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RESEARCH ARTICLE

Characterization of the Cysteine Content in Proteins Utilizing Cysteine Selenylation with 266 nm Ultraviolet Photodissociation (UVPD)

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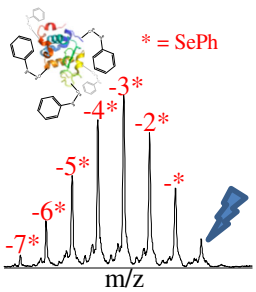

Abstract. Characterization of the cysteine content of proteins is a key aspect of proteomics. By defining both the total number of cysteines and their bound/unbound state, the number of candidate proteins considered in database searches is significantly constrained. Herein we present a methodology that utilizes 266 nm UVPD to count the number of free and bound cysteines in intact proteins. In order to attain this goal, proteins were derivatized with *N*-(phenylseleno)phthalimide (NPSP) to install a selectively cleavable Se–S bond upon 266 UVPD. The number of Se–S bonds cleaved upon UVPD, a process that releases SePh moieties, corresponds to the number of cysteine residues per protein.

Keywords: Cysteine, Photodissociation, Selenylation

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Introduction

Cysteine is involved in both stabilizing tertiary protein structure through disulfide bonding and modulation of protein redox activity [1]. There have been numerous studies aimed at characterization of the redox states of cysteines in proteins owing to the inherent importance of cysteine-mediated chemistry in countless biological processes. Methods such as UV absorption spectroscopy [2], fluorescent labeling [3], and X-ray absorption spectroscopy [4] have been used to quantify or characterize the cysteine content of proteins. Characterizing cysteine content by mass spectrometry has also become a popular option as a consequence of the development of well-established bottom-up proteomics approaches [5–7] often in combination with various clever cysteine-selective derivatization methods [8]. MS strategies for determination of cysteines have been based on utilization of mass tags [9] or isotopic labels [10, 11], differential monitoring of ESI mass spectra after cysteine-selective reactions [12], proteolysis in isotopically heavy solvents [13], characterization by high resolution top down MS/MS [14], or utilization of selective ion/ion reactions [15, 16], all of which have proven to be versatile methods for either qualitative or quantitative characterization of cysteine content of proteins and peptides. Electrochemical tagging reactions of cysteines have also been implemented via coupling an electrochemical cell in an on-line fashion to a mass spectrometer [17–21]. The latter methodologies have even shown promise for mapping reactivities of cysteine residues in different locations of proteins [22]. Chemical derivatization of cysteine residues in peptides and proteins by reactions with quinone has been shown to differentiate free thiols versus disulfide bonds [23]. For this prior study, 266 nm UVPD was used to promote homolytic cleavage of C–S bonds of quinone-derivatized proteins and peptides, generating neutral loss products that were subjected to CID to achieve radical-directed dissociation (RDD) [23]. Selective cleavage of disulfide bonds to elucidate disulfide-linked peptide pairs has also been shown to be effective using 266 nm photodissociation [24].

N-(phenylseleno)phthalmide (NPSP) is a well-known selenylating reagent [25, 26]. Fast, efficient, and selective selenylation of cysteine-containing species has been achieved previously using NPSP (Scheme 1) [27–29]. Additionally, on-line electrolytic cleavage of disulfides via an electrochemical cell prior to reactions with NPSP and mass spectrometry analysis has been reported as a means to identify disulfide-containing peptides in digests [30]. Herein we present a strategy for counting cysteine residues in proteins based on NPSP-based selenylation followed by 266 nm UVPD to selectively and exclusively cleave the tags. The cysteine content information can be utilized in conjunction with informatics engines such as UniProt to generate a list of

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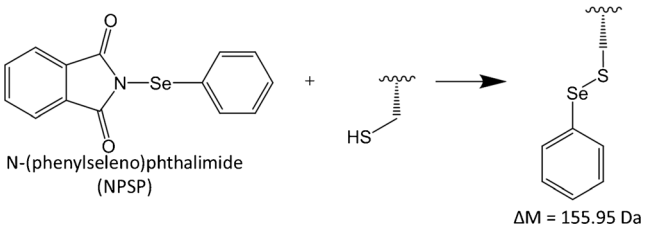

Scheme 1. Scheme showing reaction of *N*-(phenylseleno)phthalimide (NPSP) with a free thiol

candidate proteins from a proteome that match the cysteine content observed experimentally.

Methods

Sample Preparation

Proteins containing up to eight cysteines were analyzed in three ways: (1) as intact proteins prior to NPSP derivatization, (2) derivatization with NPSP without reduction of intrinsic disulfide bonds, and (3) derivatization with NPSP after reduction of disulfide bonds. For both (1) and (2), proteins were suspended at a concentration of 10 μM in 50:50 H_2O:acetonitrile and 1% formic acid. For (2), the proteins were reacted with 10 mM NPSP dissolved in dry acetonitrile (typically using 200 uL of protein solution and 2 uL of NPSP solution) for 30 s prior to analysis by mass spectrometry (without clean-up). For (3), proteins were reduced by incubation with 10 mM DTT in 150 mM NH_4HCO_3 for 3 h at 55 °C (typically using 200 uL of protein solution and 2 uL of DTT solution). Following reduction, proteins were buffer-exchanged three times into 1% formic acid using Amicon Ultra Centrifugal Filters (Merck Millipore, Billerica, MA, USA). Proteins were then diluted by addition of one volume 1% formic acid in acetonitrile and incubated with 10 mM NPSP for 30 s. The final protein solutions were analyzed without further clean-up.

Mass Spectrometry

Mass spectrometry experiments were undertaken in positive mode on a Thermo Scientific Velos Pro dual-pressure linear ion trap mass spectrometer (San Jose, CA, USA) equipped with CID, HCD, ETD, and 266 nm UVPD capabilities. Each spectrum consisted of 3 μ scans averaged. The applied ESI spray voltage was 3 kV. Tandem mass spectrometry was carried out in the high pressure trap for all activation strategies using the most abundant ion of the charge state envelope. For CID and HCD a normalized collision energy of 35 NCE was used (during an activation period of 10 ms for CID and 2 ms for HCD), whereas for ETD a reaction time from 40 to 120 ms was utilized. UVPD was implemented in a manner described previously [31] and was performed using the fourth harmonic of a Continuum Minilite Nd:YAG laser (San Jose, CA, USA) with an energy output of approximately 6 mJ per pulse. Ions were subjected to an increasing number of 266 nm laser pulses until either the precursor was completely eliminated or no additional neutral losses were observed. Post-acquisition, data analysis was assisted by charge state deconvolution software, MagTran [32]. Bovine β-lactoglobulin, bovine α-lactalbumin, chicken lysozyme, bovine ribonuclease A., bovine aprotinin, horse cytochrome *c*, and bovine serum albumin (BSA) were used in this study.

Results and Discussion

ESI-MS Analysis

Derivatization of each cysteine residue with NPSP results in a mass shift of 156 Da by the addition of a SeC_6H_5 moiety through formation of a S–Se bond as depicted in Scheme 1. Cysteine sites can be counted in several ways using this reactive tag. First, the net mass shift of a protein is readily observed in the MS1 mass spectrum. In Figure 1, this is observed for the β-

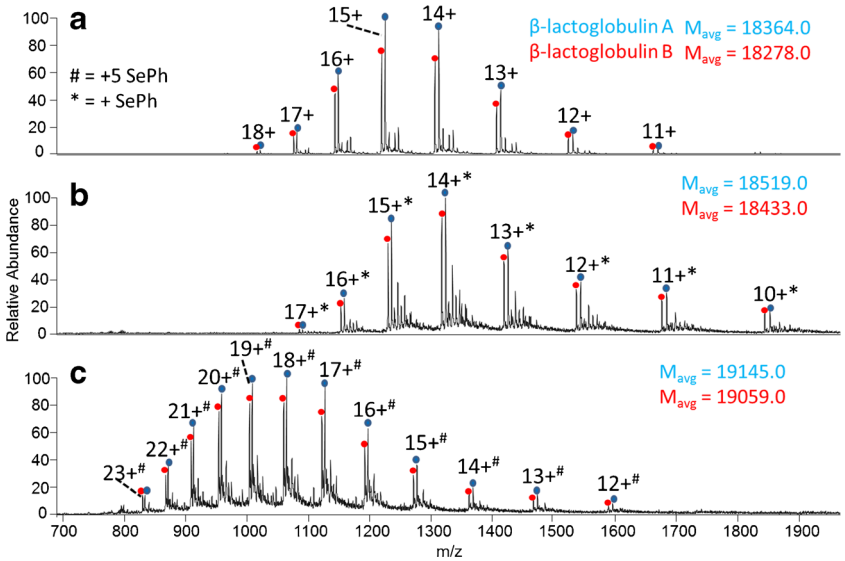

Figure 1. β-Lactoglobulin A (blue) and B (red) for **(a)** unmodified, **(b)** NPSP-modified, and **(c)** NPSP-modified after reduction. (# = + 5 SePh, * = + SePh)

lactoglobulin variants A and B. Upon deconvolution of the charge state envelope, an average molecular weight was determined. Compared with the underivatized proteins in Figure 1a, the ones in Figure 1b showed a mass shift of approximately 155 Da per protein, indicative of one reactive site (one free cysteine) for both A and B variants of β-lactoglobulin. When reduction was performed prior to derivatization of the proteins (Figure 1c), a more prominent mass addition was observed along with a shift to higher charge states. The shift to higher charge states was indicative of the cleavage of disulfide bonds that facilitated more extensive protonation of the protein. The net mass shift observed in Figure 1c for reduced β-lactoglobulin was 781 Da, corresponding to addition of five SePh tags. From these observations, it is deduced that there are five cysteine residues of which four are inaccessible until after reduction.

The same strategy was used for lysozyme (Supplementary Figure S1 in the electronic supporting information), ribonuclease A (Supplementary Figure S2), aprotinin (Supplementary Figure S3), α-lactalbumin (Supplementary Figure S4), cytochrome *c* (Supplementary Figure S5), and BSA (Supplementary Figure S6), and the findings are summarized in Table 1. In each case, the number of free cysteines and disulfides was correctly deduced based on monitoring the mass shifts upon reaction with NPSP prior to or after reduction of the protein. Ribonuclease A and aprotinin exhibited no reactions with NPSP prior to reduction, indicating the absence of free cysteines. After reduction and reaction with NPSP, ribonuclease A displayed a mass shift consistent with addition of eight SePh tags; for aprotinin it was addition of six SePh tags. For both ribonuclease A (Figure S2a, b) and aprotinin (Figure S3a, b), a +98 Da mass addition to the intact protein was observed in the mass spectra prior to NPSP reactions. This adduct was attributed to noncovalently bound sulfuric or phosphoric acid, which is known to interact strongly with surface accessible basic sites of the protein [33]. This adduct was easily dislodged in the cleanup step performed on each protein after reduction and prior to selenylation (Figure S2c, c) and did not impede the NPSP tagging strategy. For aprotonin, the smallest protein used in this study, a distinct broadening of the peaks in the charge envelope was observed in Figure S3c. This was due to the broad isotopic distribution of the selenium component of the tag and was not especially noticeable for the larger proteins because of their significantly higher charge states (and thus more compressed peak widths).

The NPSP tagging method indicated that α-lactalbumin had four disulfide-bound cysteines and no free cysteines and that cytochrome *c* had two disulfide-bound cysteines and no free cysteines (Table 1). The NPSP strategy was unsuccessful for characterization of BSA because of incomplete tagging, an outcome not unexpected owing to the fact that BSA has 35 cysteines. The method showed mixed success for lysozyme. Lysozyme has eight cysteines, all engaged in disulfide bonds. Upon reaction with NPSP, the resulting mass spectrum displayed a mass shift corresponding to attachment of one SePh tag, which suggested the presence of one free cysteine. This odd result may correspond to partial degradation of lysozyme, thus leading to cleavage of one disulfide bond in the intact protein. Although addition of two SePh tags might be expected for this scenario, it is possible that one cysteine remains inaccessible owing to the six other disulfide bonds in the protein. Upon

Table 1. Comparison of Theoretical and Experimental Cysteine Counting Results

Protein (AC) [Species]	Free cys (Uniprot)	Free cys (exp)	Total cys (Uniprot)	Total cys (exp)	Figures	% Proteome match
β-Lactoglobulin (P02754) [*Bos taurus*]	1	1	5	5	1, 2	0.11% (28 out of 24113)
Lysozyme (P00698) [*Gallus gallus*]	0	1	8	8	S1, 3	0.03% (6 out of 17691)
Ribonuclease A (P61823) [*Bos taurus*]	0	0	8	8 by mass 7 by UVPD	S2, 4	0.15% (36 out of 24113)
Aprotinin (P00974) [*Bos taurus*]	0	0	6	6	S3, S7, S10	0.31% (74 out of 24113)
α-Lactalbumin (P00711) [*Bos taurus*]	0	0	8	8	S4, 5	0.15% (36 out of 24113)
Cytochrome *c* (P00004) [*Equus caballus*]	0	0	2	2	S5, S8, S11	0.08% (19 out of 22718)
BSA[a] (P02769) [*Bos taurus*]	1	1	35	N/A	S6, S9	<0.01% (2 out of 24113)

All calculations are done based on the chain region of the protein as defined by UniProt. The percent proteome match signifies the percentages of proteins in each proteome that have the same cysteine content (free versus bound cysteines) as the protein listed in that row. The percent proteome match was calculated using a custom python script.

[a] Only the oxidized form of BSA was evaluated because it has only one free cysteine out of 35 total cysteines; the products formed after reduction of BSA would not be resolvable.

reduction of lysozyme and reaction with NPSP, the resulting mass shift was consistent with eight cysteines as expected.

Analysis by 266 nm UVPD

Sulfur-selenium bonds have been shown previously to be photolytically cleavable in solution, producing radical products [34]. Implementation of this type of photoreaction for ions in the gas phase using 266 nm photons in the present study resulted in exclusive cleavage of the S–Se bonds and loss of the SePh tags. Monitoring the loss of SePh tags was used as a second facile means to count the number of cysteine residues per protein (1) prior to reduction and (2) post-reduction.

Examples of the mass spectra obtained upon UVPD of the SePh-tagged proteins are shown in Figure 2 for β-lactoglobulin A and B. Irradiation of unmodified β-lactoglobulin with 266 nm photons (2 laser pulses, 6 mJ) resulted in no significant dissociation, as shown in Figure 2a and b for the A and B forms of the protein (15+ charge state), respectively. However, the SePh-modified proteins (Figure 2c–f) dissociated by loss of one SePh tag for the non-reduced proteins or by loss of five SePh tags for the reduced proteins upon 266 nm UVPD (two laser pulses, 6 mJ). Cleavage of the S–Se bonds is both extremely efficient and highly selective upon absorption of 266 nm photons, yielding a neutral loss of 156 Da per cleaved tag. For both variants of β-lactoglobulin, 266 nm UVPD indicated one free and four bound cysteine residues. This UVPD method was extended to lysozyme (Figure 3), ribonuclease A (Figure 4), aprotinin (Supplementary Figure S7), α-lactalbumin (Figure 5), cytochrome *c* (Supplementary Figure S8), and BSA (Supplementary Figure S9). Using multiple laser pulses facilitated removal of the SePh tags as shown for α-lactalbumin in Figure 5. The results of the UVPD strategy for counting free and bound cysteines are summarized in Table 1.

Among the proteins examined, ribonuclease A yielded discrepancies in the characterization of cysteine content via the UVPD method compared with the result obtained from the mass shift observed in the MS1 mass spectrum. For ribonuclease A, only seven neutral losses were observed with high confidence upon UVPD (Figure 4b), whereas the NPSP-modified protein contains eight tags and is thus expected to lose eight tags. One hypothesis to explain the fact that only seven out of eight Se–S bonds were cleaved in ribonuclease A arises from the presence of the aromatic amino acids tyrosine, tryptophan, and phenylalanine, all known to absorb 266 nm photons (with tryptophan and tyrosine having significantly larger photoabsorption cross-sections than phenylalanine). Consider the comparison of 266 nm UVPD spectra for NPSP-tagged β-lactoglobulin, lysozyme, ribonuclease A, and α-lactalbumin (Figures 3, 4, and 5). Each of these proteins has a number of aromatic residues (tryptophan/tyrosine/phenylalanine) that may absorb 266 nm photons: 2/4/4 (β-lactoglobulin), 6/3/3 (lysozyme), 0/6/3 (ribonuclease A), and 4/4/4 (α-lactalbumin) for tryptophan/tyrosine/phenylalanine residues. Based on absorbance profiles of amino acids in solution, it is anticipated that the photoabsorption cross-section for tryptophan in the gas phase is likely greater than that of tyrosine at 266 nm, and the photoabsorption cross-section for phenylalanine is expected to be rather low at 266 nm (these remarks are derived from solution profiles, not the gas phase [35]). Excitation energy transfer has been shown to occur between tryptophan or tyrosine and disulfide bonds, ultimately resulting in homolytic cleavage of the disulfide bond via an excited state [36]. We speculate that a similar phenomenon may occur for the NPSP-tagged proteins. For example, lysozyme and α-lactalbumin contain multiple tryptophan residues, which may

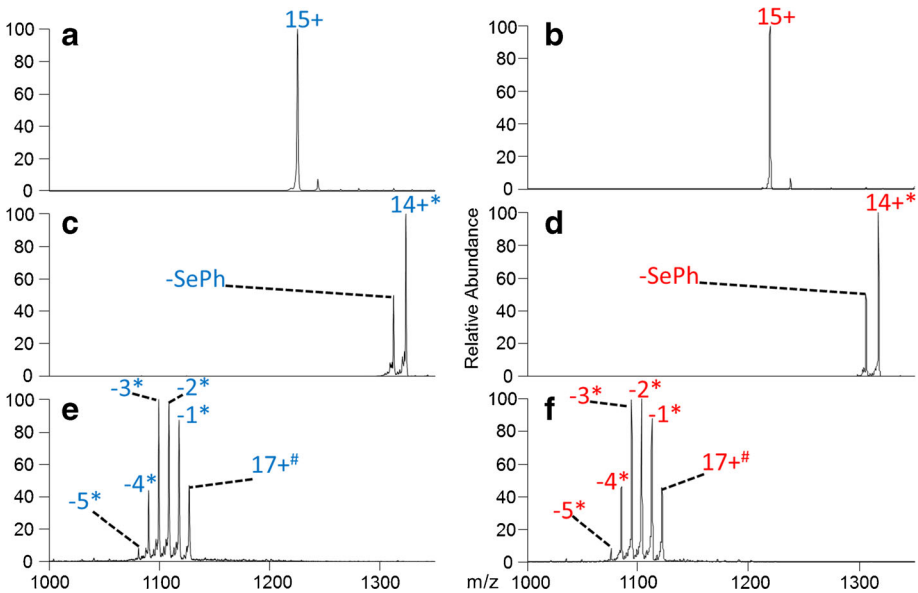

Figure 2. β-Lactoglobulin A (blue) and B (red) activated by two laser pulses (266 nm) as follows **(a)**, **(b)** unmodified, **(c)**, **(d)** modified with NPSP without reduction, and **(e)**, **(f)** modified with NPSP after reduction. (* = 1 SePh group, # = 5 SePh groups)

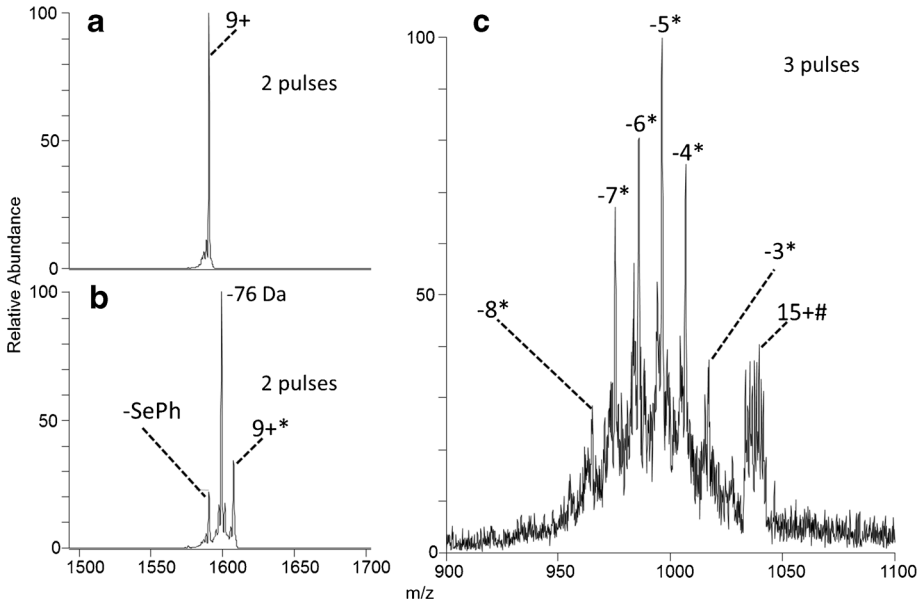

Figure 3. 266 nm UVPD mass spectra of lysozyme for **(a)** unmodified (9+), **(b)** NPSP-modified before reduction (9+), and **(c)** NPSP-modified after reduction (15+). (* = SePh, # = 8 SePh)

enhance S–Se bond cleavage from an excited state induced by absorption of 266 nm photons (similar to that shown for disulfide bonds [36]). The lack of tryptophan residues in ribonuclease A may explain the inhibition of tag loss for the protein. Additionally, lysozyme appears to have a less efficient SePh tag loss series than α-lactalbumin, yet both proteins contain the same number of cysteine residues. Lysozyme has six tryptophans in its primary sequence compared with α-lactalbumin, which has only four tryptophans, and this may contribute to a greater absorption cross-section for lysozyme and may lead to fragmentation by other pathways. While our results indicate that the photoabsorption cross-section of the benzeneselenol group is significantly greater than that of the aromatic side-chains at 266 nm, the availability of other absorbing moieties may inhibit S–Se cleavage by affording access to other fragmentation pathways, specifically pathways caused upon photoabsorption by the aromatic side-chains. The availability of other fragmentation pathways for lysozyme was further supported by activation of non-reduced SePh-tagged lysozyme (Figure 3b). For this protein, in addition to the characteristic SePh loss, an unexpected loss of 76 Da was also observed suggesting an alternative fragmentation route.

Other Activation Methods (HCD, ETD, CID)

It was previously reported that NPSP-derivatized peptides undergo Se–S cleavage upon ETD or CID [28]. Thus, for comparative purposes, collision-based (CID and HCD) and

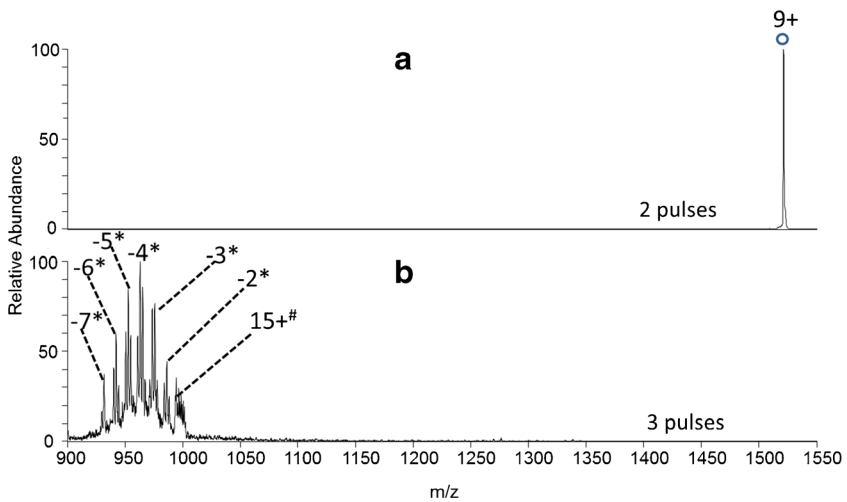

Figure 4. 266 nm UVPD mass spectra of ribonuclease A for **(a)** unmodified (9+) and **(b)** NPSP-modified after reduction (15+)

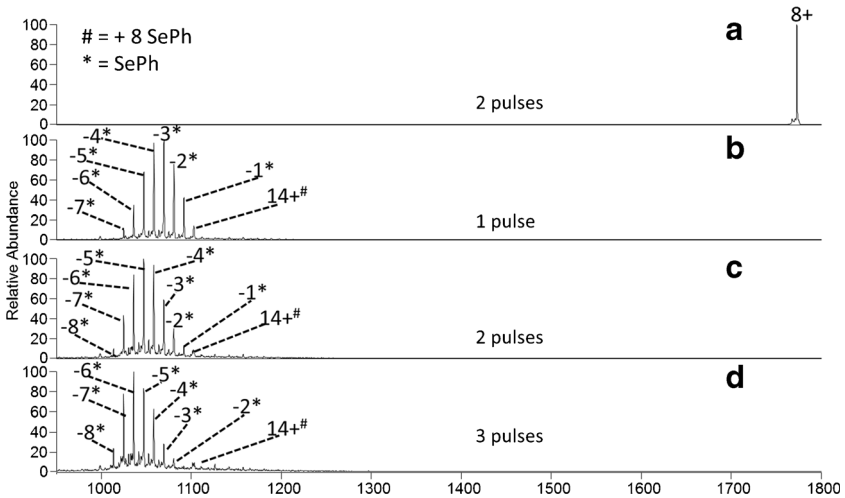

Figure 5. UVPD mass spectra of α-lactalbumin for **(a)** unmodified (8+), and for NPSP-modified after reduction (14+) using **(b)** one pulse, **(c)** two pulses, and **(d)** three pulses

electron-based (ETD) methods were also used to activate the SePh-tagged proteins in the present study. Examples of the resulting MS/MS spectra are shown in Supplementary Figure S10 for aprotinin (with six SePh tags) and Supplementary Figure S11 for cytochrome *c* (with two SePh tags). Neither HCD nor CID nor ETD resulted in efficient Se–S cleavage. ETD promoted cleavage of up to two S–Se cleavages in conjunction with charge reduction for tagged aprotinin; the analogous MS/MS spectra for cytochrome *c* were not readily interpretable. Based on this comparison, 266 nm UVPD showed remarkably high efficiency and selectivity for Se–S cleavage relative to the other activation methods.

Conclusion

The NPSP-derivatization strategy and 266 nm UVPD proved to be successful as a new means to count free and bound cysteines in proteins. Proteins containing up to eight cysteine residues were successfully characterized. The SePh tag served as an excellent chromophore for absorption of 266 nm photons, and the selective cleavage of the Se–S bond was striking. Tracking free and bound cysteines has numerous applications in proteomics and offers opportunities for incorporation in informatics engines. For example, the last column of Table 1 shows the percentages of proteins in each proteome that have the same cysteine content (free versus bound cysteines) as each protein included in this study. On average, about 0.1% of all possible proteins match each combination of free and bound cysteines, thus illustrating that characterizing cysteine content offers a significant way to constrain protein identification in database searches.

Acknowledgments

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