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Article

Exploring the Potential of Ultrafast Arylation for Capping Cysteine Residues with Fixed Charge Modifications

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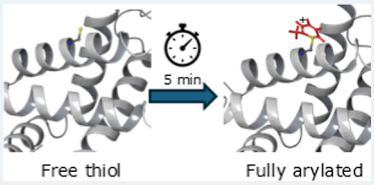
ABSTRACT: Reduction of disulfide bonds in proteins followed by selective modification of Cysteine (Cys) residues is a common practice in proteomics experiments to facilitate digestion into peptides and ultimately identification by mass spectrometry (MS). Due to its high nucleophilicity, a variety of reaction pathways can be used to block Cys; however, arylation is not often employed. Recently, Lipka and co-workers reported an electrophilic Cys arylation reagent, *N*-methyl-2-methylsulfonylpyridinium (CAP4), that can arylate free thiols with high selectivity and rate (Lipka, B. M.; Honeycutt, D. S.; Bassett, G. M.; Kowal, T. N.; Adamczyk, M.; Cartnick, Z. C.; Betti, V. M.; Goldberg, J. M.; Wang, F. Ultra-Rapid Electrophilic Cysteine Arylation. J. Am. Chem. Soc. 2023, 145 (43), 23,427−23,432). Additionally, CAP4 modification includes the addition of a fixed charge to the side chain that could potentially influence MS experiments. The fixed charge could be useful if it affords better sensitivity or detrimental if it negatively affects fragmentation. Herein, we show that CAP4 modification leads to favored fragmentation pathways involving the modified side chain in both higher-energy collisional dissociation (HCD) and electron-transfer dissociation (ETD). Abundant side chain losses from CAP4 are observed in HCD, while in ETD, electron transfer leads to the formation of a hydrogen deficient beta radical at the Cys residue. Formation of the beta radical is shown to be charge-state dependent, and collisional activation of the radical leads to radical-directed dissociation (RDD)-derived fragments. In addition, we evaluated the potential of CAP4 in a bottom-up proteomics workflow using a model protein mixture. Despite its influence on fragmentation, rapid arylation (5 min) by CAP4 resulted in comparable sequence coverage and total modified Cys-containing peptide IDs when compared to 1 h iodoacetamide modification.

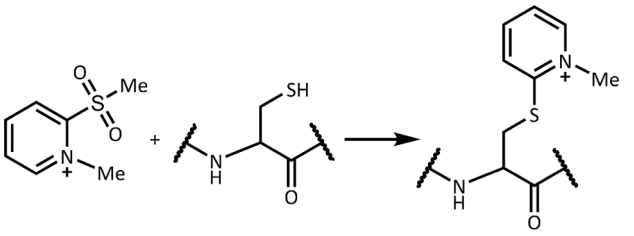


KEYWORDS: *fragmentation, isomer, photodissociation, click reaction, epimer*

INTRODUCTION

With the recent expansion of development in protein-based pharmaceuticals,[1] synthetic chemists have taken increased interest developing novel reactions that modify amino acid side chains.[2−4] For example, Lipka and co-workers recently reported the development of a variety of electrophilic cysteine arylation reagents.[5] These reagents can be employed in aqueous solvents and proceed with high rate constants, sometimes affording arylation within seconds. In addition, the reported selectivity for targeting free thiols is excellent, thus making these reagents potentially suitable for capping cysteine residues in the context of proteomics experiments.[6] Of particular interest is *N*-methyl-2-methylsulfonylpyridinium (CAP4), which offers an excellent balance of fast reaction times and selectivity. As illustrated in Scheme 1, the arylation of Cys by CAP4 also adds a fixed charge to the side chain. The fixed charge has the potential to increase the detection of Cys-containing peptides,[7,8] which can be challenging in bottom-up proteomics.[6] However, the fixed charge could also potentially impact the dissociation behavior of the resulting peptides (in either favorable or unfavorable fashion) when fragmented by collisional activation[9,10] or electron-capture or transfer.[11,12]

Scheme 1. CAP4 Structure and Reaction Product with Cys



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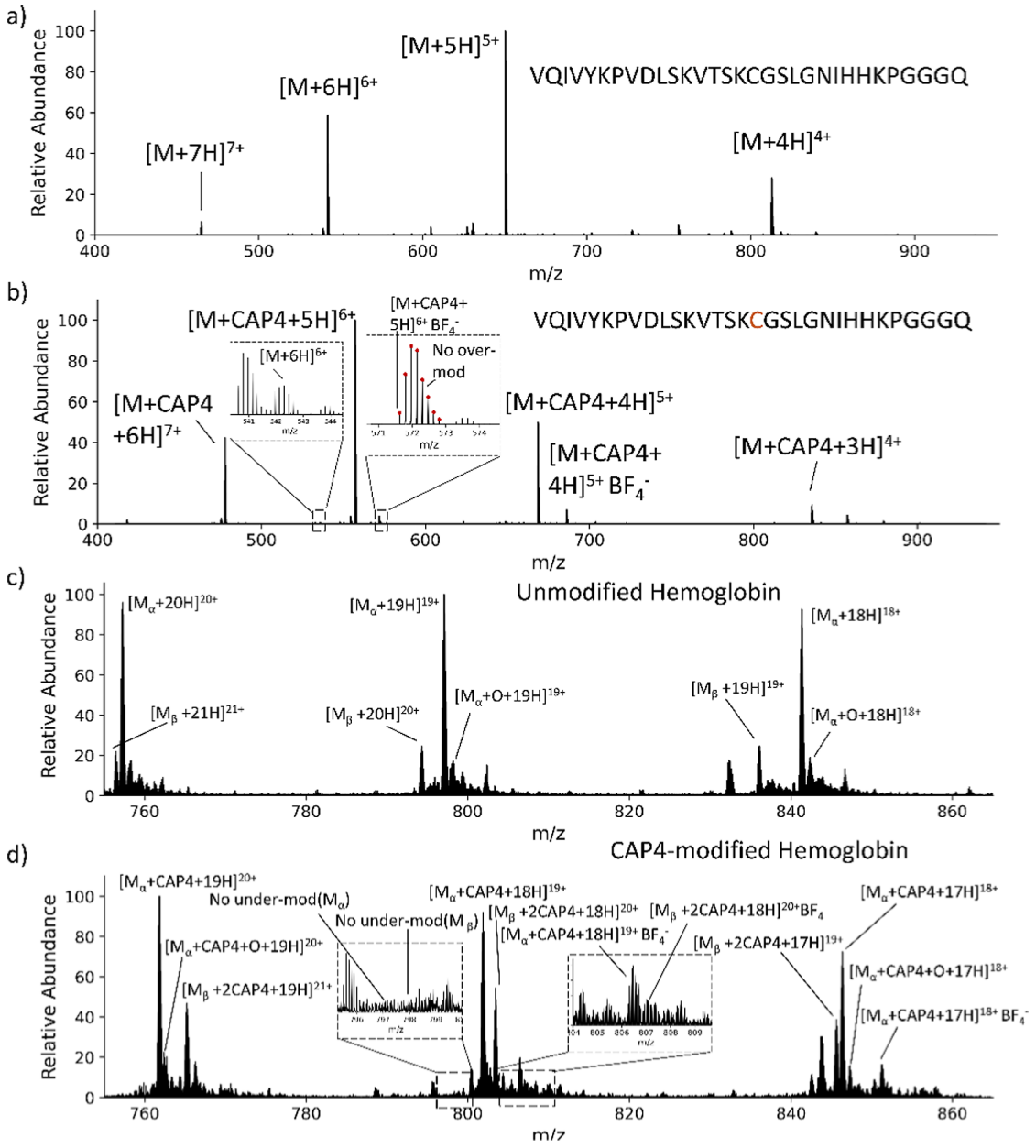

Figure 1. Mass spectra of tau-derived VQIV peptide: (a) unmodified and (b) modified with CAP4, and mass spectra of human hemoglobin: (c) unmodified protein and (d) CAP4-modified protein.

To examine the potential of CAP4 as a useful Cys blocking reagent, we modified model compounds ranging in size from small peptides to intact proteins and subjected them to higher-energy collision dissociation (HCD)[13] and electron-transfer dissociation (ETD).[14] Interestingly, the fixed charge significantly impacts the resulting dissociation in both instances. We also examined the selectivity and rate of modification by CAP4 by blocking all Cys residues from tryptic digest derived from nine test proteins, followed by evaluation of the results by liquid chromatography−mass spectrometry (LC−MS). The selectivity for reaction with Cys residues is excellent, but precautions must be taken to afford identification due to facile losses associated with CAP4 modification. Overall, our results suggest that CAP4 may have utility in proteomics, particularly for top-down experiments, where off-target modifications are particularly problematic.

MATERIALS

A fragment of the protein Tau (VQIVYKPVDLSKVTSKCGSLGNIHHKPGGGQ) was purchased from BACHEM. RAAACGVLK was synthesized using solid-phase peptide synthesis.[15] Albumin from chicken egg white, lysozyme from chicken egg white, K-casein from bovine milk, superoxide dismutase from bovine erythrocytes, cytochrome C from equine heart, bovine serum albumin, and human hemoglobin were purchased from Sigma-Aldrich. 0.2 μm cellulose acetate filters were from Avantor (product number 03CP020AS). TOHO-1 was prepared according a previously reported procedure.[16] All other reagents and solvents were purchased from Sigma-Aldrich and Fisher Scientific and used without purification. *N*-methyl-2-methylsulfonylpyridinium (CAP4) tetrafluoroborate was synthesized as previously described.[5]

Free-Thiol Modification with CAP4

Tris(2-carboxyethyl)phosphine (TCEP) was added at a 1:1 molar ratio to Cys and allowed to react for 30 min at room temperature to cleave disulfide bonds. Water was used for peptide reduction, while

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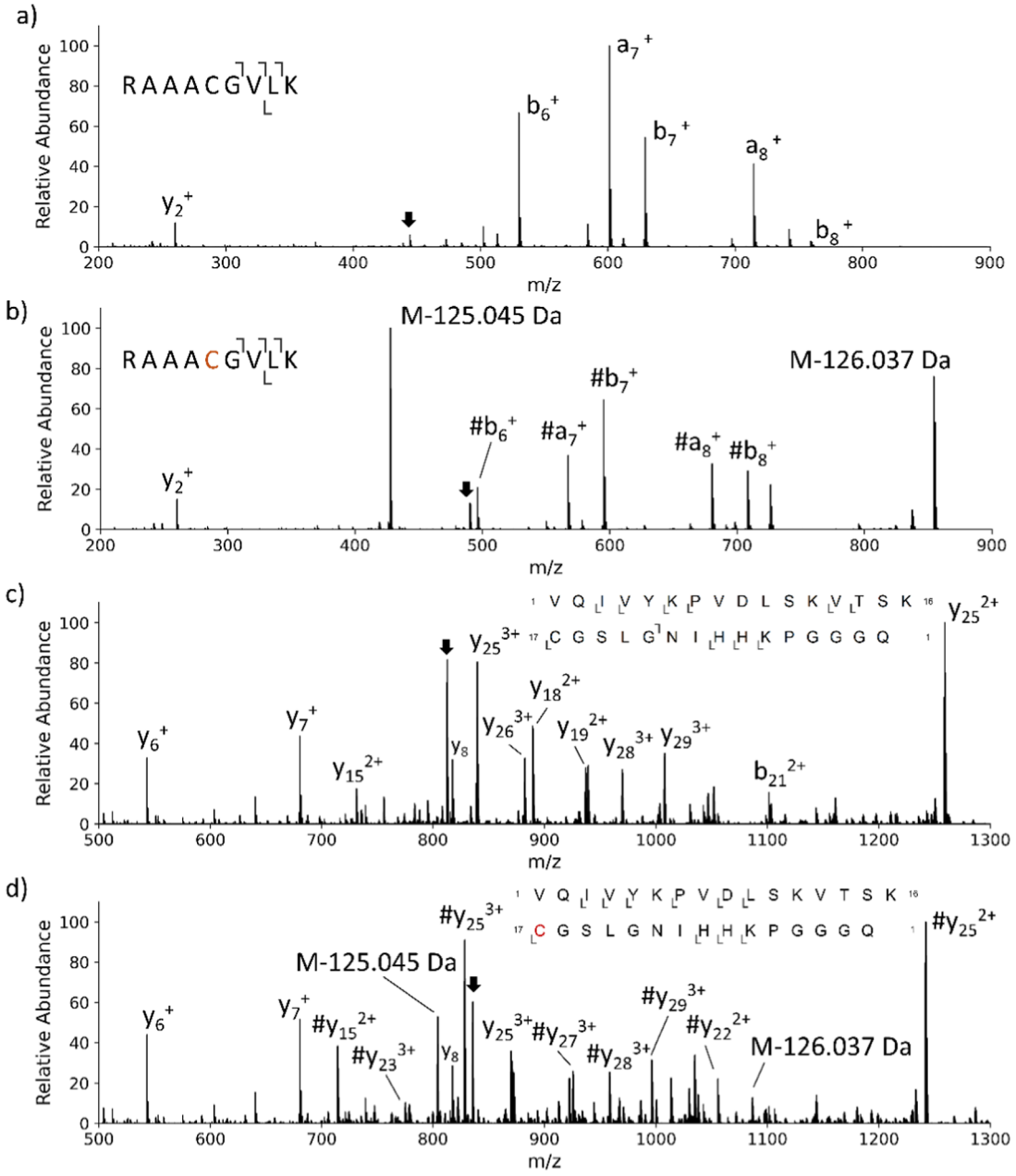

Figure 2. HCD fragmentation spectra of (a) unmodified and (b) CAP4-modified RAAACGVLK peptide, 2+ charge state; (c) unmodified and (d) CAP4-modified tau peptide, VQIV, 4+ charge state. The precursor for each MS^2 spectrum is designated with a bold downward black arrow. #: fragment ions with a 125.045 Da mass loss.

80/20 water/acetonitrile was used for protein reduction. After disulfide bond reduction, free thiols were arylated by using a 2:1 molar ratio of CAP4 to Cys for 5 min at room temperature.

Mass Spectrometry

All experiments were performed on a Thermo Orbitrap Fusion Lumos. Samples were introduced into the instrument via a nano flex source from Thermo Scientific. The nano flex source was modified with a platinum wire to allow use of tips pulled from borosilicate glass (Harvard Apparatus GC100T-10). Peptides and proteins were electrosprayed using the nano flex source with 0.1% acetic acid (AA). Peptides and proteins were isolated using the quadrupole for fragmentation with HCD or ETD. For radical-directed dissociation experiments, radicals were generated using ETD in MS^2 and were subsequently reisolated for CID MS^3. All mass spectra were acquired in the Orbitrap mass analyzer using a resolution of 120,000, RF lens at 60%, and 10 microscans being collected for 10 total scans.

All data were analyzed using Xcalibur (v4.4.16.14). Deconvolution was performed in FreeStyle (v1.7). For Xtract deconvolution, analyzer type was set to "OT," isotope table was set to "protein," and the relative abundance threshold was set to 1%.

9 Protein Mixture Arylation and Digestion

A mixture of nine proteins was diluted with 8 M urea as a final concentration of 0.1 μg/μL of each protein in the solution. 4.5 μg of protein mixture was transferred to a low-binding microcentrifuge tube. The pH of the urea solution was maintained at ~5 using 0.006% formic acid (FA). Protein solutions were reduced by adding 1.91 μL of 5 mM TCEP for 30 min at room temperature. For alkylation by iodoacetamide (IAA), samples were treated with 1.91 μL of 10 mM IAA for 60 min in the dark at room temperature, and IAA was quenched with 1.91 μL of 20 mM DTT for 15 min. For arylation by CAP4, samples were incubated with 1.91 μL of 2.5 mM CAP4 for 5 min in the dark at room temperature and quenched with 1.91 μL of 5

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J. Am. Soc. Mass Spectrom. 2026, 37, 894−901

mM DTT for 15 min. All samples were then diluted with 150 μL of Tris buffer to reach a final concentration of 2 M urea, and the pH was adjusted to 7.8 (verified by pH paper). 0.1 μg of sequencing-grade modified trypsin from PROMEGA (1:50 trypsin: protein) was used for digestion overnight at 37 °C for 18 h. Tryptic digestion was quenched by adding 10 μL of formic acid (FA) to pH 2. Samples were filtered using 0.2 μm filters to remove any particulates.

Column Preparation and LC–MS/MS

Monophasic C18 analytical columns were pulled using 100 μm diameter fused silica columns with a P-2000 laser tip puller (Sutter Instrument Co., Novato, CA) and packed to a length of 30 cm with Reprosil-Pur 120 C18-AQ 3 μm resin.

50 ng portion of each sample with 250 fmol Pierce Retention Time Calibrant (PRTC) was analyzed using a Thermo UltiMate 3000 RSLC interfaced to a Thermo Fisher Scientific Orbitrap Fusion Lumos Tribrid Mass Spectrometer. Samples were eluted using 0.1% formic acid in water as mobile phase A and 0.1% formic acid in 80% acetonitrile (80:20:0.1 ACN:H_2O:FA) as mobile phase B. Samples were desalted by loading onto an Acclaim PepMap 100 C18 3 μm 75 μm × 2 cm trap cartridge at 1% B for 2.2 min. Peptides were then separated with a 90 min LC gradient consisting of 1% B for 3 min, 1%–7% B in 7 min, 7%–15% B in 20 min, 15%–50% B in 40 min, 50%–98% B in 5 min, 98% B for 10 min, and a wash of 1% B for 5 min. The analytical column was then equilibrated at 1% B for 100 min. The instrument was operated in a data-dependent acquisition mode. MS^1 scans were collected with a resolution of 60,000 and a scan range of 375–1500 m/z. MS^2 scans used stepped HCD with 25%, 28%, and 31% collision energy, an automatically determined AGC target, a resolution of 15,000, an isolation window of 1.6 m/z, and an automatically determined scan range.[17]

Data Analysis

Thermo Raw files were converted into mzML format using Proteowizard MSConvert (version 3.0.22,288) using vendor peak peaking and zeroSamples settings. The mzML files were then imported into Skyline (version 25.1.0.237) using the DDA with MS1 filtering workflow.[18,19] Modifications on peptides were added for alkylation at Cys and oxidation at Met residue as variable; 2 missed cleavages were allowed. To provide a sufficiently complex FASTA to allow for appropriate usage of an FDR, a bacterial FASTA from *Arabidopsis thaliana* with added 9 proteins of interest was used. MSFragger (version 4.3) was used as the search engine.[20]

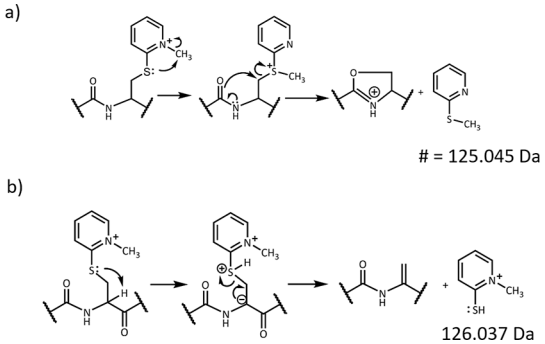
RESULTS AND DISCUSSION

In the original report on CAP4, the authors noted rapid arylation of Cys residues (within 5 min) without measurable modification of other residues for a variety of peptide targets. To further explore the reactivity of CAP4, we examined a large peptide (VQIV, corresponding to residues 306–336 of human tau) and two proteins, alpha and beta human hemoglobin. Collectively, these targets contain at least one residue from all of the canonical amino acids. The results for VQIV are shown in Figure 1a and b. The unmodified peptide in Figure 1a converts cleanly to the fully modified form after 5 min reaction time, as shown in Figure 1b. The insets in Figure 1b highlight that over- and undermodification are minimal. The undermodified peak is observed at 0.12% relative intensity, while the overmodified peak overlaps with a BF_4 anion adduct (the counterion for the fixed charge group in CAP4). The isotopic envelope for the BF_4 anion adduct matches closely with the predicted values (red dots), suggesting that minimal, if any, overmodification occurs. For VQIV, these results confirm that selective modification can be completed within 5 min. In addition, comparison of the charge state distributions between Figure 1a and b reveals that the relative intensity of the higher charge states increases significantly with CAP4 modification, likely due to the addition of the fixed charge. This suggests that the detection of tryptic peptides would benefit from the addition of CAP4, particularly in cases where the peptide contained few or no arginine residues.

Results for alpha- and beta hemoglobin are shown in Figure 1c and d. As is commonly observed with intact proteins, the spectrum in Figure 1c shows that the primary expected mass for the protein is accompanied by a variety of low-level mass-shifted proteoforms, including an oxidized form of alpha hemoglobin. After reaction with CAP4, the most abundant products correspond to singly modified alpha hemoglobin and doubly modified beta hemoglobin. The intensity of the oxidized form is also noted to increase, which has also been observed previously with alkylation using iodoacetamide.[21] The insets again illustrate that minimal over- or under-modification is observed, even with these intact proteins. Overall, our results confirm the original reports that CAP4 can rapidly and selectively arylate Cys residues in both peptides and proteins.

To illustrate the effect of CAP4 modifications on collisional activation experiments, representative HCD results for the two peptides are shown in Figure 2. Fragmentation of unmodified RAAACGVLK yields a series of mobile-proton initiated fragments, as expected. However, collisional activation of CAP4-modified RAAACGVLK is dominated by two abundant losses derived from the CAP4-modified Cys side chain. One of these losses (−126.037 Da) is accompanied by a charge state shift, which is easily rationalized due to the presence of a fixed charge within CAP4. However, the most abundant loss (−125.045 Da) occurs with retention of the charge state despite what appears to be loss of the CAP4 modification. Mechanisms that rationalize these observations are listed in Scheme 2 and Figure S4. For the charge-reduction pathway,

Scheme 2. Proposed Mechanisms for (a) 125.045 Da and (b) 126.037 Da Losses Following HCD



the Cys sulfur attacks the alpha hydrogen, followed by cleavage of the carbon–sulfur bond. The Cys residue is converted to dehydroalanine, and a fixed charge is lost. This mechanism bears some similarity to previous work where loss of protonated vinylpyridine was observed.[22] It has further been suggested that such charged losses can be used to independently identify Cys-containing peptides.[23]

In the charge-retention pathway, the methyl group from CAP4 first migrates to the Cys sulfur atom, shifting the position of the fixed charge. This intermediate resembles previously studied systems where Met residues were modified to yield fixed-charges at sulfur.[24] In that study, the fixed charge Met residues underwent facile loss of the side chain following ring formation with the *N*-terminal carbonyl oxygen. An

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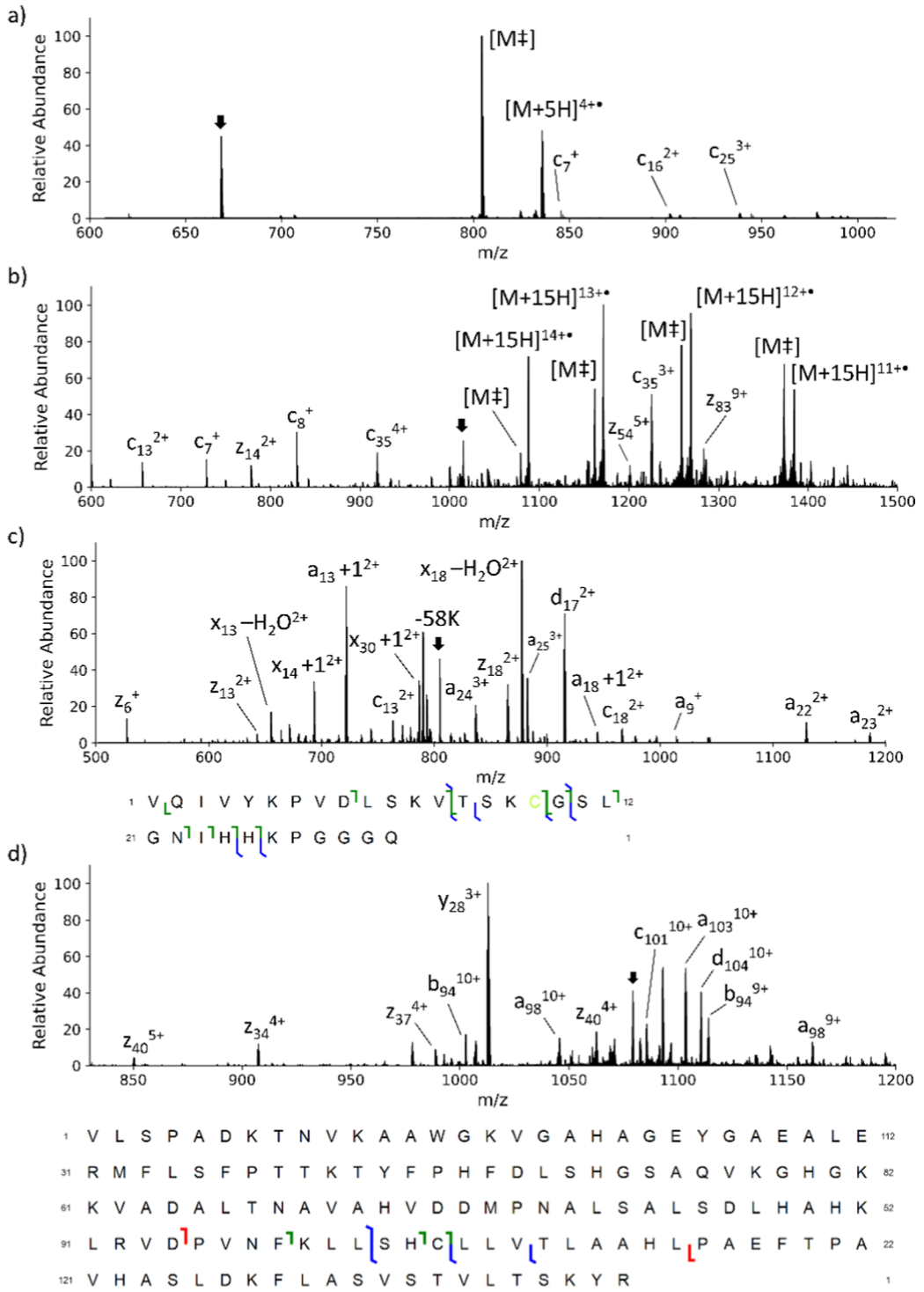

Figure 3. ETD spectra for (a) CAP4-modified VQIV peptide (5+) and (b) CAP4-modified α-Hemoglobin (15+). RDD spectra for (c) ETD-derived beta radical of the VQIV peptide and (d) ETD-derived beta radical of 15+ α-Hemoglobin. The precursor for each MS^2 spectrum is designated with a bold downward black arrow. In the sequence ladders, b/y fragments are shown in red, c/z fragments are in blue, and a, d/x fragments are in green. M‡: beta radical product as illustrated in Scheme 3.

analogous pathway is possible for Cys, as illustrated in Scheme 2, affording CAP4 loss with the conversion of the fixed charge into a proton initially located on the backbone. Although these dissociation channels are intense for RAAACGVLK, we note that all of the major backbone fragments observed in Figure 2a are also observed in Figure 2b, although many are mass shifted by the CAP4 side chain loss in Figure 2b.

Results for HCD on VQIV are shown in Figure 2c and d. Again, the unmodified peptide produces a series of primarily b−y ions, as expected. For the larger peptide, the CAP4 side chain losses from the precursor ion are present but at a significantly lower fractional abundance relative to what was observed for RAAACGVLK. The b/y ions that yield sequence information are also present, although again many are shifted by losses of the CAP4 modification. These results suggest that

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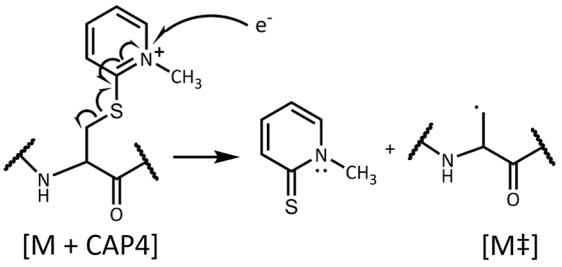
J. Am. Soc. Mass Spectrom. 2026, 37, 894−901

CAP4 should not significantly reduce sequence coverage in HCD experiments, but data assignment will be more complicated, because the favorable CAP4 side chain loss will result in many mass-shifted products that must be accounted for. To more fully explore these possibilities, additional experiments on protein digests were conducted (see below).

To evaluate whether the fixed charge in CAP4 influences fragmentation initiated by electrons, we conducted ETD experiments with VQIV and alpha hemoglobin. ETD on the +5 charge state of unmodified VQIV yields a series of c/z ions, as expected (Figure S1). However, modification with CAP4 significantly alters the behavior of VQIV by favoring electron transfer to the fixed charge group, facilitating dominant side chain loss in the modified Cys (Figure 3a). Electron transfer to the fixed charge leads to rearrangement of electrons and cleavage of the distal carbon−sulfur bond, producing a beta radical (Scheme 3). The intensity and number of c/z ions is also reduced for the CAP4-modified peptide, suggesting that the electron transfer preferentially occurs at CAP4 despite the fact that four protons are present elsewhere on the precursor ion. To explore whether preferential electron transfer to CAP4 can be overwhelmed by a greater number of competing protons, we examined ETD for CAP4-modified alpha hemoglobin. When we performed ETD on higher charge states (19+), the dominant product following transfer of a single electron corresponded to the charge-reduced precursor ion, suggesting that electron transfer to CAP4 was no longer preferred for such high charge states (see Figure S3). For lower charge states, capture at CAP4 is again disproportionately favored. For example, in Figure 3b, ETD on the +15 charge state leads to CAP4 loss for ∼25% of the first charge reduced precursor, despite CAP4 accounting for only 1/15th of the charges present.

Scheme 3. Mechanism for β-Radical Formation on ETD



The efficient conversion of precursor ions into hydrogen-deficient beta radicals[25] at CAP4 presents an opportunity. We have previously utilized photochemistry or ETD to generate hydrogen deficient radicals that were then subjected to collisional activation.[26,27] The subsequent fragmentation is often dominated by radical chemistry, which we refer to as radical-directed dissociation (RDD). To explore the potential for CAP4 in RDD experiments, we collisionally activated the beta radical products in Figure 3a and b. The results are shown in Figure 3c and d. In both instances, the observed product ion types are consistent with known RDD mechanisms, including favored dissociation to produce a d-ion at the site where the original beta radical was initially created. Therefore, while the addition of CAP4 may negatively impact the outcome of

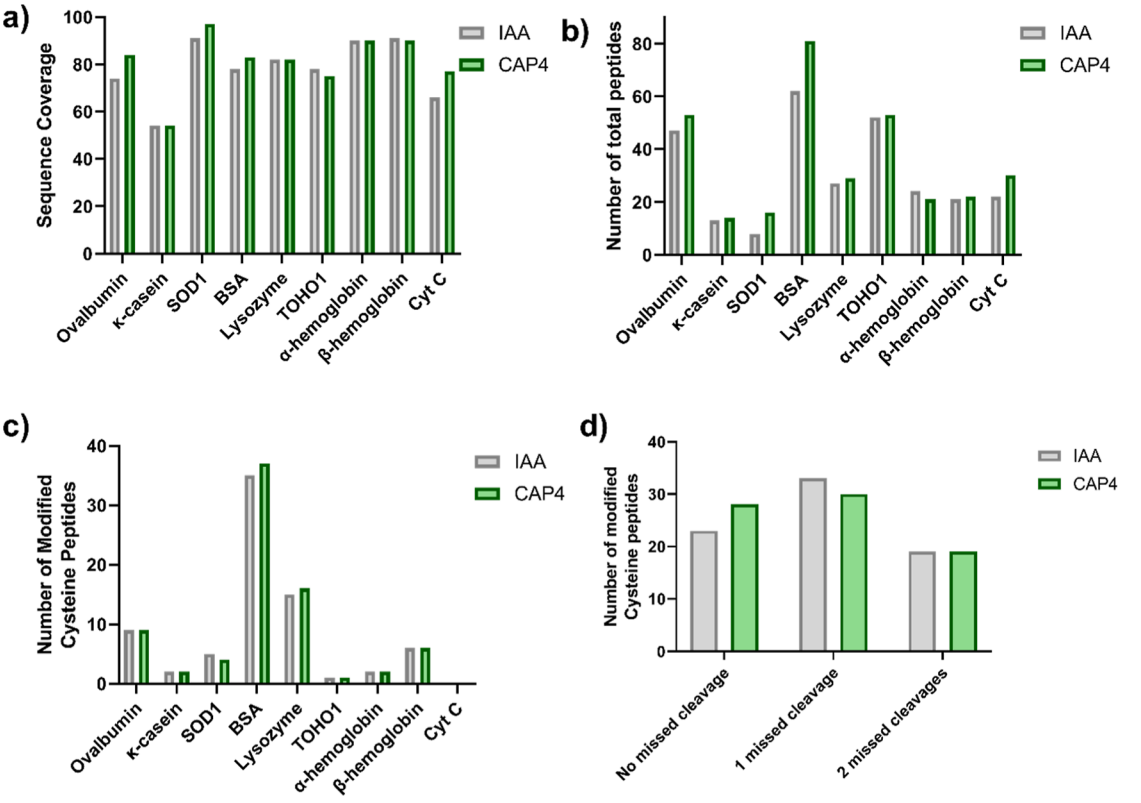


Figure 4. Results for alkylation with IAA and arylation with CAP4 of a 9-protein mixture examined by LC−MS: (a) sequence coverage, (b) total peptides detected, (c) Cys-containing peptides detected, and (d) Cys-containing peptides sorted by number of missed cleavages. Although the outcomes are comparable, CAP4 arylation is completed in 5 min versus 1 h for IAA alkylation.

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J. Am. Soc. Mass Spectrom. 2026, 37, 894−901

traditional ETD experiments, it also affords an efficient means of conducting RDD experiments without a laser.

Based on our examination of the model systems, we revealed that CAP4 significantly affects the behavior of peptides in both collision- and electron-based dissociation experiments. To evaluate whether these effects would outweigh the potential benefits of rapid and more selective modification with the potential for increased sensitivity due to the addition of a fixed charge, we examined a mixture of nine proteins using a bottom-up proteomics workflow. The results are shown in Figure 4. Relative to iodoacetamide (IAA), CAP4 modification of Cys residues leads to comparable or slightly higher sequence coverage for all proteins. The number of Cys-containing peptides observed is also comparable (77 for CAP4, 75 for IAA). Additionally, a higher proportion of CAP4-modified Cys peptides have no missed cleavage compared to IAA, but additional work is needed to confirm that CAP4 modification results in fewer missed cleavages. Importantly, the CAP4 results were obtained in significantly less time, 5 min versus 1 h. We also examined the extent of overmodification for both reagents. No abundant overmodified peptides were detected with either blocking reagent.

CONCLUSION

In summary, we arylated peptides and hemoglobin to examine the CAP4 modification efficiency and gas-phase fragmentation behavior. We observed very little under-modification and essentially no off-target modification. Our results show that two distinct mechanisms lead to abundant mass losses following collisional activation. Additionally, in ETD, electron transfer to the fixed charge of CAP4 is favored in low charge states and forms a beta radical by cleaving the carbon−sulfur bond. This product can be useful for subsequent analysis by RDD without the need for photoactivation. Furthermore, results from a model nine-protein digest bottom-up workflow were used to evaluate the potential of utilizing CAP4 instead of IAA as a Cys capping reagent prior to tryptic digestion. Given the high specificity, comparable sequence coverage, extremely fast modification, and ability to generate hydrogen-deficient radicals, CAP4 is a promising new reagent for use in both top-down and bottom-up proteomics.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/jasms.5c00400.

MS^2 ETD spectrum of unmodified VQIV peptide, 5+ charge state; unmodified α-Hemoglobin, 15+ charge state; CAP4-modified α-Hemoglobin, 19+ charge state; and alternative proposed mechanism for 125.045 Da loss following HCD (PDF)

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Notes

The authors declare no competing financial interest.

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