Published in final edited form as:

J Am Soc Mass Spectrom. 2014 October ; 25(10): 1739–1746. doi:10.1007/s13361-014-0962-4.

Label Scrambling During CID of Covalently Labeled Peptide Ions

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Abstract

Covalent labeling along with mass spectrometry is finding more use as a means of studying the higher order structure of proteins and protein complexes. Diethylpyrocarbonate (DEPC) is an increasingly used reagent for these labeling experiments because it is capable of modifying multiple residues at the same time. Pinpointing DEPC-labeled sites on proteins is typically needed to obtain more resolved structural information, and tandem mass spectrometry after protein proteolysis is often used for this purpose. In this work we demonstrate that, in certain instances, scrambling of the DEPC label from one residue to another can occur during collision-induced dissociation (CID) of labeled peptide ions, resulting in ambiguity in label site identity. From a preliminary study of over 30 labeled peptides, we find that scrambling occurs in about 25% of the peptides and most commonly occurs when histidine residues are labeled. Moreover, this scrambling appears to occur more readily under non-mobile proton conditions, meaning that low-charge state peptide ions are more prone to this reaction. For all peptides, we find that scrambling does not occur during electron transfer dissociation, which suggests that this dissociation technique is a safe alternative to CID for correct label site identification.

Introduction

Covalent labeling along with mass spectrometry is being increasingly used to study higher order protein structure and protein-protein complexes [1-9]. Covalent labels that are typically used either label specific amino acid residues (e.g. succinimides for lysines) or a range of amino acid residues (e.g. hydroxyl radicals). We have recently shown that diethylpyrocarbonate (DEPC) is a promising reagent molecule because of its ability to modify a wide range of amino acids [1, 10-14]. This potential to modify numerous amino acids enables DEPC to probe approximately 30% of the average protein [14]. Usually, proteins that are labeled with DEPC are then subjected to proteolysis so that the modification sites can be pinpointed to individual residues, thereby improving the resolution of this method.

While DEPC has been successfully used to study the structures of proteins and protein complexes, this labeling reagent introduces an electrophilic site into the side chains of the residues it modifies, opening up the possibility for some unwanted chemistry. For example,

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we recently demonstrated that, in solution, cysteine residues have the ability to capture carbethoxy groups from other residues that were modified by DEPC. This unwanted label transfer can be eliminated by deactivating cysteine's strong nucleophilic character via alkylation just after DEPC labeling of the protein is finished [15]. The presence of a new electrophilic site might also affect the gas-phase chemistry of DEPC-labeled peptides especially when subjected to slow collisional activation in a quadrupole ion trap mass spectrometer. Indeed, Reid and co-workers and others have reported that phosphorylated peptide ions can have their phosphate group transferred from one amino acid to another upon CID [16-18]. The result is the incorrect assignment of the phosphorylation site on the peptide. In addition to the scrambling of phosphate groups in peptide ions, methyl [19], acetyl, and formyl groups [20] have also been documented to undergo transfer from one amino acid to another during CID in the gas phase.

With the known chemistry of DEPC and these previous studies in mind, we set out to investigate whether DEPC label scrambling can occur during CID of DEPC labeled peptide ions. Through the study of numerous peptides, we find that label transfer can occur in DEPC-labeled peptides, and it occurs with similar characteristics to phosphate group transfer in phosphorylated peptides.

Methods

Materials

Diethylpyrocarbonate (DEPC), imidazole, iodoacetamide, and tris(2-carboxyethyl)phosphine (TCEP) were obtained from Sigma Aldrich (St. Louis, MO). Angiotensin 1-10 (DRVYIHPFHL), angiotensin 1-13 (DRVYIHPFHLVIH), apelin 13 (QRPRLSHKGMPA), β amyloid (YEVHHQKLVEF), semastatin (SPWTKCSATCGGGHYMRTR), neuromedin-C (GNHWAVGHLM) and adrenocorticotrophic hormone (ACTH) 1-13 (SYSMEHFRWGKPV) were obtained from the American Peptide Company (Sunnyvale, CA). Human β -2-microglobulin (β 2m) was obtained from Lee Biosolutions (St. Louis, MO). Immobilized chymotrypsin and triethylamine acetate (pH 8.0) were obtained from Princeton Separations (Adelphia, NJ). Ammonium acetate, methanol, formic acid, acetonitrile, and water were purchased from Fisher Scientific (Fair lawn, NJ). Centricon molecular weight cutoff (MWCO) filters were obtained from Millipore (Burlington, MA).

DEPC Labeling Reactions

Peptide solutions with a concentration of 100 μ M in 10 mM ammonium acetate were reacted with DEPC at either a 1:1 or 1:4 (peptide:DEPC) ratio for 2-5 min at 37°C. A 6 mM stock solution of DEPC in acetonitrile was added to obtain the final DEPC concentration. The final DEPC concentration resulted in a total acetonitrile concentration that was less than 2% (v/v) of the reaction mixture. The reaction time and ratio were chosen to ensure that the unmodified and the singly modified peaks were the most prominent products. The protein β 2m was reacted with DEPC at a 1:4 (protein:DEPC) ratio for 1 min at 37°C. In all cases, the DEPC reactions were quenched by the addition of 10 mM imidazole [11].

Proteolytic Digestion

Prior to proteolytic digestion of $\beta 2m$, the quenching agent, imidazole, was removed using a 10,000 MWCO filter, resulting in a 40 μ L solution of 250 μ M $\beta 2m$. $\beta 2m$ was then incubated in a buffer solution (100 mM triethylamine at pH 8.0) with 10% (v/v) acetonitrile at 50 °C for 45 min. Next $\beta 2m$ was reacted with TCEP (1:40 ratio) and iodoacetamide (1:80 ratio) in the dark for 30 min to reduce its disulfide bond and alkylate the resulting free thiols. Immobilized chymotrypsin was then added to achieve a 1:10 ratio of enzyme to substrate. The digestion reaction was allowed to proceed at 37 °C for 2 hours.

HPLC Separation

HPLC separations were performed using a Supelco Discovery C18 column (15 cm $\times$ 2.1 mm, 5 μ m particle size, St. Louis, MO) on an HP1100 HPLC system (Agilent, Wilmington, DE). To achieve sufficient separation of DEPC-labeled peptide isomers, an isocratic elution was used (40% acetonitrile in water with 0.1% formic acid) for 20 min. This was followed by a 0.5%/min increase of acetonitrile for an additional 20 min. A flow rate of 0.35 mL/min was used, and the effluent from the column was split by four prior to introduction into the mass spectrometer. The proteolytic digests were eluted using a linear gradient of methanol ranging from 10 – 100% methanol over 30 min. This mobile phase contained 0.1% acetic acid and had a flow rate of 0.25 mL/min.

Mass Spectrometry

Mass analysis was performed using either a Bruker Esquire-LC or a Bruker AmaZon (Billerica, MA) quadrupole ion trap mass spectrometer. These instruments are equipped with electrospray ionization sources, and the needle voltage was typically set to 3000 V. The capillary temperature was set to 300°C. Both CID and ETD were used to obtain tandem mass spectra. The ion isolation width for both methods was set to 1.0 Da. CID voltages were typically between 0.65 and 1.1 V and were chosen to achieve optimal dissociation efficiency. For ETD experiments, the low m/z cutoff was typically set to 135, and the reaction time was typically set to 150 ms.

Results and Discussion

CID for Label Site Identification

CID is commonly used to determine label locations in covalently labeled peptides and is typically successful. As an example, CID of the peptide apelin 13 (QRPLSHKGPMMPA) can readily enable the identification of the DEPC-labeled residues upon a 2 minute reaction with this reagent (Figure 1). LC/MS analysis of the labeled peptide suggests that there are three labeled sites as indicated by the chromatogram in Figure 1a. By examining the sequence ions upon CID of each peak in the chromatogram, the specific amino acid residues labeled by DEPC can be assigned. For DEPC-labeled apelin 13, the CID spectrum of the second chromatographic peak shows a series of b ions that are all labeled, and several y ions that are not labeled, indicating that the N-terminus is the labeled site (Figure 1b). The CID spectrum of the third chromatographic peak is consistent with a label on Lys8, as suggested by labeled y_6 , y_7 , y_9 , and y_{11} product ions, unlabeled y_4 and y_5 ions, labeled b_8 and b_{11} ions, and unlabeled b_2 and b_7 ions (Figure 1c). The final chromatographic peak corresponds to a

labeled His7 product. This assignment is evident from the labeled y_7 , y_9 , and y_{11} product ions, unlabeled y_4 , y_5 , and y_6 ions, labeled b_7 , b_8 and b_{11} ions, and an unlabeled b_2 ion (Figure 1d).

Ambiguous Label Assignment by CID

While CID often provides correct labeling site information, as exemplified by the data for apelin 13, we have also found evidence that covalent labels can be scrambled from one site to another during the CID process. The CID data of DEPC labeled-angiotensin I illustrates this phenomenon. Upon labeling this peptide with DEPC for 5 min, on average a single modification site per peptide is measured, resulting in four isomers as indicated by the chromatogram in Figure 2a. Each peak in the chromatogram corresponds to a chemically distinct structure, and indeed the CID data for the first (Figure 2b) and second (Figure S1 in the Supplemental Information) labeled peaks are consistent with this assertion, as Tyr4 and the N-terminus, respectively, are identified as the modified sites.

The identity of the labeled sites for the modified peptides associated with the final two chromatographic peaks, however, are not as clear. This fact is most evident from the presence of both labeled and unlabeled versions of the b_6 , b_8 , y_4 , and y_2 product ions in both spectra (Figures 2c and S2). One possible explanation for this is that these chromatographic peaks correspond to two unseparated isomers – one modified at His6 and the other at His9. This possibility is unlikely considering the great care taken to identify LC conditions to separate any isomers present. Moreover, the ETD spectra of these peaks (Figures 2d and S3) indicate that only His6 is modified with no evidence for the modification of His9. This conclusion comes from the series of c and z product ions in which ions that include His6 are labeled and all that do not are unlabeled. Incidentally, the ETD spectra of peaks 1 and 2 lead to the same conclusions about modification sites as the CID data (see Figures S4 and S5). Another possible explanation is that the DEPC label is lost as a neutral. This would, for example, explain the presence of both labeled and unlabeled b_6 and b_8 product ions in the CID spectrum; however, the absence of an unlabeled b_9 ion and no evidence for label loss during the CID of other DEPC-labeled peptides (e.g. apelin 13 in Figure 1) suggests that label loss is not the cause. Moreover, label loss cannot explain the presence of labeled y_2 and y_4 ions. These latter ions can only arise from the label being transferred to His9.

Upon considering the data for DEPC-labeled angiotensin I, we propose that the CID data for the final two chromatographic peaks of modified angiotensin I arise via a collision-induced transfer of the DEPC label from one His residue to another. Such a gas-phase rearrangement of a side chain modification has been seen before [16-20] during CID analyses, as indicated in the introduction. The migration of the DEPC label from His6 to His9 during collisional activation of angiotensin I can explain the existence of all observed ions and is consistent with differences in dissociation mechanisms between ETD and CID. Unlike label loss, transfer of DEPC from His6 to His9 explains, for example, why the b_9 product ion is only observed as a labeled species. The fact that label migration does not take place during ETD makes sense due to the non-ergodic nature of its dissociation mechanism. We suspect that the scrambling of the label is driven by the fact that addition of DEPC to a histidine side chain results in an electrophilic site that is vulnerable to attack by other nucleophilic sites on

the peptide. In the case of angiotensin, this nucleophilic site could be the other, unlabeled histidine residue. A possible mechanism is illustrated in Figure 3.

Another peptide that appears to undergo label scrambling during CID is the peptide adrenocorticotrophic hormone (ACTH). LC/MS analysis of DEPC labeled ACTH indicates that two amino acids are labeled in this peptide (Figure 4a). In this case, the label sites associated with both chromatographic peaks from the labeled peptide are ambiguous when CID is used. The tandem mass spectrum of the first labeled peak suggests scrambling between the N-terminus, His6, and Lys11 (Figure S6) as indicated by the presence of labeled and unlabeled versions of the $b_2 - b_{10}$ series of product ions and the $y_6 - y_{11}$ series of product ions. The fact that the labeling site is clearly identified as the N-terminus when dissociated by ETD (Figure S7) suggests that unseparated isomers are not the source of this ambiguity. CID of the second labeled chromatographic peak suggests both His6 and Lys11 as the labeled sites based on the existence of labeled and unlabeled versions of the $b_6 - b_{10}$ series of product ions and $y_3 - y_7$ series of product ions (Figure 4b). In contrast, the ETD spectrum of this labeled peptide readily identifies His6 as the labeled site (Figure 4c).

Effect of Charge State on Labeling Scrambling

Previous work has shown that the gas-phase rearrangement of phosphate and methyl groups in peptide ions is dependent on the precursor ion's charge state and the subsequent proton mobility within the ion [16,19]. Phosphorylated and methylated peptide ions with non-mobile and partially mobile protons were found to have a higher propensity to rearrange. Proton mobility in this context refers to the number of net protons on the peptide ion as compared to the number of basic residues in the peptide. Non-mobile protons are present when the number of net protons is lower than the number of basic residues in the peptide. To test whether proton mobility influences DEPC label scrambling, we compared the CID spectra of the $[M+2H+DEPC]^{2+}$ and $[M+3H+DEPC]^{3+}$ ions of the fifth chromatographic peak of labeled angiotensin (i.e. peaks in Figure 2a). Interestingly, the different charge states result in drastically different dissociation patterns with regard to label scrambling. There are no longer any labeled y_2 or y_4 product ions observed upon dissociation of the +3 charge state (Figure 5a); these ions were key indicators of label scrambling for the +2 ion (see Figure 2c). In addition, the unlabeled b_6 and b_8 ions, which also implied label scrambling in the +2 ion, are substantially reduced in relative abundance.

A similar influence of proton mobility on label scrambling is also observed for ACTH. As shown earlier in Figure 4b, the +2 charge state of third chromatographic peak exhibits scrambling upon activation with CID. When the +3 charge state of this same labeled peptide is dissociated using CID, no evidence for scrambling is observed (Figure 5b). The absence of unlabeled b_6 , labeled y_3 , and y_7 product ions in this spectrum removes all ambiguity about the label assignment. The dramatic influence of charge state on the dissociation behavior of these DEPC-modified peptides further supports the notion that label scrambling is occurring.

Prevalence of Label Scrambling

To test the pervasiveness of label scrambling, we examined several other labeled peptides, including ones from a proteolytic digest of the protein β -2-microglobulin that had been labeled with DEPC for 1 minute. Each collection of peptides was separated by LC and then subjected to both CID and ETD. In all, 34 different labeled sites were probed (Table 1). The criteria for concluding that label scrambling had occurred were: (i) unlabeled and labeled versions of product ions were observed during CID and (ii) CID and ETD identified different labeled sites in a given peptide. Of these 34 labeled peptides, nine showed evidence for scrambling. Interestingly, all nine had a histidine residue as either the originally labeled site or the site to which the labeled was transferred, indicating the lability of labeled histidine residues. Another interesting observation from this limited data set is that an acidic residue is present in all the peptides that undergo scrambling. Because of the effect of proton mobility on the prevalence of this scrambling reaction (see Figure 5), it is possible that intramolecular interactions between charged groups may be necessary to facilitate this reaction. Further work is clearly needed, though, to more fully understand how amino acid sequence affects this scrambling reaction.

Conclusions

Covalent labeling is increasingly used to study the surface structure of proteins and protein complexes, and DEPC is a labeling reagent that has shown great promise in these experiments because of its ability to label multiple residues simultaneously. Identifying the labeled sites is often done using CID after proteolytic digestion of the protein, but in this work, we show that scrambling of the DEPC label to another site on a peptide can occasionally occur during CID, resulting in ambiguous labeling site assignments. Like previous studies that reported the scrambling of functional groups (e.g. phosphate groups) on modified peptides, this scrambling occurs most readily under low proton mobility conditions. Also, scrambling does not appear to occur when ETD is used to dissociate the labeled peptides; this is consistent with the known mechanistic differences between CID and ETD. Based on the analysis of over 30 labeled peptides, we find evidence for scrambling about 25% of the time. In all the cases where scrambling is observed, histidine residues are involved. The increased tendency of histidine residues to undergo scrambling has important consequences as this residue is the most reactive amino acid with DEPC. A solution to this problem may be to produce larger peptides upon proteolysis so that the labeled peptides have higher charge states and are thus more amenable to ETD. Alternatively, supercharging agents could be used to produce higher peptide charge states to avoid the low proton mobility conditions that foster label scrambling during CID.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by NIH grant RO1 GM015092. NB was partially supporting by a NIH training grant (T32 GM008515).

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Figure 1.

(a) Total ion chromatogram after HPLC separation of DEPC-labeled apelin 13. (b) CID spectrum of the $(M+2H)^{2+}$ ion of the second chromatographic peak from the DEPC-labeled sample of apelin 13, indicating that the N-terminus is modified. (c) CID spectrum of the $(M+2H)^{2+}$ ion of the third chromatographic peak from the DEPC-labeled sample of apelin 13, indicating that Lys8 is modified. *Interfering ions make assignment of the labeled and unlabeled versions of the y_6 ion somewhat ambiguous. (d) CID spectrum of the $(M+2H)^{2+}$ ion of the fourth chromatographic peak from the DEPC-labeled sample of apelin 13, indicating that His7 is modified.

Figure 2.

(a) Total ion chromatogram after HPLC separation of DEPC-labeled angiotensin. (b) Example CID spectrum of the $(M+2H)^{2+}$ ion of the second chromatographic peak from the DEPC-labeled sample of angiotensin I, indicating that Tyr4 is modified. Unlabeled y_2 to y_6 ions, labeled y_7 to y_9 ions, labeled b_5 to b_9 ions, and an unlabeled b_2 ion indicate that Tyr4 is labeled. (c) CID spectrum of the $(M+2H)^{2+}$ ion of the final chromatographic peak from the DEPC-labeled sample of angiotensin I. The presence of both labeled and unlabeled b_6 , b_8 , y_4 , and y_2 product ions causes the labeled site to be ambiguous. (d) ETD spectrum of the $(M+3H)^{3+}$ ion of the final chromatographic peak from the DEPC-labeled sample of angiotensin I, indicating that His6 is labeled.

Figure 3.
Possible mechanism of rearrangement, where the DEPC label on one histidine side chain is transferred to another via a nucleophilic attack.

Figure 4.

(a) Total ion chromatogram after HPLC separation of DEPC-labeled ACTH. (b) CID spectrum of the $(M+2H)^{2+}$ ion of the first labeled chromatographic peak from the DEPC-labeled sample of ACTH. The presence of both labeled and unlabeled b_{10} , b_9 , b_8 , and y_7 product ions suggests label scrambling. (c) ETD spectrum of the $(M+3H)^{3+}$ ion of the second labeled chromatographic peak from the DEPC-labeled sample of ACTH, indicating that His6 is labeled.

Figure 5.

(a) CID spectrum of the (M+3H)³⁺ ion of the final labeled chromatographic peak from the DEPC-labeled sample of angiotensin I. The absence of labeled y₂ and y₄ product ions indicates label scrambling is not occurring. (b) CID spectrum of the (M+3H)³⁺ ion of the second labeled chromatographic peak from the DEPC-labeled sample of ACTH. The absence of unlabeled b₆, labeled y₃, and y₇ product ions in this spectrum indicates label scrambling is not occurring.

Table 1

DEPC-labeled sites on peptides as identified by ETD and CID and whether scrambling has occurred.

Sequence	ETD of (M+3H) ³⁺	CID of (M+3H) ³⁺	Scrambling in (M+3H) ³⁺ ?	CID of (M+2H) ²⁺	Scrambling in (M+3H) ²⁺ ?
DRVYIHPFHL peak 1	Y4	Y4	No	Y4	No
DRVYIHPFHL peak 2	N-term	N-term	No	N-Term	No
DRVYIHPFHL peak 3	H6	H6	No	H6, H9	Yes
DRVYIHPFHL peak 4	H6	H6	No	H6, H9	Yes
GNHWAVGHLM peak 1	N-Term	N-Term	No	N-Term	No
GNHWAVGHLM peak 2	H8	H8	No	H8	No
GNHWAVGHLM peak 3	H3	H3	No	H3	No
GNHWAVGHLM peak 4	H8	H8	No	H8	No
GNHWAVGHLM peak 5	H3	H3	No	H3	No
DRVYIHPFHLVIH peak 1	Y4		No	Y4	No
DRVYIHPFHLVIH peak 2	N-term		No	N-term	No
DRVYIHPFHLVIH peak 3	H9		No	H6, H9, H13	Yes
DRVYIHPFHLVIH peak 4	H9		No	H6, H9	Yes
DRVYIHPFHLVIH peak 5	H13		No	H9, H13	Yes
DRVYIHPFHLVIH peak 6	H6		No	H6, H9	Yes
SYSMEHFRWGKPV peak 1	N-term	N-term	No	N-term, H6, K11	Yes
SYSMEHFRWGKPV peak 2	H6	H6	No	H6, K11	Yes
YEVHHQKLVEFF peak 1	H4	H4, H5	Yes	H4, H5	Yes
YEVHHQKLVEFF peak 2	N-term	N-Term	No	N-term	No
YEVHHQKLVEFF peak 3	H4	H4	No	H4	No
YEVHHQKLVEFF peak 4	H5	H5	No	H5	No
YEVHHQKLVEFF peak 5	K7	K7	No	K7	No
SPWTKCSATCGGGHYMRTR peak 1	Y15		No	Y15/H14*	No
SPWTKCSATCGGGHYMRTR peak 2	H14		No	H14	No
SPWTKCSATCGGGHYMRTR peak 3	Y15/H14*		No	Y15/H14*	No
SPWTKCSATCGGGHYMRTR peak 4	N-term		No	N-Term	No
SPWTKCSATCGGGHYMRTR peak 5	K5		No	K5	No
SRHPAENGKSNF	S1/R2		No	N-term	No
SRHPAENGKSNF	H3		No	H3	No
IQRTPKIQVY	K6		No	Y3	No
IQRTPKIQVY	N-term		No	N-term	No
KNGERIEKVEHSDL	K1		No	K1	No
SQPKIVKW	K4		No	K4	No

Sequence	ETD of (M+3H) ³⁺	CID of (M+3H) ³⁺	Scrambling in (M+3H) ³⁺ ?	CID of (M+2H) ²⁺	Scrambling in (M+3H) ²⁺ ?
SRHPAENGKSNFLNCY	C15		No	C15	No

* Tandem mass spectrometry is unable to identify which of the listed residues is labeled due to the absence of the appropriate product ions.