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Utilization of LC-MS/MS Analyses to Identify Site-Specific Chemical Protein Adducts *In Vitro*

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Abstract

Biologically reactive intermediates are formed following metabolism of xenobiotics, and during normal oxidative metabolism. These reactive species are electrophilic in nature and are capable of forming stable adducts with target proteins. These covalent protein modifications can initiate processes that lead to acute tissue injury or chronic disease. Recent advancements in mass spectrometry techniques and data analysis has permitted a more detailed investigation of site-specific protein modifications by reactive electrophiles. Knowledge from such analyses will assist in providing a better understanding of how specific classes of electrophiles produce toxicity and disease progression via site-selective protein-specific covalent modification. Hydroquinone (HQ) is a known environmental toxicant, and its quinone–thioether metabolites, formed via the intermediate generation of 1,4-benzoquinone (1,4-BQ), elicit their toxic response via the covalent modification of target proteins and the generation of reactive oxygen species. We have utilized a model protein, cytochrome *c*, to guide us in identifying 1,4-BQ- and 1,4-BQ-thioether derived site-specific protein modifications. LC-MS/MS analyses reveals that these modifications occur selectively on lysine and glutamic acid residues of the target protein, and that these modifications occur within identifiable “electrophile binding motifs” within the protein. These motifs are found within lysine-rich regions of the protein and appear to be target sites of 1,4-BQ-thioether adduction. These residues also appear to dictate the nature of post-adduction chemistry and the final structure of the adduct. This model system will provide critical insight for *in vivo* adduct hunting following exposure to 1,4-BQ-thioethers, but the general approaches can also be extended to the identification of protein adducts derived from other classes of reactive electrophiles.

Keywords

Cytochrome *c*; 1,4-Benzoquinone; LC-MS/MS; 2-(*N*-acetylcystein-S-yl)-1,4-benzoquinone; Post-translational modifications; Trypsin solution digest

1. Introduction

Metabolism of xenobiotics leads to formation of a variety of reactive intermediates. Similar reactive intermediates can be formed as by-products of cellular metabolism, including reactive oxygen and nitrogen species, as well as reactive dicarbonyl degradation products, such as methylglyoxal (1). Additionally, many of these reactive intermediates damage cellular membranes, resulting in lipid peroxidation, which results in the formation of several

α,β -unsaturated aldehydes, including 4-hydroxynonenal (4-HNE) (2). Many reactive aldehydes are generated endogenously during glycation, amino acid oxidation, and lipid peroxidation, and contribute to disease progression, including diabetes and atherosclerosis (3, 4). The majority of reactive intermediates are electrophilic in nature, which allow them to adduct nucleophilic residues within target proteins. α,β -Unsaturated aldehydes form stable covalent adducts with cysteine, histidine, and lysine residues (2, 5, 6). Other reactive electrophiles, such as quinones, produce toxic effects as a result of covalent protein adduction and the generation of reactive oxygen species. It is important to note that these two effects of quinone are not mutually exclusive, since protein-bound quinones can remain redox active. Quinones form protein adducts with nucleophilic residues within target proteins, including lysine, cysteine, and histidine residues (7–10). Because reactive electrophiles are capable of forming covalent adducts with proteins, they may subsequently alter the structure and function of target proteins. Such functional alterations may include interference with protein–protein interactions and subcellular protein localization, and disruption of cellular signaling pathways.

To assess the impact of electrophile-derived covalent protein adducts on protein structure and function, electrophiles with known toxicity were utilized. Hydroquinone (HQ), and its thioether metabolites, produce renal proximal tubular cell necrosis and are nephrocarcinogenic in rats. The adverse effects of these chemicals are in part a consequence of their oxidation to the corresponding electrophile 1,4-benzoquinone (1,4-BQ), the electrophilic nature of which facilitates conjugation with glutathione (GSH) via the nucleophilic cysteine free sulfhydryl (10–13). The subsequent metabolism of 1,4-BQ-GSH conjugates via the mercapturic acid pathway results in the eventual formation of 2-(*N*-acetylcystein-*S*-yl)hydroquinone (NAC-HQ), which upon oxidation gives rise to 2-(*N*-acetylcystein-*S*-yl)-1,4-benzoquinone (NAC-BQ) (14).

Cytochrome *c* has been studied as a model protein to characterize NAC-BQ-mediated site-specific covalent adduction. We have determined that specific motifs within target proteins (“electrophile binding motifs”) predispose these proteins to chemical adduction. We have chemically reacted NAC-BQ with cytochrome *c*, purified the protein adduct solution, and subsequently utilized LC-MS/MS and data analysis to identify the specific sites at which amino acid modifications occur. The analysis also reveals insights into the resulting post-adduction chemistry. This model system will provide critical insight for *in vivo* adduct hunting following exposure to 1,4-BQ-thioethers, but the general approaches can also be extended to the identification of protein adducts derived from other classes of reactive electrophiles.

2. Materials

2.1. Single Protein Reacted with Chemical in Solution

2.1.1. Single Protein Reaction

1. NAC-BQ synthesized and purified (see Chapter 18).
2. Cytochrome *c* reaction buffer: 10 mM Tris-HCl, pH 7.5 used with horse heart cytochrome *c* (Sigma). Store cytochrome *c* at -20°C in a dessicator.

3. Microcon 3,000 Da molecular weight cut-off centrifugal filter (Millipore).
4. Voyager MALDI-TOF sample plate laser etched stainless steel, 100-position (Applied Biosystems).

2.1.2. LC-MS/MS Sample Preparation

1. pH-indicator strips (EMD chemicals).
2. Digestion buffer(s): 50 mM Tris-HCl, pH 7.5 or 50–100 mM NH_4HCO_3 , pH 8.0.
3. 2 M DTT stock solution: 154.3 mg DTT (Sigma) in 500 μl deionized distilled water (see Note 1).
4. 10 mM DTT solution for reduction: 5 μl 2 M DTT in 1 ml of 0.1 M NH_4HCO_3 (Sigma).
5. 55 mM Iodoacetamide (Sigma) stock solution: 10 mg in 1 ml 0.1 M NH_4HCO_3 .
6. Trypsin modified sequencing grade (Promega): 20 μg lyophilized powder. Store at -20°C . Dilute to 0.1 $\mu\text{g}/\mu\text{l}$ and can be stored in solution at -20°C for several weeks.

2.1.3. LC-MS/MS Analysis

1. ThermoFinnigan LTQ mass spectrometer (San Jose, CA) equipped with a Michrom Paradigm MS4 HPLC, a SpectraSystems AS3000 autosampler, and a nanoelectrospray source.

3. Methods

3.1. Single Protein Reacted with Chemical in Solution

Purified quinone–thioether compounds, including NAC-BQ, can be used in a reaction with pure cytochrome *c* from horse heart to determine site-specific adductions by these compounds, specifically, NAC-BQ on cytochrome *c* (see Note 2). Furthermore, this will guide us in identifying target residues and any resulting post-adduction chemistry as a result of NAC-BQ adduction.

3.1.1. Chemical Reaction with Single Protein

1. Horse heart cytochrome *c* (1 mg) is dissolved in 10 mM Tris-HCl pH 7.5 (1 ml) (see Note 3). The cytochrome *c* solution is aliquoted (100 μl) prior to NAC-BQ reaction for use as a control sample.

¹The 2 M DTT stock solution can be stored at -20°C for 4 weeks or can be made fresh for each use.

²Alternative pure proteins can be used to study these site-specific quinone–thioether modifications; however, cytochrome *c* was used here because its small size and structural characterization is ideal for mass spectral analysis.

³A buffering system is used that consists of 10 mM Tris-HCl, pH 7.5, and NAC-BQ adduct formation is much more efficient in this buffer than in water. This is likely because many of the NAC-BQ-adducts form on Lys residues and these residues are more nucleophilic in buffered systems. NH_4HCO_3 (0.1 M, pH 8.0) is used by other researchers doing proteomics work because this is the ideal buffer for trypsin digestion; however, because of the high salt concentration in addition to the high pH of this buffer, the *N*-acetylcysteine bond to the benzoquinone ring is not stable in these conditions for periods of time exceeding 6 h. This compound degradation results in adduction profiles that may not be accurate, and may also inhibit certain residues, including Cys, as being target residues for modifications by these compounds.

2. The cytochrome *c* solution is reacted with dry NAC-BQ at a 1:10 molar ratio at room temperature for 30 min to 1 h (see Note 4).
3. The mixture is filtered once through a Microcon 3,000 Da molecular weight cut-off centrifugal filter for 20 min at $13,000 \times g$ to remove excess NAC-BQ. The reaction mixture is then washed with 1 ml of 10 mM Tris-HCl, pH 7.5, and centrifuged as above (see Note 5).
4. Once complete, the filter is turned upside down in a new filter tube and centrifuged for 2 min at $2,000 \times g$ to collect the remaining protein solution that was on the filter. Measure the volume to determine the protein concentration, as all of the NAC-BQ-reacted protein and the native protein should remain on top of the filter because these have a mass of greater than 3,000 Da. The unreacted NAC-BQ should pass through the filter as it has a molecular mass of 269 Da. By collecting the solution remaining on the filter, this will be the solution containing the reacted protein and will be used for further analysis (see Note 6).
5. The control cytochrome *c* solution and the NAC-BQ-reacted cytochrome *c* solution will be spotted on the MALDI target plate to determine the adduction profile before proceeding forward with LC-MS/MS analysis (described in detail in Chapter 18).

3.1.2. Solution Digest of Single Protein Samples

1. Use both control and NAC-BQ-adducted samples and treat them equally throughout. Because these samples are approximately 1 mg/ml, it is sufficient to take 10 μ l from these to proceed with the following steps. This will provide approximately 10 μ g of protein for each sample (see Note 7).
2. Most successful proteins digests conducted using trypsin as the proteolytic enzyme occur using 50 mM Tris-HCl, pH 7.5 or 50–100 mM NH_4HCO_3 as the buffering system. These buffers are usually in the pH range of 7.4–8.0. This is ideal for trypsin digestion to occur (see Note 8).
3. The samples described here are in 10 mM Tris-HCl pH 7.5 buffering system, so in this case, the buffering system should be sufficient. However, in the event where the proteins of interest are in different buffering systems, it is important to spike in NH_4HCO_3 or Tris-HCl pH 7.5 to a final concentration of 50 mM Tris-HCl pH 7.5

⁴The reaction can proceed for longer than 1 h; however, because these quinol-thioether compounds are not stable in high salt or high pH conditions for periods of time exceeding 6 h, it is safe to keep the reaction under 1 h and the adduction efficiency will be sufficient. Therefore, sample preparation time is critical for accurate adduct mass analysis. When the NAC-BQ compound is stable, it will form adducts with a mass of 268 Da. Following NAC-BQ adduction and thioether bond elimination due to microenvironment instability, the resulting adduct mass will be 105 Da. This mass is indicative of the benzoquinone ring (7).

⁵This centrifugation procedure can be modified depending upon whether it is conducted at room temperature or 4°C, where the centrifugation can be extended in conditions of 4°C. Additionally, to ensure complete removal of excess NAC-BQ, the reaction mixture can be washed repeatedly with buffer and further centrifugation can be conducted.

⁶Alternative methods can be used to remove excess NAC-BQ, including dialysis and HPLC purification; however, the method used here seems to minimize sample preparation time which is critical for use with quinol-thioether adduction studies.

⁷It is important to make sure that all samples are free of detergents before proceeding with trypsin digestion and this can be achieved by protein precipitation methods.

⁸Additional enzymes can be used for protein digestion, including chymotrypsin, endoproteinase Asp-N, and Glu-C, all of which have specific pH ranges where they work best. Trypsin is the most common choice for proteomic procedures, so this is described here.

or 50–100 mM NH_4HCO_3 pH 8. Try to keep the volume as low as possible in this step, so use small amounts of buffer when possible.

4. For proteins that have disulfide bonds, a reduction/alkylation process will be necessary prior to trypsin digestion. These reduction/alkylation steps need to take place in the dark (see Note 9).
5. Add 40 μl of 10 mM DTT stock solution to both control and treated samples and incubate at 56°C for 45 min.
6. Remove samples from heat and allow cooling to room temperature.
7. Make a 55 mM Iodoacetamide stock solution and add 40 μl of this stock solution to both control and treated samples. Incubate at room temperature for 30 min.
8. The ratio of trypsin to sample should be 1:50 to 1:20 (1 mg trypsin to every 50–20 mg protein). Add the appropriate amount of trypsin to control and treated samples. Gently flick the tubes to mix.
9. Place the samples in 37°C water bath for 2 h and after 2 h, add an additional amount of trypsin to each sample, equal to the first amount added.
10. Continue to incubate at 37°C for 17–18 h to ensure complete digestion (see Note 10).
11. Trypsin digestion reaction can be stopped by freezing samples at –20°C, or 4 μl of glacial acetic acid can be added.
12. Reduce the volume of the samples to approximately 10 μl by vacuum concentration.

3.2. LC-MS/MS Analysis

1. LC-MS/MS analyses of protein digests are carried out using a linear quadrupole ion trap ThermoFinnigan LTQ mass spectrometer (San Jose, CA) equipped with a Michrom Paradigm MS4 HPLC, a SpectraSystems AS3000 autosampler, and a nanoelectrospray source (15). Peptides are eluted from a 15 cm pulled tip capillary column (100 μm I.D. $\times$ 360 μm O.D; 3–5 μm tip opening) packed with 7 cm Vydac C18 (Hesperia, CA) material (5 μm , 300 Å pore size), using a gradient of 0–65% solvent B (98% methanol/2% water/0.5% formic acid/0.01% trifluoroacetic acid) over a 60-min period at a flow rate of 350 nl/min. The LTQ electrospray positive mode spray voltage is set at 1.6 kV, and the capillary temperature at 180°C.
2. Dependent data scanning is performed by the Xcalibur v 1.4 software (16) with a default charge of 2, an isolation width of 1.5 amu, an activation amplitude of 35%, activation time of 30 ms, and a minimal signal of 100 ion counts. Global dependent

⁹Reduction steps improve digestion efficiency by preventing disulfide formation, which helps the protein to be unfolded, exposing more tryptic sites for digestion. In cases like cytochrome *c*, reduction is not necessary, as there are no free cysteine residues present in the protein. The reduction and alkylation steps in this case can be skipped.

¹⁰As stated previously, the quinone–thioether compounds have been found to be unstable in basic conditions, as well as conditions with high pH. As a result, when using compounds of this nature, the digestion procedure needs to be limited to approximately 3 h, which will minimize quinone–thioether compound exposure to the basic buffering system necessary for trypsin digestion. Efficient digestion can be accomplished in 3 h, especially when using a small pure protein such as cytochrome *c*.

data settings are as follows, reject mass width of 1.5 amu, dynamic exclusion enabled, exclusion mass width of 1.5 amu, repeat count of 1, repeat duration of 1 min, and exclusion duration of 5 min. Scan event series includes one full scan with mass range 350–2,000 Da, followed by three dependent MS/MS scans of the most intense ion.

3.3. Data Analysis

1. Database Searching: Tandem MS spectra of peptides are analyzed with TurboSEQUENT™ v3.1, a program that allows the correlation of experimental tandem MS data with theoretical spectra generated from known protein sequences (17). The peak list (dta files) for the search is generated by Bioworks 3.1. Parent peptide mass error tolerance is set at 1.5 amu and fragment ion mass tolerance is set at 0.5 amu during the search. The criteria used for preliminary positive peptide identification are the same as previously described, namely peptide precursor ions with a +1 charge having an Xcorr > 1.0, +2 Xcorr > 2.5, and +3 Xcorr > 3.5. A dCn score > 0.08 and a fragment ion ratio of experimental/theoretical >50% were also used as filtering criteria for reliable matched peptide identification (18).
2. All matched peptides are confirmed by visual examination of the spectra. All spectra are searched against current ipiRAT v3.22 database from EMBL (see Note 11). Tandem MS spectra of peptides are also analyzed with X!Tandem (<http://www.thegpm.org>; version 2007.01.01.1), which is similar to Sequest, and correlates the MS/MS spectra with amino acid sequences in a user-specified NCBI database (19, 20). X!Tandem is set up to search a subset of the ipi.RAT.v3.31 database also assuming trypsin. Sequest is set up to search the ipi.RAT.v3.31.fasta database assuming the digestion enzyme trypsin. Sequest and X!Tandem are searched with a fragment ion mass tolerance of 0.50 Da and a parent ion tolerance of 1.5 Da. Modifications including +105 (BQ), and +268 (NAC-BQ), at Lys, Arg, and Glu are specified in Sequest and X!Tandem as variable modifications (see Note 12).
3. P-Mod software is used to confirm X!Tandem and Sequest data, including the identification of spectra displaying characteristics of 1,4-BQ or NAC-BQ adductions. This program is extremely beneficial when using pure proteins and can provide reliable data without use of additional programs; however, having multiple routes to find the same data is preferred. The protein sequence of cytochrome *c*, CYC_HORSE P00004, is obtained from the NCBI database (<http://www.ncbi.nlm.nih.gov>). P-Mod is an algorithm that screens data files for MS/MS

¹¹Because cytochrome *c* used here is a pure protein, data analysis can be done against the cytochrome *c* database rather than the entire rat database. The trypsin is sequencing grade and there should not be much interference.

¹²Again, as stated previously, the sample preparation can influence the adduction profile of these quinone–thioether compounds. Additionally, results have indicated that the microenvironment of the protein can also influence the adduction profile of these quinone–thioether compounds. As a result, after NAC-BQ adduction to cytochrome *c*, regions of the protein with higher pKa have directed the post-adduction chemistry from a full NAC-BQ adduct (268-Da adduct) to a 1,4-BQ adduct (105-Da adduct), indicating elimination of the thioether bond under these types of conditions (7). Also, additional modifications can be searched depending upon the residues of interest. Typically, cysteine is a target for modification; however, in the case of cytochrome *c*, there are no free cysteines, and as a result, this residue is not included in the modification list shown here. Modifications including oxidation of methionine residues and phosphorylation of serine or threonine residues can also be included.

spectra corresponding to peptide sequences in a search list. Modification of the primary peptide sequence shifts the peptide mass, which may be experimentally observed as a difference between the measured mass of the modified peptide precursor ion (adjusted for charge state) and the predicted mass of the unmodified peptide. The mass shift also will be observed in the m/z values of fragment ions containing the modified amino acid. Scores with P -values greater than 0.01 were discarded as false positives (21). Upon collision-induced dissociation (CID) of the peptides, b- and y-ion fragments are generated: the b-ion series represents cleavage of the peptide bond with charge retention on the N-terminal piece, and y-ions result from cleavage of the amide bonds with charge retention on the C-terminal piece (22). Manual validation of MS/MS spectra was then used to confirm peptide sequence and adduct mass location. Peptides identified as being adducted by both X!Tandem, Sequest, and P-Mod were then manually validated using the program IonGen (7). IonGen generates theoretical b- and y-ions for user-specified peptides containing an adduct. This program facilitates faster, more accurate validation of adducted peptides. Only adducted peptides identified from X!Tandem, P-Mod, and IonGen, are used. Figure 1 shows a NAC-BQ-modified peptide from cytochrome *c*.

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Fig. 1.

LC-MS/MS spectra of the NAC-BQ modified cytochrome *c* peptide. The LC-MS/MS raw data was analyzed by Sequest, X!Tandem, and P-Mod, followed by manual validation. The peptide $^{39}\text{KTGCQAPGFYTDANK}^{53}$ was identified with a 268-Da adduct on K^{39} .