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Complexation between Cu(II) and curcumin in the presence of two different segments of amyloid β

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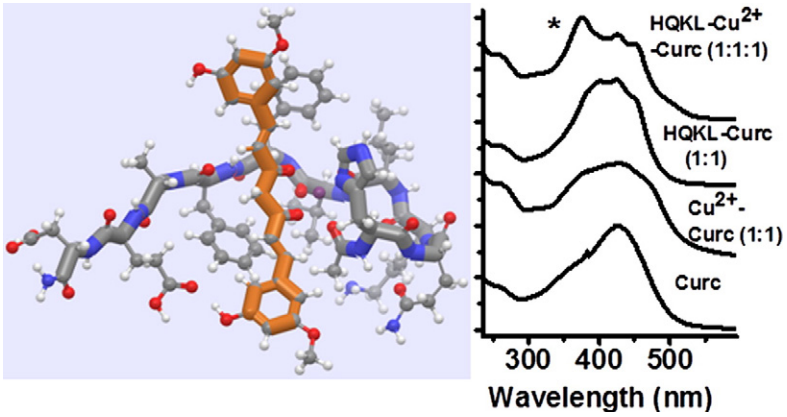
HIGHLIGHTS

- Competitive Cu^{2+} binding by curcumin was studied in the presence of two segments of Aβ protein.
- Absorbance and fluorescence spectroscopy of mixed solutions monitored binary and ternary complex formation.
- Mass spectrometry isolated ternary peptide–Cu^{2+}–curcumin complexes.
- Results showed that curcumin can simultaneously bind to Cu^{2+} and the Aβ peptide.

GRAPHICAL ABSTRACT



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ABSTRACT

The natural product curcumin has been shown to play a role in preventing Aβ amyloid fibril formation. This role could include chelation of transition metal ions such as Cu^{2+}, known to accelerate amyloid aggregation, and/or curcumin-binding directly to the Aβ protein. To investigate these different roles, curcumin complexation to Cu^{2+} was investigated in the presence and absence of two different segments of the Aβ protein including the copper-binding (Aβ6–14) and curcumin-binding (Aβ14–23) domains. Absorbance and fluorescence spectroscopy in 90% water/10% methanol solutions showed that curcumin can bind Cu^{2+} to some extent in the presence of both segments despite strong peptide–ion interactions. Estimated Cu^{2+}–curcumin binding affinities in the absence ($1.6 \times 10^5\ M^{-1}$) and presence ($7.9 \times 10^4\ M^{-1}$) of the peptide provide quantitative support for this Cu^{2+} chelation role. With the Aβ14–23 segment, the curcumin simultaneously binds to Cu^{2+} and the peptide, demonstrating that it can play multiple roles in the prevention of amyloid formation. The stabilities of ternary peptide–Cu^{2+}–curcumin complexes were evaluated using ESI mass spectrometry and support the conclusion that curcumin can act as a weak metal ion chelator and also bind directly to the Aβ14–23 peptide segment.

1. Introduction

The cause of Alzheimer's Disease (AD) is not completely understood, but it is well known that AD features insoluble amyloid fibrils in the cerebral cortex of patients [1]. These fibrils form when the amyloid β (Aβ) peptide, cleaved from the amyloid precursor protein (APP), misfolds

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into structures with high β-sheet content, which then aggregate together via cross-β interactions into protein oligomers forming insoluble fibrils [2–11]. The crucial and/or dangerous steps in this process are the initial protein misfolding/aggregation and oligomer formation [8,12–16]. Transgenic mice overexpressing APP showed decreased synchronization of neuronal stimuli, and dodecamer complexes of Aβ have been found to decrease long term memory in rats [17,18]. Hence, a great deal of biomedical research is now devoted to designing drugs for blocking the protein misfolding and peptide aggregation [19–23]. Clinical trials support this research direction and have shown that the progression of AD can be halted with drugs that block intracellular amyloid formation [22,24].

Transition metal ions such as Cu^{2+} and Zn^{2+} significantly affect protein misfolding and aggregation with direct implications to amyloid diseases [5,25–27]. These metals are found accumulated in Alzheimer's plaques [11,28] indicating disrupted metal ion homeostasis, which is tightly regulated in healthy tissue. While it is unclear exactly how the ions affect Aβ, and at what point during amyloid formation/oligomerization the ion binding is most important, EPR measurements [29] and theoretical simulations [15] have provided clues. Metal ions bind to peptide side chains and backbones, inducing fundamental peptide/protein conformational changes and accelerating amyloid fibril formation [30–35]. Furthermore, the reduction of Cu^{2+} generates reactive oxygen species, such as H_2O_2 or $OH^{\bullet}$, causing oxidative damage to proteins, lipids, and DNA [36–38]. As a result, chelation of metal ions bound to Aβ is a potential target for developing therapeutics [39–42] and clinical trials support their use and further development [23].

The natural product curcumin (Fig. 1), a known Cu^{2+} and Zn^{2+} chelator through the keto (or resonance-stabilized deprotonated) carbonyls [43,44], has been shown to be a possible candidate for preventing and reversing amyloid fibrillization [40,42,45–48]. The roles of chelator drugs in treating neurodegenerative diseases, reviewed by Baldrick and Jones in 2011, include complete removal of the metal ions as well as metal ion redistribution (which may not require complete ion chelation) [49]. An effective chelator should compete with the ion–peptide binding and also cross the blood–brain barrier [49]. If curcumin functions as a chelator drug, then it must compete with the strong Cu^{2+}–Aβ complexation.

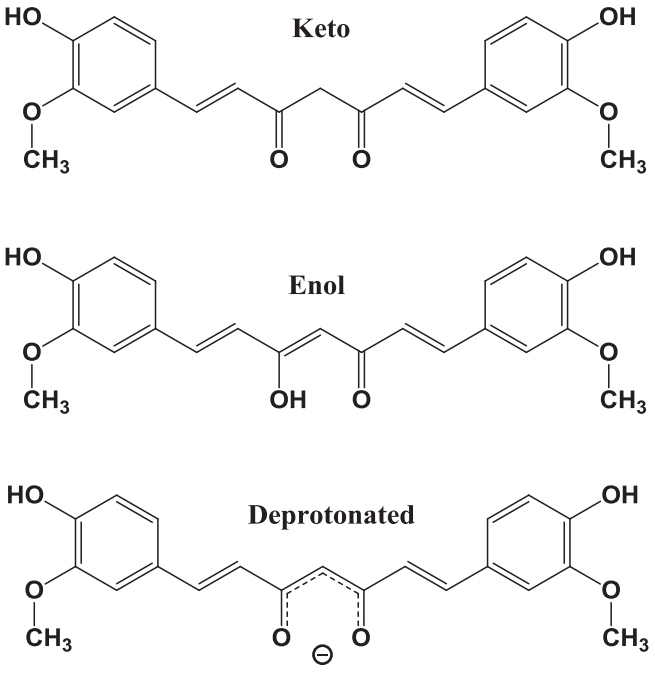

Fig. 1. Curcumin shown in the keto, enol, and deprotonated forms.

Cu^{2+} binds to the Aβ peptide via three histidine residues in the histidine-rich segment HDSGYEVHH (Aβ6–14) [29,50,51] and also to the N-terminus [52]. It is important to characterize the relative Cu^{2+}-binding interactions between curcumin and this peptide segment in order to evaluate curcumin's role as a chelator drug. However, recent studies have shown that curcumin can bind directly to a different segment of the Aβ peptide. Curcumin can bind to the KLVFF segment at the phenylalanine residues 19 and 20 via π–π interactions [46,53], and could play a role in blocking amyloid fibril formation that is different from a chelation role, or the role could be cooperative between metal ion chelation and π–π interactions. Computational studies have shown that the full Aβ protein can bind curcumin in a similar manner as the truncated KLVFF peptide but neglect metal ion binding [46,54]. Other computations have shown that transition metals bind to the Aβ6–14 region and cause the remaining peptide (Aβ15–42) to adopt a β-sheet conformation, but these studies do not include curcumin binding [5,15]. In this work we consider these regions separately. Detailed information about competitive Cu^{2+} chelation by curcumin on the different Aβ peptide segments will help to elucidate how curcumin functions with respect to the full system.

In this fundamental work, we carry out such a "piecewise" investigation with two different Aβ peptide segments and focus on characterizing the Cu^{2+}–curcumin interactions in the presence of the Cu^{2+}–peptide interactions. The two peptides chosen are HDSGYEVHH, which incorporates three of the four Cu^{2+} binding sites, and HQKLVFFAED, which incorporates the curcumin-binding region. The two segments are shown in Fig. 2 in relation to the full Aβ sequence. Both peptides are capped to prevent binding to the N-terminus, which notably neglects the fourth Cu^{2+} binding site [52] for simplicity of investigation. By evaluating the competition between Cu^{2+}–curcumin, Cu^{2+}–peptide, and curcumin–peptide interactions using absorption and fluorescence spectroscopy, and identifying simultaneous complexation using mass spectrometry, we can characterize the different roles curcumin may play as an anti-amyloid drug with different peptide segments.

2. Experimental

The capped peptides Ac-HDSGYEVHH-NH_2 (HDS) and Ac-HQKLVFFAED-NH_2 (HQKL) were obtained from Celtek Peptides (Nashville, TN, USA) and were purified by adding a small amount of 0.01 M HCl and then overnight lyophilization. After purification, purity was estimated at greater than 98% by mass spectrometry. Curcumin was obtained from TCI America and was used without further purification.

All samples were prepared from 1 mM stock solutions of the peptides, $CuCl_2$, and curcumin dissolved in HPLC-grade methanol. Stock solutions were prepared in methanol because of curcumin's low water solubility. A particular drawback of curcumin is that its solubility in water is extremely low (~1 μM). While this is a pharmaceutical problem, it is not a fundamental chelation problem as the concentrations of peptides and ion–peptide complexes in physiological systems are also very low (on the order of 1 μM). Sample solutions were prepared for analysis by mixing aliquots of the stock solutions in appropriate molar ratios and then diluting with DI water to a final analyte concentration (peptide, Cu^{2+}, and/or curcumin) of 33.3 μM. After solution preparation, we deduced that the solvent was 90% water and 10% methanol. No precipitation was observed. We note here that the experiments were performed in a predominantly aqueous environment.

Absorption spectra were recorded on a PerkinElmer Lambda 35 UV/VIS Spectrometer using a 90%/10% water/methanol blank while fluorescence spectra were recorded on a Perkin Elmer LS55 fluorimeter. All spectra were recorded with excitation and emission slit widths of 2.5 mm. Fluorescence experiments were performed for each sample. The fluorescence of curcumin was measured by excitation at the curcumin absorption wavelength (determined from absorption spectroscopy) in the presence of Cu^{2+}, peptide, or both.

Fig. 2. Full sequence of amyloid-β. The two different sequences studied in this work correspond to residues 6–14 (circled in blue), and residues 14–23 (circled in red). The residues are numbered below the sequence.

Ternary complex formation was evaluated using electrospray (ESI) mass spectrometry, using an Agilent 1100 Series ESI ion-trap mass spectrometer. The 1:1:1 molar-ratio solutions were injected directly into the ESI needle with a syringe pump at 0.3 mL/h. The ESI needle was held at +3.5 kV and the ion trap was filled with helium buffer gas and heated at a constant 325 °C. Collision-induced dissociation (CID) MS/MS experiments were performed by isolating the parent ion at 25% compound stability and applying the variable CID voltage for 20 ms.

3. Results and discussion

The electronic absorbance spectroscopic results of the 90%/10% water/methanol solutions of different peptide–metal ion–curcumin molar ratios are summarized in Fig. 3 for both peptides. The absorbance intensities for each spectrum are normalized to unity and the spectra are offset to focus the analysis on the differences in spectral shapes rather than the slight absolute absorbance differences. Above 300 nm, the curcumin-only spectrum (lower trace in Fig. 3A) exhibits a single broad band with a peak at 430 nm. This spectrum is unchanged for the HDS–curcumin solution (Fig. 3A), suggesting that the curcumin does not significantly interact with the HDS peptide.

The spectrum of the Cu^{2+}–curcumin mixture (prepared in a 1:1 molar ratio) is noticeably broader than the curcumin spectrum, and while it still has a peak at 430 nm, shoulders are evident at higher wavelength (475 nm) and at lower wavelength (375 nm). This is consistent with previous results that show metal ion chelation by curcumin results in a change in the curcumin absorption spectrum [43]. In the ternary HDS–Cu^{2+}–curcumin mixture in Fig. 3A, prepared in a 1:1:1 molar ratio, the spectrum is visibly similar to the Cu^{2+}–curcumin spectrum. The peak at 430 nm is slightly more intense and the shoulders are slightly less prominent, but the spectrum clearly indicates Cu^{2+} chelation by the curcumin in the presence of the HDS peptide, and hence effective and competitive curcumin chelation despite the strong histidine–Cu^{2+} binding [29,50,51].

The results for the HQKL peptide are shown in Fig. 3B. The curcumin and Cu^{2+}–curcumin spectra are the same as those shown in Fig. 3A. The spectrum of the HQKL peptide–curcumin mixture (1:1 molar ratio) is significantly different from the curcumin-only spectrum. The peak wavelength is around 400 nm and the peak is broader and shifted to shorter wavelengths relative to curcumin. This indicates that, unlike with the HDS peptide, curcumin interacts with the HQKL peptide. As has been reported previously (with different peptide segments) the curcumin binds to the two phenylalanine residues through π–π interactions [46,53].

In the ternary mixture in Fig. 3B (1:1:1 molar ratios), the absorbance spectrum appears different from either the Cu^{2+}–curcumin or HQKL–Cu^{2+} spectra as it exhibits a relatively intense band at 375 nm (denoted by * in Fig. 3B) that is not clearly present in other spectra. This band may correspond to a ternary complex where the curcumin simultaneously binds to the Cu^{2+} ion (likely bound to a histidine residue) through the carbonyl groups and binds the peptide at the phenylalanine residues. This would suggest that curcumin can chelate Cu^{2+} at the histidine peptide binding site and also bind to the peptide at the phenylalanine site.

For further insight, we turn to the fluorescence spectroscopic results, presented in Fig. 4. The fluorescence spectrum of the curcumin is shown as the second-to-top trace in Fig. 4A. From the spectrum of the Cu^{2+}–curcumin mixture (bottom trace in Fig. 4A), it is seen that the Cu^{2+}-chelation significantly reduces the curcumin fluorescence intensity, likely due to a heavy-atom quenching mechanism [55]. In the 1:1:1 HDS–Cu^{2+}–curcumin mixture, the fluorescence is intermediate between the curcumin and Cu^{2+}–curcumin spectra. Thus, the HDS peptide partially removes Cu^{2+} from the curcumin (partially restoring its uncomplexed fluorescence), immediately providing qualitative insight into the relative strengths of the HDS–Cu^{2+} and Cu^{2+}–curcumin interactions. Curcumin cannot completely chelate the metal ion, but it does exhibit effective partial chelation even in the presence of the strong HDS–peptide binding. Notably, the fluorescence of the HDS–curcumin mixture (top trace of Fig. 4A) is moderately higher than the curcumin fluorescence, which could suggest some modest peptide–curcumin interaction (perhaps through the tyrosine residue).

From Fig. 4A we can estimate quantitative data on the relative HDS–Cu^{2+} and Cu^{2+}–curcumin interactions by following the analysis of

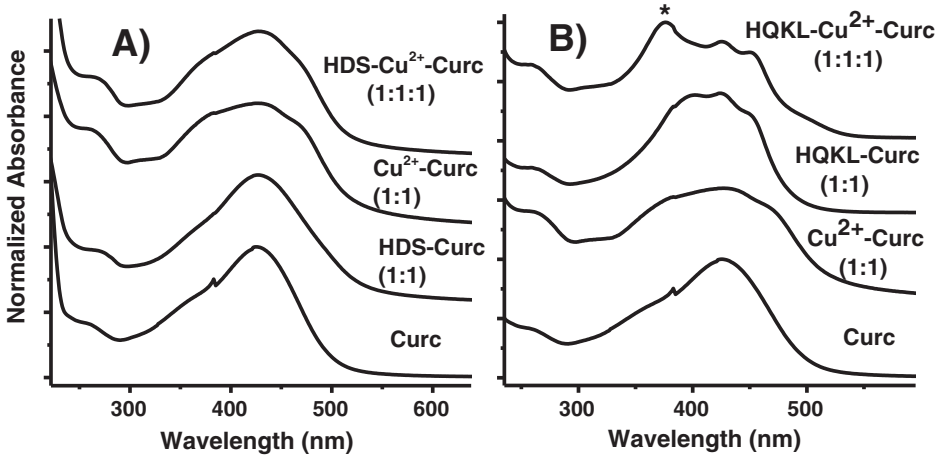

Fig. 3. Absorbance spectra of peptide–Cu^{2+}–curcumin mixtures in 90%/10% water/methanol solutions. The peptide and/or curcumin concentrations are 33.3 mM in all solutions and the molar ratios are shown next to each spectrum. Absorbance intensities are reported as normalized to unity and offset for clarity. A) HDS peptide results; B) HQKL peptide results, in which the * denotes the possible identification of a ternary peptide–Cu^{2+}–curcumin complex.

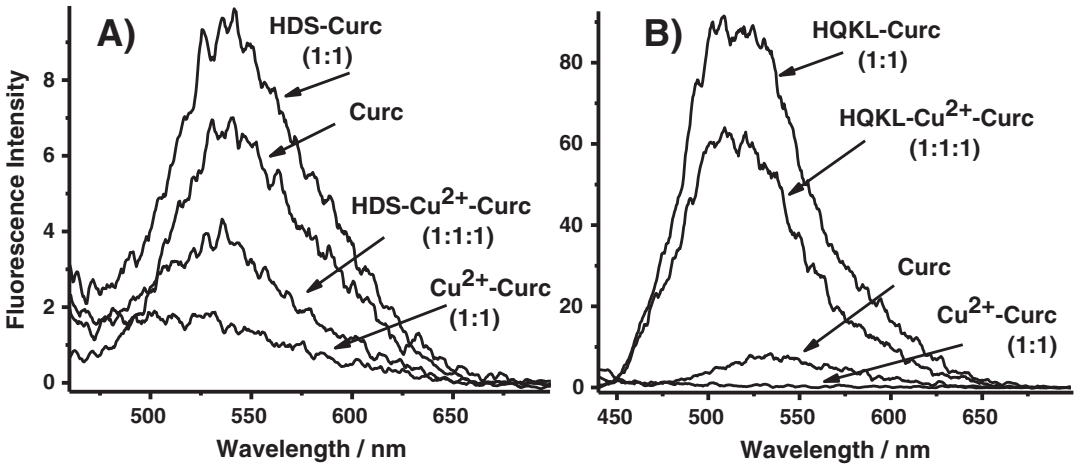

Fig. 4. Fluorescence spectra of the peptide–Cu^{2+}–curcumin solutions from Fig. 3. For all samples, the curcumin was excited at 430 nm. A) HDS peptide results; B) HQKL peptide results.

Maiti et al. [50], and using Eq. (1) to relate fluorescence intensities to the apparent Cu^{2+}–curcumin binding affinity in the presence and absence of the HDS peptide:

$$\Delta F = F_0 - FL = \frac{F_0 - F_\alpha}{2[M_0]}\left[([L] + [M_0] + K_D) - \left(([L] + [M_0])^2 - 4[L][M_0]\right)^{1/2}\right]. \quad (1)$$

In Eq. (1), $[M_0]$ is the molar concentration of curcumin, [L] is the concentration of Cu^{2+}, F_0 is the fluorescence of the curcumin molecule in absence of any quenching, F_L is the fluorescence of the curcumin molecule in the presence of Cu^{2+}, and F_α is the background (fully quenched) fluorescence. K_D is the Cu^{2+}–curcumin dissociation constant, and the inverse of K_D is K_A, the Cu^{2+}–curcumin binding affinity.

From Eq. (1), K_A of the Cu^{2+}–curcumin complex is $1.6 \times 10^5\ M^{-1}$, with a K_D value of 6.3×10^{-6} M. K_A values reported for the Cu^{2+} binding affinity of the Aβ peptide range from $1.4 \times 10^6\ M^{-1}$ [50] to ~$10^8\ M^{-1}$ for the N-Acetylated Aβ and ~$10^9\ M^{-1}$ for the un-Acetylated protein [56]. The Cu^{2+}–curcumin binding affinity thus compares favorably with the values at the low end of this spectrum. The K_A value of the Cu^{2+}–curcumin binding affinity in the presence of the binding to the HDS peptide (using Eq. (1) with data from Fig. 4A is ~$7.9 \times 10^4\ M^{-1}$, with a K_D value of 1.3×10^{-5} M. The apparent binding affinity of Cu^{2+} to curcumin is lessened by the peptide moiety, by roughly 50%. Thus the Cu^{2+}–curcumin interaction is relatively strong enough to competitively chelate Cu^{2+} in the presence of the HDS peptide.

For the HQKL peptide (Fig. 4B), a similar trend is not observed. The fluorescence spectra of the curcumin and Cu^{2+}–curcumin mixtures (same as those in Fig. 4A) are shown at the bottom of the figure. With the HQKL peptide present in 1:1 molar ratios (upper traces), the fluorescence spectra appear significantly different from the curcumin spectra. The spectrum of the HQKL–peptide mixture (top trace) exhibits much higher fluorescence intensity and is shifted to shorter wavelength by ~50 nm, which is indicative of significant changes to the curcumin excited electronic state. Both 375 nm (not shown) and 430 nm excitations produce this same result. Note that this indicates the fluorescence is not simply quenched by the peptide, and we cannot use Eq. (1) to estimate K_A. In any case, taken together with the absorbance spectrum (Fig. 3B), the results verify that the curcumin binds to the HQKL peptide.

The fluorescence spectrum of the ternary mixture is similar to the HQKL–curcumin spectrum but with ~25% decreased intensity. This is likely due again to heavy-atom quenching [55] of curcumin by the Cu^{2+} ion. The similarity of the HQKL–Cu^{2+}–curcumin fluorescence spectral shape to the HQKL–curcumin spectrum and the quenching by Cu^{2+}, along with the absorbance spectroscopic results (Fig. 3B), provides strong evidence that the Cu^{2+} simultaneously binds to the peptide and the curcumin in a ternary complex, demonstrating the ability of curcumin to simultaneously act as a chelator molecule and bind to the peptide.

Fundamental information about the competitive peptide–Cu^{2+}–curcumin interactions can be further investigated using ESI coupled to an ion-trap MS. Both sets of results in Figs. 3 and 4 suggest interaction between the peptides and peptide–metal ion complexes and the curcumin molecule. With the HDS peptide this interaction may be slight or non-existent, while with the HQKL peptide this interaction appears to be very significant. The identification of ternary complexes in mass spectrometry serves as verification of complexation in solution, and the relative ion intensities provide some measurement of complex stability. We injected the ternary (1:1:1 molar ratio) solutions into an ion-trap mass spectrometer. The results are shown in Fig. 5. With the HDS peptide (Fig. 5A), the most intense peak corresponds to the doubly-charged peptide ion. Notably, the signal intensity from the peptide–Cu^{2+} complex is very weak. The curcumin-containing species are shown clearly in the inset. The intense peak at m/z 799 corresponds to a ternary complex of deprotonated curcumin (see Fig. 1), neutral curcumin, and Cu^{2+}, based on the mass and isotope pattern. The small peak at m/z 776 is identified as a doubly-charged ion with copper based on its isotope pattern and based on its mass corresponds to the ternary peptide–Cu^{2+}–curcumin complex. The other peaks in the inset are unidentified but likely do not contain peptide components as they are present in both mass spectra in Fig. 5. While present, the intensity of this ion species appears to be relatively low.

The mass spectroscopic results for the ternary system with the HQKL peptide are shown in Fig. 5B. The doubly-charged peptide ion is observed as the most intense peak, and notably the peptide–Cu^{2+} complex ion is observed to be much more intense than with the HDS–Cu^{2+} species in Fig. 5A. The curcumin-containing species are shown in the inset. This inset is similar to that shown in Fig. 5B except that the ternary HQKL–Cu^{2+}–curcumin complex ion is observed at m/z 851 and in significantly higher relative intensity than the HDS–Cu^{2+}–curcumin species. This immediately and quantitatively verifies that the HQKL peptide forms a stable ternary complex and is in general more amenable to forming this stable ternary complex than the HDS peptide. This conclusion is consistent with the solution-phase absorption and fluorescence experiments.

Taken together, the results demonstrate that curcumin is able to competitively chelate Cu^{2+} in the presence of different segments of the Aβ peptide in aqueous environments. We have presented electronic absorption and fluorescence spectroscopic results along with ESI–ion trap mass spectrometry results for mixed peptide–Cu^{2+}–curcumin systems for two peptides representing two different segments of the Aβ protein known to be important for either Cu^{2+}-binding, curcumin

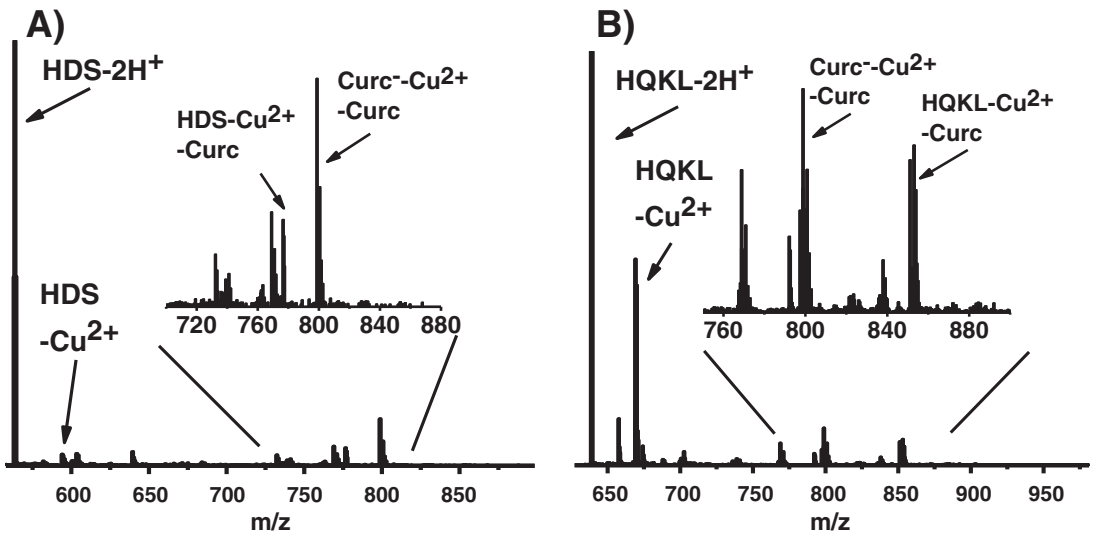

Fig. 5. ESI-mass spectra of the 1:1:1 molar-ratio solutions presented in Figs. 3 and 4. The results provide evidence of ternary complex formation and qualify the relative complex stabilities. A) HDS–Cu^{2+}–curcumin mixture, with an arrow to designate the (very weakly present) ternary complex; B) HQKL–Cu^{2+}–curcumin mixture, with an asterisk to designate the ternary complex. The higher relative intensity of this peak compared to the results in panel A suggests that the HQKL forms a more stable ternary complex.

interaction, or both. The HDS peptide contains three histidine residues known to bind Cu^{2+}, and the HQKL peptide contains two phenylalanine residues known to bind curcumin. The characterization of the relative interaction strengths in binary and ternary peptide–Cu^{2+}–curcumin systems provides insight into how curcumin can compete and/or cooperate with the metal ion–peptide interactions.

With the HDS peptide, the curcumin molecule chelates Cu^{2+} in the presence of the peptide–ion binding, albeit with reduced binding affinity. In this isolated context, we note that even with reduced binding affinity curcumin could function as a transition metal ion redistributor drug [49], which would not require complete chelation. In the case of the HQKL peptide, the results show that the curcumin interacts with the peptide and the Cu^{2+} ion simultaneously. This is supported by the absorption and fluorescence spectroscopic results as well as the mass spectrometry identification of a stable gas-phase ternary complex. It is interesting to further note that in the ternary complex, the curcumin is the less strongly bound molecule. Collision-induced dissociation MS/MS experiments verify that for both ternary complexes in Fig. 5, the ions dissociate by losing the curcumin molecule. Nonetheless, the identification of the ternary complex elucidates the special ability of the curcumin molecule to interact with the Aβ protein in different roles.

The HDS peptide models the transition metal ion-binding region of Aβ. While it neglects the N-terminus, it contains the three histidine residues expected to interact with Cu^{2+} in vivo. Miller et al. have shown that metal ions bind to this region and leave the rest of the Aβ protein (including the HQKL peptide region, Aβ14–23) as a β-sheet [15]. Thus, curcumin binding as a chelating agent must be able to effectively interact with this region of the Aβ protein. We have shown that curcumin can competitively chelate Cu^{2+} bound to the HDS peptide. Inclusion of the N-terminal region may affect the quantitative effectiveness of curcumin as a metal ion chelator, as it may participate in metal ion binding [52]. However, curcumin also binds to the phenylalanine residues F19 and F20 [53]; the HQKL peptide models this region along with one histidine residue to take into account both the curcumin–peptide interaction and the metal ion interaction. Our results qualitatively show that curcumin can simultaneously interact with the metal ion and the peptide. In the full Aβ protein, the curcumin could either interact simultaneously with both peptide regions, by binding to the Cu^{2+} at the histidine-binding region and also to the peptide at the phenylalanine residues (a possibility based on the present results), or it could interact primarily as a chelator at the histidine-binding region, or it could interact primarily as a β-sheet disruptor [46] at the phenylalanine region. We considered the two regions separately here because differentiating the two mechanisms, or simultaneous mechanism, would require very detailed structural measurements. The results presented provide insight into the different mechanisms of curcumin chelation and peptide binding and show that curcumin can both bind to Aβ and also chelate Cu^{2+}.

4. Conclusion

Curcumin has been shown to be effective in preventing or reversing amyloid fibril formation of Aβ. We have investigated the possible mechanisms of curcumin binding to Aβ by studying peptide–Cu^{2+}–curcumin mixed systems with two different segments of the Aβ protein, incorporating a Cu^{2+}-binding segment and a curcumin-binding segment. We have found that curcumin is capable of competitively chelating copper in the presence of either Aβ peptide segment in an aqueous environment. Curcumin binds to Cu^{2+} in the presence of the HDS peptide (Aβ6–14). The Cu^{2+}-binding affinity of curcumin, estimated from the fluorescence spectroscopic results, is reduced by the peptide–ion interaction by roughly 50%. This result shows that curcumin could act as a weak transition metal ion chelator. However, the results with the other segment of the Aβ protein, the HQKL peptide (Aβ14–23), provide further insight into the role of curcumin in preventing Aβ amyloid fibril formation. Curcumin can bind to the HQKL segment in the absence of Cu^{2+}, illustrating its role in disrupting Aβ aggregation, but also forms a ternary peptide–Cu^{2+}–curcumin complex, as evidenced by the absorbance and fluorescence spectroscopic results and confirmed by ESI-MS isolation of the complex. This result demonstrates that curcumin can simultaneously bind to Cu^{2+} and Aβ and thus can function both as a chelator and an Aβ binding partner. Thus, curcumin may be able to participate in the treatment of Alzheimer's Disease with respect to the Aβ protein through several different molecular mechanisms.

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