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Tunable Charge Tags for Electron-Based Methods of Peptide Sequencing: Design and Applications

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Abstract

Charge tags using basic auxiliary functional groups 6-aminoquinolinylcarboxamido, 4-amino-pyrimidyl-1-methylcarboxamido, 2-aminobenzoimidazolyl-1-methylcarboxamido, and the fixed-charge 4-(dimethylamino)pyridyl-1-carboxamido moiety are evaluated as to their properties in electron transfer dissociation mass spectra of arginine C-terminated peptides. The neutral tags have proton affinities that are competitive with those of amino acid residues in peptides. Charge reduction by electron transfer from fluoranthene anion-radicals results in peptide backbone dissociations that improve sequence coverage by providing extensive series of N-terminal ***c***-type fragments without impeding the formation of C-terminal ***z*** fragments. Comparison of ETD mass spectra of free and tagged peptides allows one to resolve ambiguities in fragment ion assignment through mass shifts of ***c*** ions. Simple chemical procedures are reported for N-terminal tagging of Arg-containing tryptic peptides.

Key words: Peptide sequencing, Electron transfer dissociation, Charge tags, Proton affinities, Recombination energies

Introduction

Dissociations of gas-phase peptide ions are known to depend on the number and nature of the charging particles. In particular, electron-based methods of peptide dissociation (ExD) require the presence of at least two charges (Z) in the precursor ion to accomplish detection of charge-reduced species by mass spectrometry. Electron-transfer dissociation (ETD) [1] of peptides further benefits from multiple charging ($Z \geq 3$) to achieve efficient backbone fragmentation [2]. Charging by electrospray ionization depends on the number and nature of basic amino acid residues in the peptide. With tryptic peptides, there is a C-terminal Lys or Arg residue that is readily protonated, but the other charge sites are mostly restricted to protonation at the N-terminal amino groups and His residues. Multiple protonation of peptides containing acidic amino acids or phosphate groups can be inefficient, lowering the population of ion species which are suitable for ETD.

Multiple charging in electrospray can be enhanced by chemical derivatization to introduce fixed-charge or readily chargeable groups [3]. Several charge tags have been investigated on the basis of their chemical availability or synthetic convenience, e.g., 2,4,6-trimethylpyridinium [4], 2,2'-bipyridyl [5], tris(2,4,6-trimethoxyphenyl)phosphonium (TMPP) [6–8], and trimethylammoniumalkyl [9, 10]. Enhanced charging can also be achieved by introducing into

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the peptide the basic guanidine group by various guanidination strategies [11–17]. There are several criteria that the charge tags should meet. One aspect concerns the effect of the auxiliary functional group on backbone fragmentations in ETD. For example, functional groups capable of trapping hydrogen atoms have been shown to efficiently quench backbone dissociations [5, 18–20]. The electronic properties of charge tags also play an important role in assisting or hampering peptide backbone dissociations. Tags with high intrinsic ion-electron recombination energies can work as electron traps that quench peptide-based dissociations, as shown for some transition metal ions [21, 22]. Tags that fragment readily upon electron attachment, such as TMPP, steer the dissociation to channels that bring no information on the peptide backbone [7, 8]. The efficiency of derivatization [23] and extent of side reactions are some other important aspects of charge tagging. An ideal charge tag would be such if it (1) can be readily introduced into the peptide in a high yield, (2) increased the charge state of the ions formed by electrospray, and (3) did not hamper peptide backbone fragmentations by undesired side reactions.

The role of the charge-tag electronic properties can be illustrated with the schematic state diagram (Figure 1) in which the tag and peptide electronic state manifolds are drawn separately [24, 25]. The left panel a shows a situation where the tag recombination energy to reach its ground (***X′***) state is much greater than the recombination energies for the peptide electronic states (***X″***, ***A″***, etc.). Under these conditions, the incoming electron can be sequestered in the tag ***X′*** state, resulting in a non-reactive state of the charge-reduced cation-radical. An internal conversion to a reactive state of a peptide-centered radical, ***X′→X″***, ***X′→A″***, etc., is hampered by the

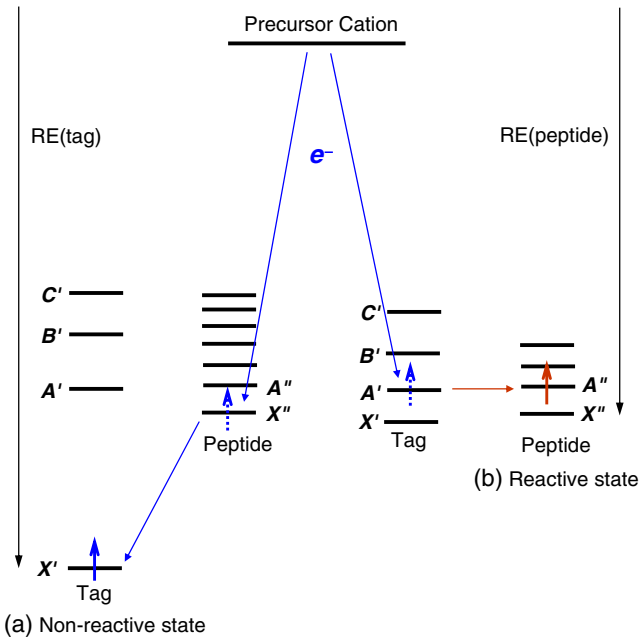


Figure 1. Manifold of electronic states in a charge-tagged peptide ion

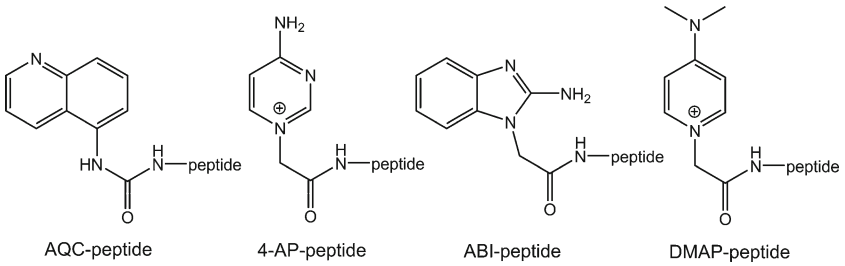
vastly greater vibronic state density of the ***X′*** state compared with those of the peptide electronic states. The right panel of Figure 1 shows a situation where the energy levels of the tag's electronic states match those of the peptide states. This may facilitate electron transfer to some of the peptide reactive electronic states to drive backbone dissociation. Hence, matching the tag's electronic state manifold with that of the peptide appears to be advantageous for facilitating electron attachment in the peptide and accessing dissociative states. Although useful, simple schemes such as that in Figure 1 do not predict the probability for electron attachment in the peptide, which depends on structure details, such as the nature and sequence of amino acid residues and the tagged peptide's secondary structure. We use the Figure 1 scheme as a guiding principle for the rational design of charge tags and report on the progress in developing new derivatives for peptide ExD. Four charge tags are investigated (Scheme 1) that are based on 6-aminoquinoline (AQC), 4-aminopyrimidine (4-AP), 2-aminobenzoimidazole (ABI), and 4-dimethylaminopyridine (DMAP).

Experimental

Materials and Procedures

All peptides were custom-synthesized by NEOPeptide Laboratories (Cambridge, MA, USA) or CHI Scientific (Maynard, MA, USA) and used as received. The purities and amino acid sequences were checked by ESI-MS/MS. Common chemicals (reagent grade) were purchased from Sigma-Aldrich (Milwaukee, WI, USA). Two N-terminal tagging procedures were developed. The first procedure used a conjugation of N-hydroxysuccinimidyl iodoacetate (Sigma-Aldrich) with the peptide in an aqueous buffer, followed by in situ alkylation with the neutral heterocyclic tag, such as 4-dimethylamino pyridine (DMAP), 4-aminopyrimidine (4-AP), or 2-aminobenzoimidazole (ABI) [26]. The following procedure is representative. A solution containing the peptide (100 µL of 3 mM peptide in acetonitrile/water (1/1) (vol/vol) and triethylamine (300 µL of 30 mM in water) was vortexed with 100 µL of 12 mM N-hydroxysuccinimidyl iodoacetate for 30 min. Then 50 µL of a 120 mM heterocyclic tag solution in acetonitrile was added and the resulting mixture was incubated at 75 °C for 2 h. After cooling to room temperature, the solution was diluted with acetonitrile/water/acetic acid (48.5/48.5/1) to a 10 µM concentration and used for direct infusion into the nano-electrospray ion source of the mass spectrometer.

The second procedure is based on a conjugation of *N*-carboxymethyl-4-dimethylaminopyridinium bromide with the peptide using dicyclohexylcarbodiimide and *N*-hydroxybenzotriazole in dimethylformamide. *N*-carboxymethyl-4-dimethylaminopyridinium bromide [27]. A solution of 4-dimethylaminopyridine (0.336 g, 2.75 mmol) in acetone (8.0 mL) was added with stirring to a solution of ethyl bromoacetate (0.330 g, 2 mmol) in acetone (1.5 mL) and the

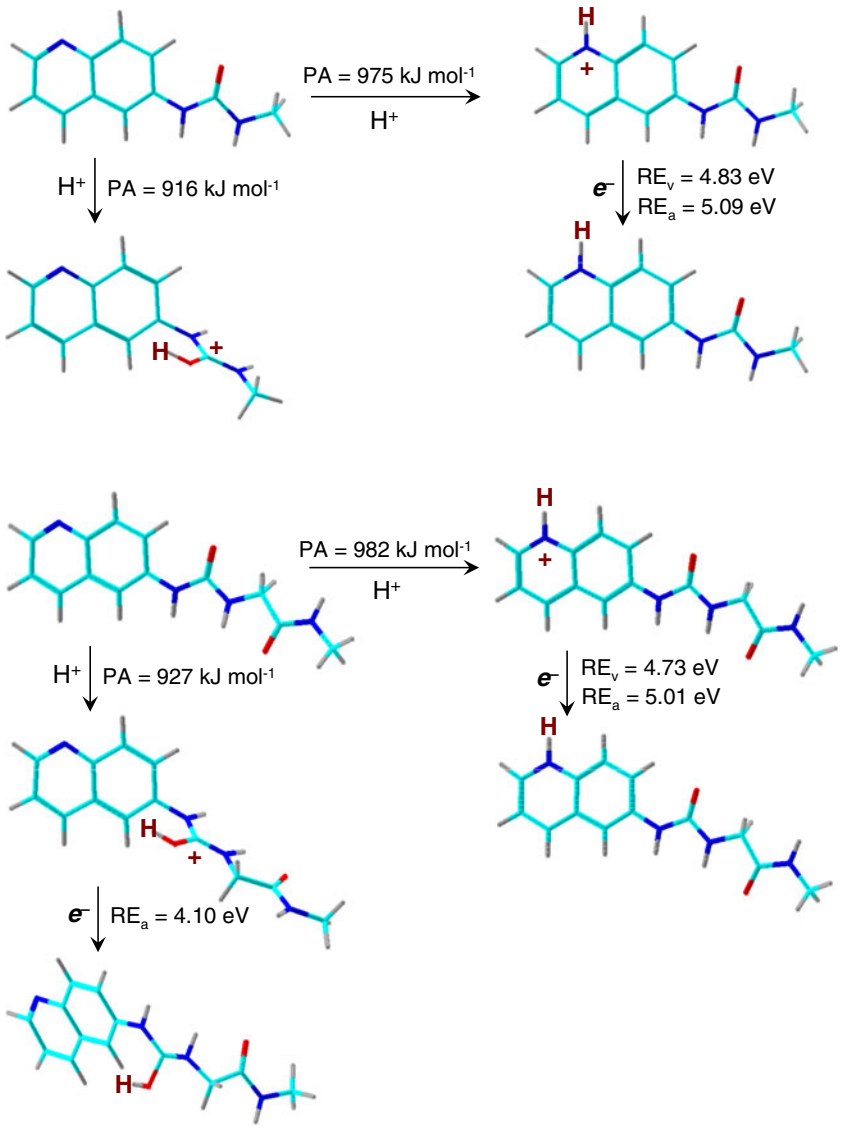


Scheme **1.** Charge tags chemical structures

mixture was refluxed for 1 h. The white needles that precipitated after cooling were filtered off and washed with acetone or ether. *N*-carbethoxymethyl-4-dimethylaminopyridinium bromide from the previous step was dissolved in water (6.5 mL), concentrated hydrobromic acid (1.6 mL) was added, and the mixture was refluxed for 2 h. Then it was cooled and evaporated to dryness in vacuo (1 Torr, 20 °C). The resulting white crystals of carboxymethyl-DMAP were washed with acetone and used without further purification; yield 200 mg.

Tagging Reagent

The tagging reagent solution was prepared as follows. Carboxymethyl-DMAP (3.2 mg), dicyclohexylcarbodiimide



Scheme **2.** Structures, protonation, and reduction of 6-aminoquinoline conjugates

(DCC, 4.1 mg), and *N*-hydroxybenzotriazole (HOBt, 2.7 mg) were mixed in 10 mL of dry *N*,*N*-dimethylformamide at 0° C for 30 min. *N*-hydroxysuccinimide (NHS, 2.3 mg) was added and the solution continued to be mixed at room temperature for 1 h. The tagging reagent was immediately used for peptide derivatization as it slowly decomposes upon standing.

Peptide Charge Tagging

Peptides were tagged by adding 10 µL of 3.0 mM peptide stock solution in water/acetonitrile and 10 µL of the above-described freshly prepared tagging solution to 50 µL of triethylamine/acetic acid buffer (pH 9.5). The solution was vortexed for 1 h at 60 °C. The product was diluted 10-fold by 50:50:1 methanol:water:acetic acid and used for direct infusion into the nanoelectrospray ion source. Tagging with 6-aminoquinolinyl-N-hydroxysuccinimidyl carbamate was performed using a Waters AccQ-Tag AccQ-Fluor Reagent Kit, cat. No. WAT 052880; Milford, MA, USA) according to literature procedures [28, 29].

Mass Spectrometry

Electron-transfer dissociation mass spectra were measured on a Thermo Fisher (San Jose, CA, USA) LTQ XL linear ion trap instrument, outfitted with a chemical ionization source for the production of fluoranthene anion radials as ETD reagent. Precursor dications were mass isolated with a window of 3–4 *m*/*z* units to accommodate nearest ^{13}C isotopologues and allowed to react for 100 and 200 ms with fluoranthene anions. Electron-capture dissociation (ECD) mass spectra were obtained on a Thermo Fisher LTQ-FT-ICR instrument. Precursor ions were isolated in the LTQ with a mass selection window of 1.8-2.5 *m*/*z* units, transferred to the ion cyclotron resonance (ICR) cell, and irradiated by electrons from an external source. The electron energy cannot be measured directly on the Thermo instrument and the corresponding electron emitter bias was set at 3 V. The irradiation pulse width was 300 ms.

Calculations

Standard ab initio and density functional theory calculations were performed using the Gaussian 09 suite of programs [30]. Structures were optimized with B3LYP/6-31+G(d,p) [31–33] and the local energy minima were confirmed by frequency calculations. Single point energies were calculated with B3LYP/6-311++G(2 d,p) and MP2/6-311++G(2 d,p) [34], and averaged to cancel out known errors in each method [35]. Calculations of open-shell systems were performed with the spin-unrestricted formalism (UB3LYP and UMP2), and higher spin states in UMP2 calculations were annihilated by standard projection procedures [36, 37].

Results and Discussion

Aminoquinoline Tags (AQC)

The tags developed for peptide ExD were designed on the basis of their charging properties and ion recombination energies. With neutral tags such as AQC, the charging

Table 1. Recombination Energies of Peptide Charged Groups

Ion	Recombination energy[a,b]			
Ion[c]	RE_a[d]	RE_v	Proton site	Ref.
(**Arg**+H)$^+$	3.39	2.19	Side chain	[38]
(**Arg**-NH_2+H)$^+$	3.22-3.51[e]	2.19	Side chain	[39]
(**His**-$NHCH_3$+H)$^+$	3.46	2.62	Side chain	[40]
{N_a-acetyl-**His**-$NHCH_3$+H)$^+$	3.63-3.74[e]	2.67-2.79[e]	Side chain	[40]
(Gly-**His**+H)$^+$	3.49	2.61	Side chain	[40]
(**His**-Gly+H)$^+$	3.48	2.79	Side chain	[40]
(Gly-**Arg**+H)$^+$	3.44-3.75[e]	2.64-2.81[e]	Side chain	[41]
(Gly-**Lys**+H)$^+$	(4.24)[f]	3.15	Side chain	[42]
(**Pro**-Gly+H)$^+$	3.36	3.07	αNH	[43]
(**Gly**-Pro+H)$^+$	(4.31)[f]	3.17	N-terminus	[43]
{**Gly**-Gly-NH_2+H)$^+$	3.71	2.77	N-terminus	[42]
{**His**-Ala-Ile+H)$^+$	3.27	2.29	Side chain	[44]
{**His**-Ala-Leu+H)$^+$	3.30	2.39	Side chain	[44]
{Ala-**His**-Leu+H)$^+$	3.45	2.46	Side chain	[44]
{Ala-Leu-**His**+H)$^+$	3.31	2.43	Side chain	[44]
{**His**-Asp-Ala-Ala-Leu+H)$^+$	2.83	2.20	Side chain	[44]
{**His**-Ala-Asp-Ala-Leu+H)$^+$	2.96	2.16–2.30[e]	Side chain	[44]
{Ala-**His**-Asp-Ala-Leu+H)$^+$	2.93	-	Side chain	[44]
{Ala-**His**-Ala-Asp-Leu+H)$^+$	3.07	2.21	Side chain	[44]

[a] From combined B3LYP and PMP2 calculations, for details see the references.
[b] In units of eV.
[c] The protonated amino acid residue is shown in bold.
[d] Adiabatic recombination energies include zero-point energy corrections for the precursor ion and the reduced radical.
[e] For different precursor ion or radical conformers.
[f] The radical rearranges by proton migration.

properties were first analyzed by establishing the probable protonation sites and proton affinities of model conjugates with amino acids. Calculations suggest that the AQC moiety in a conjugate (**1**) is going to be protonated at the quinoline ring nitrogen atom (**1a**$^+$), not the carbonyl of the ureido linker (**1b**$^+$, Scheme 2). The calculated proton affinities of the quinoline nucleus in **1** (975 kJ mol^{-1}) and in the glycine conjugate **2** (982 kJ mol^{-1}) are comparable to those of N-terminal amino groups in amino acids to give a quinoline-protonated ion (**2a**$^+$). The potential advantage of the AQC group is that it is not readily accessible for internal solvation by the peptide backbone carbonyls and thus does not seriously affect the secondary structure of the peptide conjugate to hamper protonation at the peptide basic sites. This was reflected in the electrospray ionization mass spectra of AQC-labeled pentapeptides HAAAR, AHAAR, AAHAR, and AAAHR that showed mainly triply charged ions at *m/z* 232.5 formed by attachment of three protons, whereas doubly charged ions at *m/z* 348.2 were weak (<10%). In contrast, the underivatized peptides in the same series gave mainly doubly charged ions at *m/z* 263.1 while triple charging was less efficient. The *m/z* 263.1 ions appeared in some electrospray spectra of these AQC-labeled pentapeptides because of incomplete derivatization.

The ion-electron recombination energies (RE) of the protonated AQC moiety were calculated to range from 4.73 eV vertical to 5.01 eV adiabatic. In general, these are greater than the intrinsic recombination energies of charged groups in amino acid and peptide ions, which range between 2.8 and 3.7 eV for RE$_a$ (Table 1) [38–44] and depend on the charge internal solvation and thus the ion secondary structure. The RE for the AQC group indicate that electron attachment to doubly charged AQC-derivatized peptides should form a cation-radical in which the radical site is in the quinoline ring in the ground electronic state. This is undesirable, because peptide backbone fragmentations rely on radical sites being introduced into the amide groups [45]. The (quinoline+

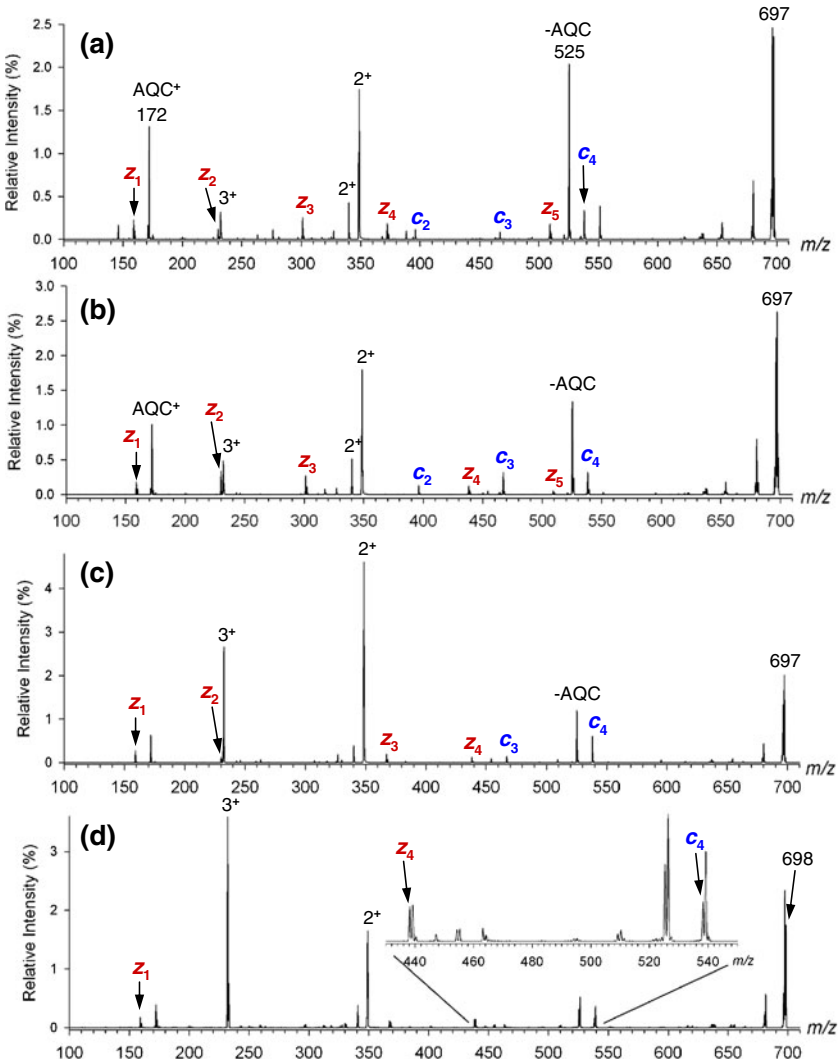


Figure 2. ETD mass spectra (100 ms ion-ion interaction time) of AQC-tagged pentapeptides. **(a)** HAAAR, **(b)** AHAAR, **(c)** AAHAR, **(d)** AAAHR

H) radical moiety is expected to be stable by analogy with other heterocyclic radicals studied previously [46, 47]. This conclusion is corroborated by the ETD spectra of doubly charged AQC-pentapeptides (HAAAR, AHAAR, AAHAR, and AAAHR, *m/z* 348.2), which showed mainly the charge-reduced ions at *m/z* 696.3 and a few low-abundance sequence fragments (z_2 and z_3). The sequence coverage in ETD of the doubly charged ACQ-derivatized peptides was lower than in ETD of the parent doubly protonated peptides [48].

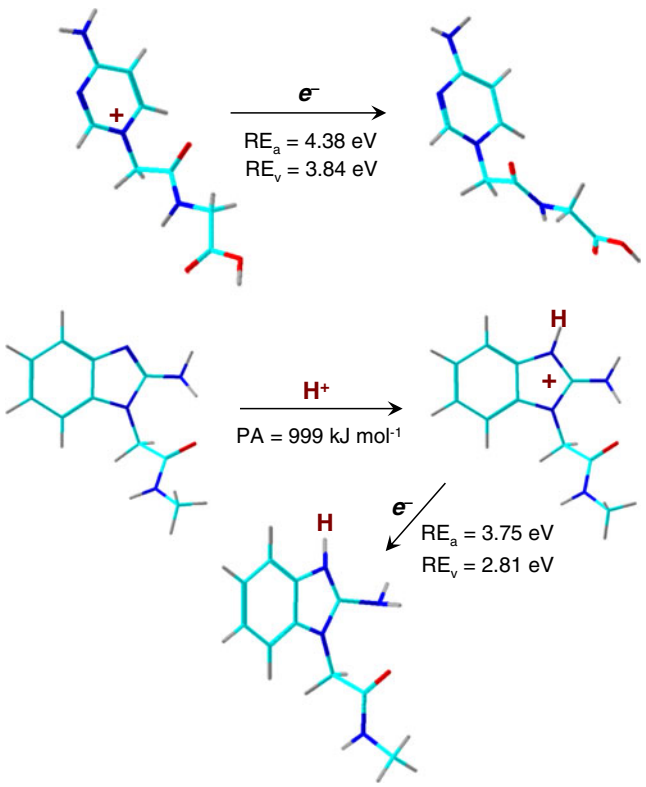
Electron attachment to triply charged AQC-pentapeptides resulted in extensive backbone dissociations yielding series of singly charged ***c*** and ***z*** fragment ions (Figure 2). A common by-product fragmentation was loss of the tag ($C_{10}H_8N_2O$, 172 Da) giving the *m/z* 525 fragment ion. The elemental composition of the *m/z* 525 ion was established from its accurate mass in the ECD spectrum where it represented the by far most abundant fragment. Interestingly, the complementary *m/z* 172 fragment, which also appears in the ETD and ECD spectra of AQC-tagged peptides, is a cation-radical although its structure (classical or distonic) has not been established. Another common feature of the ETD spectra in Figure 2 is the presence of abundant *m/z* 697 ions corresponding to the attachment of two electrons and loss of one hydrogen atom and resulting in the formation of cation-radicals.

The ETD mass spectra in Figure 2 show some interesting sequence dependence. The sequence coverage is best for the HAAAR and AHAAR sequences, which show z_1 through z_5 and c_2 through c_4 sequence fragments. This is an improvement compared to ETD of the respective underivatized peptides where arginine-containing *z*-type fragments ions are mostly observed. The electron transfer efficiency and backbone fragment ion relative intensities decrease for AAHAR and AAAHR. The last peptide is the only one that forms abundant singly and doubly reduced ions without loss of hydrogen that appear at *m/z* 348.7 and 697.4, respectively. Most of the fragment ions in the ETD spectrum of ACQ-AAAHR appear as doublets, e.g., c_4 and c_4+H, z_4 and z_4+H shown in the inset of Figure 2(d), and likewise for the others. This effect cannot be due to hydrogen atom migration between the complementary fragments because both are hydrogen rich. Rather, the backbone and other dissociations compete with hydrogen atom loss to give fragments differing by one Da. The unusual behavior of tagged AAAHR is probably due to a stabilization of the *m/z* 697 ion by rearrangements in the His and Arg radicals that have been reported previously to form stable radical intermediates [41, 48].

The abundant charge-reduced *m/z* 697 ions and the *m/z* 525 fragment ions in the ETD spectra retain all the original amino acid residues and presumably could provide further sequence information upon CID in MS^3 mass spectra. The ETD-CID MS^3 spectra of the *m/z* 697 ions showed mainly loss of $C_9H_{10}N_2$ (146 Da) and $C_{10}H_8N_2O$ (172 Da) neutral fragments from the AQC tag and did not provide sequence information (Figure S1, Supplementary Material). This indicated that these charge-reduced ions contained the extra hydrogen atom in the AQC moiety to promote its radical-induced fragmentations. The ETD-CID MS^3 spectra of the even-electron *m/z* 525 ions did show extensive series of ***b*** and ***y*** ions (Figures S2 and S3, Supplementary Material). Interpretation of these ETD-CID MS^3 spectra should be done with caution, because of the presence of internal fragments, e.g., *m/z* 209 from AAHAR, which is isobaric with the b_2 ion from AHAAR and HAAAR but is not a sequence fragment.

Amidine Tags

Heterocyclic molecules containing the amidine group, such as 4-aminopyrimidine and 2-aminobenzoimidazole can serve as potential ionizable tags to be attached to a peptide via a linker. We used the -CH_2-CO- linker to attach these tags to the N-terminus of the peptide to be derivatized. The tags are denoted as 4AP and ABI for the 4-aminopyrimidylmethylcarboxamido (4AP) and 2-aminobenzoimidazolylmethylcarboxamido (ABI) groups, respectively. Both the 4-aminopyrimidyl and 2-aminobenzoimidazolyl groups can presumably be attached via one of the ring nitrogens or the amino group. 4-Aminopyrimidine is known to be alkylated at N-1 [49, 50] to produce a fixed-charged pyridinium tag.



Scheme 3. Structures, protonation, and ion-electron recombination of 4-aminopyrimidine and 2-aminobenzoimidazole conjugates

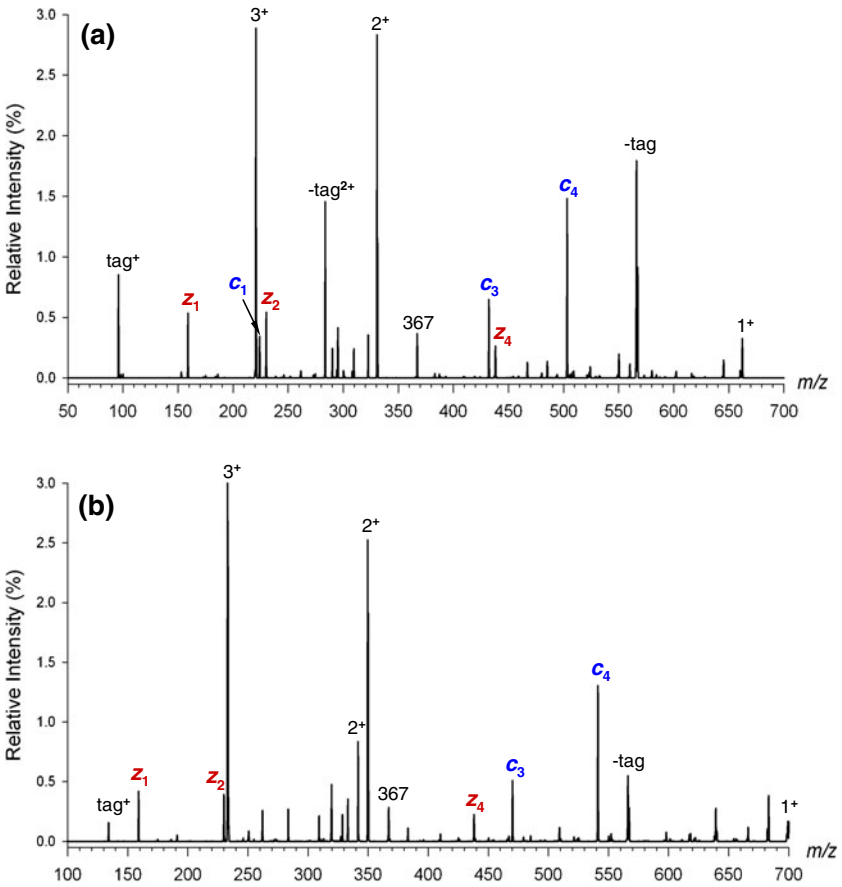


Figure 3. ETD mass spectra (100 ms ion-ion interaction time) of triply charged AHAAR tagged with **(a)** 4-aminopyridyl-1-methylcarboxamido (4AP), and **(b)** 2-aminobenzoimidazolyl-1-methylcarboxamido (ABI) groups

The ABI group is also likely to be attached by an imidazole ring nitrogen [51] to produce a neutral tag.

The gas-phase basicity and recombination energies of the 4AP and ABI tags were assessed from the calculations and published data for the parent heterocycles. 4-Aminopyrimidine prefers protonation at the ring amidine nitrogen with the respective proton affinity of 943 kJ mol^{-1} [47]. The vertical recombination energy of ring protonated 4-aminopyrimidine is 3.72 eV, when based on the reported ion and radical PMP2 energies [47]. The adiabatic and vertical recombination energies of a 4AP-glycine conjugate (Scheme 3) was calculated as RE_a=4.38 eV and RE_v=3.84 eV. 2-Aminobenzoimidazole is another basic group for which we calculate a proton affinity PA=999 kJ mol^{-1}, which is comparable to or greater than those of amino acid residues in peptides, indicating that the tag can be protonated in ABI-derivatized peptides. The 2-aminobenzoimidazole ion recombination energies, RE_v=2.81 eV and RE_a=3.75 eV

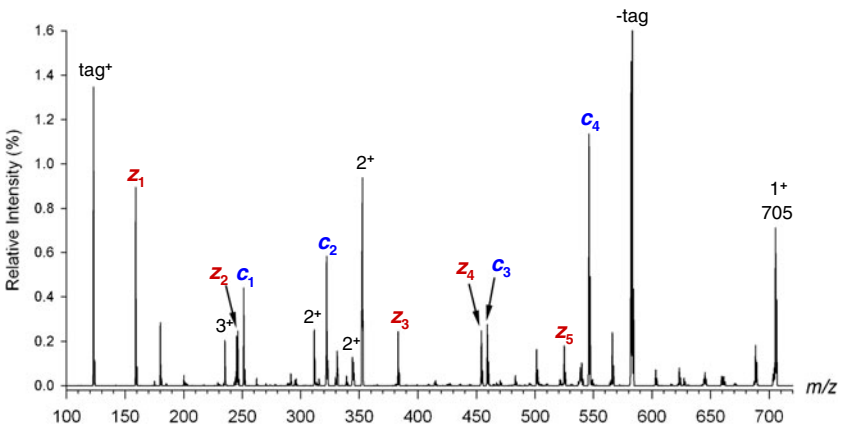


Figure 4. ETD mass spectrum (100 ms ion-ion interaction time) of triply charged DMAP-AAHSR ion

for the ring nitrogen protonated structure $\mathbf{3}^{+}$ (Scheme 3), are low enough to prevent electron trapping in nonreactive benzoimidazole radicals in tagged peptides.

The ETD mass spectra of triply charged 4AP-AHAAR and ABI-AHAAR are shown in Figure 3. Both peptide conjugates show backbone fragments due to cleavages close to the Arg residue, e.g., the complementary z_1/c_4 and z_2/c_3 ions. Loss of the tag also occurs, but it is not a dominant dissociation. ETD of the doubly protonated 4AP and ABI derivatives resulted in charge reduction and very little dissociation. Overall, the 4AP and ABI tags do not appear to be particularly advantageous. The derivatization had a rather low conversion that depended on the peptide composition, and the ETD spectra did not show improved sequence coverage compared to that achieved by ETD of the underivatized peptides.

Fixed-Charge Pyridinium Tags

The *N,N*-dimethylaminopyridyl-1-methylcarboxamido (DMAP) group has recently been shown to substantially improve ETD sequence coverage of small peptides [52]. We therefore focused on studying a broader series of penta-, nona-, and decapeptides tagged with DMAP at the N-termini. The sequences we investigated all had an arginine residue at the C-terminus and were developed systematically by modifying the position or nature of one amino acid residue, e.g., in pentapeptides AAAAR, AAAHR, AAHAR, AHAAR, HAAAR, AAHHR, AAHMR, AAHSR, AAHWR, AAMAR, AASAR, AAWAR, AAYAR, nonapeptides AAHAAXXAR, and decapeptides AAHAAXXAAR, where X were combinations of D, E, N, and Q. Representative ETD spectra are shown below and the sequence coverage as well as issues with the derivatization procedures are discussed.

The DMAP-tagged pentapeptides not containing histidine produced doubly charged ions by electrospray. The ETD spectra of the doubly charged precursor ions showed two major types of fragments. One type was the single dominant peak (80%–90% of fragment ion count) due to loss of the DMAP label (122 Da neutral loss). The other type was a

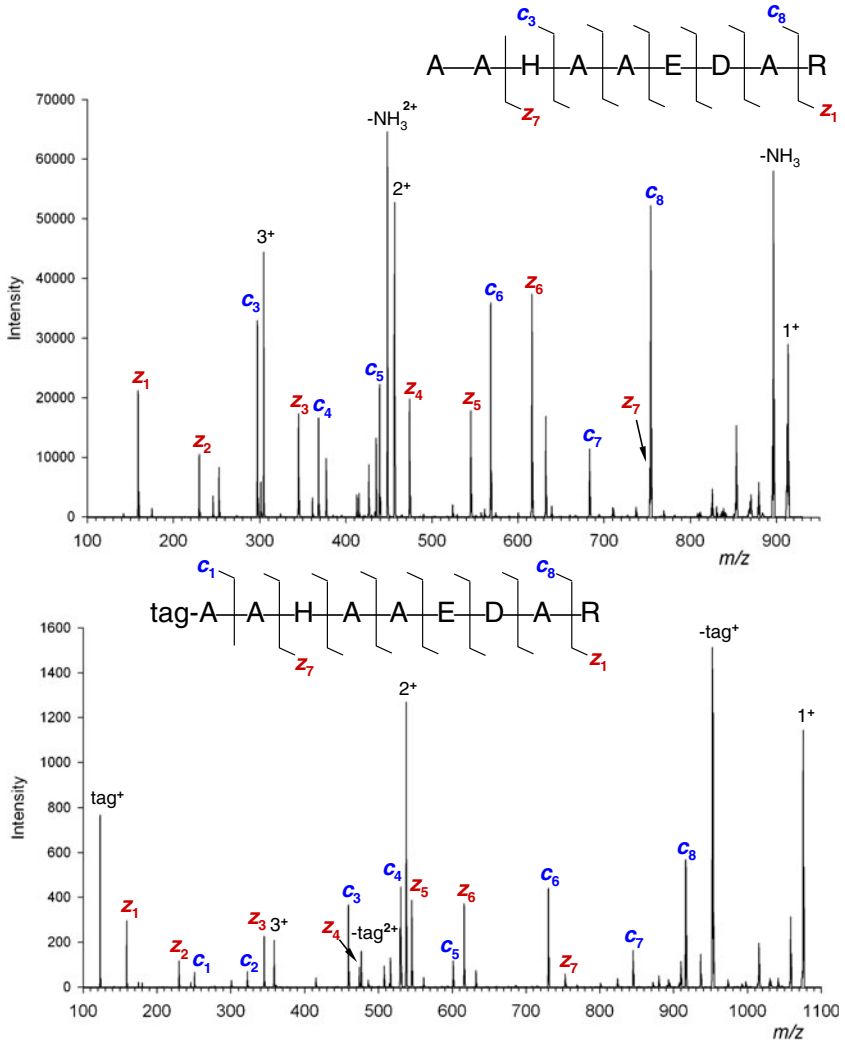


Figure 5. ETD mass spectra of triply charged (*top*) AAHAAEDAR and (*bottom*) DMAP-AAHAAEDAR ions

complete series of DMAP-containing c_1-c_4 ions (10%–20% of fragment ion count), as illustrated for DMAP-AASAR and DMAP-AAWAR (Figure S4, Supplementary Material). The abundant fragments due to the loss of DMAP, *m/z* 516 and 615 from DMAP-AASAR and DMAP-AAWAR, respectively, were further analyzed by CID-MS^3, which gave the series of z and y-2 ions (Figure S5, Supplementary Material). These are most likely formed by radical-induced dissociations accompanied by hydrogen atom migrations. The radical nature of these dissociations was further corroborated by the competing losses of CH_3S and $CH_3SCH{=}CH_2$ from the methionine residue in AAMAR and the loss of a 106 Da neutral fragment from the tyrosine residue in AAYAR [53–59].

The DMAP-tagged pentapeptides containing histidine produced triply charged ions by electrospray. The ETD spectra of the triply charged precursor ions showed three types of fragments of which the sequence ions predominated. For example, the ETD spectrum of DMAP-AAHSR showed complete series of c_1-c_4 (35%) and z_1-z_5 (22%) fragment ions (Figure 4). Loss of DMAP was also present (*m/z* 583, 43%) and this fragment can be used for obtaining or confirming the sequence information through CID-MS^3.

The competing dissociations by backbone N–C_α bond cleavages and loss of DMAP can be attributed to different electronic states in the charge reduced ions. Thus, loss of DMAP is triggered by electron attachment to the fixed-charge group which weakens the pyridinium N–CH_2 bond in the radical intermediate [25]. This is a major process in ETD of doubly charged precursor ions where the intrinsic recombination energy of the DMAP group is greater than that of the Arg guanidinium group [38, 42], and thus electron attachment in the former produces the ground electronic state of the charge-reduced ion. Conversely, the significant formation of DMAP containing sequence c ions is evidence of the involvement of low-lying excited electronic states arising by electron attachment to the peptide backbone and followed by proton transfer from the Arg residue. This behavior is perfectly consistent with the Utah-Washington model of electron based dissociations [60, 61].

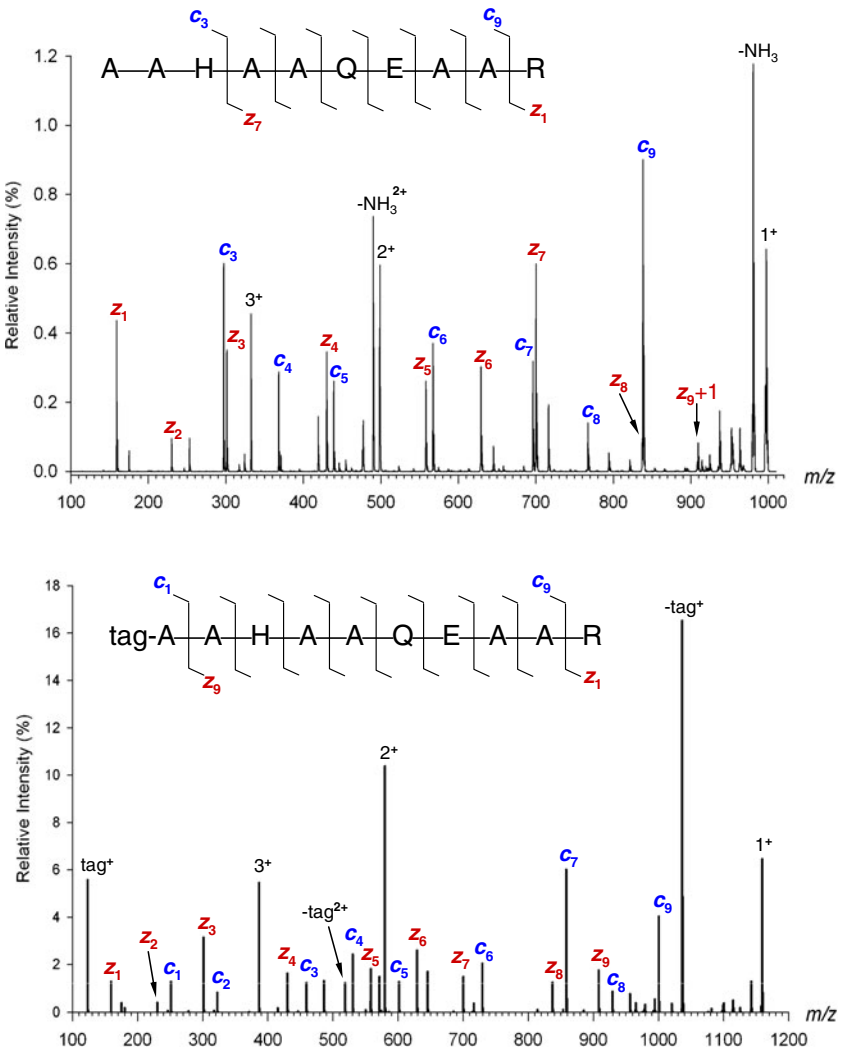


Figure 6. ETD mass spectra of triply charged (*top*) AAHAAQEAAR and (*bottom*) DMAP-AAHAAQEAAR ions

The DMAP-tagged nona- and decapeptides produced mainly triply charged ions by electrospray. The ETD spectra of the triply charged free peptides and their tagged derivatives can be illustrated with those of nonapeptide AAHAAEDAR (Figure 5) and decapeptide AAHAAQEAAR (Figure 6). The ETD mass spectrum of the nonapeptide (Figure 5) shows a very good sequence coverage ranging from z_1 through z_7 and from c_3 through c_8. A potential caveat in a de novo sequence assignment is the adjacent *m/z* values for the z_7 and c_8 fragments, which in the absence of high resolution data could be read as $c_8 - 1$ and z_7+1, respectively, due to hydrogen transfer accompanying the fragmentation [62]. The absence of small c fragments makes it difficult to characterize the residues close to the N-terminus. These difficulties are absent in the ETD spectrum of the DMAP-tagged peptide (Figure 5(b)), which shows a complete series of tag-containing c fragment ions at *m/z* values that are shifted by 162 Da compared with those from the free peptide. The presence of the DMAP tag does not impede the formation of the z ions which show a z_1 through z_7 series. Since the DMAP tagging is N-terminus specific, one can apply the mass shift rule [63] in comparing the ETD spectra of the free and tagged peptides and unequivocally assign the c and z fragment ion series. Similar results were obtained with ETD of the other nonapeptides, the representative decapeptide AAHAAQEAAR, and its analogs. The ETD spectrum of triply charged AAHAAQEAAR showed a z_1 through z_7 series of sequence fragments (Figure 6(a)). The z_8 ion is obscured by the abundant c_9 fragment on an adjacent *m/z*. The c ion series runs from c_3 through c_9 and does not cover all of the N-terminal residues. The ETD spectrum of the DMAP-tagged peptide (Figure 6(b)) shows complete series of c_1 through c_9 and z_1 through z_9 ions, allowing redundant sequence determination. Owing to mass shifts of the c ions, there are no overlaps with the z ions.

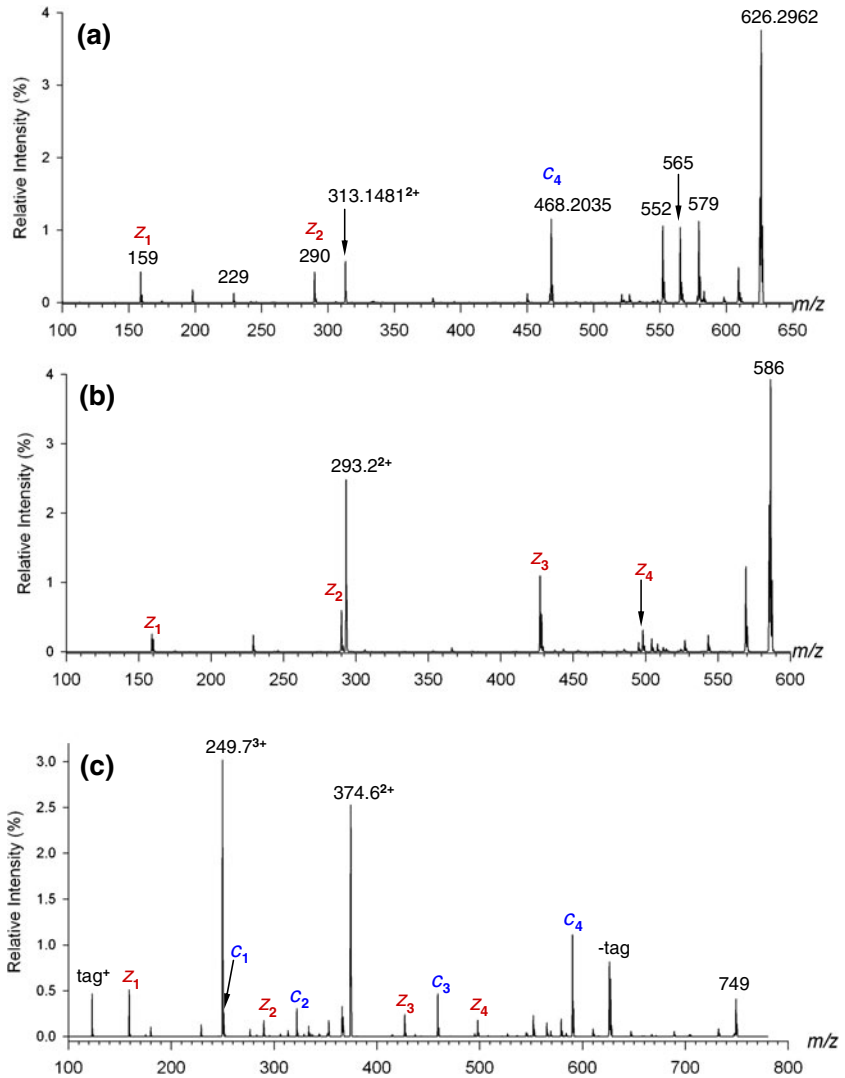


Figure 7. ETD mass spectra of doubly charged ions of **(a)** the byproduct of alkylation of AAHMR, **(b)** AAHMR, and **(c)** triply charged DMAP-AAHMR

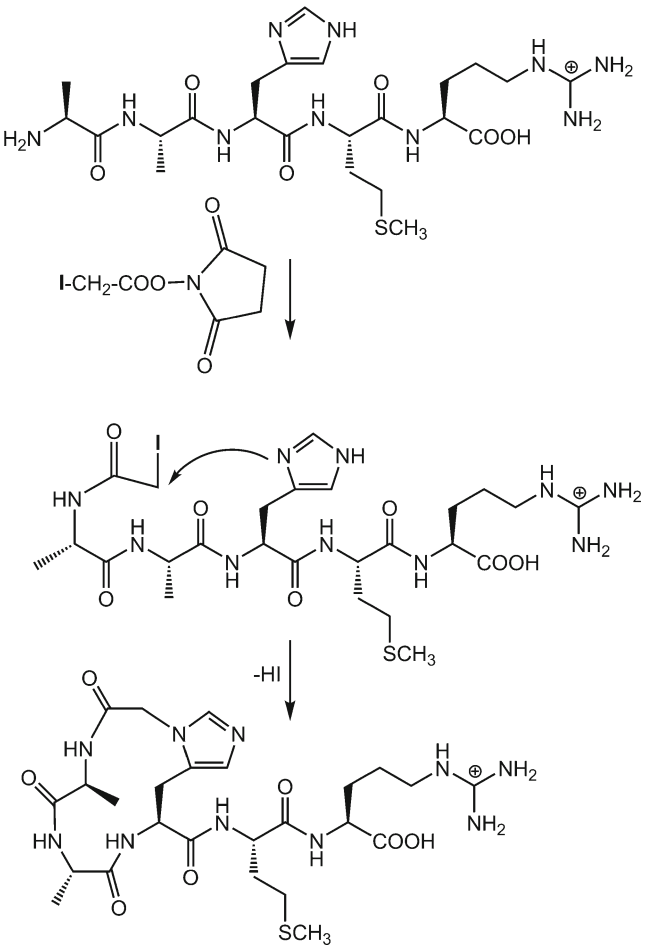
The efficiency of sequence coverage in the ETD spectra can be assessed by considering ions that carry no sequence information. Those are the charge-reduced ions (both singly and doubly charged) and fragment ions formed by loss of ammonia and the tag from the free and tagged peptide ions, respectively. The combined relative intensities in ETD spectra of these non-sequence ions ranged between 30% and 40% for free nona- and decapeptides compared with 40%–50% for the same DMAP-tagged peptides. Thus, loss of the tag has a relatively minor effect on the fragmentation efficiency providing sequence information.

Products of Side Reactions

The derivatization procedures produced a few byproducts that were identified by their MS/MS spectra. In particular, alkylation with DMAP resulted in the formation of peptide derivatives that were 40 Da heavier than the free peptides. Figure 7 shows the ETD mass spectra of ions produced from AAHMR. The top spectrum (Figure 7(a)) shows a doubly charged ion at *m/z* 313.1481 and its charge-reduced counterpart at *m/z* 626.2962. This corresponds to an addition of 39.998 Da to AAHMR, indicating a C_2NO increment. ETD fragmentation shows the $\boldsymbol{z_1}$ and $\boldsymbol{z_2}$ ions indicating unchanged Arg and Met residues. The *m/z* 468.2035 ion corresponds to a $\boldsymbol{c_4}$+C_2HNO fragment, which is complementary to $\boldsymbol{z_1}$. However, there are no ions from backbone fragmentation within the AAH segment. The free peptide (Figure 7(b)) as well as the DMAP-tagged one (Figure 7(c)) show unimpeded backbone fragmentations covering the entire sequence. We interpret the data as showing the formation of an internally cyclized byproduct, whereby the imidazole nitrogen of the His residue in the ICH_2CO-AAHMR intermediate undergoes nucleophilic attack at the N-terminal iodoacetamide group (Scheme 4). Analogous (M+40) adducts were present in the mixtures after derivatization with DMAP that used the alkylation step. However, they were absent when the tagging was performed by acylation with the in situ generated DMAP-CH_2CO-N-hydroxysuccinimide ester using the above-described alternative procedure.

Overall Evaluation of Charge Tags

The functional groups used as charge tags in this study have been selected primarily on the basis of their intrinsic ion-electron recombination energies. The calculated RE_a values run from 3.8 eV for ABI through 5.0 eV for ACQ, whereas the RE_v values are in the 2.8–4.7 eV range. The ETD data show that the 4-dimethylaminopyridinium tag of RE_a= 4.08 eV has the broadest applications in increasing peptide sequence coverage. This indicates that an intrinsic RE_a around 4.0 eV may be a favorable figure for an efficient charge tag. However, there are other factors affecting ETD, which are due to interactions between the tag and peptide electronic states in a charge-reduced ion [24]. In order to facilitate the formation of ***c***-type fragment ions, the tag must not impede electron attachment to the peptide amide groups that drives N–C_α bond dissociations. Such an attachment is enabled by Coulomb effects of the charge groups that stabilize the amide -$C^{\bullet}(-O^{-})$-NH-anion radicals in zwitter-ionic intermediates [48, 60, 61]. The Coulomb effects in turn depend on the secondary structure of the charge-reduced peptide ion. This combination of electronic and conformational effects cannot be solely accounted for on the basis of the tag intrinsic recombination energy. The recombination energies can be viewed as a useful yet imperfect guide in optimizing peptide charge tags for ETD.



Scheme 4. Formation of a 40-Da byproduct by alkylation of AAHMR

Conclusions

The rational design of peptide charge tags for ETD takes into account the chemical, charge-carrying, as well as electronic, properties of the auxiliary groups. Groups having low intrinsic ion-electron recombination energies have the potential of enhancing the sequence coverage in peptide ETD spectra. However, the selection of the optimal peptide charge tag remains empirical. At the same time, chemical derivatization increases the complexity of peptide mixtures by forming side products. The main benefit of derivatization

with charge tags is seen in de novo sequencing where a comparison of ETD spectra of the free and derivatized peptide allows an unambiguous assignment to be achieved through a complete sequence. The reported chemical procedure can be adopted for tagging lysine-containing peptides that can be first guanidinated to protect the ε-amino group and increase its basicity.

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