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Detection and identification of 4-hydroxy-2-nonenal Schiff-base adducts along with products of Michael addition using data-dependent neutral loss-driven MS³ acquisition: method evaluation through an *in vitro* study on cytochrome c oxidase modifications

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Abstract

We report a data-dependent neutral-loss driven MS³ acquisition to enhance, in addition to abundant Michael adducts, the detection and localization of Schiff-base adducts of proteins and 4-hydroxy-2-nonenal, a reactive end-products of lipid peroxidation. *In vitro* modification of cytochrome c oxidase, a mitochondrial protein complex, was used as a model to evaluate the method. The technique allowed for a confident validation of modification sites and also identified a Schiff-base adduct in subunit Vb of the protein complex.

Keywords

4-hydroxy-2-nonenal; Protein adducts; Schiff-base; Michael addition; Electrospray ionization; Liquid chromatography–tandem mass spectrometry; Neutral loss-driven MS³

4-Hydroxy-2-nonenal (HNE) exerts a potentially detrimental effect to proteins by forming covalent adducts, resulting in diminished protein function, altered physicochemical properties [1] and induction of antigenicity [2]. The modification can take place by the 1,4-addition (Michael addition) of the nucleophilic groups in cysteine (Cys), histidine (His) or lysine (Lys) residues of the protein, respectively, onto the electrophilic double bond of HNE, giving an increase in the protein's molecular mass by 156 Da with each molecule of HNE being added. Alternatively, Schiff-bases are formed with ϵ -NH₂ groups of Lys residues, which yield an increase of 138 Da in molecular weight as the reaction involves the loss of a water molecule (Scheme 1). Michael adducts generally represent >99% of HNE protein modifications while Schiff-base adduct formation is less prevalent even in presence of excess HNE [3,4]. As Schiff-base formation is a reversible process, it is difficult to characterize such modification in proteins. In addition to being a short-lived species, the MS/MS spectra of Schiff-base adducts may have neutral loss ions that can preclude their identification by mass spectrometry as neutral loss limits further fragmentation that would provide sequence information to facilitate identification of the modified peptide. A neutral loss (NL)-driven MS³ (NL-MS³) technique that exploits the neutral loss feature of collision-

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induced dissociation (CID) and performs fragmentation on the neutral loss ion exhibiting a difference of 156 Da from the precursor ion (corresponding to Michael addition of HNE) has been reported by our group [5] and Roe *et al.* [6]. Here, the loss of HNE observed upon MS/MS of HNE-modified peptides will trigger MS³ analysis of the neutral-loss product ion to reveal the sequence of the peptide.

To our knowledge, the implementation of a NL-driven MS³ method for characterizing Schiff-base adducts of HNE modification has not been reported. In this paper, we describe the application of this technique to facilitate the identification of Schiff-base modification site(s) in tryptic peptides of HNE-modified proteins. Cytochrome *c* oxidase (Complex IV, COX) was used for this study, as Chen *et al.* [7] have shown that HNE generated during oxidative stress induced by *tert*-butylhydroperoxide is inhibitory to COX activity in a concentration-dependent manner. In a separate study, they have also shown that ethanol administration to rats enhanced the formation of HNE in liver mitochondria which, in turn, decreased the activity of COX by forming adducts with the enzyme complex [8]. Furthermore, myocardial reperfusion following short-term ischemia in rats was seen to cause enhanced formation of HNE adducts with COX subunits, in particular subunit IV, resulting in significant decrease in the enzyme activity [9]. HNE treatment was also found to inhibit activities of COX and aconitase in PC12 cells thereby inducing apoptosis [10]. COX subunits susceptible for HNE-modifications *in vitro* by Michael addition have been identified [11]. However, modification sites other than His-36 of subunit VIII have not been localized, and Schiff-base adducts have not been reported.

COX is the terminal component of the mitochondrial respiratory chain and catalyzes transfer of electrons from cytochrome *c* to molecular oxygen, generating a proton gradient required for ATP synthesis. COX is an integral inner membrane protein of mitochondria and its dimeric assembly is necessary for H⁺ pumping. Each monomeric mammalian COX consists of 13 protein subunits of which ten subunits (IV, Va, Vb, VIa, VIb, VIc, VIIa, VIIb, VIIc and VIII) are encoded by nuclear DNA and the remaining three largest subunits I, II and III that comprise the catalytic core of the enzyme, are mitochondrial in origin. The subunits are associated in a 1:1 stoichiometry except subunit VIIIb which is present in two copies [12].

As an integral membrane-protein complex, in addition to being surrounded by other phospholipids, COX contains tightly bound cardiolipin which is also required for full electron transport activity [13]. Eighty percent or more of the fatty acids of cardiolipin constitutes of linoleate that are vulnerable to damage by reactive oxygen species generated upon “leakage” of electrons during coupling of electron transfer to oxidative phosphorylation [14]. Consequently, reactive lipid products including α,β -unsaturated aldehydes such as HNE and 4-hydroxy-2-hexenal are produced as end products. These aldehydes act as toxic second messengers of oxidative stress and can endanger the integrity of enzyme complexes [15].

To characterize the structural modification of COX by HNE, crude preparation of COX from bovine heart mitochondria was purchased from Worthington Biochemical Corp. (Lakewood, NJ) and used without further enrichment. COX was modified *in vitro* with HNE (Cayman Chemical, Ann Arbor, MI). One hundred microliters of COX (60 u/ml) was incubated in 100 μ l of 20 mM Tris-sulfate plus 2 mM dodecyl maltoside or 8 M Urea buffer and 2 mM HNE at 37 °C for 5 h followed by washing three times with ethyl acetate (100 μ l aliquots) to remove the excess HNE. The proteins remaining in the aqueous phase were then reduced with 30 mM dithiothreitol at 60 °C for 30 min and alkylated with 55 mM iodoacetamide at room temperature in dark for 30 min. After acetone precipitation to remove the excess dithiothreitol and iodoacetamide, the proteins were resuspended in 100 mM ammonium bicarbonate and subsequently digested with 3 μ g of trypsin (TPCK treated,

Applied Biosystems, Foster City, CA) for 18 h at 37 °C. The digestion was terminated by adding formic acid (1% of total volume), and the peptide solution was lyophilized using FreeZone freeze dry systems (Labconco, Kansas City, MO).

HNE-modified COX tryptic digests were then used for reversed-phase (RP) HPLC-tandem mass spectrometric analysis employing the neutral loss-driven MS³ technique described previously [5] and modified to detect, in addition to the Michael adducts, Schiff-bases as well. Online RP-HPLC-tandem mass spectrometric analysis was performed on a hybrid linear ion trap (LTQ) – 7-Tesla Fourier transform ion cyclotron resonance (FTICR) mass spectrometer (LTQ-FT, Thermo Finnigan, San Jose, CA) equipped with a nanoelectrospray ionization source and operated with the Xcalibur (version 2.2) data acquisition software. Five microliters of COX protein digest was loaded onto a ProteoPep™ II C18 capillary trap (New Objective, Woburn, MA) and desalted with an aqueous washing phase containing 3% (v/v) acetonitrile and 1% (v/v) acetic acid for 5 min prior to injection onto a 15 cm × 75 μm PepMap C18 column (LC Packings, Sunnyvale, CA). Following peptide desalting and injection onto the analytical column, elution by a linear gradient was carried out by raising acetonitrile to 40% (v/v) in 90 min at 250 nl/min flow rate using an Eksigent nanoLC-2D system (Dublin, CA). The mobile phase were mixed from solvent A (0.1% acetic acid and 99.9% water, v/v) and B (0.1% acetic acid and 99.9% acetonitrile, v/v). First, conventional data-dependent mode of acquisition was utilized in which an interim accurate *m/z* survey scan (giving resolving power of 12500 about 0.1 s after the start of recording) performed in the FTICR cell was used to trigger consecutive MS/MS analysis of the top five most intense precursor ions in the linear ion trap (i.e., MS/MS spectra were recorded at low mass resolving power, <1000). FTICR full-scan mass spectra were acquired at 100000 mass resolving power (*m/z* 400) from *m/z* 350 to 1500 using the automatic gain control mode of ion trapping (10⁶ target ion count). CID in the linear-ion trap was performed using a 3.0 Th isolation width and 35% normalized collision energy with helium as the collision gas. During CID, the dissociation of the precursor ion was induced using an activation time window of 30 ms. Charge state rejection was enabled to exclude data dependent MS/MS scans of precursor ions with a single charge. For NL-driven MS³ data-dependent acquisitions, isolation and subsequent fragmentation of ions exhibiting a *m/z* 69 or 46 difference (representing NL of HNE from doubly- or triply-charged precursor ions, respectively) from the precursor ion were conducted to detect Schiff-base adducts, if the NL fragment ions passed specified selection criteria (i.e., they were among the three most intense ions in the MS/MS spectra, which was determined empirically considering the employed separation speed and our instrument's acquisition rate) as shown in Figure 1. In addition, MS³ of precursor ions exhibiting a difference of *m/z* 78 or 52 corresponding to doubly or triply charged precursor ion species was used to enable detection of Michael adducts (+156 Da) [5].

MS/MS data generated by conventional and NL-driven MS³ data-dependent acquisition via the LTQ-FT were extracted by BioWorks version 3.3 and were not combined for the subsequent database search that relied on the IPI bovine (version 3.21, 32872 entries) protein sequence database and the Mascot version 2.2 (Matrix Science, Boston, MA) search algorithm. Mascot was searched with parent-ion and fragment-ion mass tolerances of 10.0 ppm and 0.80 Da, respectively, and specifying trypsin as digestion enzyme. For NL-driven MS³ experiments, a parent-ion tolerance of 1.5 Da was utilized since measurement of the neutral loss fragment ion *m/z* occurred in the linear ion trap (LTQ). Carbamidomethylation of Cys, oxidation of methionine, carbamylation of Lys and the N-terminal amino acid residue, HNE-Schiff-base adducts on Lys, as well as HNE-Michael adduct formation on Cys, His and Lys were specified as variable modifications among the Mascot options. In general, probability-based MOWSE scores corresponding to a significance threshold of *p* < 0.05 were considered for peptide identification.

Scaffold (version_Scaffold_2_00_06, Proteome Software Inc., Portland, OR) was used to validate MS/MS-based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 20.0% probability as specified by the Peptide Prophet algorithm [16]. Manual validation of tandem MS data of each HNE-modified peptide was performed to discard false positives and accept false negatives, if any. Protein identifications, where protein probabilities were assigned by the Protein Prophet algorithm [17], were accepted if they could be established at greater than 99.0% probability and contained at least 2 identified peptides.

Mass spectrometric analysis of HNE-modified COX tryptic digests allowed for the detection of all subunits except VIc and VIII of the enzyme complex. *In vivo* incubation of COX with HNE resulted in the modification of several sites occurring in different subunits. Figure 1 shows the schematic representation of the NL-MS³ method for the analysis of Schiff-base and Michael adducts. In this scheme, the neutral loss ion showing the designated difference (m/z 46 or 69 for Schiff-base and m/z 52 or 78 for Michael adducts) is selected for an additional round of fragmentation giving MS³ spectra. The neutral loss ion present in MS/MS spectra provides a signature tag of modification type (Schiff-base or Michael addition), whereas the extensive fragment ions observed in MS³ spectra facilitates correct identification of the peptide sequence.

The results in Figure 2 obtained by data-dependent acquisition and NL-driven MS³ technique show that implementation of the latter technique improved the detection and identification of a Schiff-base modified site in the tryptic peptide KGQDPYNILAPK of COX subunit Vb. Figure 2a shows the MS/MS spectrum of $[M + 2H]^{2+}$ ion at m/z 672.4 of the unmodified peptide KGQDPYNILAPK. The Mascot ion score and identity score were 48.5 and 34.3, respectively, corresponding to a 95% peptide identification probability in Scaffold software. Figure 2b shows the MS/MS spectrum of $[M + 2H]^{2+}$ ion at m/z 741.4 of the HNE-modified peptide KGQDPYNILAPK. The Mascot ion score and identity score were 10.0 and 34.0, respectively, corresponding to an 88% peptide identification probability with the site of modification localized at the N-terminal lysine. In the MS/MS spectrum, the peak at m/z 672.47 was falsely labeled as an internal fragment (PYNILA) instead of a neutral loss ion by the Scaffold program, which was apparently the reason for the lower Mascot ion score. However, the neutral loss ion at m/z 672.47 indicated the presence of a Schiff-base adduct, as the mass difference between the neutral loss ion and precursor ion was 138 Da (Figure 2b). Implementation of the NL-driven MS³ technique allowed for an additional round of CID-based fragmentation on the neutral loss ion at m/z 672.47 yielding enough fragment ions (Figure 2c) to improve the search algorithm score to an acceptable probability threshold for correct peptide identification. The Mascot ion score of the MS³ spectrum was 49.2 with the Mascot identity score being 47.2 corresponding to a 95% peptide identification probability. Although there was no direct evidence for the site of modification in the MS/MS and MS³ spectra, the site-chain amino group of the N-terminal Lys was the only plausible site for HNE attachment in this tryptic peptide according to the chemistry of HNE modifications in proteins (Scheme 1) and considering the trypsin-based bottom-up methodology. Localization of a Schiff-base on the C-terminal residue had not been an acceptable alternative in this regard, because trypsin would have been unable to cleave after a modified Lys. On the other hand, the N-terminal Lys in KGQDPYNILAPK is preceded by arginine (Arg, R) in the COX subunit Vb; therefore, the observed cleavage between Arg and the Schiff-base modified Lys complied with the selectivity of the proteolytic enzyme employed.

We observed only a single site of a Schiff-base HNE adduct in the COX enzyme complex. However, the reaction of COX with HNE is dominated by Michael addition products (+156 Da) corroborating the prevalence of the latter type of adducts observed previously [3].

Thirteen sites of Michael addition were identified in eight different COX subunits (II, IV, Va, Vb, VIa, VIb, VIIa and VIIc; Table 1) after our *in vitro* experiment. MS/MS and/or MS³ spectra for the corresponding modified peptides were provided in the Supporting Information. HNE modification of subunits II, IV, Vb, VIIa and VIIc by Michael addition were in agreement with a previous report [11]. We were not able to detect subunit VIII (modified or unmodified) by our method. The 4,962-Da COX subunit VIII have been reported to undergo HNE modification at its His-36 residue, which is associated with the loss of activity for COX [11]. However, the assignment was made by off-line “top-down” technique after extensive effort to isolate the HNE-modified protein. On the other hand, our “bottom-up” shotgun approach involving online LC–tandem mass spectrometry has identified, along with 12 additional sites for covalent attachment of the lipid peroxidation product, site-specific modification in subunit VIIc (His-2 of the mature protein [18] based on identifying the HNE-modified tryptic peptide SH*YEEGPGK, Table 1) also shown to contribute to the significant loss of COX activity upon exposure to HNE [11].

Michael addition of HNE mostly targeted His residues of COX proteins, except for subunit Va where modifications occurred at Lys and Cys residues, respectively, based on our identification of WVTYFNK*PDIDAWELR and SLC*PISWVSTWDDR among the modified tryptic peptides. From the mass spectrometric characterization of HNE modification sites in COX, nearly all modified peptides produced neutral losses and, hence, the NL-driven MS³ technique was helpful in increasing the search algorithm scores to acceptable probability thresholds for correct peptide identification.

Some HNE-modified peptides could be identified based only on the MS³ spectra, while others were identified by MS/MS spectra in addition to the corresponding MS³ spectra, ultimately increasing the identity score assigned by the Mascot search algorithm. Two HNE-modified peptide fragments from subunit Va of COX, WVTYFNKPDIDAWELR and SHGSHETDEEFDAR[@] (Table 1), were identified solely by the NL-MS³-based technique. However, the major limitation of the NL-driven MS³ technique is that HNE provides no diagnostic mass difference upon neutral loss that may result in ambiguity when more than one possible candidate sites are present within the peptide. In the tryptic peptide WVTYFNK*PDIDAWELR, the HNE modification site can be assigned to Lys, as this is the only probable site of modification. However, the MS³ spectrum does not identify which His is modified in the tryptic peptide SHGSHETDEEFDAR. Electron capture dissociation (ECD) that provides complementary fragmentation information and retains labile modifications during fragmentation could potentially overcome this limitation through neutral-loss triggered ECD (NL-ECD-MS/MS) [19]. In this technique, CID would provide sequence information for unmodified peptides and, in the case of HNE-modified peptides that exhibit a HNE-neutral loss, ECD is initiated which in turn facilitates the identification of the exact site containing the HNE group. Nevertheless, the Schiff-base adduct in the tryptic peptide K*GQDPYNILAPK of subunit Vb is a novel HNE modification site identified by our NL-MS³ technique reported here.

In conclusion, our results show that the NL-driven MS³ data dependent acquisition technique can be a valuable tool in “bottom-up” characterization of even low-abundance Schiff-base adducts of HNE to proteins, in addition to the predominant Michael addition products. The method can possibly be equally effective in identifying these type of adducts involving other reactive carbonyl compounds such as 4-hydroxy-2-hexenal or 4-oxo-2-nonenal. Modified peptides of low abundance could apparently be missed by the reported data-dependent acquisition strategy, albeit the functional significance of the corresponding modifications would probably be diminished. Beyond proving the applicability of NL-driven MS³ to detect Schiff-base adducts, we propose that our approach may also add an important control to the experiments probing posttranslational protein modifications by HNE or related

lipid peroxidation end-products. Specifically, identification of low-prevalence Schiff-base adducts [3,4] among the modified peptides could indicate that the majority of the abundant modification sites bearing potential consequences on function are found for the proteins and sequences covered by the employed shotgun analysis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

COX	Cytochrome c oxidase
HNE	4-Hydroxy-2-nonenal
NL	neutral loss

Figure 1.
Schematic representation of the NL-MS³ method for the MS analysis of Schiff-base and Michael adducts.

Scheme 1.
Chemistry of HNE-modifications for proteins.

Table 1

HNE-modified Cytochrome c oxidase tryptic peptides derived from bovine heart mitochondria and identified by online RP-HPLC combined with conventional data-dependent and NL-driven MS³ acquisition on a hybrid LTQ-FT mass spectrometer.

IPI accession number	COX subunits	HNE-modified peptide sequence ^{a)}	Method of identification ^{b)}	Mechanism of reaction ^{c)}
IPI00708779	II	MLVSSEDVLH*SWAVPSLGLK	++	MA
IPI00714240	IV	AH*GSVVK	+	MA
		DYPLPDVAH*VK	++	MA
IPI00691338	Va	WVTYFNK*PDIDAWELR	+++	MA
		SHGSHETDEEFDAR@	+++	MA
IPI00694849	Vb	LVPH*QLAH	++	MA
		(alkC)PS(alkC)GTH*YK	++	MA
		K*GQDPYNILAPK	++	SB
IPI00689990	VIa	GDH*GGTGAR	+	MA
		ERPAFIPYH*HLR	++	MA
IPI00709627	VIb	N(alkC)WQNYLDFH*R	++	MA
		SLC*PISWVSTWDDR	++	MA
IPI00717407	VIIa	LFQEDNGLPVH*LK	++	MA
IPI00694475	VIIc	SH*YEEGPGK	++	MA

^{a)} Abbreviations:

* Site of HNE-modification

@ MS³ spectra were unable to identify which His was modified.

^{b)} Abbreviations:

+ HNE-modified peptide identified only by data dependent acquisition.

++ HNE-modified peptide identified both by data-dependent and NL-driven MS³ acquisition method.

+++ HNE-modified peptide identified only by NL-driven MS³ method.

^{c)} Refer to Scheme 1.