RESEARCH ARTICLE

Tandem Mass Spectrometric Characterization of Thiol Peptides Modified by the Chemoselective Cationic Sulfhydryl Reagent (4-Iodobutyl) Triphenylphosphonium—

Effects of a Cationic Thiol Derivatization on Peptide Fragmentation

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Abstract

Fixed charge chemical modifications on peptides and proteins can impact fragmentation behaviors in tandem mass spectrometry (MS/MS). In this study, we employed a thiol-specific cationic alkylation reagent, (4-iodobutyl)triphenylphosphonium (IBTP), to selectively modify cysteine thiol groups in mitochondrial proteome samples. Tandem mass spectrometric characteristics of butyltriphenylphosphonium (BTP)-modified peptides were evaluated by comparison to their carbamidomethylated (CAM) analogues using a quadrupole time-of-flight (Q-TOF) instrument under low energy collision-induced dissociation (CID) conditions. Introduction of the fixed charge modification resulted in the observation of peptide and fragment (b_n and y_n) ions with higher charge states than those observed for CAM-modified analogues. The charged BTP moiety had a significant effect on the neighboring amide bond fragmentation products. A decrease in relative abundances of the product ions at the corresponding cleavage sites was observed compared with those from the CAM-modified derivatives. This effect was particularly noticeable when an Xxx-Pro bond was in the vicinity of a BTP group. We hypothesized that the presence of a phosphonium moiety will reduce the tendency for protonation of the proximal amide bonds in the peptide backbone. Indeed, calculations indicated that proton affinities of backbone amide bonds close to the modified cysteine residues were generally 20–50 kcal/mol lower for BTP-modified peptides than for the unmodified or CAM-modified analogues with the sequence motif -Ala-Cys-Ala_n-Ala₂-, -Ala-Cys-Ala_n-Pro-Ala-, and -Ala-Pro-Ala_n-Cys-Ala-, $n=0-3$.

Key words: Peptide fragmentation, Fixed charge modification, Thiol-specific modification, Proline effect

Brian Arbogast, 1945–2010. The authors are immensely grateful for his contribution to this work.

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Introduction

Many proteomics workflows include reduction and subsequent modification of cysteine residues with diverse derivatization reagents to enhance the efficiency of the proteolytic step necessary for achieving high sequence coverage and high confidence protein identifications [1–6].

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Most cysteine alkylations are performed under denaturing conditions after a complete reduction of the disulfide linkages [2, 4]. However, in some experimental strategies, cysteine modifications are performed under non-denaturing conditions to obtain information regarding surface accessibility of specific cysteine residues or to map cysteine residues with changed reactivities [7–9]. Murphy and colleagues introduced the thiol-specific alkylation reagent (4-iodobutyl)triphenylphosphonium (IBTP) to facilitate redox state changes of thiol proteins in respiring mitochondria [10]. IBTP forms a stable thioether bond with cysteine via a S_N2 -type alkylation reaction (Scheme 1). As a lipophilic and cationic reagent, IBTP is able to penetrate the lipid bilayers and accumulate inside the mitochondria due to the large membrane potential present across the inner mitochondrial membrane. Our group has explored the use of stable isotopically-coded IBTP probes in gel-free proteomics approaches to obtain site-specific information on reactive thiol groups in mitochondrial proteins [11]. During the course of these studies, we observed that the introduction of the butyltriphenylphosphonium (BTP)-moiety affected the collision-induced dissociation (CID) behaviors of BTP-modified peptides, which warranted further investigations.

Use of fixed charge derivatization to improve peptide fragmentation behaviors in mass spectrometry (MS) was first reported by Sadagopan and Watson [12]. This experimental strategy has been extended by several other groups with the goal to obtain simplified fragmentation patterns for highly efficient peptide sequence analyses by tandem mass spectrometry (MS/MS) [1, 13–15]. A series of *tris*(2,4,6-trimethoxyphenyl)phosphonium (TMPP) analogues were used to modify

the N-terminus of peptides, which resulted in the increased formation of a_n - or b_n -ions [16–20]. The TMPP-modified peptides were also used to investigate Asp-containing peptide fragmentation mechanisms [20–22]. Besides targeting the N- and/or C-termini of peptides, ‘fixed charge’ chemical reagents have been described for selectively labeling certain amino acid side chains, such as cysteine and lysine [23, 24]. In the case of IBTP, a chemoselective thiol probe, the introduction of the fixed charge usually occurs at an internal position. The effect of the fixed charge on the CID behavior of peptide ions will therefore be sequence-dependent. Here, we report a comparative study of fragmentation characteristics of thiol-containing peptides modified by a BTP or a carboxyamidomethyl (CAM) group using electrospray ionization (ESI) and low energy CID conditions on a quadrupole time-of-flight (Q-TOF) instrument. We observed a significant effect on the fragmentation behaviors at cleavage sites in the vicinity of a BTP group. This influence was particularly noticeable for the cleavage of Xxx-Pro bonds in proximity to the modification sites.

Experimental

Materials

Iodoacetamide (IAM), trifluoroacetic acid (TFA), and 1,4-diiodobutane were purchased from Sigma-Aldrich (St. Louis, MO, USA). Triphenylphosphine was obtained from Spectrum (Gardena, CA, USA). *Tris*(2-carboxyethyl)-phosphine hydrochloride (TCEP·HCl) was from Pierce (Rockford, IL, USA). Sequencing-grade modified trypsin was from Promega Corp. (Madison, MI, USA). The solvents

Scheme 1. Modification reactions of protein thiolates with cysteine specific reagents (a) (4-iodobutyl)triphenylphosphonium (IBTP) and (b) iodoacetamide (IAM)

used for high-performance liquid chromatography (HPLC) were from VWR (West Chester, PA, USA). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Synthesis of IBTP

IBTP was synthesized as previously described [10]. Briefly, 1,4-diiodobutane (8.5 mmol, 5-fold molar excess) was reacted with triphenylphosphine (1.5 mmol) at 100 °C in the dark for 1.5 h. The raw product was washed with diethyl ether, and then dissolved in 5 mL dichloromethane and precipitated with 50 mL diethyl ether. The solvents were discarded after precipitation. Residual solvent was then removed under reduced pressure to give a pale yellow solid (yield 72%). IBTP was stored at −20 °C and kept from light.

Derivatization of Mitochondrial Thiol Proteins with IBTP and IAM

The alkylation of mitochondrial proteins with IBTP was described previously [11]. Briefly, rat cardiac mitochondria were isolated according to Suh *et al.* and stored at −80 °C [25]. Aliquots of frozen mitochondria were suspended in 20 mM potassium phosphate buffer, pH 8.4. Mitochondria were disrupted by several freeze–thaw cycles. Protein concentration was determined by the Pierce-Coomassie protein assay. Mitochondrial protein samples were reduced using TCEP at a final concentration of 5 mM at room temperature for 30 min and then IBTP was added using a final concentration of 2 mM. Alkylation occurred at 37 °C for 1.5 h. The reaction solution was digested in 100 mM ammonium bicarbonate with trypsin (1:40) at 37 °C overnight. The tryptic peptides were centrifuged, and the supernatant containing the BTP-modified peptides was analyzed by nanoLC-ESI-MS/MS. The modification of mitochondrial proteins with IAM followed the same procedure.

Mass Spectrometry (MS)

Tryptic digests of BTP- and CAM-modified mitochondrial proteins were analyzed using a quadrupole orthogonal acceleration time-of-flight mass spectrometer (Q-TOF Ultima Global; Micromass/Waters, Manchester, UK) coupled to a nanoAcquity Ultra Performance LC system (Waters, Milford, MA, USA) using similar conditions as described in the previous work [26]. The tryptic peptide mixtures were trapped and washed on a C₁₈ nanoAcquity column (Symmetry, 5 µm, 180 µm×20 mm) using 2% acetonitrile containing 0.1% formic acid at a flow of 5 µL/min for 3 min. Peptide mixtures were separated using an ethylene bridged hybrid (BEH) column (Waters, 1.7 µm, 75 µm×150 mm) with a binary gradient consisting of 2% acetonitrile in 0.1% formic acid (solvent A) and acetonitrile in 0.1% formic acid (solvent B) over 72 min. Under identical chromatographic conditions, as expected, longer retention times were observed for BTP-

modified derivatives compared with their respective CAM-modified peptides (data not shown).

The ESI source of the Q-TOF mass spectrometer was operated in the positive ion mode using a spray voltage of 3.5 kV. The data-dependent MS/MS mode was used with 0.6 s survey scans and 2.4 s MS/MS scans on the three most abundant ion signals in the MS survey scan with previously selected *m/z* values excluded for 60 s. The collision energy for MS/MS ranged from 25–65 eV and was dynamically selected based on the charge state of precursor ion selected by the first quadrupole. Lock mass correction was performed every 30 s per scan using the doubly charged ion of Glu¹-fibrinopeptide B ([M+2H]²⁺ 785.8426 Da, monoisotopic mass). Mass spectra were calibrated using fragment ions of Glu¹-fibrinopeptide B (MH⁺ 1570.6774 Da, monoisotopic mass). Peak list (pkl) files were generated using ProteinLynx Global Server 2.3 (PLGS; Waters, Manchester, UK).

Database Searching

Mascot software (Matrix Science, London, UK) was used to facilitate the interpretation of tandem mass spectral data. MS/MS data were searched against the mammalian Swiss-Prot database (*Taxonomy rodentia*) with variable peptide modifications including Met oxidation (147.04 Da, monoisotopic mass), carboxyamidomethylated Cys (160.03 Da, monoisotopic mass), and BTP-modified Cys (419.15 Da, monoisotopic mass). Trypsin/P was selected as the digesting enzyme allowing for one missed cleavage site. Mass tolerance was set to ±0.1 Da and ±0.12 Da for precursor and fragment ions, respectively. In addition, peptide identifications were confirmed by manual inspections and fragment ion annotations were added as necessary.

Calculation of Proton Affinities

Structures of unmodified, CAM-modified, BTP-modified peptides and their corresponding protonated peptides were optimized at the PM6 semi-empirical level of theory to identify the global minimum using software MOPAC2009 [27]. Proton affinities (PAs) of all peptides and their derivatives were calculated according to the definition [28]. Briefly, PA equals the negative enthalpy change of a protonation reaction, Equation (1):

i.e., $-\Delta H = PA$, where, Equation (2):

$$\Delta H = \Delta H_f(MH^+) - \Delta H_f(M) - \Delta H_f(H^+) \quad (2)$$

ΔH_f is the enthalpy of formation of the respective chemical species. The heat of formation of H⁺ in the gas phase, $\Delta H_f(H^+)$, is 365.7 kcal/mol [29]. Heats of formation of all optimized structures were calculated by MOPAC2009. For protonated peptides, the protons were placed at the N-terminal amino group of Pro (for proline-containing peptides) or Ala (for non-proline-

containing peptides). Peptides with the following sequences were calculated with both CAM and BTP modifications: -Ala-Cys-Ala_n-Ala₂-, -Ala-Cys-Ala_n-Pro-Ala-, and -Ala-Pro-Ala_n-Cys-Ala-, where $n=0-3$.

Results and Discussion

Comparative Tandem Mass Spectral Study of BTP- and CAM-Modified Tryptic Peptides Derived from Mitochondrial Proteins

The use of IBTP reagent to selectively modify mitochondrial thiol proteins has been successfully demonstrated by our group and others [10, 11]. With the potential of being a mitochondria-targeted analytical thiol proteomics tool, we felt it was of general importance to gain a better understanding of the fragmentation behaviors of the resultant BTP-modified peptide derivatives in low energy CID experiments. For this purpose we evaluated the fragmentation patterns of peptides modified by the cationic BTP moiety and the corresponding peptides modified by the neutral carboxyamidomethyl group. The modification reactions of protein thiols with IBTP and IAM, respectively, are shown in Scheme 1.

The rat heart mitochondrial samples were treated with IBTP and IAM reagents, respectively, trypsinized and subjected to nanoLC-MS/MS using a Q-TOF instrument operated in the data-dependent acquisition mode. A comparison of the two datasets yielded nine peptide pairs for which fragmentation information was available encompassing the same cysteine residues modified by IBTP and IAM, respectively. The tandem mass fragmentation patterns of these peptides are summarized in Table 1. The majority of CAM-modified mitochondrial peptides were doubly charged ions, $[M+2H]^{2+}$, with charging arising from a mobile or partially mobile proton along the peptide backbone and a localized proton at the C-terminal basic residue (Arg or Lys). Consequently, most fragment ions were singly charged since no permanently charged moiety was attached to the peptides. Occasionally, doubly charged y_n ions were detected with low intensity, when either a His residue was located internally in the peptide or the y_n ions consisted of a relatively long partial sequence of the peptide, which can potentially carry an additional mobile proton along the backbone. In contrast, with only mono-BTP-modified tryptic peptides identified from the mitochondrial sample, the majority of BTP-modified peptides were observed as triply charged ions, $[M+2H]^{3+}$, in agreement with that the phosphonium ion in BTP, a localized proton at the C-terminal Arg or Lys, and a mobile or partially mobile proton on the N-terminus of a peptide contribute to the observed charge state. Occasionally, an internal basic residue, likely a protonated His residue, may contribute an additional charge to the peptide ion (Figure S3). For the fragmentation patterns of BTP-modified peptides, the introduction of the fixed

charge modification resulted in the presence of more doubly charged fragment ions in the tandem mass spectra. The location of the modified Cys residue within the peptide sequence determined the predominant types of the observed fragment ions which allowed us to group the peptides into three categories: (1) Peptides with a Cys(BTP) residue located near the N-terminal end, i.e., Cys within the first third of the peptide, yielded MS/MS spectra in which doubly charged b_n ions were clearly visible. (2) Peptides with a modified Cys residue located in the middle of the peptide, i.e., Cys within the second third of the peptide, gave MS/MS spectra in which doubly charged b_n ions gradually disappeared and, instead, doubly charged y_n ions started to appear. (3) Peptides with a Cys(BTP) residue located near the C-terminal end, i.e., Cys within the last third of the peptide, in general, resulted in MS/MS spectra in which y_n^{2+} fragment ions encompassing the Cys(BTP) residue were clearly displayed.

The Effect of a Thiol-Specific Fixed Charge Chemical Modification on the Fragmentation Behavior of Cysteine-Containing Peptides

The impact of the fixed charge of the phosphonium group was observed on the cleavages at peptide backbones under low energy CID conditions. For instance, the peptide NCAQFVTGSQAR from ATP synthase subunit d was detected as BTP- (m/z 533.25, 3+) and CAM-modified (m/z 669.81, 2+) derivatives (Figure 1). A comparison of the MS/MS spectra revealed that although many fragment ions were observed for both peptide derivatives, the y_9 and y_{10} ions were absent in the MS/MS spectrum of the BTP derivative. We speculated that the reduced formation of the y ions in proximity to the cationic BTP-modified residue was at least partly due to hampered protonation of the amide bonds in proximity to the BTP moiety caused by charge repulsion between the cationic phosphonium ion and the mobile proton. Moreover, this hypothesis was supported by differences in the relative intensities of the y_7 and y_8 ions. In contrast, the high intensities of the y_9 and y_{10} ions observed for the CAM-modified analogue implied uninterrupted proton mobility along the peptide backbone in this peptide. The high intensity of the b_2 ion in the protonated CAM-modified peptide fragment spectra is likely due to the relatively stable protonated oxazolone structure of the b_2^+ ion [30, 31]. Retention of the fixed charge on the N-terminal fragment may likely contribute to the relatively high abundance of the b_2 ion in this BTP-modified peptide. However, the mechanism of formation and the structure of the b_2 ion are not clear for this BTP-modified peptide ion.

Suppression of y_n -fragment ion formation in the vicinity of a BTP-modified cysteine residue was observed in other peptides as well, including YFAGNLSGGAAGATSLCF-VYPLDFAR from ADP/ATP translocase 1 (Figure 3),

Table 1. Comparison of Fragmentation Patterns of BTP- and CAM-Modified Peptide Pairs Identified from Mitochondrial Samples

Cys located at	Protein name (Accession and fig. no.)	Precursor ion (m/z , z)	BTP- and CAM-modified peptides and observed fragmentation ions indicated
1st third of peptide (N- terminal end)	ATP synthase subunit d (P31399, Figure 1)	533.25, 3+	N ^{b₁} ^{b₂} ^{b₃} ^{b₄} ^{b₅} ^{b₆} ^{b₇} ^{b₈} ^{b₉} ^{b₁₀} ^{b₁₁} ^{b₁₂} ^{b₁₃} ^{b₁₄} ^{b₁₅} ^{b₁₆} ^{b₁₇} ^{b₁₈} ^{b₁₉} ^{b₂₀} ^{b₂₁} ^{b₂₂} ^{b₂₃} ^{b₂₄} ^{b₂₅} ^{b₂₆} ^{b₂₇} ^{b₂₈} ^{b₂₉} ^{b₃₀} ^{b₃₁} ^{b₃₂} ^{b₃₃} ^{b₃₄} ^{b₃₅} ^{b₃₆} ^{b₃₇} ^{b₃₈} ^{b₃₉} ^{b₄₀} ^{b₄₁} ^{b₄₂} ^{b₄₃} ^{b₄₄} ^{b₄₅} ^{b₄₆} ^{b₄₇} ^{b₄₈} ^{b₄₉} ^{b₅₀} ^{b₅₁} ^{b₅₂} ^{b₅₃} ^{b₅₄} ^{b₅₅} ^{b₅₆} ^{b₅₇} ^{b₅₈} ^{b₅₉} ^{b₆₀} ^{b₆₁} ^{b₆₂} ^{b₆₃} ^{b₆₄} ^{b₆₅} ^{b₆₆} ^{b₆₇} ^{b₆₈} ^{b₆₉} ^{b₇₀} ^{b₇₁} ^{b₇₂} ^{b₇₃} ^{b₇₄} ^{b₇₅} ^{b₇₆} ^{b₇₇} ^{b₇₈} ^{b₇₉} ^{b₈₀} ^{b₈₁} ^{b₈₂} ^{b₈₃} ^{b₈₄} ^{b₈₅} ^{b₈₆} ^{b₈₇} ^{b₈₈} ^{b₈₉} ^{b₉₀} ^{b₉₁} ^{b₉₂} ^{b₉₃} ^{b₉₄} ^{b₉₅} ^{b₉₆} ^{b₉₇} ^{b₉₈} ^{b₉₉} ^{b₁₀₀} ^{b₁₀₁} ^{b₁₀₂} ^{b₁₀₃} ^{b₁₀₄} ^{b₁₀₅} ^{b₁₀₆} ^{b₁₀₇} ^{b₁₀₈} ^{b₁₀₉} ^{b₁₁₀} ^{b₁₁₁} ^{b₁₁₂} ^{b₁₁₃} ^{b₁₁₄} ^{b₁₁₅} ^{b₁₁₆} ^{b₁₁₇} ^{b₁₁₈} ^{b₁₁₉} ^{b₁₂₀} ^{b₁₂₁} ^{b₁₂₂} ^{b₁₂₃} ^{b₁₂₄} ^{b₁₂₅} ^{b₁₂₆} 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Figure 1. Tandem mass spectra of (a) BTP-modified (533.25, 3+) and (b) CAM-modified (669.81, 2+) peptide NCAQFVTGS-QAR (aa 100–111) from ATP synthase subunit d acquired on an ESI Q-TOF instrument. $\text{C}^\ddagger$ = BTP-modified Cys residue; $\text{C}^\#$ = CAM-modified Cys residue

cytochrome *c* oxidase subunit 6b1 is a typical example. This peptide was observed with both BTP- and CAM-modifications at m/z 646.31 (3+) and m/z 839.37 (2+), respectively (Figure 2). According to the proline effect, a strong y_{11} fragment ion resulting from the selective cleavage of the Cys–Pro bond is expected. Indeed, the proline effect was observed for the CAM-modified derivative; a strong y_{11} ion is visible in the tandem mass spectrum depicted in Figure 2b. However, the y_{11} ion was absent in the tandem mass spectrum of the BTP-modified SLCPVSWVSAWDDR peptide (Figure 2a). The impact of the fixed charge modification extended to the y_{10} , y_9 , and y_8 ions as well: no y_{10} fragment was detected for the BTP-modified peptide, and the relative abundances of y_9 and y_8 ions in the BTP-modified peptide were significantly lower than those observed for the CAM-modified derivative.

Similar observations were also made for the following peptides listed in Table 1: LMCPQEIVDYIADK from the acyl carrier protein (Figure S1) and LGYILTCPSNLGTGLR from sarcomeric creatine kinase (Figure S2). In both cases, the BTP-derivates showed fragmentation patterns that contradicted the expected trend for proline-containing peptides, indicating at least a partial suppression of the proline effect by the fixed charge modification.

The impact of a BTP moiety on the proline effect was also observed in peptides with the general sequence of -Cys(BTP)-Xxx_n-Pro-, where $n=1-3$ (Table 2). For example, in the tryptic peptide YFAGNLSGGAAGATSLCFVYPLDFAR from ADP/ATP translocase 1, three amino acid residues are present between the cysteine and proline residues. This peptide was observed as BTP-modified (m/z 1019.19, 3+) as well as CAM-modified (m/z 1398.63, 2+) derivative (Figure 3). The fragment ion y_6 , associated with the proline effect at Pro-22, was the most intense fragment ion signal in the MS/MS spectrum of the CAM-modified peptide. However, the corresponding y_6 ion was not observed in the BTP-modified analogue. Furthermore, the BTP moiety displayed a significant impact on the protonation of up to four successive neighboring amide bonds, i.e. none of the y ions from y_6 to y_9 were detected in this peptide.

A less pronounced proline effect was also observed for peptides with a sequence of -Pro-Xxx_n-Cys(BTP)-, where $n=1-3$ (Table 2). For example, the peptide, GEVPCTVTAFPLDEAVLSELK from ATP synthase subunit O, yielded fragment ion spectra for both CAM- and BTP-modified derivatives (Figure 4). The y_{19}^{2+} ion was detected as the base peak for the CAM-modified peptide in accord with a favored cleavage at Val-Pro bond, whereas with a much lower relative abundance in the BTP-modified analogue. Again, the product ions, $y_{15}-y_{17}$, resulting from the cleavages of amide bonds proximal to the BTP moiety, all displayed a relatively lower abundance in the BTP-modified analogue.

Proton Affinity Calculations

The above described qualitative observations prompted us to examine to what extent the cationic BTP group affects the

proton affinities of the amides in peptide backbones. Proton affinities of derivatized amino acids have been calculated and experimentally measured by several groups. Their studies have pointed out the importance of hydrogen bonding for structure stabilization and the degree of charge stabilization and delocalization for the local proton affinity of an amino acid residue [32, 33]. We hypothesized that the decreased abundances of fragment ions derived from the cleavages of amide bonds in the proximity of a BTP group were caused by the reduced propensity for the protonation of the backbone amides in vicinity of a fixed charge phosphonium ion. Therefore, lower proton affinities at those amide bonds were expected compared with the ones in unmodified or CAM-modified analogues without influence of a charged BTP group. To support this hypothesis, we calculated proton affinities of several N-terminal amino groups close to a cysteine residue with a BTP- or CAM-modification in a set of cysteine-containing peptides: -Ala-Cys-Ala_n-Ala₂-, where $n=0-3$.

To better understand the impact of a BTP moiety on the proline effect, we also calculated the proton affinities of backbone amides in proline-containing peptides with the general sequences of -Ala-Cys-Ala_n-Pro-Ala- and -Ala-Pro-Ala_n-Cys-Ala-, where $n=0-3$, with BTP and CAM modifications in our calculations. As one of the few selective collision-induced gas-phase fragmentations observed for peptide ions, the proline effect has been extensively investigated. Studies by many groups have led to the conclusion that the proline effect (1) results from a charge-directed peptide fragmentation pathway, i.e., this cleavage is initiated by a charge (a proton) that is transferred to the vicinity of the cleavage site, i.e., a carbonyl oxygen or amino nitrogen [34]; (2) proceeds via an intermediate oxazolone structure; this pathway explains why this cleavage typically occurs N-terminal rather than C-terminal to the proline residue since C-terminal cleavage would require an unfavorable 5–5 bicyclic ring structure transition state [21, 35]; and (3) is facilitated via proton transfer governed by the PA of heteroatoms (O, N, S, etc.). The high PA of the amino group in proline may explain why y_n -ions are the predominant fragment ions observed in the MS/MS spectra during low energy CID experiments of proline-containing peptides [36, 37].

In our PA calculations, all protons were placed at nitrogen atoms of backbone amide bonds. For non-proline containing thiol peptides, protonations are more likely to occur at carbonyl oxygen of the peptide backbone prior to cleavages. However, for the dissociation of amide bonds to result in y_n -ions, the predominant ions observed in our data, the protons have to be transferred to the nitrogen of the amide bonds [38]. For proline-containing peptides, the protons are likely to migrate to the amino nitrogen of proline residues due to the high basicity (or high PA). The spatial effect of a BTP moiety on PAs was also estimated by placing up to three alanine residues between a Cys(BTP) and a protonated amide site. The results of our PA calculations

Figure 2. Tandem mass spectra of **(a)** BTP-modified (646.31, 3+) and **(b)** CAM-modified (839.37, 2+) peptide SLCPVSWVSAWDDR (aa 63–76) from cytochrome *c* oxidase subunit 6B1 acquired on an ESI Q-TOF instrument. The y_{11} ion produced as a result of preferential cleavage from the proline effect is only observed in the CAM-tagged peptide. C[‡] = BTP-modified Cys residue; C[#] = CAM-modified Cys residue

Table 2. BTP-Modified Proline-Containing Mitochondrial Thiol Peptides

-Cys-(Xxx) _n -Pro- or -Pro-(Xxx) _n -Cys- <i>n</i>	Protein name	Precursor ion (<i>m/z</i> , <i>z</i>)	BTP-modified Pro-containing thiol peptide ^{a,b}	Observed fragments from Xxx-Pro (Figure no.)
<i>n</i> = 0	Acyl carrier protein (NP_001099764)	651.98, 3+	LMC [†] PQEIVDYADK ^a	Suppressed <i>y</i> ₁₁ (Figure S1)
	Cytochrome <i>c</i> oxidase subunit 6B1 (NP_001138745)	646.31, 3+	SLC [†] PVSWVSAWDDR ^a	No <i>y</i> ₁₁ (Figure 2)
	Creatine kinase S-type (P09605)	665.34, 3+	LGYLTC [†] PSNLGTGLR ^a	Suppressed <i>y</i> ₉ (Figure S2)
	Cytochrome <i>b</i> - <i>c</i> 1 complex subunit 1 (Q68FY0)	995.79, 3+	YFYDQC [†] PAVAGYGP [†] IEQLSDYNR	Observed all related fragments (Figure S6)
<i>n</i> = 1	ATP synthase subunit O (Q06647)	879.13, 3+	GEVPC [†] TVTTAFPLDEAVLSELK ^a	Suppressed <i>y</i> ₁₉ ²⁺ (Figure 4)
	Thioredoxin (P0AA25)	785.7, 3+	ADGAILVDFWAEWC [†] GPC [#] K ^b	No <i>y</i> ₃ [11]
	3-ketoacyl-CoA thiolase (P13437)	983.5, 3+	VVGIFYVSGC [†] DPAIMG [†] PVPAITGALK ^b	High <i>y</i> ₈ and <i>y</i> ₁₀ and no <i>y</i> ₁₇ [11]
	Cytochrome <i>c</i> 1, heme protein (EDM15989)	971.83, 3+	HGGEDYVFSLLTGYC [†] EPPTGVSLR	Low <i>y</i> ₈ and <i>b</i> ₁₆ ²⁺ (Figure S7)
<i>n</i> = 2	Isocitrate dehydrogenase [NADP] (P56574)	659.35, 3+	NILGGTVFREPIIC [†] K	High <i>y</i> ₅ (Figure S8)
<i>n</i> = 3	ADP/ATP translocase 1 (Q05962)	1019.19, 3+	YFAGNLASGGAAGATSLC [†] FVYPLDFAR ^a	No <i>y</i> ₆ (Figure 3)
	Creatine kinase S-type (P09605)	719.61, 4+	LIDHFLFDKPVSP [†] LLTC [†] AGMAR	Moderate <i>y</i> ₁₀ ²⁺ and high <i>y</i> ₁₃ ²⁺ (Figure S9)

^aPeptides also identified with CAM-modification.^bPeptides identified from the earlier study [11].[†]indicates BTP-modified Cys residue; [#] marks CAM-modified Cys residue.

are summarized in Table 3 and indicate that BTP-modified peptides consistently show PAs that were 20–50 kcal/mol lower compared with the unmodified or CAM-modified analogues. This difference indicates that higher energies are required in order to protonate the amide bonds in peptide backbones in the vicinity of a Cys(BTP) residue. With the suppressed propensities for protonating those amide bonds, reduced relative abundances of the product ions (mainly *y*_{*n*} ions) corresponding to the cleavages at those sites are expected, which is consistent with the experimental observations obtained in the current study. As assumed, PAs of the BTP-modified derivatives increased gradually when enlarging the distances between the protonation sites (Ala or Pro) and Cys(BTP) residues (*n*=0 versus *n*=3). The optimized three-dimensional structures display more compact conformations for CAM-tagged and unmodified peptides, while the BTP-modified derivatives show more open structures. This is most likely due to the charge repulsion between the cationic phosphonium ion and the protonated amide bond (Figure S10).

The Proline Effect in BTP-Modified Multiple-Proline-Containing Peptides

The selective cleavages arising from specific amino acid residues in protonated peptides, i.e., Asp, His, and Pro residues, have been statistically evaluated using large datasets of “real world” MS/MS spectra [39–41]. The Wysocki and Yates III groups have determined the average relative cleavage ratios of Xxx-Pro bonds from a dataset of 516 peptides acquired under low energy CID conditions on an ion trap MS [40]. The relative cleavage ratio of an Xxx-Pro bond was defined as the total relative abundance of the sum of a, b and y ions formed at the Xxx-Pro bond divided by the sum of the total ions, a, b and y, observed for that peptide. In

their study, average Xxx-Pro cleavage ratios were reported for 18 combinations, and the cleavage ratios for Cys-Pro and Met-Pro bonds were not summarized because these sequence motifs were under represented in the dataset evaluated. Here, we used the average Xxx-Pro cleavage ratios reported in their study to evaluate the impact of a BTP moiety on the proline effect in peptides containing multiple proline residues.

Several BTP-modified thiol peptides identified from heart mitochondria contain multiple proline residues. The BTP moiety exerted its influences on the respective Pro residues proximate to a Cys(BTP) residue. For example, the previously mentioned peptide, GEVPC[†]TVTTAFPLDEAVLSELK from ATP synthase subunit O, contains two Pro residues, Pro-4 and Pro-12, and was detected with BTP- and CAM-modified derivatives at *m/z* 879.13 (3+) and *m/z* 1188.59 (2+), respectively (Figure 4). The fragment ions related to the proline effect in this peptide were the singly and/or doubly charged *b*₃/*y*₁₉ and *b*₁₁/*y*₁₁ ions resulting from the amide bond cleavages at Val-Pro and Phe-Pro, respectively. The average cleavage ratios for those two bonds have been reported as 0.383±0.223 (for Val-Pro) and 0.282±0.199 (for Phe-Pro), respectively [40]. The *y*₁₉²⁺ ion was the predominant ion in the tandem mass spectrum of the CAM-modified peptide, and the higher relative intensity of *y*₁₉²⁺ ion compared with that of *y*₁₁ ion is in accord with the reported cleavage ratios for those two motifs. In contrast, for the BTP-modified peptide, the relative intensities of the *y*₁₉²⁺ and *y*₁₁ (and *y*₁₁²⁺) ions showed an opposite trend reflecting the impact of the BTP group on the proton affinity of the proline residue at position 4.

Similarly, the fragmentation behavior observed for the BTP-modified peptide VVGIFYVSGCDPAIMGIGPVPAITGALK (*m/z* 983.5, 3+) from acetyl-CoA-acyltransferase from our early study further underscored the impact of the fixed cationic derivatization on the cleavage propensities of

Figure 3. ESI Q-TOF MS/MS spectra of **(a)** BTP-modified (m/z 1019.19, 3+) and **(b)** CAM-modified (m/z 1398.63, 2+) peptide YFAGNLASGGAAGATSLCFVYPLDFAR (aa 112–138) from ADP/ATP translocase 1. The fragment ion expected from preferential cleavage N-terminal to Pro, y_6 , is only observed in the CAM-modified derivative. C[‡] = BTP-modified Cys residue; C[#] = CAM-modified Cys residue

Figure 4. Tandem mass spectra of **(a)** BTP-modified (m/z 879.13, 3+) and **(b)** CAM-modified (m/z 1188.59, 2+) peptide GEVPCVT^TTA^FPLDEAVLSELK (aa 137–158) from ATP synthase subunit O acquired on an ESI Q-TOF. The fragments produced from the selective cleavage N-terminal to Pro are encircled. C⁺ = BTP-modified Cys residue; C[#] = CAM-modified Cys residue; P, precursor ion

Table 3. Proton Affinities of Thiol Peptides with BTP or CAM Modifications

		PA (kcal/mol)			
		<i>n</i> = 0	<i>n</i> = 1	<i>n</i> = 2	<i>n</i> = 3
Non-Pro-containing-peptide	-Ala-Cys(BTP)-Ala _n -Ala ₂ -	181.6	177.6	178.3	193.4
	-Ala-Cys(CAM)-Ala _n -Ala ₂ -	217.9	225.8	222.7	234.8
ΔPA (BTP vs. CAM)		36.3	48.2	44.4	41.1
Pro-containing-peptide	-Ala-Cys(BTP)-Ala _n -Pro-Ala-	178.8	187.9	196.0	190.4
	-Ala-Cys(CAM)-Ala _n -Pro-Ala-	218.7	221.9	216.9	219.7
	-Ala-Cys-Ala _n -Pro-Ala-	214.2	213.8	232.5	222.7
ΔPA (BTP vs. CAM)		39.9	34.0	20.9	29.3
	-Ala-Pro-Ala _n -Cys(BTP)-Ala-	182.0	190.2	187.3	202.0
	-Ala-Pro-Ala _n -Cys(CAM)-Ala-	218.2	233.8	227.9	232.6
ΔPA (BTP vs. CAM)		36.2	43.6	40.6	30.6

the multiple Xxx-Pro bonds present in this peptide [11]. This tryptic peptide has three Pro residues and the reported average cleavage ratios for those three sites are 0.367 ± 0.235 (for Asp-Pro), 0.383 ± 0.223 (for Val-Pro), and 0.068 ± 0.071 (for Gly-Pro). But, only two Pro-related y_n fragment ions, y_8 and y_{10} (cleavages at Val-Pro and Gly-Pro), were detected with high intensities. The expected y_{17} ion (cleavage at Asp-Pro) was not observed although this particular amide bond is considered as an enhanced cleavage site [40]. The absence of the y_{17} ion in the BTP-derivative is a strong experimental evidence of the suppressing effect of a BTP group on the protonation and fragmentation of the proximate Asp-Pro bond. Other BTP-modified multiple-proline-containing peptides and their fragmentations at the corresponding Xxx-Pro bonds are described in Table 2.

In summary, our study demonstrates the impact of the cationic BTP moiety (versus a neutral CAM group) on the fragmentation behaviors of protonated peptides under low energy CID conditions. The CID-MS/MS studies of cysteine-containing peptides generated from a biological sample revealed that a large cationic moiety in a cysteine side chain can hamper the protonation of proximal amide bonds, including the Xxx-Pro bond. The decreased relative intensities of y -type fragment ions were in accord with the proposed reduced propensities of protonation at those sites. PA calculations supported our experimental observations and confirmed lower PAs for the backbone amide bonds proximal to a modified cysteine residue for BTP-modified peptides compared with the CAM-modified or unmodified analogues. For proline-containing thiol peptides, a hindered proline effect was observed in our studies. PA calculations also supported the dependence of the protonation propensities of Xxx-Pro bonds on the spatial vicinity to a Cys (BTP) residue.

Conclusions

We report a tandem mass spectrometric study of a cysteine thiol-specific charged modification applied to heart mitochondrial samples. The alkylation reagent IBTP is a lipophilic and membrane-permeable cationic probe typically used to label thiol proteins in mitochondria. The introduction of a charged moiety had a significant impact on the relative

abundances of fragment ions resulting from the cleavages at proximal amide bonds of a BTP group. We speculated that a very likely reason for this observation is that a large positively charged moiety in the side chain of Cys residues may interfere with the protonation of neighboring amide bonds. Proton affinity calculations supported the experimental observations in the current study. Our study emphasized the effect on the proton affinities of peptide backbones and the related MS/MS fragmentation behaviors caused by a charged chemical modification on a peptide side chain functional group.

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