Published in final edited form as:

J Am Soc Mass Spectrom. 2015 January ; 26(1): 174–180. doi:10.1007/s13361-014-1002-0.

Gas Phase Reactivity of Carboxylates with *N*-Hydroxysuccinimide Esters

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Abstract

N-hydroxysuccinimide (NHS) esters have been used for gas phase conjugation reactions with peptides at nucleophilic sites, such as primary amines (N-terminus, ϵ -amine of lysine) or guanidines, by forming amide bonds through a nucleophilic attack on the carbonyl carbon. The carboxylate has recently been found to also be a reactive nucleophile capable of initiating a similar nucleophilic attack to form a labile anhydride bond. The fragile bond is easily cleaved, resulting in an oxygen transfer from the carboxylate-containing species to the reagent, nominally observed as a water transfer. This reactivity is shown for both peptides and non-peptidic species. Reagents isotopically labeled with O^{18} were used to confirm reactivity. This constitutes an example of distinct differences in reactivity of carboxylates between the gas-phase, where they are shown to be reactive, and the solution-phase, where they are not regarded as reactive with NHS esters.

Keywords

Ion/ion reaction; carboxylate reactivity; *N*-hydroxysuccinimide ester

INTRODUCTION

Analytes are commonly modified in solution to facilitate ionization¹, quantification^{2, 3}, or structural characterization^{4, 5} via mass spectrometry. A variety of reagents have been utilized for selective modification of various reactive functionalities in solution⁶. Among those, *N*-hydroxysuccinimide (NHS) and *N*-hydroxysulfosuccinimide (sulfo-NHS) based reagents have been commonly used for the covalent derivatization of primary amine groups in peptides and proteins, such as the N-terminus or the ϵ -NH₂ group of a lysine residue. These condensed phase reactions result in the formation of amide bonds through the acylation of the nucleophilic amines. This targeted modification at native functional groups allows the development of site-specific methods to extract useful information from biomolecules.

Reactions analogous to those observed in solution that result in selective covalent bond formation have been explored in the gas phase using ion/ion reactions. Gas-phase ion/ion

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reactions, in general, are effective in transforming gaseous ions from one ion type to another, and can be categorized into two groups. The first group involves simple charged particle transfer, e.g., proton transfer, metal ion transfer, and electron transfer. Such reactions have been demonstrated to be useful for manipulating charge states (e.g., proton transfer), dissociating ions^{7, 8} (e.g., electron transfer), and for switching metal ions for protons for subsequent fragmentation⁹. Small charged particle transfer can occur via a long-lived complex or at crossing points on the energy surface of the collision partners. The second category, which proceeds via the formation of a long-lived electrostatic complex, involves the formation of covalent bonds between reactants. Formation of a long-lived complex allows for the more complex mechanisms generally associated with rearrangement reactions that lead to new covalent bonds. For biomolecules, the selective formation of covalent bonds via a long-lived electrostatic complex has been used for such tasks as tagging peptides with chromophores¹⁰, cross-linking peptides and proteins^{11, 12}, and increasing sequence information^{13, 14, 15}. This gas phase approach of selective covalent modification is attractive from the standpoint of speed, selectivity, and flexibility. Consequently, we have embarked on an effort to develop gas-phase derivatization techniques that can be used as part of an overall MSⁿ strategy for the characterization of analyte ions. It has been demonstrated that NHS ester-based reagents can be used for targeted gas phase conjugation reactions with peptides and proteins at the N-terminus or ϵ -NH₂ group of lysine in a fashion that is similar to solution phase reactions^{8, 16}. The initial step of these gas phase reactions is the formation of a long-lived electrostatic complex via the attachment of the NHS ester-based reagent ion to a charged site of the peptide ion. Upon activation of the complex, nucleophilic attack of an unprotonated amine on the carbonyl carbon of the ester results in the formation of an amide bond and loss of a neutral NHS-based molecule, which is a signature of the covalent modification in the gas phase.

Interestingly, some moieties, such as the guanidinium group of arginine, have been observed to show reactivity in the gas-phase that is not generally noted in solution. The guanidinium group shows reactivity similar to that of unprotonated primary amines in the gas phase^{17, 18}. In aqueous solution, on the other hand, the arginine residues tend to be protonated under physiological conditions due to the high pK_A value of guanidino groups, rendering them unreactive with NHS esters. The gas phase provides an environment where arginine residues are not necessarily protonated, depending on the charge state, polarity, and amino acid composition of the peptide, allowing for the acylation of arginine to take place.

In aqueous solutions, the carboxylate functionality displays rather low nucleophilicity and is typically regarded as unreactive toward the majority of bioconjugation reagents that couple through a nucleophilic attack process¹⁹, including NHS ester-based reagents. It has been reported that these esters can react with a sulfhydryl or hydroxyl group, forming labile thioesters or ester linkages, respectively¹⁷. Tyrosine, serine, and threonine residues have been reported to form stable products upon reactions with NHS esters^{20, 21, 22}. Histidine side-chain nitrogens of the imidazolyl ring may also be acylated with an NHS ester reagent, but they hydrolyze very rapidly in aqueous environments²³. Thus, another possible reason for the lack of observed carboxylate reactivity in condensed phase is that the generated anhydride undergoes rapid hydrolysis to regenerate unaltered reactants in an aqueous environment.

In this work we demonstrate that in the gas phase, the carboxylate group is a reactive site capable of initiating a nucleophilic attack on the carbonyl carbon of NHS ester-based reagents, resulting in the formation of a labile anhydride bond. The gas phase provides an environment in which the nucleophilicity of the carboxylate may be enhanced in the absence of polar solvent, allowing both the formation of the anhydride moiety and subsequent dissociation without regeneration of the carboxylate. This report explores the reactivity of the carboxylate functionality with NHS and sulfo-NHS esters under several conditions, and the subsequent dissociation behavior of the formed anhydride. The work here constitutes an example of distinct reactivities of a functional group between the gas and solution phases.

EXPERIMENTAL

Materials

Methanol, acetic acid, and acetonitrile were purchased from Mallinckrodt (Phillipsburg, NJ). The peptide AADAADAA was custom synthesized by NeoBioLab (Cambridge, MA). The (–)-(18-crown-6)-2,3,11,12-tetracarboxylic acid and ethylenediaminetetraacetic acid tetrasodium salt dihydrate were obtained from Sigma Aldrich (St. Louis, MO). The bis(sulfosuccinimidyl) suberate (BS³) cross-linker was obtained from Thermo Fisher Scientific Inc. (Rockford, IL). The *N*-hydroxysuccinimide ester of 4-trimethylammonium butyrate (TMAB-NHS) reagent was a generous donation from Prof. Fred Regnier. All peptide solutions for positive nanoelectrospray (nESI) were prepared in 49.5/49.5/1 (v/v/v) methanol/water/acetic acid, while the peptide solutions for negative nESI were mixed in 49.5/49.5/1 (v/v/v) methanol/water/ammonium hydroxide solution (~50 μM). The BS³, ethylenediaminetetraacetic acid (EDTA) and (–)-(18-crown-6)-2,3,11,12-tetracarboxylic acid (18C6-TCBA) reagents were dissolved in water (~2 mM), while the TMAB-NHS was dissolved in acetonitrile (~2 mM).

Peptide acetylation

N-terminal acetylation was performed as described previously¹⁸. Briefly, approximately 1 mg of solid peptide was dissolved in a 5 mM pH 9 sodium borate buffer in water. To this, 20 μL of acetic anhydride was added and the mixture was allowed to react to completion, which was approximately 2 hours.

O¹⁸ labeling

C-terminal O¹⁸ labeling was performed by dissolving approximately 1 mg of solid peptide in H₂O¹⁸ with 1% (v) trifluoroacetic acid as described previously²⁴. The mixture was allowed to react under room temperature for 3 days followed by lyophilization to dryness. The O¹⁸-labelled peptides were resuspended in water prior to use.

Mass Spectrometry

All experiments were performed using a prototype version of a triple quadrupole/linear ion trap mass spectrometer²⁵ (QTRAP®, AB Sciex, Concord, ON, Canada), previously modified for ion/ion reactions. Alternately pulsed nESI²⁶ allows for sequential injections of the analyte and reagent ions into the q2 reaction cell for mutual storage ion/ion reactions²⁷. The reagents and singly protonated peptide cation (or doubly deprotonated dianion) are

sequentially isolated in the Q1-mass filter prior to their injection into the q2 reaction cell. After a defined mutual storage reaction time of 500 to 1000 *ms*, the product ions are then transferred to Q3 where the electrostatic complexes are mass-selected. Resonant excitation was used to first activate the complexes to effect covalent modifications and later to activate the modified peptides to fragmentation in Q3. The product ions were then mass analyzed by mass-selective axial ejection (MSAE)^{25, 28}.

Calculations

Geometry optimization and harmonic frequency calculations were carried out using the Gaussian09 program to calculate the zero point energies at the B3LYP/6-31+G(d) level of theory for EDTA and TMAB-NHS.

RESULTS AND DISCUSSION

The doubly-deprotonated ethylenediaminetetraacetic acid [EDTA-2H]²⁻ ion was allowed to react with the NHS ester of the 4-trimethylammonium butyrate cation [TMAB-NHS]⁺. The fixed charge associated with the quaternary amine group of TMAB-NHS can engage in strong electrostatic interactions with the EDTA anion, thereby promoting the formation of a long-lived electrostatic complex [EDTA+(TMAB-NHS)-2H]⁻ (Figure 1a). Collision-induced dissociation (CID) of the complex results in a loss of neutral NHS (115 Da), suggesting the formation of a covalent bond, as well as an abundant loss of 260 Da to nominally form [EDTA-H₂O-H]⁻. Isolation and activation of the product formed via NHS loss, [EDTA+TMAB-2H]⁻, showed exclusive loss of 145 Da (Figure 1c), generating a product with the same nominal mass of [EDTA-H₂O-H]⁻. This result suggests that the 260 Da loss noted in Figure 1b proceeds via facile loss of NHS followed by the loss of 145 Da. The latter loss corresponds to the mass of 3-carboxypropyl-trimethylammonium (CP-TMA) as a zwitterion, which is essentially the TMAB with an oxygen atom transferred from the EDTA. This covalent reaction channel is unexpected in the absence of either an amine or guanidinium group. Given that the most nucleophilic site on the precursor is the carboxylate group, and the most likely origin of the transferred oxygen is one of the carboxyl groups, the data of Figure 1 suggest that a covalent reaction between TMAB-NHS and the carboxylate has occurred. Note that in both Figures 1a and 1b, a nominal [EDTA+14-H]⁻ peak was observed, corresponding to a methyl cation transfer from the TMAB to the carboxylate, in analogy with alkyl ion transfers to carboxylate groups from other alkyl ‘onium’ ions^{29, 30, 31}. Doubly deprotonated (–)-(18-crown-6)-2,3,11,12-tetracarboxylic acid (18C6-TCBA) has also been tested to show the same reaction channel with TMAB-NHS (Figure S1), suggesting that this is a general process for analytes containing free carboxylates. We note that carboxylic acid groups (i.e., -COOH groups) have previously been shown to be reactive in the gas phase with carbodiimide based reagents³².

A schematic diagram for the reaction between a carboxylate group and the NHS ester is proposed in Scheme 1. In the long-lived complex, the carbonyl carbon of TMAB-NHS undergoes a nucleophilic attack by the carboxylate in the absence of reactive primary amines. Similar to reactions between NHS esters and amines, the loss of neutral NHS is indicative of the formation of a covalent bond; however, in the case of the carboxylate reacting as a nucleophile, the newly-formed bond generates an anhydride between the

carboxylate and the carbonyl from the ester. The anhydride bond is very labile and is susceptible to dissociation, with the resulting formation of a carboxylic acid being possible at either side of the bond. In this case, cleavage at the peptide side of the linkage is favored, presumably because it facilitates the loss of the residual reagent as a neutral zwitterion. The net result is a water transfer from the precursor anion to the reagent to yield the [EDTA-H₂O-H][−] anion.

Figure 2 shows the relative stabilities of the reactant (i.e., the initially formed ion/ion complex), intermediates, and products, determined by DFT calculations at the B3LYP/6-31+G(d) level of theory, for the two observed reaction pathways, i.e. anhydride formation and methyl cation transfer. The channel on the right, A→B→C illustrates the formation of an anhydride bond and loss of a neutral NHS molecule from the complex. As expected, the formation of B is energetically downhill by 5.2 kcal/mol. The extent to which collisional activation is necessary to overcome barriers to generate B is unclear. The detailed mechanism for the reaction could involve several steps with distinct entropic and energetic requirements that can be expected to be unique for each analyte ion structure. In any case, activation is required to separate the NHS from complex B since it requires 39.2 kcal/mol (B→C). In contrast, the methyl cation transfer channel A→D is also energetically downhill (−15 kcal/mol), and the loss of a neutral requires 12.3 kcal/mol (D→E). These values are consistent with the experimental data in Figure 1 in that both channels are observed. That is, collisional activation of complex A gives rise to both products C and E. The relative contributions of the two channels are difficult to determine, however, because the main sequential fragmentations from each, viz., methanol loss in the case of methyl cation transfer (A→D→E pathway) and loss of CP-TMA in the case of anhydride formation (A→B→C pathway), yield [EDTA-H₂O][−] products.

This reaction with TMAB-NHS was applied to carboxylate-containing peptides. One example is the doubly deprotonated peptide Ac-AADAADAA, which is expected to have two carboxylate groups. Figure 3 shows the reaction spectrum with TMAB-NHS and subsequent CID of the complex. The predominant loss of NHS and the subsequent loss of 145 Da suggests that the complex that survives the ion/ion reaction reacts predominantly via the mechanism illustrated in Scheme 1. Since the anhydride bond is weaker than amide bonds, the loss of the CP-TMA zwitterion dominates under low-energy activation conditions and no backbone fragmentation of the peptide is observed. The similar reaction channel is observed on several other peptides, including Ac-DGAIL, Ac-GAIDDL (Figure S2), Ac-AADAADAA methyl esters (Figure S3), and Ac-AAEAAEAA (data not shown).

To further validate the reaction site, O¹⁸ labels were used with the carboxylates of Ac-AADAADAA. All of the 6 oxygen atoms (O¹⁶) in the 3 carboxylate groups were exchanged to O¹⁸. The comparison of Figure 4a (6 O¹⁶ atoms) with Figure 4b (6 O¹⁸ atoms) shows that CID of the respective electrostatic complexes yields exclusive loss of NHS with no O¹⁸. Subsequent cleavage yields a loss of 145 Da when only O¹⁶ is present (Figure 4a) and loss of 147 Da when only O¹⁸ is present (Figure 4b). These data are consistent with the carboxylate reacting to form an anhydride bond with subsequent cleavage that transfers a carboxylate oxygen to yield the neutral zwitterionic product (see Scheme 1).

To probe the structure of the product ion of this reaction, CID of the $[\text{Ac-AADAADAA-H}_2\text{O-H}]^-$ ion produced via ion/ion reaction was compared with that of the water loss ion generated via CID of the deprotonated peptide $[\text{Ac-AADAADAA-H}]^-$ ion (Figure S4). The same comparison was also done for several other peptides (Figure S5). In some cases the two spectra are the essentially the same while in other cases they differ to some extent. Unfortunately, CID of the peptide anions does not yield rich backbone cleavage, which hampers detailed structural characterization. Nevertheless, these comparisons suggest that the two distinct routes to generating water loss products (viz., ion/ion reaction versus direct CID of the $[\text{M-H}]^-$ species) may or may not lead to different mixtures of ions on a case-by-case basis. Too few comparisons of this type have been conducted to draw insights into when the two approaches are likely to generate different ion structures.

The reactivity of carboxylates and primary amines toward TMAB-NHS is compared in Figure 5. The non-acetylated species, $[\text{AADAADAA-2H}]^{2-}$, is expected to contain one unprotonated primary amine, viz., the N-terminal amine, and two carboxylate groups. If the fixed cationic charge of the TMAB-NHS reagent forms an electrostatic interaction with one of the carboxylate groups, the free carboxylate and the primary amine can compete for reaction with the NHS ester. As reflected in Figure 5a, the reaction between $[\text{AADAADAA-2H}]^{2-}$ and $[\text{TMAB-NHS}]^+$ results in spontaneous and complete loss of NHS. That is, no long-lived complex is observed, although it presumably existed as an intermediate to covalent bond formation. This spontaneous covalent bond formation is suggestive of higher reactivity with the presence of the unprotected N-terminus (see results of the comparable experiment for the reaction of $[\text{Ac-AADAADAA-2H}]^{2-}$ with $[\text{TMAB-NHS}]^+$ in Figure 3a). Activation of the NHS loss product yields extensive small molecule loss, such as water loss and the loss of trimethylamine (59 Da). Such losses are largely absent in the product ion spectrum of modified $[\text{Ac-AADAADAA+TMAB-2H}]^{2-}$ (see Figure 3c), which suggests that these losses take place from the peptide modified at the N-terminus. The presence of the relatively low abundance peak arising from 145 Da loss is strongly suggestive that a small fraction of the reactions proceed via the process shown in Scheme 1. (The O^{18} labeling experiment was also performed to validate that the 145 Da loss observed in Figure 5b truly includes an oxygen atom from the modified carboxylate. A loss of 147 Da is noted from the O^{18} labelled precursor (Figure 5c), which is fully consistent with the result obtained with the O^{18} -labelling experiment of Figure 4). In this case, the N-terminus appears to be more reactive than the carboxylate group. While the reactivities of the various potential nucleophiles in a peptide ion can vary based on intramolecular interactions, it is clear from this result that the presence of an unprotonated primary amine can significantly reduce the extent to which a carboxylate group reacts with an NHS ester.

To examine the reactivity of a carboxylic acid ($-\text{COOH}$), rather than the carboxylate group ($-\text{COO}^-$), a charge inversion reaction between the singly protonated Ac-AADAADAA and the doubly charged anionic homobifunctional cross-linker bis(sulfosuccinimidyl) suberate (BS^3) was conducted. In this case the carboxyl groups in the peptide are presumed to be carboxylic acids rather than carboxylates. One of the sulfonates of the reagent is expected to associate with the protonation site while the other is expected to carry the negative charge due to its lower proton affinity relative to a carboxylate group. Figure 6 illustrates that CID of the $[\text{Ac-AADAADAA+BS}^3\text{-H}]^-$ complex does not produce a sulfo-NHS loss. Rather, proton

transfer from the peptide to the doubly deprotonated reagent results in the observation of the singly deprotonated reagent. This result is analogous to what has been observed for amines and guanidines^{10, 16, 17}, wherein the protonated, and therefore non-nucleophilic, sites are unreactive towards NHS ester-based reagents while unprotonated amines and guanidines have been found to be highly reactive. The carboxylic acid is not regarded as being nucleophilic, yet the carboxylate is shown above to participate in nucleophilic reaction chemistries.

CONCLUSIONS

This work demonstrates reactivity of the carboxylate group towards N-hydroxysuccinimide esters in the gas phase. The carboxylate group can form an anhydride bond with the reagent resulting in the loss of N-hydroxysuccinimide. When other nucleophiles are present in the analyte, however, such as an unprotonated primary amine, reactivity can be dominated by the other nucleophile. When a counter-ion is associated with the carboxylate group, as is the case with the carboxylic acid functionality, no reactivity is observed. Unlike the strong amide bond formed between amines and NHS esters, the anhydride bond formed through the reaction of a carboxylate and NHS esters is extremely labile and cleavage tends to result in an oxygen transfer from the carboxylate to the reagent, nominally observed as a water loss from the original carboxylate-containing species. This reaction of carboxylates with NHS-based esters has not been observed in aqueous solution. The observation of this reactivity in the gas phase is likely a result of increased nucleophilicity in the absence of solvent effects. Furthermore, unlike in solution, the anhydride formed in the gas phase cannot undergo a hydrolysis process to regenerate two carboxylic acids. This result constitutes another example of distinct behaviors of reactivity between the gas phase and solution phase, and adds to the growing diversity of NHS chemistry in the gas phase in addition to the previously reported primary amine- and guanidine-specific reactions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Dr. Mingji Dai for helpful discussions on a potential gas-phase scrambling mechanism of the anhydride. This work was supported by the National Institutes of Health under Grant GM 45372.

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Figure 1. Product ion spectra derived from (a) ion/ion reaction between $[\text{EDTA-2H}]^{2-}$ and $[\text{TMAB-NHS}]^+$, (b) CID of $[\text{EDTA-2H}+(\text{TMAB-NHS})]^-$ and (c) CID of $[\text{EDTA-H}+\text{TMAB}]^-$

Figure 2.

Energy diagram depicting the anhydride formation channel and the methyl cation transfer channel that the electrostatic complex undergoes. The energies of B and D are calculated as a complex, whereas the energies of C and E are the sum of the energies of individual products, in order to illustrate the energy required to separate the two products.

Figure 3. Product ion spectra derived from (a) ion/ion reaction between $[\text{Ac-AADAADAA-2H}]^{2-}$ and $[\text{TMAB-NHS}]^+$, (b) ion trap CID of $[\text{Ac-AADAADAA}+(\text{TMAB-NHS})-2\text{H}]^-$ complex and (c) ion trap CID of the NHS loss from the complex, $[\text{Ac-AADAADAA}+\text{TMAB-2H}]^-$. (Lightning bolt indicates ion subjected to activation)

Figure 4.

Ion trap CID spectra of the electrostatic complex generated via an ion/ion reaction between $[TMAB-NHS]^+$ and (a) unlabeled, (b) or O^{18} -labeled $[Ac-AADAADAA-2H]^{2-}$.

Figure 5.

Product ion spectra derived from (a) then ion/ion reaction between $[AADAADAA-2H]^{2-}$ and $[TMAB-NHS]^+$, and CID of the covalently-modified peptide, (b) unlabeled and (c) O^{18} labeled $[AADAADAA+TMAB-2H]^-$.

Figure 6.

Ion trap CID spectra of the complex formed via an ion/ion reaction between $[BS^3-2H]^{2-}$ and $[Ac-AADAADAA+H]^+$.

Scheme 1.

Formation and dissociation of the anhydride formed via the ion/ion reaction between a dianion of a carboxylate-containing species and [TMAB-NHS]⁺ leading to the characteristic 145 Da loss