Aβ1-40. (A) Average MS spectrum of Aβ6-16 displays a predominant abundance of the +2 unoxidized ion (669.1) over the +1 ion (1336.5). The inset displays an enlargement of the spectrum around the +2 ion to show the ratio of relative abundances of unoxidized to oxidized forms of the peptide. (B) Average MS spectrum of Aβ29-40 displays a predominant peak at 1101.6, indicating mono-oxidation of the fourth tryptic fragment (+1 ion). The inset displays an enlargement of this area, which indicates that the average MS spectrum contains an extremely low relative abundance (<5%) of the unoxidized form of the peptide detected (+1 ion = 1086.4) and a small abundance (<10%) of the di-oxidized form (1117.5).

685.0, respectively). Di-oxidized peptides correspond to a 32-Da shift in the m/z value of the unmodified peptide [m/z: 1369.2 (+1), 685.2 (+2)]. For Aβ29-40, a high relative abundance of the mono-oxidized species was predominantly found in the +1 ion (m/z = 1102.4), whereas a low relative abundance of the di-oxidized, sulfone methionine form of the peptide was detected (+1 ion m/z = 1117.5) (Fig. 2B).

MS/MS Identification of Unmodified Aβ6-16

In order to determine the SALSA search parameters needed for oxidized Aβ6-16 (ox-Aβ6-16), an analysis of the MS/MS spectrum of the unmodified tryptic fragment must initially be carried out. Figure 3A displays a MS/MS spectrum of unmodified Aβ6-16 (+2 ion, m/z = 669.2); the b- and y-ions associated with the fragmentation of this peptide are labeled. b- and y- fragment ions are produced by the cleavage of the peptide bond between adjacent amino acids (27). b-ions refer to the fragment ions containing the N-terminus of the peptide, and y-ions refer to the fragment ions containing the C-terminus (27). In order to predict the b- and y-ions associated with ox-Aβ6-16, 16 Da, the equivalent to the addition of one oxygen atom, was added to the monoisotopic masses of the b- and y-ions after the amino acid site of modification. Figure 4 displays the values of b- and y-ions associated with the unoxidized and possible oxidized forms of Aβ6-16.

This same approach was taken to determine the values of the oxidized b- and y-ions of the fourth tryptic fragment, Aβ29-40. The equivalent of one oxygen molecule (16Da) was added to the monoisotopic masses of the b- and y-ions after the amino acid site of oxidation (Met35). Figure 5 displays b- and y-ion values of the unoxidized and oxidized forms of Aβ29-40.

Construction of SALSA Search Parameters

Specific SALSA searches were created for each of the four possible oxidation sites of the second tryptic fragment,

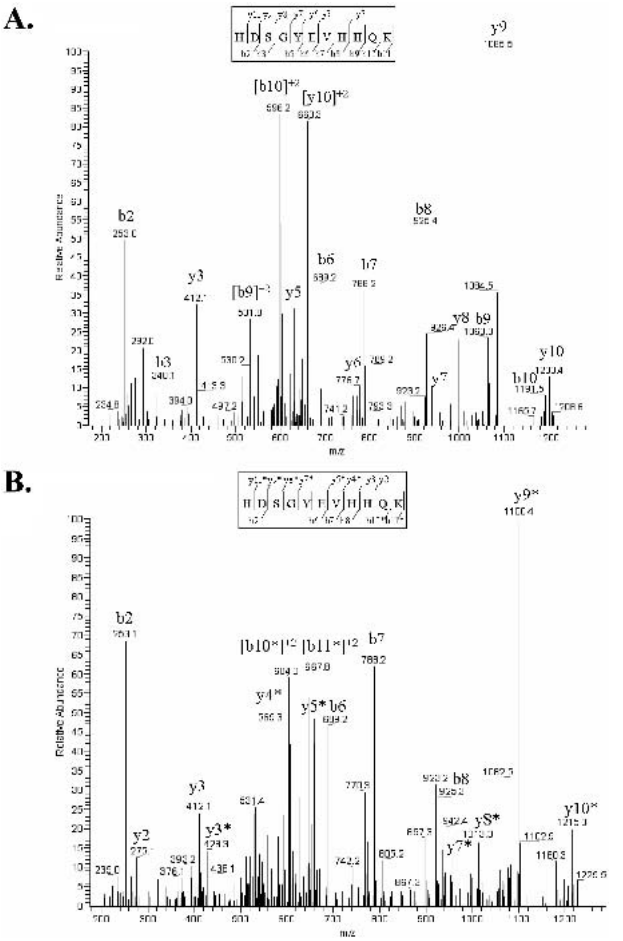

Fig. 3. Unoxidized and oxidized MS/MS spectra of Aβ6-16 tryptic fragment. (A) MS/MS spectrum of unmodified Aβ6-16 (parent mass = 669.2) with abundant b- and y-ions labeled. The inset displays CID fragmentation ions resulting from the Aβ6-16 parent ion. (B) MS/MS spectrum of mono-oxidized Aβ6-16 (parent mass = 677.2) with abundant b- and y-ions labeled. This spectrum displays a mixture of His13 and His 14 oxidation. An asterisk (*) indicates the oxidative form of the ion fragment. The inset displays CID fragmentation ions resulting from the Aβ6-16 parent ion.

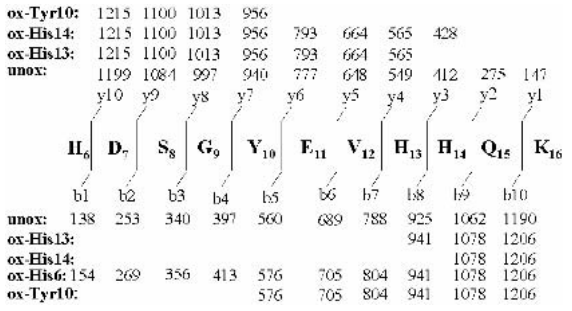

Fig. 4. Theoretical b- and y-ions of oxidized and unoxidized Aβ6-16. B-ions refer to the fragment ions containing the amino-terminus, whereas y-ions refer to the fragment ions containing the carboxyl-terminus. The oxidized form of the b- and y-ions contain a 16-Da shift in fragment ion masses after the amino acid site of modification.

Aβ6-16: His6, Tyr10, His13, and His14 (designated as ox-His6, ox-Tyr10, ox-His13, and ox-His14) (Table I). The possible oxidation sites of Aβ6-16 were chosen based on the susceptibility of these amino acids to oxidative attack (26) and also based on research by Atwood *et al.* that suggested that copper coordinates with the histidine residues (His6, His13, and His14) and possibly the tyrosine residue (Tyr10) of Aβ1-40 (19). The metal-catalyzed oxidation of histidine residues results in the formation of oxo-histidine residues (Fig. 6A) (26).

The SALSA search files for the above-mentioned sites of oxidation of Aβ6-16 were created as follows. First, the unoxidized Aβ6-16 amino acid sequence was inputted in either the amino- to carboxyl-terminus direction to search for y-ions or the carboxyl- to amino-terminus direction to search for b-ions associated with the amino acid sequence. This information is entered into the algorithm as an "ion series." Based on the spacing of the masses of the amino acids in the sequence, the algorithm will generate a corresponding "theoretical ruler" with which to search various MS/MS spectra. If the Aβ6-16 sequence was entered N- to C-terminus, the "ruler" created would consist of the mass differences between the y-ion values associated with the unoxidized amino acid sequence (Fig. 4: unoxidized y-ion series: 147, 275, 412, 549, 648, 777, 940, 997, 1084, 1199). Thus, for this example, the SALSA algorithm would search MS/MS spectra containing ions that are 128, 137, 137, 99, 129, 163, 57, 87, and 115 Da apart (275 – 147 = 128, 412 – 275 = 137, and so forth). Likewise, if the sequence were entered C- to N-terminus, the mass difference between the unoxidized b-ions of Aβ6-16 (Fig. 4: unoxidized b-ion series) would be used to search the MS/MS spectra. To allow the algorithm to also search for an oxidized form of the peptide, a secondary search called "product ion" can be linked to the "ion series" search. "Product ions" refer to specific masses of y- or b-ions, rather than mass differences, and thus can allow for searches of masses associated with a given post-translational modification of a specific amino acid, such as oxidation. For example, to search for ox-His13, the ion series "HDSGYEVHHQK" corresponding to the unmodified Aβ6-16 y-ion series (Fig. 4: unox) was first entered. Then, the "product ions" corresponding to ox-His13 y-ions (Fig. 4: ox-His13 y-ions: 565, 664, 793, 956, 1013, 1100, 1215) were entered and linked to the above ion series. Table I summarizes the search parameters for each of the possible Aβ6-16 copper-oxidation sites: ox-His6, ox-Tyr10, ox-His13, ox-His14. By linking the product ions (with a 16-Da increase in the corresponding b- and y-ions) to the ion series of Aβ6-16, the SALSA algorithm can simultaneously search for the oxidized and unoxidized forms of the peptide sequence. MS/MS spectra displaying the oxidized form of the peptide can be deciphered from MS/MS spectra displaying unoxidized forms by parent ion mass and SALSA identification of the specific product ions associated with the oxidized form.

Another SALSA search file was created to look for possible oxidation products of the fourth tryptic fragment, Aβ29-40. This fragment contains a single methionine residue (Met 35), which is extremely susceptible to oxidative attack (26–28). Under oxidative conditions, ROS can cause the conversion of the sulfur of the methionine residue to a sulfoxide, and upon further oxidative attack, the methionine sulfoxide can irreversibly be modified to a methionine sulfone (28) (Fig. 6B). The SALSA search for Met35 oxidation listed in Table I consisted of 2 ion series searches for both the y- and b-ions associated with the unmodified form of Aβ29-40 (Fig. 5: unox) entered as the ion series "GAIIGLMVGGVV" and "VVGGVMLGIIAG." Linked to each of the ion series were the product ions associated with oxidized b- or y-ions (Fig. 5: ox-Met35).

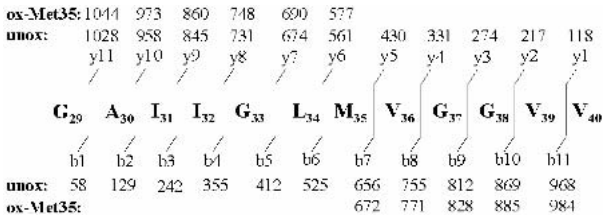

Fig. 5. Theoretical b- and y- ions of unoxidized and oxidized forms of Aβ29-40. B-ions refer to the fragment ions containing the amino-terminus, whereas y-ions refer to the fragment ions containing the carboxyl-terminus. The oxidized form of the b- and y-ions contain a 16-Da shift in fragment ion masses after the amino acid site of modification (Met35).

Identification of Oxidized Aβ6-16 and Aβ29-40 by SALSA Analysis

When SALSA searches are performed, MS/MS spectra are assigned X-correlation scores based on the initial parameters entered into a given SALSA search file. The MS/MS spectra of copper-oxidized Aβ6-16 showed preferential oxidation of His13 and His14 over His6 and Tyr10. Oxidation was determined by the observation of a 16-Da shift, indicative of an oxygen molecule addition, in b- and y- ions at the amino acid site of the modification (Fig. 3). The amino acid oxidation site was determined based on SALSA scores and manual examination of the specific MS/MS spectrum associated with the highest scores.

In a SALSA search for oxidized Aβ6-16, the algorithm scored all of the MS/MS spectra from a given LC/MS/MS analysis of copper-oxidized Aβ based on the search parameters listed in Table I. The top 20 SALSA scores were reviewed for possible oxidation products. For the Aβ6-16 tryptic fragment, two LC/MS/MS acquisitions, one with and one without isolation of the specific ion masses 1353.2 and 677.2 (the +1 and +2 ions of oxidized Aβ6-16) programmed into the method file, were analyzed using the previously described

Table I. SALSA Search Parameters for the Detection of Aβ1-40 Amino Acid Sites of Oxidation

Oxidation site	Ion series[a] (amino acid sequence)	Product ions[b]
His6	KQHHVEYGSDH	154, 269, 356, 413, 576, 705, 804, 941, 1078, 1206
His13	HDSGEYVHHQK	565, 664, 793, 956, 1013, 1100, 1215
His14	HDSGYEVHHQK	428, 565, 664, 793, 956, 1013, 1215
Tyr10	HDSGYEVHHQK	956, 1013, 1100, 1215
	KQHHVEYGSDH	576, 705, 8043, 941, 1078, 1206
Met35	GAIIGLMVGGVV	577, 690, 747, 860, 973, 1044
	VVGGVMLGIIAG	672, 771, 828, 885, 984, 1083

SALSA, scoring algorithm for spectral analysis.

[a] Ion series corresponds to the amino acid sequence entered as the primary search criterion. The sequence inputted is direction-specific, therefore if the sequence is entered from N- to C-terminus, SALSA will search for y-ions, and if the sequence is written from C- to N-terminus, the software will search for b-ions.

[b] Product ions are entered as a secondary search criterion linked to the ion series. After searching and an initial scoring of MS/MS spectra for the ion series, the SALSA algorithm will then adjust the scores based on this secondary product ion criterion. The product ion masses correspond to the b- and/or y-ions of the oxidized form of the peptide (i.e., the b- or y-ion plus 16 Da).

SALSA search files. The results of the search performed on the LC/MS/MS acquisition without isolation of the specific mono-oxidized parent ion masses showed positive identification of ox-His13 and ox-His14 within the top 20 SALSA scores. For the ox-His13 search, the top-three-scoring mono-oxidized MS/MS spectra were scan numbers 842, 882, and 835 and received correlation scores of 10.68, 9.39, and 8.71, respectively (Table II). The same three scans were also identified in the ox-His14 search as the top-three-scoring mono-oxidized spectra with SALSA scores of 13.70 (842), 12.74 (882), and 11.90 (835). Closer examination of these spectra shows that both oxidation products were present. Figure 2B shows a representative MS/MS spectrum with a mixture of ox-His13 and ox-His14. Searches for oxidized His6 and Tyr10 yielded no oxidized forms in the top 20 SALSA scores. MS/MS spectra not corresponding to the proper parent ion mass of the mono-oxidized form of the peptide (m/z = 677.2) but which received high SALSA scores were either identified as synthetic Aβ deletion products or the unmodified form of the peptide. The results of the SALSA searches for oxidation products of Aβ6-16 in which the parent ion mass of the mono-oxidized form was specifically isolated during the LC/MS/MS acquisition yielded the highest X SALSA scores for spectra that were oxidized at His13 (top score = 14.65) or His14 (top score = 21.41). SALSA searches of ox-His6 and ox-Tyr10 had top scores of 9.17 and 7.19, respectively. The same MS/MS scan numbers were seen in the top 20 of all of these searches, indicating that a mixture of ions exists in a single MS/MS spectrum with an amino acid oxidation site preference of His13 and His14 over His6 and Tyr10.

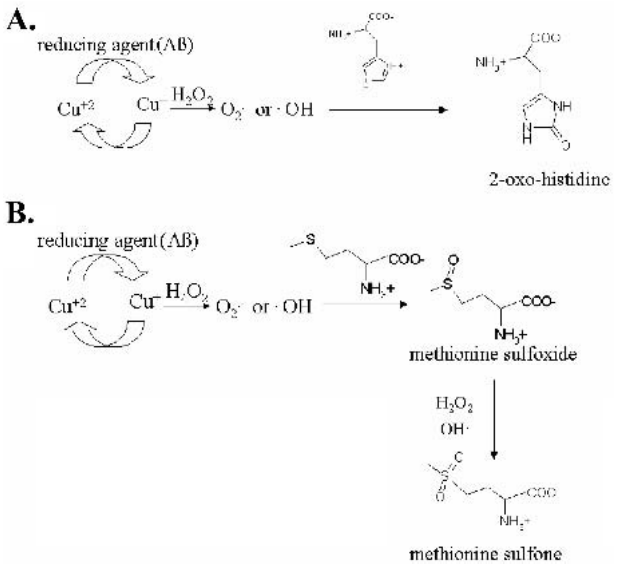

Fig. 6. Reaction schemes for copper-catalyzed oxidation. Copper is reduced in the presence of Aβ, and its reduction results in the production of reactive oxygen species (ROS), such as H_2O_2, •OH, $O_2^{\cdot-}$. (A) The ROS can then attack histidine residues (e.g., His6, His13, His14 of Aβ1-40) to convert them to oxo-histidines. (B) The ROS can also attack methionine residues (e.g., Met35 of Aβ1-40) to convert them to methionine sulfoxides. Further attack by ROS results in the formation of methionine sulfones.

Table II. Summary of SALSA, Sequest, and Mascot Scores of a Single LC/MS/MS Acquisition[a] Searching for Aβ6-16 Oxidation

	MS/MS spectra scan number		
	842	882	835
Salsa score ox-His13	10.68	9.39	8.71
Salsa score ox-His14	13.70	12.74	11.90
Salsa score ox-His6	3.56	5.67	6.46
Salsa score ox-Tyr10	3.56	4.08	4.15
Sequest Xcorr ox-His13	3.078	Not scored	3.122
Sequest Xcorr ox-His14	3.340	Not scored	3.200
Sequest Xcorr ox-His6	1.391	Not scored	1.636
Sequest Xcorr ox-Tyr10	2.268	Not scored	2.381
Mascot probability score	37[b]	40[b]	37[b]

SALSA, scoring algorithm for spectral analysis; LC/MS/MS, liquid chromatography tandem mass spectrometry.

[a] This LC/MS/MS acquisition method had no selective ion monitoring for Aβ6-16 parent ions.

[b] Statistically significant Mascot probability score for oxidized His13.

A SALSA search for ox-Met35 of Aβ29-40 of an LC/MS/MS acquisition, in which no specific parent masses were programmed into the method file, resulted in the positive identification of the oxidized form of the peptide as the top-scoring scans of the search (average highest SALSA score = 23.01; n = 3 LC/MS/MS acquisitions). Figure 7 displays a representative MS/MS spectrum of Aβ29-40 with Met35 oxidized. The SALSA search also found spectra displaying the unmodified form of the peptide and also unmodified Aβ29-39 fragments, which are most likely over-digested tryptic fragments. Despite the extremely low average abundance of unmodified Aβ29-40, the combination of LC/MS/MS and SALSA was able to detect the presence of the tryptic fragment (SALSA score = 10.73), reinforcing the sensitivity and strength of this novel approach.

Confirmation of SALSA Search Results by Sequest and Mascot

To confirm the SALSA search results, another search algorithm known as Sequest was also used in the study. Sequest is a search algorithm that matches MS/MS data to amino acid sequences from protein and nucleotide databases (29,30). Experimental spectra are given X-correlation scores (Xcorr) based on their similarity to the known peptide sequences. Sequest searches also allow the user to look for amino acid modifications, such as oxidation, on any residue.

Sequest analysis of the copper-oxidized Aβ6-16 fragment confirmed the predominance of ox-His13 and ox-His14 over ox-His6 and ox-Tyr10 (Table II). In the Sequest search, the same scan numbers that were given high scores in the SALSA searches were given high Sequest X-correlation scores, except for scan number 882, which was not detected by the Sequest algorithm. The X-correlation scores of a given scan number showed that the spectrum usually contained a mixture of the different oxidation products with oxidation site preference given to His14 and His13 followed by Tyr10. Sequest did not detect any MS/MS spectra that corresponded to Aβ6-16 oxidized at His6.

Another method of independently validating the SALSA search results is by Mascot analysis. Mascot is a probability-based database search that matches MS/MS data to primary sequence FASTA databases (31). The top-scoring MS/MS spectra from the SALSA searches for ox-His13 and ox-His14 were searched against a human FASTA database with histidine oxidation as part of the search parameters. The highest probability score identified the scans as being the second tryptic fragment of amyloid-β protein with ox-His13. The probability scores assigned to the MS/MS spectra containing the oxidized peptide were the only scores that were found to be statistically significant within the searches.

For Aβ29-40, Mascot analyses of the top-scoring SALSA scans confirmed that these spectra corresponded to the fourth tryptic fragment of the amyloid-β protein with ox-Met35. Sequest analysis of the entire LC/MS/MS data file also detected the fourth tryptic fragment, Aβ29-40, with ox-Met35. The Sequest search also detected MS/MS spectra of unmodified Aβ29-40 and the oxidized Aβ29-39 fragment that were also found in the SALSA searches.

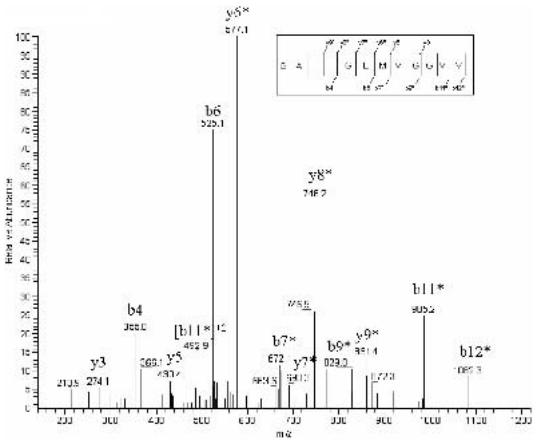

Fig. 7. MS/MS spectrum of copper-oxidized Aβ29-40 with Met35 oxidized. b- and y-ions are labeled, and the inset displays the fragmentation of specific b- and y-ions. An asterisk (*) indicates the oxidatively modified form of the b- or y-ions.

Relative Quantitation of Oxidized Aβ1-40

The levels of unoxidized, mono-oxidized, and di-oxidized Aβ6-16 and Aβ29-40 were quantitated in the following manner. First, using Xcalibur 1.3, individual base peak chromatograms were plotted for each of the ions corresponding to the unoxidized and oxidized forms of the peptides. Base peak chromatograms are a plot of the intensity of the largest peak in mass range (in this case, the peptide mass of the ion ±0.5 Da) vs. time. The base peak chromatograms were smoothed using a Gaussian algorithm, and then the chromatograms were integrated using the Genesis algorithm. The data were exported to an Excel spreadsheet, where the areas of the peaks of a single chromatogram were summed, and ratios were then calculated from the total area values for oxidized and unoxidized species.

For Aβ6-16, the average of four separate LC/MS/MS acquisitions yielded ratios of unoxidized to mono-oxidized of 1.48 (σ = 1.21), unoxidized to di-oxidized of 2.44 (σ = 1.91), and mono-oxidized to di-oxidized of 1.62 (σ = 0.68). The ranges of the ratios were 2.83–0.32, 4.36–0.26, and 2.46–0.79 for unox:mono-ox, unox:di-ox, and mono-ox:di-ox, respectively. For Aβ29-40, the average of the four separate LC/MS/MS runs yielded ratios of mono-oxidized to unoxidized of 806.5 (σ =1494.6), unoxidized to di-oxidized of 35.7 (σ = 56.0), and mono-oxidized to di-oxidized of 12.3 (σ = 13.7). The ranges of the ratios were 3045.2–1.18, 121.0–3.17, and 25.2–0.37 for mono-ox:unox, di-ox:unox, and mono-ox:di-ox, respectively.

The results of the quantitation of these two tryptic fragments show that the ratios of the different species vary greatly from run to run as seen by the large standard deviation values. This can be explained by the fact that Aβ6-16 binds to the SCX column but is eluted with the salt step and does not efficiently adsorb to the downstream C18 micro-capillary column. Therefore, this fragment is never really resolved by HPLC reversed phase (RP) chromatography and is only detected in the flow through of the salt step. On the other hand, the Aβ29-40 fragment binds tightly to the C18 RP column, and continued elution of this fragment is seen in subsequent wash steps after the actual elution step. Another factor that

can also lead to the large deviation in ratios is a differential ionization efficiency of the peptides within and between different LC/MS/MS acquisitions.

DISCUSSION

The SALSA algorithm detected His6, Tyr10, His13, His14, and Met35 as specific amino acid site modifications of copper-oxidized Aβ1-40. SALSA results indicated a preference for His13 and His14 oxidation over His6 and Tyr10 for the second tryptic fragment, Aβ6-16. Other independent search algorithms, Sequest and Mascot, also confirmed these oxidation sites. Each amino acid site of oxidation was determined by a mass shift of 16 Da, indicative of the addition of an oxygen molecule, in the b- and y-ions at the amino acid site of modification.

Oxidative stress plays a prominent role in the pathology of AD. Transition metals (Al, Cu, Fe, Zn) have been found to complex with many enzymes and proteins in the brain, including Aβ (17,18). It has also been shown that elevated levels of Fe, Cu, and Zn exist in the senile plaques of the AD brain (16). Previous studies have suggested that copper ion coordinates with Aβ at the histidine and, perhaps, tyrosine residues, thereby generating hydrogen peroxide and hydroxyl radicals (17,18). Due to the close proximity of the histidine residues to the reduced copper metal and their increased sensitivity to oxidation by metalloproteins (26), it was hypothesized and shown that the three histidine residues (His6, His13, and His14) could undergo conversion to oxo-histidines (21,22). Our MS/MS data show oxo-histidines to be present at His13 and His14 in a copper-oxidized Aβ1-40 sample, which concurs with the previously published data (21). We have also shown almost complete conversion of the Met35 residue to methionine sulfoxide in the fourth tryptic fragment, Aβ29-40.

The relevance of copper oxidation of Aβ to the pathology of AD is that the reduction of this metal ion can also lead to the aggregation of the peptide. Atwood *et al.* showed that in slightly acidic conditions (pH 6.8), copper induced the aggregation of Aβ (19). It has also been shown that a decrease in pH (6.6) is found in AD brain tissue (20), which would create an environment suitable for the reduction of copper, resulting in Aβ aggregation.

The assay developed in this study provides a unique tool to monitor and determine oxidation of Aβ1-40. SALSA can determine the amino acid site of a given post-translational modification based on user-defined parameters. The unique quality of SALSA is that MS/MS spectra are scored based on the user's criteria rather than a given database. It can search all spectra for a specific ion mass or an amino acid sequence. This advantage is lacking in other search algorithms, such as Sequest and Mascot. By creating search files for different sites of oxidation, SALSA was able to find the preferential amino acid sites of modification of copper-oxidized Aβ1-40. In general, this SALSA assay can also be used for the identification of oxidation sites or other post-translational modifications of interest in other proteins as well. The SALSA program interface already includes an option for phosporylation searches by inserting a "p" in front of the amino acid thought to be phosphorylated. Other post-translational modifications can also be searched for by simply increasing the mass of the b- and/or y-ions by the mass of the modification after the site of modification.

In addition, it is a point to note that SALSA searches can result in higher SALSA scores assigned to spectra of other forms of the peptide, such as the unmodified form or deletion products of the peptide, rather than the oxidized form of the protein. This can occur for many reasons. First, this can happen if the mass spectrometer is not instructed to isolate and collect MS/MS data solely for the parent ion masses associated with mono-oxidized Aβ6-16 (m/z = 1353.39, 677.20, 451.80). The copper-oxidation reaction does not fully convert Aβ6-16 to its oxidized forms as seen in Fig. 2A, and therefore, both species, unoxidized and oxidized, are present in the sample. Second, unoxidized forms of the protein might receive higher SALSA scores because the primary search is the "ion series" associated with the unoxidized form of the peptide. The secondary SALSA search, "product ions," allows for the specific masses of the modifications to be detected. Therefore, if MS/MS data were collected solely for the oxidized form of the peptide, then only the oxidized spectra would receive high SALSA scores. Third, deletion products might be present and identified through the SALSA searches. Deletion products arise from improper coupling of the amino acids during peptide synthesis. In the case of this study, one of the deletion products found contained the peptide fragment "DAEFDSGYEVHHQK" [SALSA Scores (scan 1072) = 13.90 (ox-His13), 18.72 (ox-His14), 8.68 (ox-His6), 23.48 (ox-Tyr10); +2ion m/z = 831.7 Da]. This deletion product is missing the His6 residue as well as the arginine residue, Arg5, which is required for the proper tryptic digest of the protein. The deletion product fragment would yield high SALSA scores because the y-ion series is almost identical to that of the Aβ6-16 y-ion series, with the exception of y11 ion, corresponding to the His6 residue, and the 4 additional ions corresponding to the Phe, Glu, Ala, and Asp residues. However, by checking the parent mass of the peptide, one can easily discern between deletion products and the proper tryptic fragments. It is a point to note that Sequest or Mascot analysis would be unable to detect these deletion products because both search algorithms use databases of known primary sequences with which to score MS/MS spectra and thus would not be able to identify sequences with missing internal amino acids. This demonstrates that SALSA can be used for a wide variety of purposes in a single search; it can detect the presence of deletion products, unmodified forms, and modified forms of the peptide. If oxidation of a specific fragment is all that is desired, it is recommended that the LC/MS/MS acquisition of data be conformed to collect only specific parent ion masses, provided that the oxidation mass is known.

Modifications to the SALSA searches were made to the approach taken in this study in order to search for di-oxidized forms of the tryptic fragments, Aβ6-16 and Aβ29-40. The modification to the algorithm consisted of an adjustment in the product ion values to incorporate the addition of two oxygen molecules (32 Da). The SALSA search created for Aβ6-16 was intended to search for MS/MS spectra containing the Aβ6-16 fragment with both His13 and His14 oxidized (parent peptide mass +2 ion m/z = 685.2). The SALSA algorithm positively identified MS/MS spectra with both His13 and His14 oxidized with an average highest SALSA score of 11.04 (σ = 2.18) over three LC/MS/MS acquisitions. For Aβ29-40, the SALSA algorithm detected MS/MS spectra

containing the methionine sulfone modification of Met35 (parent peptide mass +1 ion m/z = 1117.5). The average highest SALSA score of positively identified MS/MS spectra over three LC/MS/MS acquisitions was 18.43 (σ = 1.01). Both of the SALSA searches for doubly oxidized tryptic fragments were independently confirmed by Sequest analysis. This demonstrates the ability of SALSA to detect accurately MS/MS spectra completely based on the user's preferences.

Although synthetic Aβ1-40 was used in this study, we would like to translate this assay to Alzheimer's disease transgenic mouse models and to determine if similar oxidation occurs *in vivo*. Moreover, we would like to investigate further what occurs at His6 and Tyr10 of Aβ in the presence of copper. It has been postulated that His6 is a bridging residue in the complexing of copper in which both nitrogens of the imidizole ring of histidine are involved in preventing the oxidative attack on the residue (21), which supports the data found in this study. Because it has been shown that under metal-catalyzed oxidative conditions, tyrosine residues are subject to dityrosine formation (26), Tyr10 might form cross-links between Aβ peptides.

Initial investigations into identifying the cross-linked peptide and dityrosine formation by SALSA analysis were unsuccessful. Analysis of the search results yielded no detection of parent peptide masses corresponding to that of a dimer. However, this could possibly be due to low abundance of dimer in the sample, its low or high affinity for the column, or an inherent weak ionization capacity or efficacy. Furthermore, the folding of the dimer and fibril forms of the peptide could conceivably affect the efficiency of tryptic digest of the peptide, resulting in very little cross-linked Aβ6-16. A "top down" approach, in which LC/MS/MS acquisition is taken from intact, nondigested peptide, might provide the best method to identify the presence of cross-linked Aβ.

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