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Covalent Modification of Gaseous Peptide Ions with *N*-Hydroxysuccinimide Ester Reagent Ions

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Abstract: Covalent modification of primary amine groups in multiply protonated or deprotonated polypeptides in the gas phase via ion/ion reactions is demonstrated using *N*-hydroxysuccinimide (NHS) esters as the modifying reagents. During the ion/ion reaction, the peptide analyte ions and the NHS or sulfo-NHS based reagent form a long-lived complex, which is a prerequisite for the covalent modification chemistry to occur. Ion activation of the peptide−reagent complex results in a neutral NHS or sulfo-NHS molecule loss, which is a characteristic signature of covalent modification. As the NHS or sulfo-NHS group leaves, an amide bond is formed between a free, unprotonated, primary amine group of a lysine side chain in the peptide and the carboxyl group in the reagent. Subsequent activation of the NHS or sulfo-NHS loss product ions results in sequence informative fragment ions containing the modification. The N-terminus primary amine group does not make a significant contribution to the modification process; this behavior has also been observed in solution phase reactions. The ability to covalently modify primary amine groups in the gas phase with *N*-hydroxysuccinimide reagents opens up the possibility of attaching a wide range of chemical groups to gaseous peptides and proteins and also for selectively modifying other analytes containing free primary amine groups.

Introduction

The advent of soft ionization methods that can produce multiply charged ions, such as electrospray ionization (ESI), has enabled the study of biomolecule ion/ion reactions.[1] Gas phase ion/ion reactions have proven to be particularly effective in transforming gaseous ions from one ion type to another. Such ion transformations include, inter alia, the reduction of charge via proton transfer,[2] the inversion of charge via multiple ion transfers in a single collision,[3] the increase of charge via sequential charge inversion reactions,[4] the incorporation of metal ions into peptides either via metal transfer reactions[5] or metal cation attachment,[6] and the conversion of multiply charged bio-ions to radical species via electron transfer.[7] Coupled with mass spectrometry, the charged particle transfer/attachment reactions just listed, e.g., proton transfer, metal ion transfer, and electron transfer, have proven to be useful in the identification and characterization of proteins[7b,8] and oligonucleotides[9] as means for dissociating ions, for manipulating charge states, or for generating alternative precursor ion types for subsequent dissociation.

The mechanisms by which oppositely charged ions react in the gas phase[10] can proceed via charged particle transfer at crossing points in the interaction potentials without formation of a long-lived chemical complex, as is expected to be particularly important, for example, in electron transfer reactions,[11] or via the formation of a relatively long-lived complex. The latter process allows for relatively complex processes to take place, such as those associated with multiple charged particle transfers in a single ion/ion encounter (e.g., charge inversion reactions). The formation of covalent bonds between functional groups on the oppositely charged reagents is another example where formation of a relatively long-lived complex is

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required. An example of such an ion/ion reaction has recently been reported with the demonstration of Schiff base formation resulting from the ion/ion reaction of an aldehyde-containing reagent anion (i.e., singly deprotonated 4-formyl-1,3-benzenedisulfonic acid) with primary amine groups in multiply protonated peptide ions.[12] Recently, Schiff base formation in polypeptide ions has been implemented in conjunction with charge inversion[13] whereby singly protonated peptides are reacted with doubly deprotonated 4-formyl-1,3-benzenedisulfonic acid to yield modified anions that, upon collision-induced dissociation, yielded more informative structural information than either the singly protonated or singly deprotonated peptide. The observation of Schiff base formation via ion/ion reactions demonstrated the possibility for the specific covalent modification of gaseous peptide ions. The initial step in the ion/ion reaction was the attachment of the reagent ion to the polypeptide ion. Upon subsequent activation, water loss takes place as Schiff base is formed. Unfortunately, water loss is a common dissociation pathway for polypeptide ions. As a result, the population of species formed following water loss from the ion/ion complex is comprised of a mixture of species that includes the Schiff base product along with other species formed by dehydration.

Proteins and peptides, as mediators of specific activities and functions within living organisms, are common targets for modification or conjugation techniques in solution. In mass spectrometry, proteins and peptides are commonly modified in solution to facilitate ionization,[14] quantification,[15] or structural characterization.[16] A variety of reagents have been implemented for selective covalent derivatization of various amino acids in solution.[17] Primary amine groups in peptides and proteins, such as the N-terminus or the ε-NH_2 group of a lysine residue, are commonly acetylated or otherwise modified via reactions with *N*-hydroxysuccinimide (NHS) derivatives. The carbonyl carbons of NHS esters undergo nucleophilic attack by primary amines, resulting in loss of NHS (or sulfo-*N*-hydroxysulfosuccinimide) and formation of an amide bond. One of the earliest reports on using covalent modifications in solution with mass spectrometry examined the relative reactivities of primary amine groups in *Aplysia* egg-laying hormone with the *N*-hydroxysuccinimide ester of biotin.[18] The study revealed that the α-amino group at the N-terminus was the least reactive. Next, Novak et. al. used *N*-hydroxysuccinimidyl acetate to study reactivities of the amino groups of ubiquitin in solution under native and denaturing conditions in top−down Fourier transform ion cyclotron resonance (FTICR)−mass spectrometry (MS).[19] Also, Tsumoto et. al. have demonstrated an efficient method for identification and quantification of proteins using isotope-coded succinimide derivatives for chemical modification in matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) analysis.[20] Sulfo-NHS-biotin reagents have proven effective in cell membrane protein enrichment[21] and assessment of amino acid accessibility in protein structures.[22] Bioconjugation chemistry is also important in the covalent immobilization of peptides onto solid supports. Peptide-modified surfaces play a role in applications ranging from characterization and identification of biologically active motifs in proteins to the development of novel biosensors.[23] In this area of research, Laskin and co-workers have demonstrated covalent immobilization of peptides on NHS-terminated surfaces by soft landing of peptide ions[24] where the NHS groups were released as an amide bond formed between the surface and the peptide primary amine groups.

Given the fact that NHS esters are among the most popular reagents for bioconjugation reactions in solution, a wide variety of reagents are commercially available. Furthermore, the synthesis of various NHS derivatives is straightforward. In this work, we demonstrate that covalent modification of primary amine groups in polypeptide cations and anions occurs using either NHS or sulfo-NHS based reagents. Loss of the NHS or sulfo-NHS groups represents a characteristic signature of the chemical modification process. We also demonstrate that collisional activation of covalently modified peptides results in sequence informative fragment ions containing the modification. The work here suggests that ions derived from NHS esters can be used as reagents for the attachment of a wide variety of functional groups to gaseous polypeptide ions. Peptide ion modification in the gas phase is expected to be most useful in the near term for peptide identification/characterization purposes. For example, the addition of a chromophore can be used to enhance photodissociation efficiencies, the utility for which has been demonstrated previously via solution phase modification.[16] Another recent example of the modification of peptide ions in solution to alter fragmentation chemistry includes the incorporation of modifications that, upon activation, yield radical sites that direct subsequent fragmentation chemistry.[25] Modifications in solution that incorporate more strongly basic sites, such as the guanidination of lysine residues, have been used to alter proton mobility and thereby enhance preferred cleavages at aspartic acid residues.[26] While these solution-based modifications have already been shown to be useful, the ability to effect modifications in the gas phase would provide additional advantages, such as high selectivity, as the peptide ion to be modified can be "purified" via mass selection, and the ready comparison of the modified and unmodified forms of the peptide. In the longer term, other types of applications may develop that

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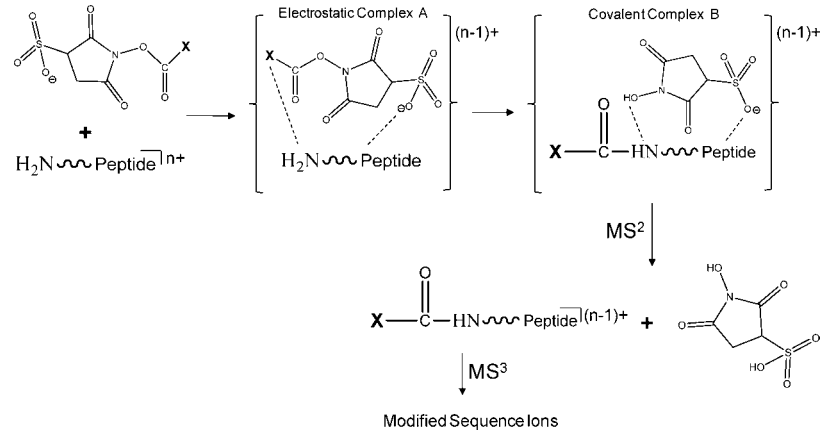
Scheme 1. Sequence of Events in the Ion/Ion Reaction between a Polypeptide Cation and Sulfo-NHS Based Reagent Anion Leading to the Covalent Modification of the Peptide in the Gas Phase



take advantage of this synthetic form of chemistry in the mass spectrometer.

Experimental Section

Materials. Methanol, glacial acetic acid, and acetonitrile were purchased from Mallinckrodt (Phillipsburg, NJ). Peptides KKKKKKKKKK and KAGKGAKAGK were custom synthesized by Pepnome Limited (China), peptides KGAILKGAILR, KGAILDGAILR, and DGAILDGAILD were synthesized by SynPep (Dublin, CA), and peptides YGGFLK, HDMNKVLDL, and VDPVNFK were synthesized by CPC Scientific (San Jose, CA). Reagents sulfo-NHS-AMCA (AMCA = aminomethylcoumarin acetate), sulfo-NHS-SS-biotin, and sulfo-NHS-acetate were obtained from Thermo Fisher Scientific Inc. (Rockford, IL) and Boc-Gln-OSu (*N*-(*N*-α-*tert*-Boc-L-glutaminyloxy)succinimide) was purchased from Sigma Aldrich (St. Louis, MO). The *N*-hydroxysuccinimide ester of 4-trimethylammonium butyrate (TMAB-NHS) reagent was a generous donation from Prof. Fred Regnier. The peptide acetylation procedure has been described previously.[27] All peptide solutions for positive electrospray were prepared in 49.5:49.5:1 (v/v/v) water/methanol/acetic acid mixture at a concentration of ~50 μM, while the peptide solutions for negative electrospray were mixed in 49.5:49.5:1 (v/v/v) water/methanol/ammonium hydroxide solution (~50 μM). All reagent solutions were prepared at a concentration of approximately 2 mM in equal parts of methanol and water, except for TMAB-NHS solution, which was made in acetonitrile.

Mass Spectrometry. All ion/ion reactions were performed on a prototype version of a triple quadrupole/linear ion trap, QTRAP mass spectrometer[28] (Applied Biosystems/MDS Sciex, Concord, ON, Canada), equipped with a home-built dual nanoelectrospray ionization source.[29] The peptide and reagent ions were independently mass selected in Q1 and subsequently transferred to Q2, the collision cell. The ions of opposite polarity were allowed to react under mutual storage mode for various times.[27,30] The ion/ion reaction products were then transferred to Q3. In this transfer step, the product ions were accelerated between Q2 and Q3 to collisionally dissociate any residual sulfo-NHS or NHS molecules. The covalently modified peptide ions were mass selected in Q3, where they were collisionally activated using ion trap collision-induced dissociation (CID). Following the ion trap collisional activation, the modified product ions were mass analyzed using mass-selective axial ejection (MSAE).[31]

Results and Discussion

Multiply Protonated Peptides. Some of the NHS esterified species of interest for conjugation to peptides have limited solubility in water. Addition of the sulfo group to the NHS ring, however, significantly increases water solubility. The sulfo-group modification provides additional advantages for ion/ion reactions involving peptide or protein cations. First, the sulfo group provides an acidic site to facilitate anion formation via negative electrospray ionization (ESI), and second, sulfonate groups engage in relatively strong acid−base interactions with protonated sites on polypeptide cations.[32] These interactions tend to stabilize ion/ion complexes, the long lifetimes of which are amenable to covalent bond formation. The chemical modification described in this paper results from the reaction of a primary amine (i.e., free N-terminus or ε-NH_2 of lysine) with NHS and sulfo-NHS based reagents. The overall process when sulfo-NHS based reagents are used is shown in Scheme 1 as a multistep process. The first step involves attachment of the sulfonate group of the NHS reagent to a protonated site, which is denoted as complex A. Note that complexes of this type are formed regardless of the presence or absence of primary amine groups. When complexes of this type are subjected to collisional activation, loss of the intact reagent as a neutral species dominates. The covalent modification occurs via nucleophilic attack of a neutral primary amine at the carbonyl carbon of the ester functionality. Note that the sulfo-NHS group remains relatively strongly bound to the peptide via the interaction between the sulfonate group and a protonated site. The species formed by the nucleophilic substitution is denoted as complex B. Subsequent activation of complex B in Scheme 1 leads to the formation of a neutral sulfo-NHS molecule and a modified peptide ion linked to the modification via an amide bond. Activation of the covalently modified peptide cation generates fragment ions containing the modification. (Note that it cannot be determined based on the data for peptide cations if rearrangement of complex A to complex B is spontaneous. Activation of the complex may be necessary to overcome any

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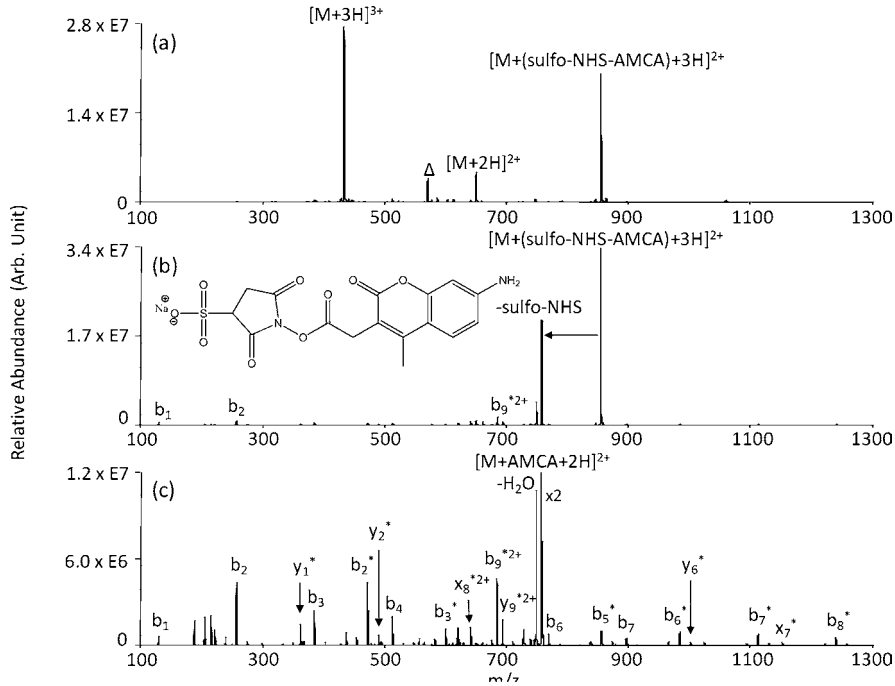

Figure 1. Product ion spectra derived from (a) ion/ion reaction between $[KKKKKKKKKK + 3H]^{3+}$ and $[(sulfo\text{-}NHS\text{-}AMCA)\text{-}Na]^-$, (b) ion trap CID of $[KKKKKKKKKK + (sulfo\text{-}NHS\text{-}AMCA) + 3H]^{2+}$, and (c) MS^3 of $[KKKKKKKKKK + AMCA + 2H]^{2+}$. * denotes ions modified with AMCA, and Δ represents ions from the isolation of the precursor ion.

barriers between A and B. However, the spontaneous loss of the NHS group from polypeptide anions, as discussed below, suggests that activation of complex A may not be necessary for the formation of complex B.)

An illustration of the overall ion/ion reaction phenomenology observed when covalent modification occurs is illustrated by the spectra in Figure 1, which relate to the reaction between triply protonated KKKKKKKKKK cations and singly deprotonated sulfo-NHS-AMCA (the sodium salt of sulfo-NHS-AMCA is shown as an inset in Figure 1b). This reagent is used to label proteins with AMCA, a blue fluorescent dye. Figure 1a shows the results obtained from mutual storage of $[KKKKKKKKKK + 3H]^{3+}$ with deprotonated sulfo-NHS-AMCA. The major ion product corresponds to the complex $[KKKKKKKKKK + (sulfo\text{-}NHS\text{-}AMCA) + 3H]^{2+}$, which may be of the complex A type, the complex B type, or a mixture of the two. A small proton transfer product (i.e., doubly protonated KKKKKKKKKK, indicated as $[M + 2H]^{2+}$ in Figure 1a) is observed as well and likely reflects a contribution from a "proton hopping" channel.[33] The ion trap CID of the complex shows loss of neutral sulfo-NHS as the main fragmentation channel (see Figure 1b), which is consistent with covalent modification of the peptide (see Scheme 1). Essentially no loss of the neutral sulfo-NHS-AMCA reagent to form $[M + 2H]^{2+}$ is observed. Taken together, these results suggest that the $[KKKKKKKKKK + (sulfo\text{-}NHS\text{-}AMCA) + 3H]^{2+}$ species that undergoes fragmentation is largely comprised of complex B species with little contribution from complex A species. Subsequent activation of the sulfo-NHS loss peak (Figure 1c) generates a significant number of b-, y-, and x-type fragment ions containing the AMCA modification. Given the large number of primary amine groups in excess of the total charge in this peptide ion, there is a variety of possible modified structures that can be formed. Evidence for the presence of a mixture of modified structures comes from the appearance of some sequence ions with and without the AMCA modification. Similar fragmentation patterns were observed in the ion trap CID spectra of $[KKKKKKKKKK + (sulfo\text{-}NHS\text{-}SS\text{-}biotin) + 3H]^{2+}$ and $[KKKKKKKKKK + (sulfo\text{-}NHS\text{-}acetate) + 3H]^{2+}$, where complexes were formed after the ion/ion reaction of $[KKKKKKKKKK + 3H]^{3+}$ with $[(sulfo\text{-}NHS\text{-}SS\text{-}biotin)\text{-}Na]^-$ or $[(sulfo\text{-}NHS\text{-}acetate)\text{-}Na]^-$ (see Figures S1 and S2, respectively, in the Supporting Information).

Deprotonated sulfo-NHS-AMCA was also reacted with triply protonated KAGKGAKAGK in the gas phase, as illustrated with the data in Figure 2. Both complex formation and proton transfer were observed as ion/ion reaction channels (Figure 2a), as also noted above. In analogy with the data for KKKKKKKKKK, sulfo-NHS loss as a neutral molecule (Figure 2b) was the dominant process upon activation of the complex. Essentially no loss of the intact neutral reagent was observed, thereby suggesting that most of the peptides in the population of complex ions underwent covalent modification. The MS^3 experiment involving dissociation of the sulfo-NHS loss product resulted in b-, y- and a-type fragment ions that contain AMCA (Figure 2c), which is fully consistent with covalent modification.

The previous two examples illustrate cases in which the number of primary amine groups in the peptide exceeds the total charge. The mechanism for modification of primary amines requires the amine to act as a nucleophile. Protonation of the amine renders it a very poor nucleophile. Hence, when all potential reactive sites are protonated or share a proton, the peptide ion is expected to show poor reactivity toward NHS based reagents.[34] The data for the reaction between triply protonated KGAILKGAILR and deprotonated sulfo-NHS-AMCA (Figure 3) provides an example in which the charge of the peptide renders it relatively unreactive. As with the previous

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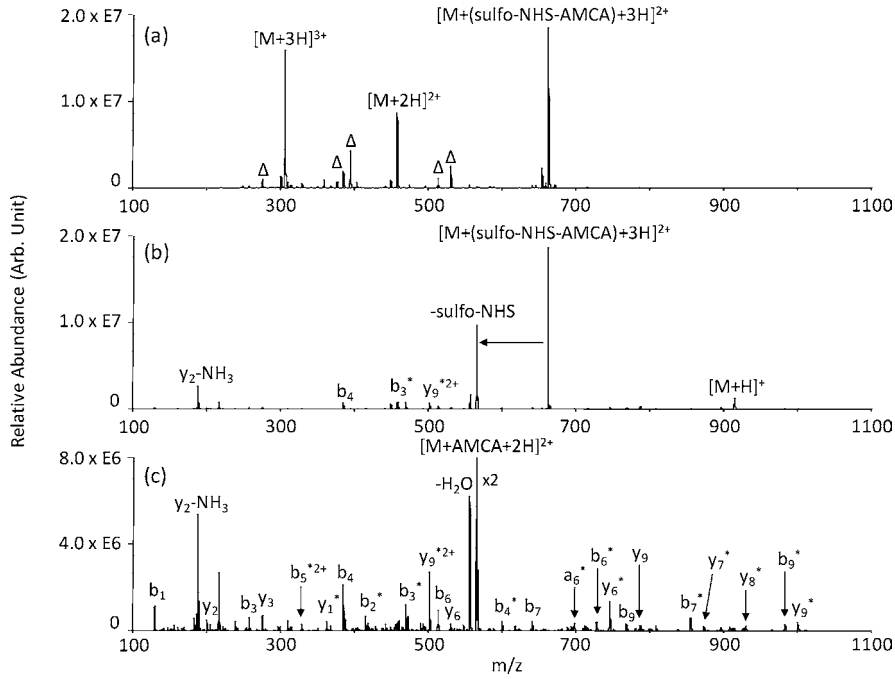


Figure 2. Product ion spectra derived from (a) ion/ion reaction between $[KAGKGAKAGK + 3H]^{3+}$ and $[(sulfo\text{-}NHS\text{-}AMCA)\text{-}Na]^{-}$, (b) ion trap CID of $[KAGKGAKAGK + (sulfo\text{-}NHS\text{-}AMCA) + 3H]^{2+}$, and (c) MS^3 of $[KAGKGAKAGK + AMCA + 2H]^{2+}$. * denotes ions modified with AMCA, and Δ represents ions from the isolation of the precursor ion.

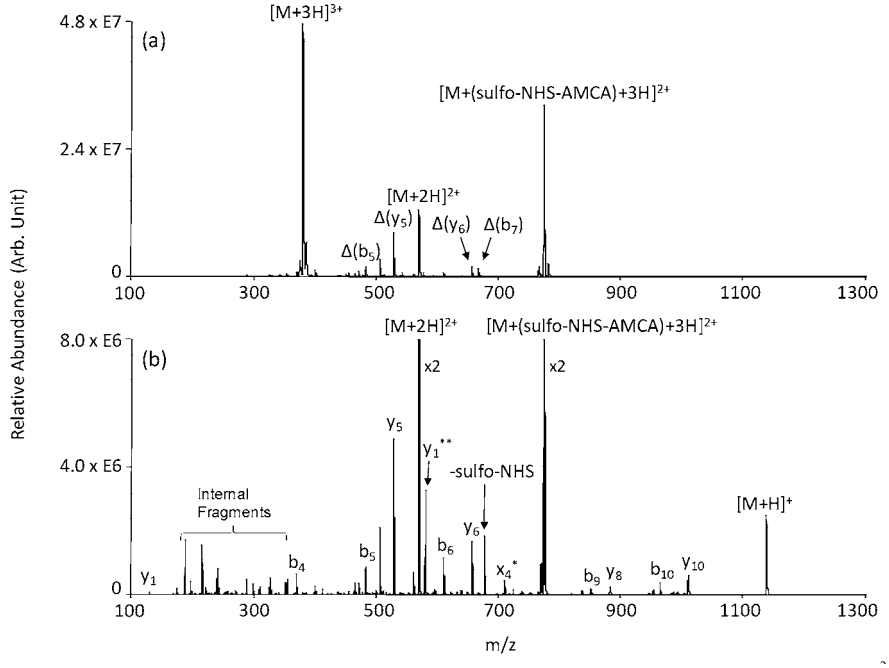


Figure 3. Product ion spectra derived from (a) ion/ion reaction between $[KGAILKGAILR + 3H]^{3+}$ and $[(sulfo\text{-}NHS\text{-}AMCA)\text{-}Na]^{-}$ and (b) ion trap CID of $[KGAILKGAILR + (sulfo\text{-}NHS\text{-}AMCA) + 2H]^{2+}$. Δ represents ions from the isolation of the precursor ion, * denotes ion modified with AMCA, and ** denotes ions with sulfo-NHS-AMCA.

examples, the ion/ion reaction leads to formation of a complex and, to a lesser extent, proton transfer (Figure 3a). In contrast to the previous two examples, ion trap CID of the complex $[KGAILKGAILR + (sulfo\text{-}NHS\text{-}AMCA) + 3H]^{2+}$ in Figure 3b resulted in a loss of sulfo-NHS-AMCA and concomitant formation of $[M + 2H]^{2+}$ as the most abundant channel. This result is consistent with most of the complex ions being noncovalently bound (complex A of Scheme 1). A minor loss of neutral sulfo-NHS suggests that a small fraction of the ion/ion complex might have rearranged to yield covalent modification (complex B). Activation of the $[KGAILKGAILR + AMCA + 2H]^{+}$ product resulting from sulfo-NHS loss (data not shown) resulted in formation of covalently modified b- and y-type sequence ions. The results from this experiment are consistent with observations made independently by Knock and Laskin, who concluded that the α-amino group at the N-terminus was involved the least in the modification. Charge that resides at the N-terminal lysine residue is most likely solvated or shared by both the side chain of Lys-1 and the N-terminus. If one of the remaining charges is associated with the lysine at residue

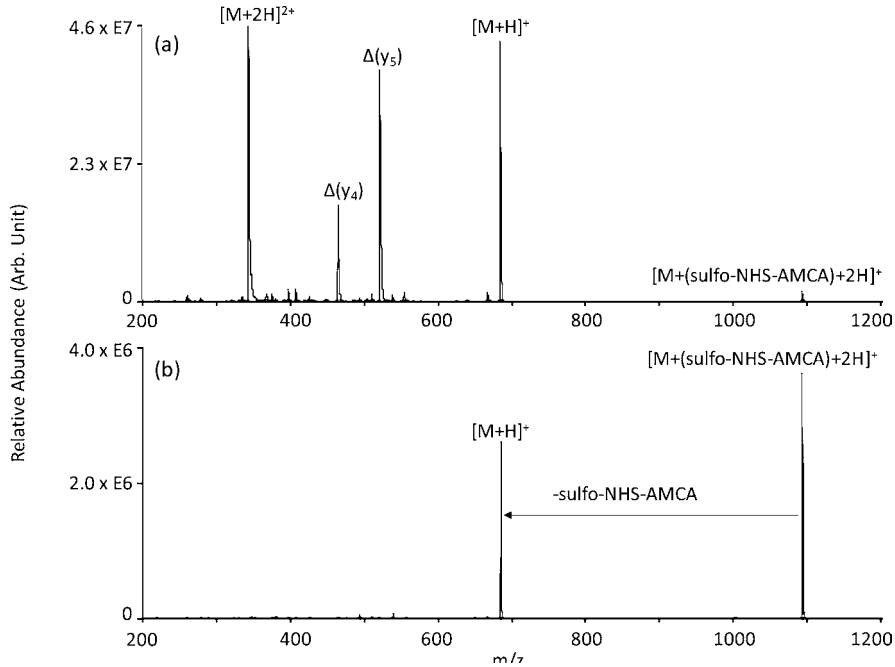


Figure 4. Product ion spectra derived from (a) ion/ion reaction between $[YGGFLK + 2H]^{2+}$ and $[(sulfo\text{-}NHS\text{-}AMCA)\text{-}Na]^{-}$ and (b) ion trap CID of $[YGGFLK + (sulfo\text{-}NHS\text{-}AMCA) + 2H]^{+}$. Δ denotes ions present in the isolation of the precursor ion.

6, all of the primary amine groups are likely to have diminished nucleophilicity, thereby accounting for the limited reactivity of this peptide ion. That is, while the peptide nominally has four basic sites, the N-terminus, two lysine residues, and one arginine residue, the N-terminus and N-terminal lysine can act as a single protonation site if they share the proton, which would make both sites less nucleophilic. If this interpretation is correct, doubly protonated KGAILKGAILR should show greater reactivity toward deprotonated sulfo-NHS-AMCA than triply protonated KGAILKGAILR, as reflected in the relative propensities for sulfo-NHS loss versus loss of the intact neutral reagent from the ion/ion complex. This is observed to be the case, as illustrated in Figure S3b in the Supporting Information, which shows that activation of the $[KGAILKGAILR + (sulfo\text{-}NHS\text{-}AMCA) + 2H]^{+}$ yields slightly more sulfo-NHS loss than loss of the intact neutral sulfo-NHS-AMCA reagent. This suggests that most of the complexes were covalent in nature (as reflected by loss of sulfo-NHS) rather than electrostatic in nature (as reflected by loss of the neutralized reagent).

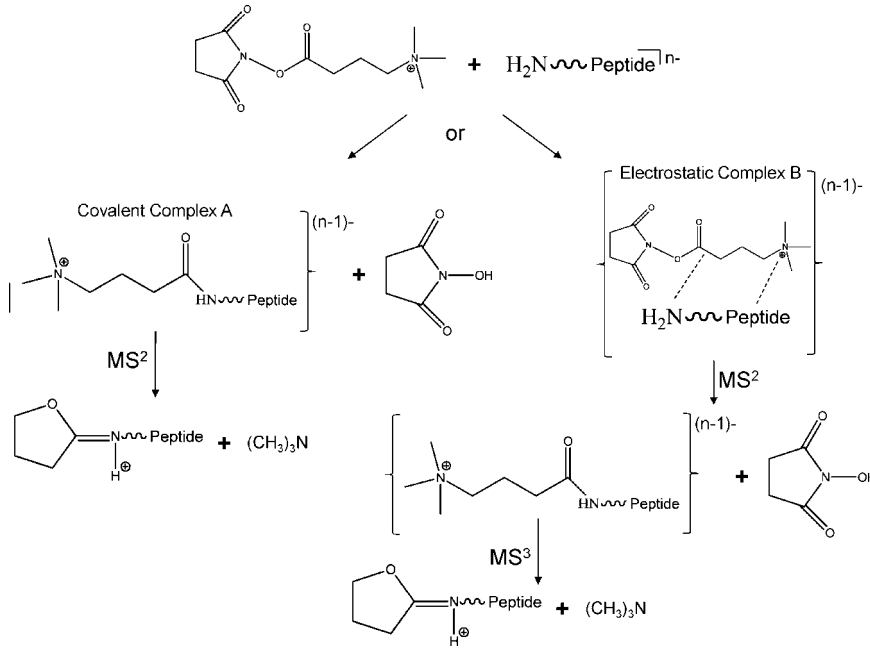
In contrast with the previously discussed peptides, the ion/ion reaction between doubly protonated YGGFLK and singly deprotonated sulfo-NHS-AMCA resulted in mostly proton transfer with minimal complex formation, as seen in Figure 4a. Activation of the complex (see Figure 4b) shows exclusive loss of the intact neutral reagent with no evidence for sulfo-NHS loss. This experiment provides further support that covalent modification requires the availability of a neutral primary amine. Proton transfer was exclusively observed in the ion/ion reaction of $[YGGFLK + 2H]^{2+}$ and sulfo-NHS-acetate, while separation of charge occurred during ion trap CID of the complex $[YGGFLK + (sulfo\text{-}NHS\text{-}SS\text{-}biotin) + 2H]^{+}$ (see Figures S4 and S5, respectively, in the Supporting Information).

Singly Protonated Peptides. Singly protonated peptides can be modified without neutralizing the peptide via the use of multiply charged reagent anions. An example is provided in the reaction of protonated KAGK with doubly deprotonated bis[sulfosuccinimidyl] suberate (BS^3), a commonly used cross-linking reagent. The reaction of these two ions results in the formation of the singly charged anionic complex $[KAGK + BS^3\text{-}H]^{-}$. Subsequent activation of this product shows a dominant loss of sulfo-NHS, which is consistent with covalent modification of one of the amino groups (see Figure S7 in the Supporting Information). This result indicates that singly charged peptides can be modified such that the modified species retains charge, provided the reagent ions are multiply charged.

Multiply Deprotonated Peptides. The data for multiply protonated peptides suggest at least two important criteria for successful covalent modification with an NHS ester reagent ion. First, the reagent must form a relatively long-lived complex with the peptide ion to allow time for the rearrangement reaction that leads to the modification to occur. For the positive ions discussed above, the sulfo group present on the NHS ring served as a "sticky" group for long-lived complex formation. Second, an uncharged primary amine must be available to serve as the nucleophile in the reaction. In the case of deprotonated peptides, the availability of neutral primary amines should be less of an issue than for protonated peptides. However, carboxylate groups, which are expected to be the major charge-bearing sites in most peptide anions, interact less strongly with protonated sites than do sulfonate groups. Hence, the formation of long-lived ion/ion complexes from reaction of multiply deprotonated peptides with protonated NHS esters is more challenging than the formation of long-lived complexes from reactions between multiply protonated peptides and deprotonated sulfo-NHS esters. For example, anions derived from DGAILDGAILD and HD-MNKVLDL in reactions with protonated *N*-(*N*-α-*tert*-Boc-L-glutaminyloxy)succinimide (Boc-Gln-OSu) showed exclusively proton transfer. The products of the ion/ion reaction between $[DGAILDGAILD\text{-}2H]^{2-}$ and protonated Boc-Gln-OSu, for example, are shown in Figure S6 of the Supporting Information.

To evaluate the reactivity of polypeptide anions with NHS esters, the *N*-hydroxysuccinimide ester of 4-trimethylammonium butyrate (TMAB-NHS) was chosen as a reagent due to the presence of the fixed charge associated with the quaternary

Scheme 2. Sequence of Events in the Ion/Ion Reaction between a Polypeptide Anion and NHS Based Reagent Cation Leading to the Covalent Modification of the Peptide in the Gas Phase



amine group. The fixed positive charge can engage in strong electrostatic interactions with the peptide anion, thereby promoting the formation of long-lived ion/ion complexes. Due to the fact that the "sticky" group is not present on the NHS ring, as it is with the sulfo-NHS esters, the overall reaction scheme differs somewhat from that of Scheme 1. If the nucleophilic attack occurs spontaneously (i.e., without subsequent ion trap collisional activation), the NHS leaving group is not expected to be strongly bound within the complex, in contrast to the case when sulfo-NHS is the leaving group, because of the absence of a "sticky" group on the NHS ring. Hence, loss of NHS can occur from an intermediate ion/ion complex without the need for subsequent activation. In this case, the intermediate complex may not be observed. Scheme 2 reflects this situation by indicating a pathway that does not show an intact intermediate complex (i.e., the pathway that passes through covalent complex A). Alternatively, a few peptides form electrostatic complexes first and additional activation steps show evidence for at least some covalent modification (see below). This scenario is reflected in the pathway that passes through electrostatic complex B.

Dianions of the peptides VDPVNFK and KGAILDGAILR showed essentially no intact complex formation. Rather, the major ion/ion reaction product reflected complex formation followed spontaneously by loss of the NHS group, as depicted by the pathway that passes through covalent complex A. Figure 5a illustrates this result for doubly deprotonated VDPVNFK and the TMAB-NHS cation. When the N-terminus and lysine residue were acetylated, thereby removing the primary amine sites, the peptide-TMAB-NHS electrostatic complex was formed exclusively (Figure 5b).

Ion activation of all $[M + TMAB\text{-}3H]^-$ ions formed "spontaneously", where M is a peptide with a primary amine group, resulted in a dominant loss of $(CH_3)_3N$ (59 Da). Figure 6a shows the results for $[KGAILDGAILR + TMAB\text{-}3H]^-$, for which loss of $(CH_3)_3N$ is the dominant process. The same was also true for the $[VDPVNFK + TMAB\text{-}3H]^-$ ion (data not shown). Trimethylamine loss is a well-known process for peptides that have been covalently modified with TMAB.[35] The dissociation pattern of the covalent complex was compared to the dissociation pattern of an electrostatically bound complex formed in the gas phase, $[KGAILKGAILR + (CP\text{-}TMA)\text{-}2H]^-$. 3-Carboxypropyl-trimethylammonium (CP-TMA) is essentially the TMAB-NHS reagent without the NHS functionality. Therefore, electrostatic binding without covalent chemistry is expected to occur with this reagent cation. Figure 6b demonstrates loss of the entire reagent as a zwitterion (i.e., the carboxyl group is deprotonated resulting in a loss of 145 Da) upon activation of the electrostatically bound complex. No 59 Da loss was observed from the precursor ion.

The data of Figure 6 illustrate that loss of trimethylamine (59 Da) is a dominant process from the covalently modified species and is not observed from electrostatically bound complexes. Collisional activation of the $[ac\text{-}VDPVNFK + (TMAB\text{-}NHS)\text{-}2H]^-$ species illustrates the behavior of an electrostatically bound complex comprised of a fixed-charge reagent with the NHS group (see Figure 7a). A small loss of NHS is noted along with an abundant loss of 259 Da. Isolation and activation of the product formed via NHS loss, $[ac\text{-}VDPVNFK + TMAB\text{-}3H]^-$, showed exclusive loss of 145 Da (see Figure 7b), which yields the same mass product as the dominant CID product in Figure 7a. This result suggests the possibility that the loss of 259 Da noted in Figure 7a proceeds via facile loss of NHS followed by the loss of 145 Da. The latter loss is the same as that noted for loss of CP-TMA as a zwitterion in the data shown in Figure 6b. This implies a net transfer of an oxygen atom from the peptide to the reagent. Taken collectively, these results illustrate the signatures for covalent reaction within the ion/ion complex (reaction 1) and exclusive electrostatic interaction in the complex (reaction 2):

(35) (a) Riggs, L.; Seeley, E. H.; Regnier, F. E. *J. Chromatogr., B* **2005**, *817*, 89–96. (b) Che, F.-Y.; Fricker, L. D. *J. Mass Spectrom.* **2005**, *40*, 238–249.

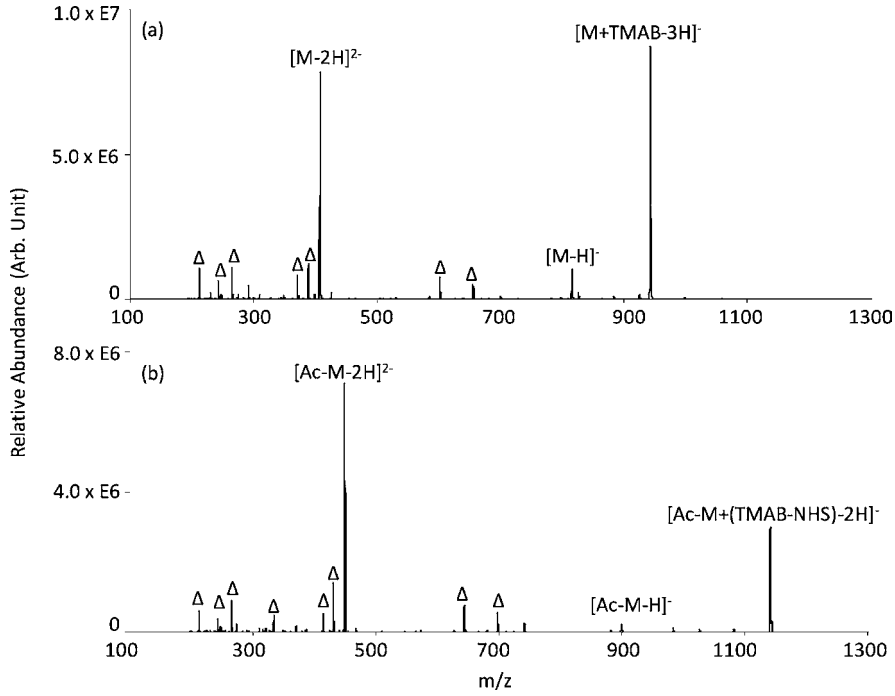


Figure 5. Product ion spectra derived from (a) ion/ion reaction between $[VDPVNFK\text{-}2H]^{2-}$ and $[TMAB\text{-}NHS]^{+}$ and (b) ion/ion reaction between [acetylated-$VDPVNFK\text{-}2H]^{2-}$ and $[TMAB\text{-}NHS]^{+}$. Δ denotes ions present in the isolation of the precursor ion.

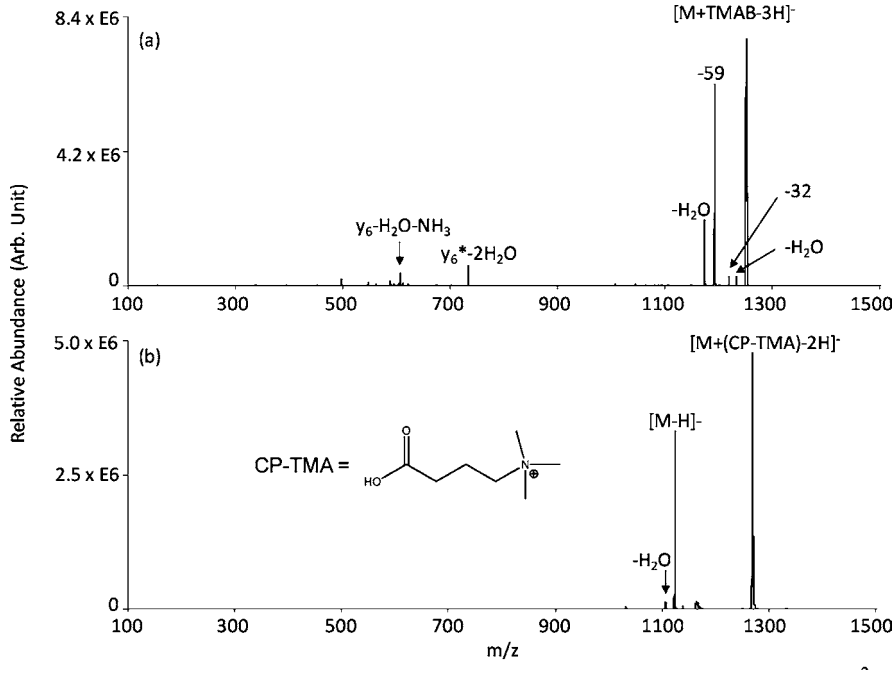


Figure 6. Product ion spectra derived from (a) ion trap CID of $[KGAILDGAILR\text{-}2H]^{2-}$ and $[TMAB\text{-}NHS]^{+}$ and (b) ion trap CID of electrostatically bound complex formed in the gas phase, [KGAILDGAILR + (CP-TMA)-2H]$^{-}$. * represents TMAB modified ions.

[covalent complex] → loss of NHS → loss of 59 Da (trimethylamine) (1)

[electrostatic complex] → loss of NHS → loss of 145 Da (CP-TMA zwitterion) (2)

The reactions of the anions derived from KGAILDGAILR and VDPVNFK appear to give rise predominantly to covalent attachment of the TMAB group by virtue of the dominance of the process summarized in reaction 1 and Scheme 2. The fully acetylated version of VDPVNFK appears to react exclusively via an electrostatic complex, as reflected by the dominance of the sequence summarized in reaction 2. Doubly deprotonated DGAILDGAILD is an example of a peptide anion that showed mixed behavior. That is, some of the ions were covalently modified and others were not. The behavior of this ion is summarized in Figure 8. The ion/ion reaction yielded primarily the intact complex formed by the attachment of $[TMAB\text{-}NHS]^{+}$ to the dianion (see Figure 8a). A minor signal due to [M + TMAB-3H]$^{-}$ was observed, which may reflect that a small fraction of the reactions proceeded via the process shown in

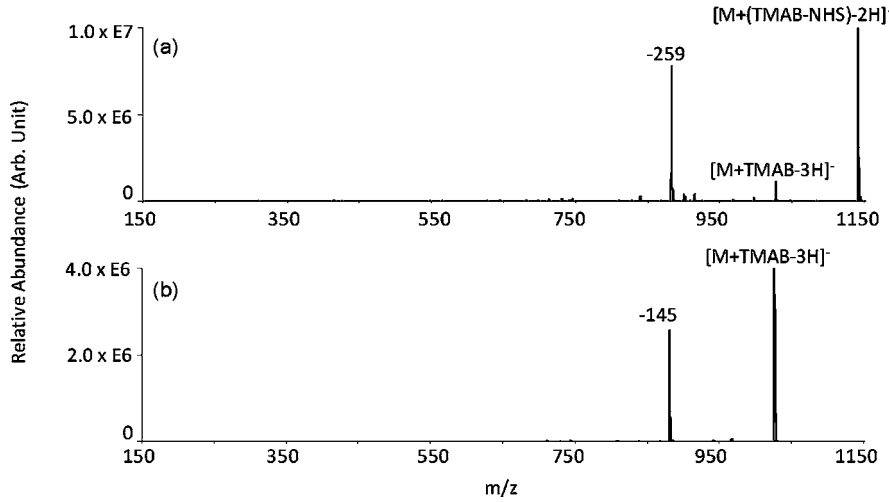


Figure 7. (a) CID of the [ac-VDPVNFK + (TMAB-NHS)-2H]$^-$ complex generated by the experiment illustrated in Figure 5b. (b) CID of the [ac-VDPVNFK + TMAB-3H]$^-$ product generated by the experiment in (a).

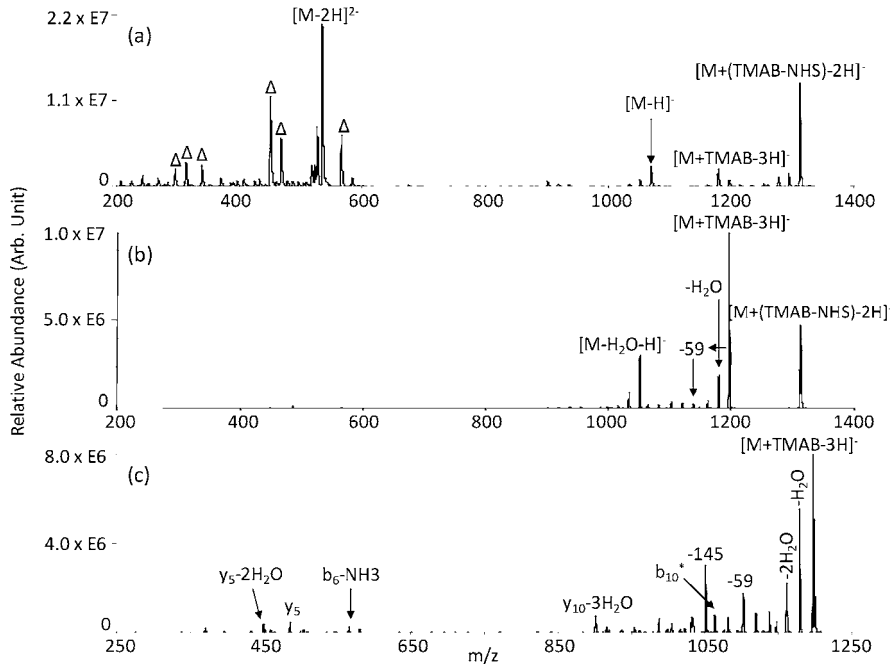


Figure 8. Product ion spectra derived from (a) ion/ion reaction between [DGAILDGAILD-2H]$^{2-}$ and [TMAB-NHS]$^+$, (b) ion trap CID of [DGAILDGAILD + (TMAB-NHS)-2H]$^-$, and (c) MS^3 of [DGAILDGAILD + TMAB-2H]$^-$. * denotes ions modified with TMAB, and Δ denotes ions present in the isolation of the precursor ion.

the left side of Scheme 2. The predominant formation of the intact complex suggests that the interaction was largely electrostatic. Activation of the intact complex showed the loss of NHS, which is a pathway common to both covalent and electrostatic complexes (see Figure 8b). There was also a small signal for 59 Da loss from the [M + TMAB-3H]$^-$ ion (consistent with reaction 1) and a much larger signal for 145 Da loss from the [M + TMAB-3H]$^-$ ion (consistent with reaction 2). Activation of the [M + TMAB-3H]$^-$ ion showed losses of one and two water molecules, which are the most abundant processes from the [M − H]$^-$ ions (data not shown), as well as significant losses of 59 and 145 Da. Taken collectively, the results suggest that some of the DGAILDGAILD anions underwent covalent modification while most probably engaged in an electrostatic interaction with the reagent cation. As the N-terminus is the only primary amine available for the reaction, the lower reactivity of this anion relative to those with one or more lysine residues might be expected.

Conclusions

Multiply protonated or deprotonated polypeptide ions can be covalently modified in the gas phase via ion/ion reactions with *N*-hydroxysuccinimide ester based reagent ions provided several criteria are met. For example, a neutral primary amine must be available to serve as the nucleophile for displacement of the NHS group. This criterion is more readily met with polypeptide anions because no excess protons are available to protonate either the N-terminus or the ε-NH_2 group of lysine. A second criterion is that the reagent ion must contain a functional group that binds strongly to the peptide so that a long-lived complex is formed that can provide time for the reaction to occur. In the case of peptide cations, sulfo-NHS esters make excellent

reagents due to the strong interactions between the sulfonate group and protonated sites on the peptide. A wide variety of sulfo-NHS based reagents are already available due to their frequent use in condensed-phase bioconjugation applications. Since the strongly interacting group is attached to the NHS leaving group, virtually any species that can be coupled with the NHS group can be incorporated into a peptide with available neutral amine sites. In the case of peptide anions, the reagent used here, TMAB-NHS, relies on the strong electrostatic interaction between the fixed-charge TMAB group and negative sites on the peptide. Upon nucleophilic displacement, the TMAB group remains attached to the peptide. It is preferred, however, to have the strongly interacting group present on the leaving group (i.e., bound to the NHS ring) as this provides greater flexibility in choosing the species to attach to the peptide. In any case, the TMAB-NHS reagent used in this work demonstrated that the nucleophilic displacement reaction occurs with peptide anions.

The initial step of the ion/ion reaction involves the formation of a long-lived electrostatically bound complex. In the case of sulfo-NHS based reagents and peptide cations, the rearrangement reaction leading to amide bond formation does not lead to the spontaneous loss of the sulfo-NHS leaving group due to the strong interaction between the sulfonate group and the protonated site to which it initially attached. Subsequent activation of the complex is required to drive off the sulfo-NHS group. Further activation of the covalently modified peptide results in modified sequence ions. When no rearrangement leading to amide bond formation occurs, activation of the complex leads to loss of the intact reagent either as a neutral or as a protonated molecule. In the case of the TMAB-NHS reagent used here for peptide anions, formation of the ion/ion complex can lead to covalent modification with spontaneous loss of the NHS group, since it does not interact strongly with the peptide. The signature reaction indicating covalent modification is the loss of trimethylamine from modified peptide. When the ion/ion complex is electrostatically bound, activation of the complex leads to NHS loss but not to subsequent loss of trimethylamine.

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Supporting Information Available: Structures of the reagents (Schemes S1 and S2) and Figures S1−S7. Figure S1 relates the reaction phenomenology for $[KKKKKKKKKK + 3H]^{3+}$ and [sulfo-NHS-SS-biotin-Na]$^-$, Figure S2 relates the reaction phenomenology for $[KKKKKKKKKK + 3H]^{3+}$ and [sulfo-NHS-acetate-Na]$^-$, and Figure S3 relates the reaction phenomenology for $[KGAILKGAILR + 2H]^{2+}$ and [sulfo-NHS-AMCA-Na]$^-$. Figure S4 relates the reaction phenomenology for $[YGGFLK + 2H]^{2+}$ and [sulfo-NHS-acetate-Na]$^-$ and Figure S5 relates the reaction phenomenology for $[YGGFLK + 2H]^{2+}$ and [sulfo-NHS-SS-biotin-Na]$^-$. Figure S6 relates the reaction phenomenology for $[DGAILDGAILD-2H]^{2-}$ and [Boc-Gln-Osu $+ H]^+$. Figure S7 shows the CID of $[KAGK + BS^3\text{-}H]^-$ after an ion/ion reaction of $[KAGK + H]^+$ with $[BS^3\text{-}2H]^{2-}$.This information is available free of charge via the Internet at http://pubs.acs.org/.

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