Curcumin Alters the Salt Bridge-containing Turn Region in Amyloid β(1–42) Aggregates

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Background: Curcumin reduces the risk of Alzheimer disease via an unknown mechanism.

Results: Curcumin-incubated Aβ42 aggregates retain the hairpin architecture but have disruptions in the turn region (surprising similarity with Zn2+ incubation).

Conclusion: Salt bridge-containing turn region is a major determinant of morphology and toxicity.

Significance: Identification of crucial structural changes provides a checkpoint for developing effective AD therapeutics.

Curcumin, a phenolic compound from the Asian spice turmeric (Curcuma longa), is of great interest for its plausible role in countering neurodegenerative diseases like Parkinson disease (1–3) and Alzheimer disease (AD) (4, 5). In vitro studies have shown that curcumin can retard the process of amyloid β (Aβ) aggregation (5, 6), which is supposed to be the initiator of AD. It disrupts the formation of the long straight Aβ fibrils (5, 7) and reduces Aβ toxicity (8, 9). Moreover, it can also disrupt the preformed Aβ fibrils (6, 7). The reduction of toxicity is possibly related to these effects. Alternative modes of action have also been suggested; it has been shown to inhibit the process that produces Aβ from the amyloid precursor protein (10).

In addition, curcumin in general can reduce the concentration of the reactive oxygen species (11), which plays a role in neurodegeneration. Whatever is its main mode of action, information about the conformational changes of Aβ induced by curcumin would be valuable in understanding its role in AD.

There are several modified curcumin analogues that have been developed that show even more potent anti-AD activity in animal models (12), and pharmaceutical development will benefit from this understanding. Curcumin is known to bind to β-sheet-rich Aβ species like protofibrils and fibrils and not to unstructured monomers (7). Theoretical studies have suggested that curcumin can interact with Aβ oligomers to disrupt the β-sheet content and possibly alter the conformation of several regions of the Aβ molecule (13). Experimental studies using curcumin analogues with different linker lengths have shown that there is an optimum linker length that interacts with Aβ, suggesting that the interaction of Aβ with curcumin is site-specific (14, 15). However, it is still not known whether curcumin induces specific structural changes in Aβ aggregates.

Like curcumin, Zn2+ ions are also known to alter the toxicity of Aβ and disrupt the Aβ fibrils (16–18). It is known that Zn2+ ions preferentially precipitate the amyloid oligomers and can stoichiometrically bind to Aβ (19–21). Zn2+ is thought to bind to the N-terminal region, and this is most likely mediated by His-6, His-13, and His-14 (22–25). Using ssNMR studies, we have previously shown that Zn2+ binding disrupts the salt bridge between Asp-23 and Lys-28 (26). Although Zn2+ and curcumin have a completely different chemical nature, there are interesting parallels between the effects induced by them. It is therefore worthwhile to compare their structural effects. If some of the structural changes are common, then those
Molecular Insights into Curcumin-induced Changes in Aβ42

EXPERIMENTAL PROCEDURES

Aβ42—Synthesis, Purification, and Sample Preparation—Synthesis and purification procedures of two different Aβ42 peptide specimens with different 13C and 15N isotopic labeling schemes (P1 and P2) are described elsewhere (26). Purified Aβ42 peptides were initially dissolved in pH 11.0 water (adjusted by NaOH) to prepare 2 mM stock solutions. A 4 mM stock solution of curcumin (Sigma) was also prepared by dissolving in pH 11.0 water to grow Aβ42 aggregates in the presence of curcumin, 1.5 ml of Aβ42 stock (2 mM) and 150 μl of freshly prepared curcumin stock (4 mM) were mixed in pH 11.0 water and immediately diluted with 5.85 ml of HEPES buffer (containing 20 mM HEPES, 146 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl2·2H2O, and 0.8 mM MgSO4·7H2O) at pH 7.4 such that the total volume was 7.5 ml. This final solution containing 400 μM Aβ42 and 80 μM curcumin was incubated at room temperature (∼24°C) for 4 days with mild rotation (10 rpm). This resulted in the formation of aggregates. The rest of the materials used in the experiments was same as described in Ref. 26.

Solid-state NMR—For ssNMR measurements, solutions containing peptide aggregates were subjected to centrifugation (16,000 × g) for 1 h. The pellets thus collected were washed with de-ionized water twice by resuspending them in water and ultracentrifuging for 1 h each time. The final pellets so obtained were rapidly frozen using liquid nitrogen and then lyophilized. The powdered samples were rehydrated (33 weight %) by adding ∼0.5 μl of de-ionized water per mg of dry peptide aggregates. The hydrated sample was then packed in a 2.5-mm magic angle spinning rotor such that it contains ∼10 mg of sample.

All the ssNMR measurements were performed using a 700 MHz Bruker AVIII NMR spectrometer at a magic angle spinning frequency of 15 kHz using a 2.5-mm triple resonance magic angle spinning probe. Cross-polarization to 13C and 15N from 1H was implemented using a linear ramped radio frequency field (27) centered around 65 kHz on the 1H channel and with a 55-kHz field on the 13C/15N channel with a contact time between 2.5 and 4.0 ms. 1H dipolar decoupling was accomplished using the swept frequency two–pulse phase modulation (ϕ = 10°) decoupling scheme (28) with a field strength of 85 kHz. Two-dimensional 13C-13C through-space NMR spectra were recorded using second-order dipolar recoupling schemes of PARIS-xy (m = 1, n = 2) (29) and PDSD (30) and mixing periods of 100 and 1000 ms, respectively. 1H irradiation of 15 kHz was used while applying PARIS-xy (m = 1, n = 2). A total of 200 points was acquired in the indirect dimension with a dwell time of 12.5 μs amounting to an acquisition time of 2.5 ms. The number of scans per free induction decay and interscan delay was fixed to 256 and 2 s, and 512 and 1.5 s in case of PARIS-xy (m = 1, n = 2) and PDSD, respectively. Frequency-selective 13C-15N rotational-echo double-resonance (REDOR) experiments were recorded using the pulse sequence designed by Jaronec et al. (31). Rotor-synchronized trains of phase-alternated π pulses following xy-8 scheme (32) were applied on the 15N channel. 466.7 μs long frequency-selective Gaussian pulses were applied at the 13C–15N frequencies of Asp-23 and Lys-28, respectively, in the middle of the REDOR-dephasing period (τm). Reference spectra (S0) were recorded after removing the frequency-selective refocusing pulse on 15N channel.

The results shown in this study for amyloid aggregates of only Aβ42 and those grown in the presence of 400 μM Zn2+ ions (Aβ42–Zn) are based on higher quality ssNMR spectra compared with those reported in our previous article (26). This is a result of better purification and degree of hydration (50 weight %), but they do not change any of the conclusions of the previous study.

NMR Data Analysis—All one-dimensional spectra were processed and analyzed using TopSpin 3.1. All two-dimensional spectra were processed with TopSpin 3.1 and analyzed using CcpNmr Analysis 2.2.2. The data were zero-filled in the t2 and t1 dimensions to 512 and 4096 points, respectively. A mixed sine/cosine (ϕ = π/3 at t = 0) apodization function was used in each dimension. All the spectra were externally referenced to tetramethylsilane in methanol (33). TALOS+ (34) was used to obtain predicted ψ and φ backbone torsion angles, based on 13C chemical shifts of α, β, and carbonyl carbons.

Calculating Average Chemical Shift Change (Δδ)—13C chemical shifts of isotopically labeled amino acids in Aβ42 fibrils (26) were subtracted from that of Aβ42–Zn (26) and Aβ42–Cur aggregates. These chemical shift differences for α, β, and carbonyl carbons (the backbone) and the remaining carbons (the side chain) were then averaged over the number of carbons constituting the backbone and side chain, respectively, to obtain an average chemical shift change (Δδ).

Electron Microscopy—10-μl solutions of Aβ42 (400 μM) and Aβ42–Cur (400 μM Aβ42 + 80 μM curcumin), aggregated for 4 days, were placed on carbon-coated 100-mesh copper grids for 2 min, followed by blotting by a filter paper. The extra salt on the grids was removed by four cycles of mild washing with double-distilled water. Then a drop of 0.1% uranyl acetate was added to each grid and left for 5 min for staining. After removing the extra uranyl acetate solution by a filter paper, the grids were dried under an infrared lamp. The samples were examined with a transmission electron microscope (LIBRA 120, EFTEM, Carl Zeiss, Germany). The width analysis of fibrils was performed with Image (open source software, rsbweb.nih.gov).

Cell Culture—Primary cortical neurons were cultured from pregnant female Wistar rats obtained from the Institute Animal Facility. All animal handling procedures were approved by the
Molecular Insights into Curcumin-induced Changes in Aβ42

animal ethics committee of the Institute. Neuronal cultures were obtained from the cortex of 17-day-old embryos. Cells were grown in Neurobasal media supplemented with 2% B-27 supplement, 0.5% penicillin/streptomycin, and 0.25% l-glutamine. Cell culture media and chemicals were obtained from Invitrogen.

Cell Viability Assay—Primary cortical neurons grown in 96-well plates were treated with Aβ42 (40 or 400 μM) on day 5. After 48 h, the cells were assessed for viability. The cells were treated with 0.01 mg/ml propidium iodide (Molecular Probes) for 10 min, washed with phosphate-buffered saline, and imaged with an epifluorescence microscope (Zeiss Axiovert 200, Germany) using a 40× objective. Propidium iodide binds to DNA but is cell-impermeable and hence can penetrate cells with damaged membrane. This gives the dead cell count. The number of live cells (propidium iodide-positive cells were subtracted from total cells counted from transmission images) expressed as a percentage of total cell count gives the percentage cell viability. Cell viability of a control set of cells treated with vehicle is normalized to 100%, and all the results are expressed with respect to that. To investigate the effectiveness of curcumin in reducing the Aβ-induced toxicity, curcumin was added at 1:5 molar ratio (i.e. 8 μM curcumin to 40 μM Aβ42 and 80 μM curcumin to 400 μM Aβ42). Each experiment was performed on six different wells.

RESULTS AND DISCUSSION

We tested the effects of curcumin on Aβ42-induced toxicity on rat primary cortical neuronal cultures. Fig. 1, A–D, shows the superimposed transmission and propidium iodide-stained (marks dead cells, green) images of neurons for different treatments. The results of this study are summarized in Fig. 1E. The cells exposed to 40 μM Aβ42, for 48 h showed a reduced viability of 62 ± 4% compared with vehicle-treated control cells. However, co-incubation with 8 μM curcumin led to an improved viability of 85 ± 2%. This shows that a sub-stoichiometric amount of curcumin (at 1:5 molar ratio to Aβ42) is able to significantly ameliorate the toxic effects Aβ42. This neuro-protective role of curcumin was more evident at a higher concentration of Aβ42. Although the cells treated with only 400 μM Aβ42 for 48 h showed very low viability (4 ± 1%), the addition of 80 μM curcumin improved the cell viability to 74 ± 3%, manifesting a very pronounced effect. Based on these results, we decided to probe the structural alterations of Aβ42 aggregates caused by curcumin, starting from a solution containing 80 μM curcumin and 400 μM Aβ42.

Aβ42 fibrils are characterized by a cross-β architecture in which each Aβ42 monomer adopts a hairpin structure (although not a β-hairpin). The two largely hydrophobic β-strands are connected by a loop, and there is an overhanging unstructured N terminus (35, 36). These β-strands polymerize in a parallel in-register orientation forming intermolecular β-sheets running perpendicular to the direction of the fibril axis. The presence of an intra and intermolecular contact between the side chains of amino acids Phe-19–Leu-34 and Gln-15–Gly-37 (36) and an intermolecular salt bridge between the COO− and NH3+ groups present in the side chains of Asp-23 and Lys-28 (35) are some other prominent features in the available structural models of Aβ42 fibrils.

Aβ42 aggregates. In our previous study (26), we investigated the effect of Zn2+ on the molecular structure of Aβ42 fibrils with ssNMR using two Aβ42 peptides, namely P1 and P2, containing 13C- and 15N-labeled amino acids at specific positions along the peptide sequence (26). P1 has uniformly 13C- and 15N-labeled Gln-15, Phe-19, Ala-30, Leu-34, Val-36, and Gly-38 and uniformly 15N-labeled His-13, whereas P2 has uniformly 13C- and 15N-labeled Ser-8, Val-12, Phe-20, Asp-23, Lys-28, Met-35, and Ile-41 and uniformly 15N-labeled His-14. We use the same peptides in this work, which allow a direct comparison.

Chemical shifts of 13C atoms in isotopically enriched amino acids were obtained from two-dimensional 13C-15N correlation spectra of Aβ42 aggregates grown in the presence of curcumin (termed as Aβ42-Cur) recorded using PARIS-xy (m = 1) (n = 2) (29) recoupling scheme with a mixing time of 100 ms. Selected regions of these spectra containing aliphatic-carbonyl and aliphatic-aliphatic cross-peaks are shown in Fig. 2, A and B. The corresponding spectra for Aβ42 are shown in Fig. 2, C and D. The chemical shift values are listed in Table 1 along with the values obtained for Aβ42-Zn aggregates. For Aβ42-Cur aggregates, multiple sets of chemical shifts were observed for amino acids Val-12 (two), Asp-23 (three), Lys-28 (two), Ala-30 (three), Val-36 (three), and Gly-38 (two), which each set represents a distinct structural conformation. The rest of the amino acids yield unique sets and hence adopt a unique structural conformation in Aβ42-Cur aggregates. Similar structural heterogeneity prevails in the molecular structure of Aβ42 aggregates (grown in absence of curcumin) with these same amino acids.
exhibiting multiple structural conformations. Even the number of multiple conformations is same in both cases, except for Val-12 and Lys-28, both of which exhibit an extra conformation in case of Aβ42 aggregates. Fig. 3, A and B, shows the average chemical shift changes (Δδ) incurred by the backbone and side-chain carbons of isotopically labeled amino acids in Aβ42 when aggregated in presence of Zn2+ ions and curcumin, respectively (see under “Experimental Procedures” for calculation of Δδ values). Only those Δδ values that are ≥0.5 ppm and at least three times larger than the error associated with them are considered as significant, i.e. represent a significant structural change. These are highlighted in gray in Fig. 3, A and B. These changes are generally associated with local structural changes, which can be caused by direct or indirect interaction of the external ligand. These values indicate that both Zn2+ ions and curcumin cause structural perturbations of the Val-12 backbone. In addition to these, curcumin also causes chemical shift changes in the Asp-23 and Leu-34 side chains (Fig. 3B).

More differences are observed when other types of spectral features, like changes in the peak intensities in both one-dimensional and two-dimensional spectra, are considered. These changes are shown in Fig. 3, C–G, which shows selective regions of one-dimensional and two-dimensional spectra of Aβ42 (black), Aβ42-Zn (blue), and Aβ42-Cur (orange) aggregates. Amino acids showing other type of spectral changes are Ser-8, His-13, and His-14 in the case of Zn2+ ions and Val-36 and Gly-38 in the case of curcumin. Our previous study has shown that the presence of Zn2+ ions imparts increased structural order to the otherwise less ordered side chains of His-13 and His-14 (26). This observation was based on observation of broad peaks around 174 and 208 ppm in 15N one-dimensional spectrum of Aβ42-Zn aggregates (Fig. 3, C and D, blue). These broad peaks arise from the imidazole ring nitrogens, ε2 and δ1, present in the side chain of histidine. The absence of signal in the case of Aβ42 aggregates grown in the absence of Zn2+ ions (Fig. 3, C and D, black) is attributed to the structural heterogeneity associated with these side chains, which become structurally more ordered due to Zn2+ binding. However, the presence of curcumin does not impart any structural order to these flexible His side chains as is evident from the absence of NMR peaks in the region of interest (Fig. 3, C and D, orange). Fig. 3E highlights the Cβ-C′ correlations in Ser-8 as observed in the PARIS-xy (m = 1) (n = 2) spectrum recorded with a mixing time of 100 ms. Clearly, a much stronger correlation is observed in the case of Aβ42-Zn aggregates (Fig. 3E, blue) compared with the Aβ42 (black) and Aβ42-Cur (orange) aggregates. The intensity of such through-space 13C-13C correlations produced by second-order recoupling schemes like PARIS-xy (m = 1) (n = 2) depends strongly on the molecular properties of the system as well as the experimental parameters used (37). Because all spectra were recorded under similar experimental conditions, this change in intensity of Cβ-C′ correlations in Ser-8 must have its origin in changes occurring at the molecular level. It most likely reflects that the Ser-8 backbone acquires more structural order when Aβ42 is aggregated in presence of Zn2+ ions but not curcumin.

Fig. 3F highlights the Cα-Cα cross-peaks observed for three conformers of Val-36 as observed in PARIS-xy (m = 1) (n = 2) spectrum using a mixing time of 100 ms. In the case of Aβ42 (Fig. 3F, black) and Aβ42-Zn (blue) aggregates, the Val-36 conformer yields the most intense cross-peak (cross signs) followed by Val-36′ (plus signs) and Val-36″ (circled cross signs), and hence it is the most populated conformer among the three. However, in the case of Aβ42-Cur aggregates (Fig. 3F, orange), cross-peaks of both Val-36 and Val-36′ conformers are nearly equally intense and that of Val-36″ was not observed at all (only observed in PDSD spectrum recorded with long mixing time of 1 s). It is thus clear that curcumin disturbs the population equilibrium of various structural conformers of Val-36. Similar observations exist in the case of Gly-38, where population distribution between the two conformers of Gly-38 (Fig. 3G), Gly-38...
Molecular Insights into Curcumin-induced Changes in Aβ_{42}

| Residue | C'  | Cα  | Cβ  | Cγ  | Cδ  | Cε  | Torsional angles (ϕ, ψ) |
|---------|-----|-----|-----|-----|-----|-----|-------------------------|
| Ser-8   | 173.3 | 57.2 | 62.7 |     |     |     | (–)                     |
|         | (174.2) | (57.4) | (62.9) |     |     |     | (–)                     |
| Val-12  | 173.5 | 59.6 | 33.3 | 19.8,19.8 |     |     | (–)                     |
|         | (173.7) | (59.7) | (33.4) | (20.1,20.1) |     |     | (–)                     |
| Val-12' | 173.2 | 59.8 | 32.1 | 19.9,19.9 |     |     | (–)                     |
|         | (173.9) | (60.5) | (31.9) | (20.1,20.1) |     |     | (–)                     |
| Val-12' | 173.8 | 59.8 | 34.4 | (20.1,20.1) |     |     | (–)                     |
|         | (174.9) | (61.3) | (30.8) | (20.3,20.3) |     |     | (–)                     |
| Gln-15  | 172.6 | 53.7 | 32.4 | 177.8 |     |     | (–)                     |
|         | (173.2) | (53.8) | (31.4) | (33.1) | 177.4 |     | (–)                     |
|         | (173.2) | (53.9) | (30.8) | (32.9) | 177.8 |     | (–)                     |
| Phe-19  | 172.4 | 54.6 | 41.0 | 137.8 | 130.6 | 129.8 | 127.3 | (–)                     |
|         | (171.9) | (54.7) | (41.4) | (137.4) | (130.4) | (129.4) | (127.3) | (–)                     |
| Phe-20  | 171.0 | 55.2 | 41.5 | 137.5 | 129.8 | 129.8 | (–)                     |
|         | (171.1) | (54.9) | (42.0) | (137.8) | (130.3) | (130.3) | (130.3) | (–)                     |
| Asp-23  | 175.2 | 52.8 | 40.1 | 179.0 |     |     | (–)                     |
|         | (173.0) | (53.3) | (36.1) | (180.2) |     |     | (–)                     |
| Asp-23' | 174.9 | 52.2 | 40.3 | 178.4 |     |     | (–)                     |
|         | (174.4) | (52.3) | (41.6) | (178.8) |     |     | (–)                     |
| Asp-23' | 174.4 | 51.9 | 42.9 | 177.7 |     |     | (–)                     |
|         | (175.1) | (52.1) | (42.0) | (177.2) |     |     | (–)                     |
| Lys-28  | 173.2 | 54.3 | 32.6 | 24.8 | 28.4 | 41.2 | (–)                     |
|         | (173.8) | (54.5) | (32.7) | (24.9) | (28.5) | (41.0) | (–)                     |
| Lys-28' | 173.2 | 54.5 | 32.7 | 25.0 | 28.6 | 41.2 | (–)                     |
|         | (173.8) | (54.5) | (32.7) | (25.0) | (28.6) | (41.2) | (–)                     |
| Ala-30  | 174.4 | 48.7 | 20.8 |     |     |     | (–)                     |
|         | (174.3) | (48.8) | (20.7) |     |     |     | (–)                     |
| Ala-30' | 174.3 | 50.1 | 21.5 |     |     |     | (–)                     |
|         | (174.6) | (49.9) | (21.0) |     |     |     | (–)                     |
| Ala-30' | 175.6 | 50.8 | 18.7 |     |     |     | (–)                     |
|         | (175.8) | (50.5) | (19.0) |     |     |     | (–)                     |
| Leu-34  | 173.1 | 52.5 | 45.0 | 26.0 | 23.0,23.0 |     | (–)                     |
|         | (172.5) | (52.5) | (44.4) | (26.7) | (24.4,24.4) |     | (–)                     |
| Met-35  | 172.5 | 53.1 | 34.2 | 30.9 | 18.1 | (18.3) | (13)                    |
|         | (172.8) | (53.2) | (34.2) | (30.9) | (18.3) | (13) | (13)                    |
| Gly-38  | 170.3 | 42.6 |     |     |     |     | (–)                     |
|         | (170.1) | (42.3) |     |     |     |     | (–)                     |
| Gly-38' | 170.2 | (42.4) |     |     |     |     | (–)                     |
|         | 169.6 | 44.6 |     |     |     |     | (–)                     |
|         | (169.5) | (44.7) |     |     |     |     | (–)                     |
| Ile-41  | 172.8 | 58.7 | 37.8 | 26.2,16.4 | 12.2 |     | (–)                     |
|         | (173.2) | (58.9) | (38.1) | (26.3,16.4) | (12.3) |     | (–)                     |
|         | (173.0) | (58.9) | (38.1) | (26.4,16.5) | (12.3) |     | (–)                     |

(cross signs) and Gly-38' (plus signs), gets perturbed only in the presence of curcumin but not in the presence of Zn^{2+} ions.

In our previous study, it was shown that Zn^{2+} ions disrupt the Asp-23–Lys-28 salt bridge in Aβ_{42} aggregates by stabilizing only the non-salt bridge-forming conformations of Asp-23 and
Molecular Insights into Curcumin-induced Changes in Aβ_{42}

Lys-28 (26). Interestingly, presence of curcumin also causes major population redistribution between different conformers of Asp-23 and Lys-28, leading to the near disappearance of the Asp-23 and Lys-28 conformers as assigned in Aβ_{42} aggregates (see Table 1). This effect is clearly shown in Fig. 4, A and B, which shows an overlay of selected regions of the one-dimensional 13C and 15N spectra of Aβ_{42} (black) and Aβ_{42}-Cur (orange) aggregates, respectively. These are two-dimensional 13C-13C PARIS-xy (m = 1) (n = 2) (100-ms mixing time) spectra of Aβ_{42} (black), Aβ_{42}-Zn (blue), and Aβ_{42}-Cur (orange) aggregates, respectively. Dashed arrows in C and D highlight peaks in 15N one-dimensional spectra of Aβ_{42}-Zn aggregates corresponding to ε nitrogen in imidazole ring of His-13 (C) and δ' and ε nitrogen in imidazole ring of His-14 (D). Correlations highlighted in two-dimensional spectra are between the following: E, C_β and C' carbons in Ser-8 (S8 (cross signs)); F, C_α and C_β carbons in Val-36 (V36 (cross signs), V36' (plus signs), and V38' (circled cross signs)); G, C_α and C' carbons in Gly-38 (G38 (cross signs) and G38' (plus signs)).

A recent ssNMR study by Masuda et al. (38) has shown that curcumin interacts with the residues from 17 to 21, as well as with residue 12 present in the N-terminal β-sheet. Masuda et al. (38) postulate that curcumin targets the C-terminal β-sheet in a nonspecific fashion. Our results substantiate this speculation with three amino acids in this region, namely Leu-34, Val-36, and Gly-38, undergoing either structural reorganization or population redistribution between different conformers in the presence of curcumin. Regarding the N terminus, our results are partially in agreement with their findings. We also found that Val-12 was affected by curcumin. However, Phe-19 and Phe-20, the only isotopically labeled amino acids in the 17–21 region, remain unaffected by the presence of curcumin. This discrepancy most likely has its origin in different way these studies have been performed. Masuda et al. (38) incubated curcumin with preformed fibrils, whereas we precipitated Aβ_{42} from the solution in the presence of curcumin. In a nutshell, curcumin does target the β-sheet-rich regions of Aβ_{42}, but these are not the only regions affected by it, as shown by our results.

Secondary chemical shifts (Δδ) of Aβ_{42} and Aβ_{42}-Cur aggregates, defined as the difference between the observed and random coil chemical shifts, were calculated for C_α, C_β', and carboxyl carbon atoms and are shown in Fig. 6, A and B, respectively. A positive value for C_α carbon and negative values for C_β and carboxyl carbon atoms is indicative of the amino acid being part of a β-sheet region. Most of the amino acids follow these criteria, thus indicating dominance of the β-sheet structural elements in both Aβ_{42} and Aβ_{42}-Cur aggregates. With the current isotopic labeling schemes, it seems that these regions run roughly between amino acids Val-12 and Phe-20.
Molecular Insights into Curcumin-induced Changes in Aβ₄₂

FIGURE 4. A, carbonyl region of one-dimensional $^{13}$C spectra of Aβ₄₂ (black) and Aβ₄₂-Cur (orange) aggregates, highlighting peaks yielded by $\gamma$ carbon of Asp-23. B, selective region of $^{15}$N-one-dimensional spectrum of Aβ₄₂ (black) and Aβ₄₂-Cur (orange) aggregates, highlighting peaks yielded by $\zeta$ nitrogen of Lys-28. Peaks in A and B are labeled according to side chains of various conformers of Asp-23 and Lys-28 as explained in Ref. 26. Selective region of two-dimensional $^{13}$C-$^{13}$C correlation spectra (PARIS-xy, 100 ms) of Aβ₄₂ (C) and Aβ₄₂-Cur (D) aggregates. Also shown is the connectivity between aliphatic carbons ($\delta$, $\gamma$, $\delta$, and $\epsilon$) of three and two conformers of Lys-28 in Aβ₄₂ and Aβ₄₂-Cur aggregates, respectively. E and F, amino acids showing structural changes in the presence of Zn²⁺ ions and curcumin are highlighted over a schematic of a hairpin model of Aβ₄₂. Structural changes causing significant chemical shift changes are highlighted using dark cyan filled circles. Amino acids showing other type of spectral changes are highlighted using circles with red outline.

(N-terminal β-sheet) and Ala-30 and Ile-41 (C-terminal β-sheet). Even though multiple conformations are observed for some amino acids in this region, all of them seem to adopt β-sheet secondary structure except Gly-38. The presence of curcumin increases the population of the non-β-sheet conformer (Gly-38′) of Gly-38. Among the remaining amino acids, Ser-8 clearly seems to be part of the unstructured N terminus in both Aβ₄₂ and Aβ₄₂-Cur aggregates. The salt bridge-forming conformer of Asp-23, denoted D23 in figures, adopts a totally random structure. Its other two conformers in Aβ₄₂ aggregates also adopt non-β-sheet conformation. However, in case of Aβ₄₂-Cur aggregates, two out of its three conformers (Asp-23’ and Asp-23”) seem to adopt the β-sheet structure. Similarly, in the case of Lys-28, the disappearing conformer, denoted K28” in figures, adopts a structural conformation that is significantly different from the remaining two conformers (K28 and K28”). The structural conformation of the other two conformers of Lys-28 is not completely like β-sheet, but this conformation is more or less retained in the presence of curcumin. It seems that in Aβ₄₂ aggregates, both Asp-23 and Lys-28 adopt a secondary structure that is partially β-sheet in nature, and hence, they are most likely part of the turn region connecting the N- and C-terminal β-sheets. The presence of curcumin results in an increase in the β-sheet content of both Asp-23 and Lys-28 in Aβ₄₂-Cur aggregates. However, as both of them still contain conformers that are not strictly β-sheet-like, they might still be part of the turn region. To summarize, even though presence of curcumin causes local disturbances in the molecular structure, Aβ₄₂-Cur aggregates still retain the basic secondary structural motifs of Aβ₄₂ aggregates.

It was seen that despite some major structural changes, Aβ₄₂ aggregates grown in the presence of Zn²⁺ ions also retain both the secondary structure as well as the hairpin structure of Aβ₄₂ fibrils (26). In the hairpin structure of Aβ (both 40 and 42) fibrils, the side chains of amino acids present in the two β-sheets interdigitate to form a hydrophobic steric zipper (35, 36, 39–43). This interdigitating pattern differs from one structural model to another (35, 36, 39, 40, 42, 43). In other words, even though each model proposes the same hairpin structure for Aβ, they differ in terms of inter-residue contacts proposed between the two β-strands. However, intramolecular Phe-19–Leu-34 side-chain contacts have been proposed in most of the Aβ₄₂ and Aβ₄₀ models (36, 40, 42, 43), and they have even been

FIGURE 5. Carbonyl regions of $^{13}$C spectra of Aβ₄₂ (A) and Aβ₄₂-Cur (B) aggregates. The topmost spectrum (one-dimensional) in each case is recorded by simply acquiring the signal after cross-polarization from protons. Other spectra are recorded using frequency selective REDOR pulse sequence, without applying the frequency selective pulse on $^{15}$N channel (reference spectra, S₀) and spin-echo periods as indicated.
used to verify the hairpin structure of Aβ (26, 40). We also looked for this contact in Aβ_{42-Cur} aggregates. Because it is a long range contact, a two-dimensional $^{13}$C-$^{13}$C through-space correlation spectrum was recorded using the PDSD recoupling scheme (30) with a long mixing time of 1 s. A selected region of this spectrum is shown in Fig. 6C. The presence of the unambiguous cross-correlation between the aromatic ($\delta$/$\epsilon$) carbons of Phe-19 and $\gamma$ carbons of Leu-34 is indicative of their side chains being spatially proximal. Hence, just like the secondary structure, the tertiary structure also seems to be conserved in Aβ_{42-Cur} aggregates.

We note that the Phe-19–Leu-34 contact by itself does not prove that the peptide is bent into a hairpin shape. This contact constrains the possibilities to either a hairpin or to an open state with the monomeric strands in antiparallel arrangement, with Phe-19 and Leu-34 in register. This alternative possibility would imply a fibrillar width that is much larger. We examined the width of the Aβ_{42} and Aβ_{42-Cur} aggregates by transmission electron microscopy. The Aβ_{42} aggregates show a fibrillar morphology with the dominant form showing long, straight, unbranched fibrils that extend frequently beyond 1 μm in length (Fig. 7A). The Aβ_{42-Cur} aggregates, however, show only short fibrils or quasi-fibrillar structures (Fig. 7B). An analysis of the width of the fibrillar Aβ_{42} and quasi-fibrillar Aβ_{42-Cur} aggregates (a few representative positions are marked in red in Fig. 7, A and B) yields similar values (7.9 ± 0.9 and 8.1 ± 1.1 nm, respectively). This implies that the Aβ_{42-Cur} aggregates have a similar hairpin architecture as the Aβ_{42} fibrils. The disruption of the mesoscopic structures observed in the presence of curcumin is consistent with those reported earlier (5, 7) and also as reported for Zn$^{2+}$ (26). It is possible that both of these agents precipitate the aggregates of Aβ_{42} at an early stage and reduce their toxicity by locking them into a nontoxic conformation, and/or by simply removing them from the solution. Evidence for such a mode of action has indeed been seen for Aβ_{40-Zn$^{2+}$} (17, 21) and also for Aβ_{40-Cur}.$^{4}$

CONCLUSION

Significant changes in the Asp-23 and Lys-28 conformation is a major feature of the curcumin-induced disruption of the Aβ_{42} conformation. We note that the Phe-19–Leu-34 contacts in Aβ_{42} are thought to be intramolecular, whereas the salt bridge connecting Lys-28 and Asp-23 is thought to be between neighboring strands (35). Thus curcumin (like Zn$^{2+}$) seems to
Molecular Insights into Curcumin-induced Changes in Aβ

preserve the gross intramolecular conformation, while disrupting the intramolecular arrangements. This is consistent with its ability to disrupt the fibrillar architecture (as observed by transmission electron microscopy in Fig. 7) (5, 7). There are also specific changes in the intramolecular structure, with the C-terminal residues mostly affected by curcumin, whereas the N-terminal residues are mostly affected by Zn2+ ions. Both of these therefore appear to stabilize a nonfibrillar or partly fibrillar family of aggregate structures whose morphology and toxic properties are very different from that of the regular fibrillar aggregates. These structural differences highlight potential target regions of the peptide for designing therapeutics for Alzheimer disease.

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