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Structural Analysis of [Cu(II)-amyloidogenic peptide] Complexes

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Abstract : Studies on the interactions of amyloidogenic proteins with trace metals, such as copper, have indicated that the metal ions perform a critical function in the early oligomerization process. Herein, we investigate the effects of Cu(II) ions on the active sequence regions of amyloidogenic proteins using electrospray ionization mass spectrometry (ESI-MS) and collision induced dissociation tandem MS (CID-MS/MS). We chose three amyloidogenic peptides NNQQNY, LYQLEN, and VQIVYK from yeast prion like protein Sup35, insulin chain A, and tau protein, respectively. [Cu-peptide] complexes for all three peptides were observed in the mass spectra. The mass spectra also show that increasing Cu(II) concentrations decrease the population of existing peptide oligomers. The tandem mass spectrum of NNQQNY shows preferential binding for the N-terminal region. All three peptides are likely to appear to be in a Cu-monomer-monomer (Cu-M-M) structure instead of a monomer-Cu-monomer (M-Cu-M) structure.

Keywords : amyloidogenic peptides, Cu(II) ions, oligomer, ESI-MS, CID-MS/MS

Introduction

Amyloid fibrils are implicated in a wide variety of diseases including Alzheimer's disease, type II diabetes, and prion related diseases. These fibrils are thought to form by nucleation-dependent polymerization through self-assembly[1-2] and present filamentous morphology, cross-β sheet structure, and pathogenic effects.[3-4] However, amyloid pathology is thought to arise from smaller oligomer complexes rather than from the fully formed fibrils.[5-6] In addition, shorter active sequences of the larger fibril-forming amyloidogenic proteins present the same characteristics as full peptides and are believed to help determine whether the larger proteins form fibrils.[7]

Previous studies have indicated that trace metals, such as Cu(II) ions, have a large influence on the structure of amyloidogenic proteins through modulation and inhibition of their aggregation.[8-9] Studies have also shown that in hIAPP,[10-11] full length PrP,[12-13] insulin monomers,[14-15] and tau protein,[16-17] oligomer formation was dependent on the concentration of Cu(II). It is also known that amyloidogenic peptide interactions with copper are dose-dependent, with three separate kinetic pathways depending on whether [Cu]<<[peptide], [Cu]=[peptide], or [Cu] > [peptide].[18-19] However, it is not known whether the interaction of metal ions such as Cu(II) with the active sequence region of other amyloidogenic proteins affects their self-assembly process. In addition, in amyloidogenic protein dimers Cu(II) has been shown to prefer bind between monomer subunits leading to a (M-Cu-M) like structures that encourages formation of reactive oxygen species (ROS)[19-20] and decrease formation and stability of peptide complexes[10,21] but Cu(II) structures for the active sequence peptides are yet unknown.

In this study, electrospray ionization mass spectroscopy (ESI-MS) and collision induced dissociation (CID) using tandem mass spectroscopy (MS/MS) were used to investigate the molecular interactions and structures of the [Cu-peptide] complexes. We selected three active sequence amyloidogenic peptides that were previously indicated to self-assemble in a tyrosine centered manner[22]: NNQQNY, from a yeast prion like protein Sup35, LYQLEN, from insulin chain A, and VQIVYK, from tau protein. We also varied the concentrations ratios of [peptide]:[Cu] using 1:0, 1:0.1, and 1:1 ratios. We report that at higher concentrations, Cu(II) ions decrease formation of the larger oligomers and we propose possible structures of the [Cu-monomer] and [Cu-dimer] complexes for further study.

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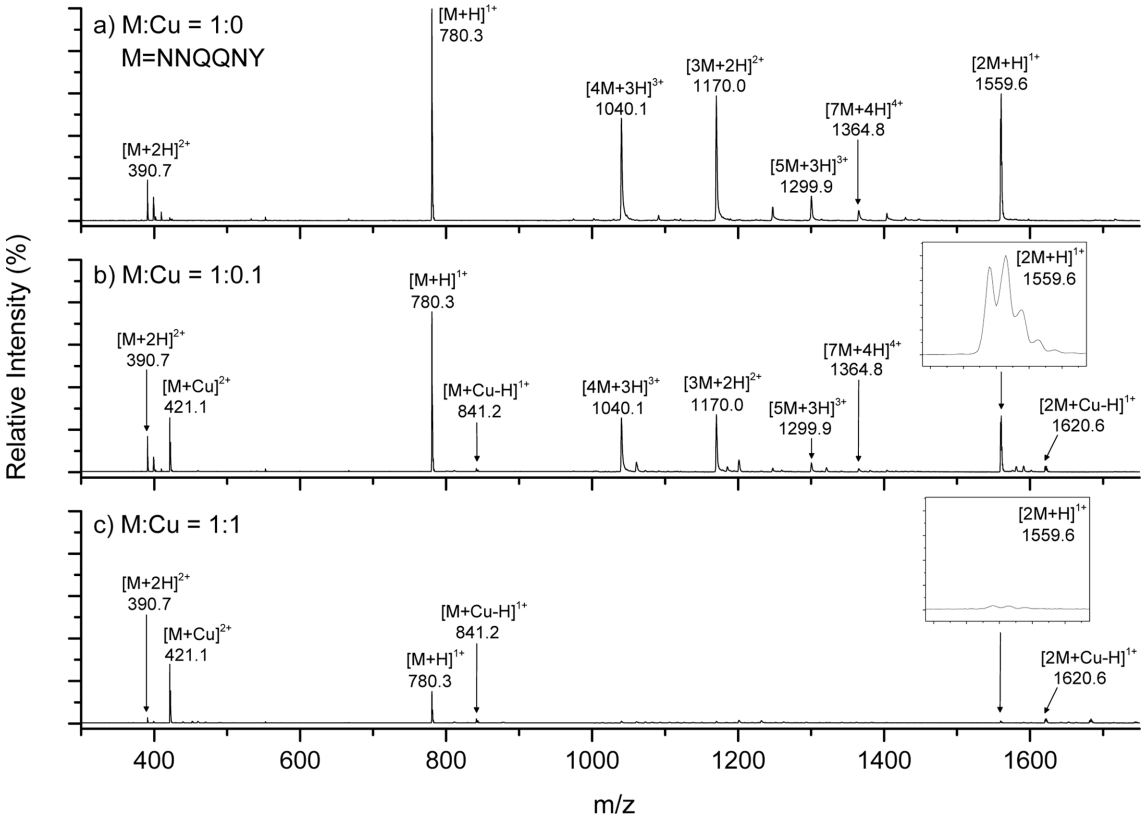


Figure 1. Comparison of full mass spectra at **a)** 1:0, **b)** 1:0.1, and **c)** 1:1 ratios of [peptide]:[Cu] of the active sequences NNQQNY. All peaks with m/z values greater than 1000 were magnified by a factor of 20. Magnified NNQQNY $[2M+H]^{1+}$ peaks are presented as insets.

Experimental

Mass Spectrometry

All spectra were acquired in the positive ion mode over an m/z range of 50-2000 by averaging 100-2000 scans. The CID-MS/MS experiments were conducted at capillary temperatures of 200°C, which resulted in the best signal/noise ratios in the MS/MS spectra. The electrospray needle voltage was set to 3.3-3.5 kV. The samples were introduced into the electrospray interface by a direct infusion method using a microsyringe pump (Hamilton, USA) at a flow rate of 1-2 μL/min. The MS/MS spectra were acquired under the following experimental conditions: an isolation width of 1-1.5 mass units, an activation time of 30 ms, and an injection time of 100-200 ms. In MS/MS, the parent ion molecules were individually and manually selected and then subjected to CID. Normalized collision energies were optimized for each MS/MS experiment using the minimal collision energy that would allow fragments to be viewed at sufficient signal to noise ratios.

Reagents

The synthetic peptides NNQQNY (>95%, Peptron, Daejeon, Korea), LYQLEN (>95%, Peptron, Daejeon, Korea), VQIVYK (>95%, Peptron, Daejeon, Korea) and $CuCl_2$ (>99%, Sigma-Aldrich, Korea) were used in the experiments. HPLC-grade H_2O (Merck Ltd., Korea) was used as a solvent. Peptides were dissolved in H_2O to prepare 5×10^{-4} M solutions and experiments were performed the next day. Powdered $CuCl_2$ was added just before the ESI-MS experiments to give 1:0, 1:0.1, and 1:1 final concentration ratios of [peptide]:[Cu].

Results and Discussion

MS Spectra

There have been conflicting reports on whether copper ions inhibit or promote amyloidogenic peptide aggregation depending on the [peptide]:[metal] ratio.[12,16,23-24] Copper ions promote amyloid dimerization leading to larger amyloid-copper aggregates.[25-26] Here we investigated the stability of amyloidogenic active sequences in the presence of Cu(II) ions using ESI-MS. Previous research indicated the importance of Y residues in the early oligomerization process[22] and so three amyloidogenic peptides with varying locations of Y residues were selected in order to observe its

Table 1. Summary of the nomenclature used for MS/MS spectra. Italics indicate conventional notation as proposed by Roepstorff and Fohlman.[28] Non-italicized b_t would conventionally be represented as a neutral species $[b_t]^0$. Non-italicized y_n would be represented as $[y_n"\text{-}2H]^0$.

Nomenclature	Notes	Conventional Notation
$(M+Cu-H-b_t)^{1+}$	$[(M+Cu-H)-b_t+H]^{1+}$, t=1-6	$[y''_{6-t}+Cu-2H]^{1+}$
$(M+Cu-H-y_n)^{1+}$	$[(M+Cu-H)-(y_n+H)]^{1+}$, n=1-6	$[b_{6-n}+Cu-2H]^{1+}$
$\{b_t$ loss$\}$	In the case of $(parent-b_t)^{1+}$, t=1-6 e.g. $(M+Cu-H-b_1)^{1+}$, $(M+Cu-H-b_2)^{1+}$, …, $(M+Cu-H-b_t)^{1+}$	N/A
$\{y_n$ loss$\}$	In the case of $(parent-y_n)^{1+}$, n=1-6 e.g. $(M+Cu-H-y_1)^{1+}$, $(M+Cu-H-y_2)^{1+}$, …, $(M+Cu-H-y_n)^{1+}$	N/A

effect on Cu(II)-peptide interactions. We present the full mass spectra of three amyloidogenic active sequence peptides: NNQQNY, LYQLEN, and VQIVYK at stoichiometric ratios of 1:0, 1:0.1, and 1:1 of [peptide]:[Cu] (Figures. 1, S1, and S2). A close up of the $[2M+H]^{1+}$ complexes are shown as insets. Higher order oligomers up to heptamers were observed as shown in a previous study without the addition of Cu(II) ions.[22] [Cu-oligomer] complexes up to trimers were also observed. m/z values assignments for peptide complex peaks are listed in Table S1.

In general, the intensity of $[M+H]^{1+}$, $[2M+H]^{1+}$, and $[3M+2H]^{2+}$ spectral peaks decreased with an increase in the concentration of Cu(II) ions indicating Cu(II) plays a role in decreasing the population of monomer and early oligomer complexes similar to other larger peptides[10,21,27]

All three peptides showed multiple [Cu-peptide] complex ions at significant intensities indicating stable Cu(II) binding by all three peptides. The [Cu-monomer or dimer] complexes were observed in a 1+ and 2+ complex form, and the mass spectra show that the 2+ complex is more easily formed compared to the 1+ complex.

MS/MS Spectra

MS/MS Nomenclature

CID experiments were conducted to provide determine the possible structures of the [Cu-peptide] complexes. For the MS/MS spectra, fragment ions here are assigned in the 'xx loss' form to emphasize the patterns of fragment loss. Conventional notation (y_n and b_t as proposed by Roepstorff and Fohlman[28] and italicized here for emphasis) for MS/MS is generally used to denote the peptide sequence in monomers[29] instead of structures of both monomers and dimers, as in this study. In addition, the neutral species $[y_n\text{-}2H]^0$ and $[b_t]^0$ are represented here as non-italicized y_n and b_t. Complexes formed by the addition of a copper ion and the loss of a proton are written as $[nM+Cu-H]^{1+}$. Complexes formed by the loss of a single fragment from a $[nM+Cu-H]^{1+}$ complex is written as $(M+Cu-H-y_n)^{1+}$ or $(M+Cu-H-b_t)^{1+}$. A series of single b_1, b_2, b_3 loss ions are referred to as a $\{b_t$ loss$\}$ series and likewise with y_n ions as a $\{y_n$ loss$\}$ series. The MS/MS nomenclature is summarized in Table 1.

MS/MS spectra of the monomers

In the NNQQNY $[M+Cu-H]^{1+}$ and $[M+Cu]^{2+}$ complexes the $\{y_n$ loss$\}$ series up to n=3 was observed (Figure 2a and b). This is in contrast to previous work by Seo et al.[22] that showed a $\{b_t$ loss$\}$ and $\{y_n$ loss$\}$ series in NNQQNY $[M+H]^{1+}$. Based on these fragment ions, we surmise that in the NNQQNY peptide the Cu(II) stably binds to the N-terminal region as shown in Scheme 1a.

The reason for the preference of the N-terminal domain over the C-terminal domain is unclear. This high affinity and site-specific binding of the Cu(II) ion is not observed in the $[M+Cu-H]^{1+}$ complexes of the LYQLEN or VQIVYK peptides.

In the fragmentation pattern of the LYQLEN $[M+Cu-H]^{1+}$ we observe a mixture of a $\{y_n$ loss$\}$ series up to n=3 and a $\{b_t$ loss$\}$ series up to t=2 (Figure 2c). The y_3 loss and b_2 loss fragment ions are observed at significant intensities indicating that Cu(II) interacts either with the N-terminal or the C-terminal regions. On the other hand, in the $[M+Cu]^{2+}$ MS/MS spectrum we only observe a $\{y_n$ loss$\}$ series up to n=3 with corresponding y_1, y_2, and y_3 ions (Figure 2d). The fragmentation pattern indicates that in $[M+Cu]^{2+}$, Cu(II) binds to the N-terminal region.

Similarly, in the VQIVYK peptide MS/MS fragmentation patterns of singly charged $[M+Cu-H]^{1+}$ (Figure 2e), a $\{y_n$ loss$\}$ series up to n=2 and a $\{b_t$ loss$\}$ series to up to t=2 was observed, implying that Cu(II) ions binds to either N-terminal or C-terminal regions. On the other hand, the

Table 2. Comparison of [Cu-peptide] MS/MS fragmentation patterns for NNQQNY, LYQLEN, and VQIVYK.

Peptides	[Cu-peptide] MS/MS fragmentation patterns			
	[Cu-monomer]		[Cu-dimer]	
	1+	2+	1+	2+
NNQQNY	$\{y_n$ loss$\}$	$\{y_n$ loss$\}$	$\{b_t$ loss$\}$	$\{b_t$ loss$\}$
LYQLEN	$\{b_t$ loss$\}$ $\{y_n$ loss$\}$	$\{y_n$ loss$\}$	$\{y_n$ loss$\}$	$\{y_n$ loss$\}$
VQIVYK	$\{b_t$ loss$\}$ $\{y_n$ loss$\}$	$\{y_n$ loss$\}$	$\{b_t$ loss$\}$	-

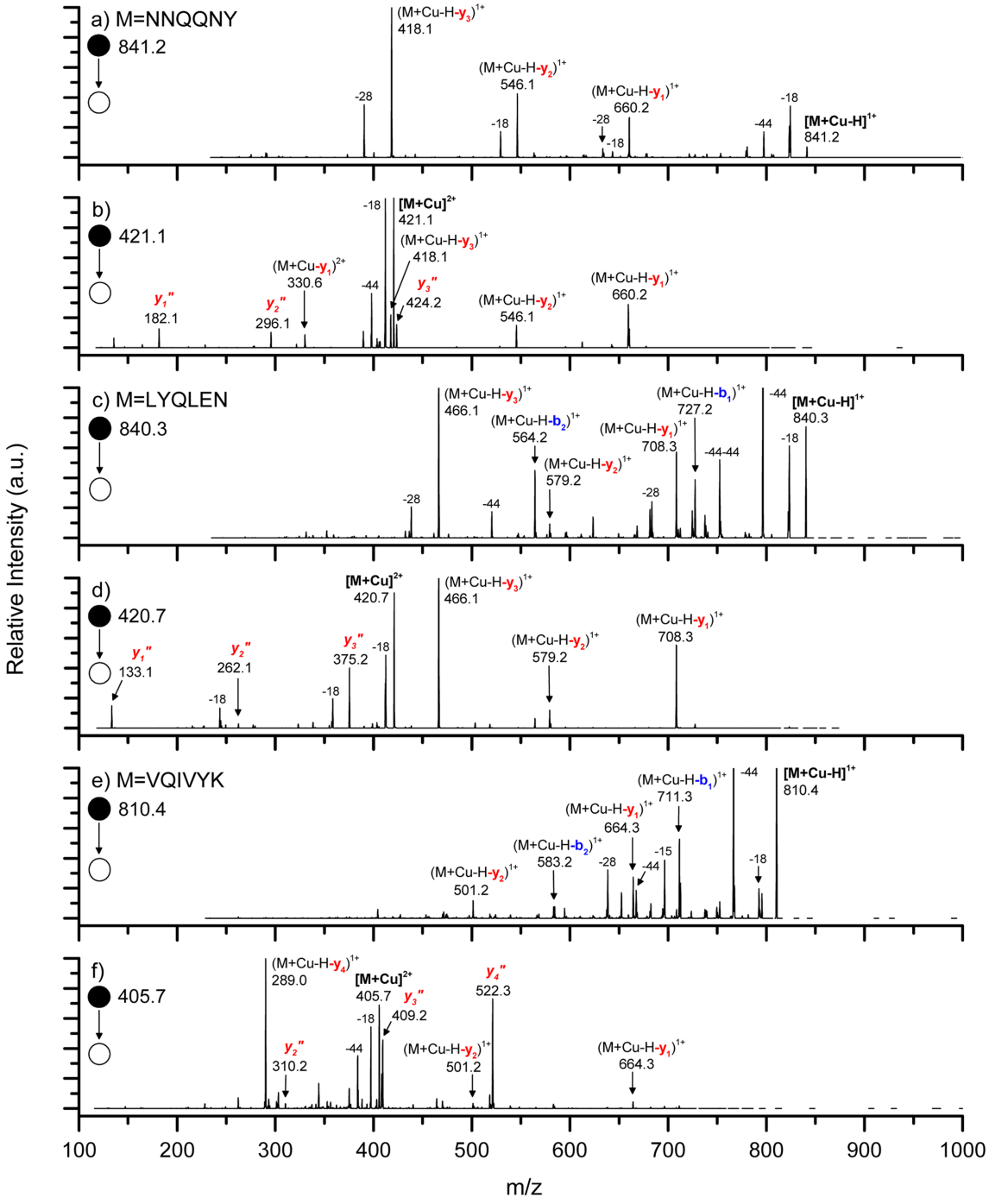


Figure 2. MS/MS spectra of singly and doubly charged monomer complexes of NNQQNY, LYQLEN, and VQIVYK peptides with a bound Cu(II) ion. $[M+Cu-H]^{1+}$ of **a)** NNQQNY, **c)** LYQLEN, and **e)** VQIVYK. $[M+Cu]^{2+}$ of **b)** NNQQNY, **d)** LYQLEN, and **f)** VQIVYK. Peaks labeled -18 likely result from the loss of an H_2O moiety. Peaks labeled -28 result from the loss of an additional CO moiety and would conventionally be labeled as a_n ions. Peaks labeled -44 likely result from the loss of a CO_2 moiety. b_t fragments are expressed in blue and bold and y_n fragments are expressed in red and bold.

$[M+Cu]^{2+}$ spectra contain only a $\{y_n$ loss$\}$ series as observed in the NNQQNY and LYQLEN peptides, with the y_4 loss fragments appearing with particularly high intensity (Figure 2f). This implies that in the $[M+Cu]^{2+}$ complex, Cu(II) ions preferentially bind to the N-terminal region.

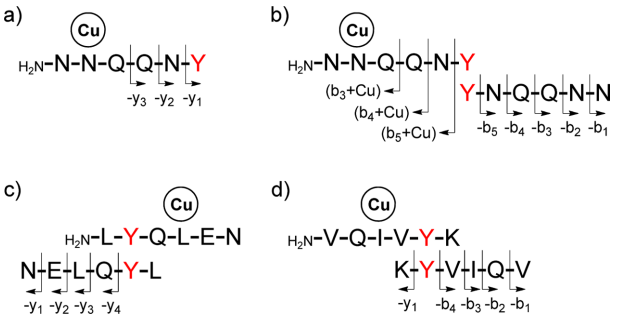
A summary of the observed MS/MS fragmentation patterns is available in Table 2.

MS/MS spectra of dimers

In the MS/MS spectra of the NNQQNY $[2M+Cu-H]^{1+}$, fragmentation patterns predominantly display a $\{b_t$ loss$\}$ series up to t=5 (Figure 3a) instead of a $\{y_n$ loss$\}$ series, as was observed in the $[M+Cu-H]^{1+}$ complexes. The preservation of Y6-Y6 interactions of the NNQQNY $[2M+Cu-H]^{1+}$ complexes was observed, which agrees with a previous study[22] which also used CID-MS/MS experiments with NNQQNY $[2M+H]^{1+}$.

In the MS/MS spectra of the NNQQNY doubly charged dimer complexes, $[2M+Cu]^{2+}$, we observe a series of $(b_t+Cu)^{1+}$ ions from $(b_3+Cu)^{1+}$ to $(b_5+Cu)^{1+}$ fragments (Figure 3b), in addition to the $\{b_t$ loss$\}$ series up to t=5. The series of $(b_t+Cu)^{1+}$ ions in addition to the corresponding $\{b_t$ loss$\}$ series indicates that the Cu(II) ion has high affinity for the N-terminal region. This presence of the $\{b_t$ loss$\}$ series up to t=5 is similar to the series found in $[2M+Cu-H]^{1+}$. As further evidence of the Cu(II) affinity for the NNQ residues, even in the dimer complexes, MS/MS/MS spectra of the $(b_5+Cu)^{1+}$ and $(b_4+Cu)^{1+}$ ions from $[2M+Cu]^{2+}$ were measured (Figure 3c and d).

Based on these results, we propose a (Cu-M-M) structure with the Cu(II) bound to the NNQ portion of the interacting monomer subunits (Scheme 1b), which plausibly explains the observed mass spectra.



Scheme 1. The proposed location of the Cu(II) ion in relation to the monomer of **a)** NNQQNY and the dimer of **b)** NNQQNY, **c)** LYQLEN, and **d)** VQIVYK. The observed dissociation channels in the MS/MS spectra are labeled and indicated with arrows.

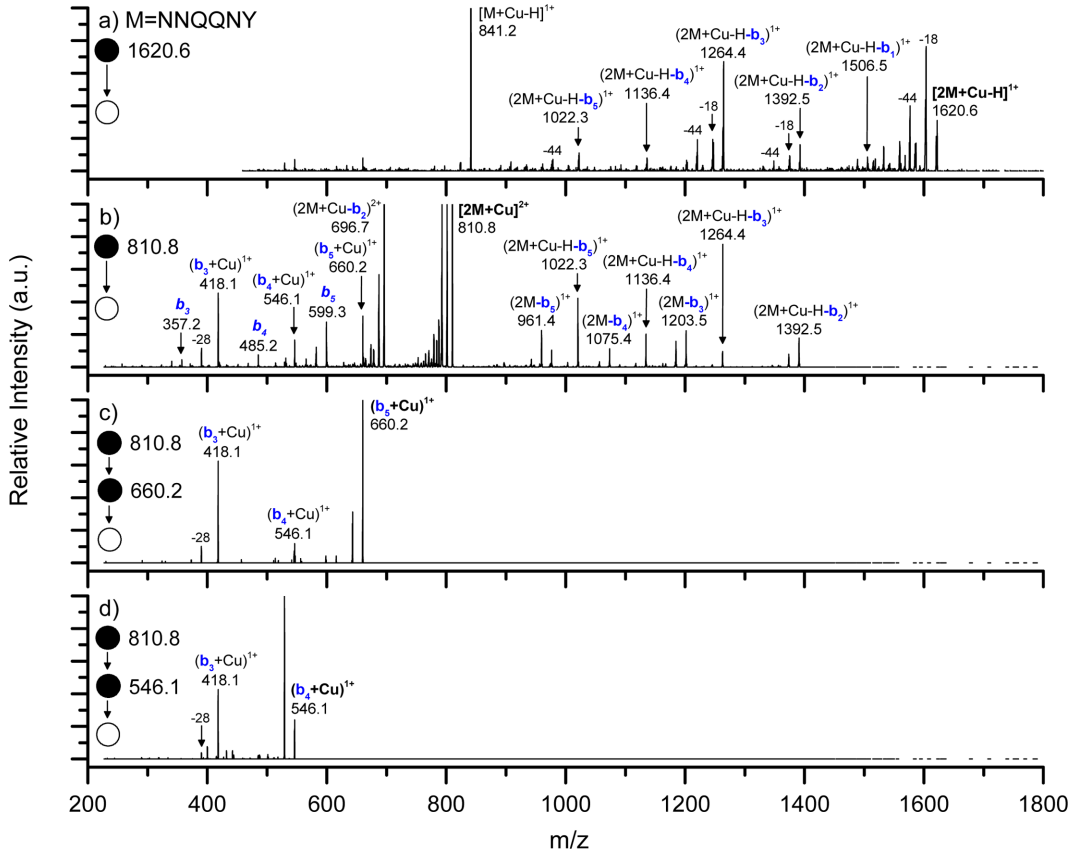


Figure 3. MS/MS spectra of singly and doubly charged dimer complexes of NNQQNY with a bound Cu(II) ion. **a)** $[2M+Cu-H]^{1+}$ and **b)** $[2M+Cu]^{2+}$. MS/MS/MS spectra of $(b_t+Cu)^{1+}$ fragments observed in b) spectrum. **c)** $(b_5+Cu)^{1+}$ and **d)** $(b_4+Cu)^{1+}$.

The LYQLEN $[2M+Cu-H]^{1+}$ MS/MS spectrum displayed only a $\{y_n$ loss$\}$ series instead of a mixture of $\{b_t$ loss$\}$ and $\{y_n$ loss$\}$ series, as was observed in the monomers (Figure S3a). A $\{y_n$ loss$\}$ series up to n=4 was observed, showing stable Y2-Y2 interactions similar to that of LYQLEN $[2M+H]^{1+}$ MS/MS spectrum.[22] Similarly, in the $[2M+Cu]^{2+}$ MS/MS spectrum (Figure S3b) a $\{y_n$ loss$\}$ series up to the n=4 was also observed. Scheme 1c shows a structure that could account for these observations.

The VQIVYK $[2M+Cu-H]^{1+}$ MS/MS spectrum, however, display mostly a $\{b_t$ loss$\}$ series instead of the $\{b_t$ loss$\}$ and $\{y_n$ loss$\}$ series mixture observed in the monomers. A $\{b_t$ loss$\}$ series up to t=4 is observed with an additional y_1 loss (Figure S3c). The observation of a $\{b_t$ loss$\}$ series without b_5 and b_6 loss and presence of y_1 loss can be explained through the increased stability of Y5-Y5 interactions of VQIVYK $[2M+H]^{1+}$, as shown by Seo et. al.[22] Scheme 1d shows the Cu(II) binding location in $[2M+Cu-H]^{1+}$. The MS/MS spectra of the VQIVYK $[2M+Cu]^{2+}$ complexes were unable to be determined due to low intensity.

In the MS/MS spectra of the [Cu-dimer] complexes of the three peptides, the Cu(II) ions were consistently observed to bind the monomer subunit of the [Cu-dimer] structures as (Cu-M-M) structures, in which Y-Y interactions were maintained instead of formation of a (M-Cu-M) structures.

The MS/MS fragmentation patterns of [Cu-dimer] are summarized in Table 2. We note that the MS/MS fragmentation patterns of [Cu-monomer] are not maintained in those of [Cu-dimer] due to the steric hindrance of Y-Y interactions of the (Cu-M-M) structures.

Conclusions

The results of the MS experiments with Cu(II) ions and the active peptide sequences NNQQNY, LYQLEN, and VQIVYK show that the [Cu-peptide] complex spectral peaks increase in intensity as the concentration of Cu(II) rises, indicating the stable binding of Cu(II) ions by all three peptides. Peptide aggregates, which readily form in solution,[30] were allowed to form before addition of Cu(II). Our data would indicate increasing concentrations of Cu(II) decrease the population of existing active sequence peptide oligomers similarly to studies of larger amyloidogenic peptides[10,21] and small peptides.[27,31]

The MS/MS spectra of [Cu-monomer] complexes show that Cu(II) ions have a clear binding preference for the N-terminal region of the NNQQNY peptide. In the LYQLEN and VQIVYK $[M+Cu-H]^{1+}$ complexes, the Cu(II) ions appear to bind non-specifically in more than one region, whereas the $[M+Cu]^{2+}$ complexes appear to bind specifically to the N-terminal regions.

The MS/MS spectra of [Cu-dimer] complexes show that for the NNQQNY, LYQLEN, and VQIVYK peptides, Cu(II) prefers to bind away from the central Y-Y interactions in a (Cu-M-M) structure and also that Y6-Y6, Y2-Y2, and Y5-Y5 interactions are maintained during CID-MS/MS. In NNQQNY dimers, the Cu(II) ion shows preferential binding to the N-terminal region similar to the [Cu-monomer] complex, which was confirmed by MS/MS/MS. However, the binding site of Cu(II) ion in the dimers of the LYQLEN and VQIVYK peptides is suggested to be at the monomer subunit, as shown in Scheme 1.

Acknowledgments

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