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Short communication

Unusual structural transition of antimicrobial VP1 peptide

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ABSTRACT

VP1 peptide, an active domain of m-calpain enzyme with antimicrobial activity is found to undergo an unusual conformational transition in trifluoroethanol (TFE) solvent. The nature of, and time dependent variations in, circular dichroism associated with the amide I vibrations, suggest that VP1 undergoes self-aggregation forming anti-parallel β-sheet structure in TFE. Transmission electron micrograph (TEM) images revealed that β-sheet aggregates formed by VP1 possess fibril-like assemblies.

1. Introduction

Calpains [1] belong to the superfamily of calcium dependent intracellular cysteine proteases that are expressed ubiquitously in mammals [2,3]. These enzymes are believed to play important roles in many processes such as cytoskeletal remodeling, cell differentiation, embryonic development, signal transduction and apoptosis [4–6]. Calpain 2 (or m-calpain) is the most studied member of the calpain superfamily. The larger subunit (80 kDa) of m-calpain consists of four domains (I–IV), while smaller subunit (30 kDa) consists of two domains (V and VI) [7]. Domain V is largely cleaved from m-calpain during autolysis [8]. It has been demonstrated that a C-terminal sequence of domain V, GTAMRILGG, is required for lipid interaction of the protease [9]. Further, domain V derived from four different mammalian (porcine, human, bovine and rabbit) m-calpains showed that each of them hold a common segment, GTAMRILGGVI, referred to as the VP1 peptide [10]. Later studies, using peptides homologous to various regions of m-calpain's domain V, showed that although the presence of TAMRIL sequence is required for m-calpain–lipid interaction, the presence of glycine units is also necessary for such interaction [11].

The conformational preferences of this important VP1 peptide are not well established. Infrared (IR) spectroscopic results on films were interpreted to suggest that VP1 peptide adopts β-sheet structure in aqueous buffer solution and varying amounts of α-helical and β-sheet structures in the presence of different anionic lipids [12]. In addition, VP1 was suggested to have ~45% helical and ~25% β-sheet structures in the presence of mixed lipid vesicles [13]. Based on IR spectra, a similar level of α-helicity was proposed in 90% trifluoroethanol (TFE) [13]. Although the crystal structure was reported for m-calpain [14,15], it did not include domain V, so the three-dimensional structure for domain V are not currently available.

Nuclear magnetic resonance (NMR) and X-ray crystallography are generally used to determine the three-dimensional structures of proteins and peptides. However it is not easy to utilize NMR when a protein/peptide aggregates lead to fibril formation. In such situations, electronic circular dichroism (ECD) and IR spectroscopy are often used for the elucidation of secondary structures and transitions between them in solution state. The spectra–structure correlations in ECD depend on observing characteristic ECD bands associated with a particular type of structure. For proteins and peptides containing substantial number of aromatic amino acids, particularly phenylalanine, such correlation can sometimes be misleading because of the overlap of ECD bands originating from the peptide back bone with those from aromatic groups [16]. Furthermore, in some cases, spectra–structure correlation deduced from ECD spectra may not be consistent with the structure determined from other methods [17,18]. The spectra–structure correlation from IR spectra depends predominantly on observing the characteristic amide carbonyl group stretching (amide I) band in the 1700–1600 cm^{-1} region. Even though the resolution enhancement methods, such as Fourier self deconvolution and second derivatives, have been used to separate the

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overlapping bands, the structural deductions based on IR spectra alone are not unambiguous. The combined application of multiple spectroscopic methods is useful to avoid such uncertainties.

In recent years, vibrational circular dichroism (VCD) and Raman optical activity (ROA) have emerged as powerful tools to determine the secondary structure of proteins and peptides [19,20]. The ability to measure VCD spectra under a broad range of sample conditions (including dried films, protein aggregates, protein fibrils, gel, and adsorbed to different solid surfaces) makes VCD a useful tool for probing the protein structure [21–24].

The structure of VP1 in TFE solvent is investigated here using multiple spectroscopic methods: VCD, IR, and ECD. TFE solvent has been used as a membrane mimicking environment for studying proteins and peptides. Since the m-calpain activity [12,13] and antimicrobial property of VP1 peptide [25] are often correlated with VP1–lipid interaction, the choice of TFE solvent is practically relevant for VP1 structural study.

2. Experimental

VP1 peptide was obtained from Genscript Inc. with >95% purity. HPLC and Mass spectrometry were used to assess the purity of the peptide. TFE was purchased from Acros (USA). Phosphotungstic acid was obtained from Aldrich (USA). The fixed path length IR cell (100 μm) with BaF_2 windows was purchased from International Crystal Labs (USA).

2.1. VCD and FTIR spectroscopy

All VCD and IR absorption spectra were recorded on a commercial Chiralir spectrometer (BioTools, USA). This instrument was modified to minimize the spectral artifacts. IR absorption spectra were acquired simultaneously with VCD measurements. All spectra were recorded at room temperature. For time dependent VCD spectra, 1 mg of VP1 peptide was dissolved in 100 μL of TFE solvent. This solution was left at room temperature for 0.5 h before VCD measurements. VP1 solution was loaded into the 100 μm fixed path length cell containing BaF_2 windows. The VCD spectra were collected over a period of four days, each with 1 h data collection time at resolution of 8 cm^{-1}. The VCD spectrum of solvent (TFE) itself was recorded under similar conditions to obtain the VCD baseline and subtracted from the VCD spectra of VP1. The IR absorption spectra of VP1 are also solvent subtracted.

2.2. ECD spectroscopy

All ECD spectra were recorded on a Jasco J720 spectropolarimeter at room temperature. The instrument was calibrated with ammonium D-camphor-10-sulfonate as described by the instrument manufacturer. For time dependent ECD spectra, similar to VCD, 1 mg of VP1 peptide was dissolved in 100 μL of TFE. This solution was left at room temperature for 0.5 h before ECD measurements. About 20 μL of the VP1 solution was loaded into the 0.01 cm circular quartz cell. All ECD spectra are averages of three individual scans. A scan speed of 50 nm/min, time constant of 0.125 s, resolution of 1 nm, and sensitivity of 100 mdeg were used. The ECD spectrum of solvent was subtracted from those of VP1 solution. The ECD spectra were collected over a period of three days.

2.3. Electron microscopy

Aged solution (96 h) of VP1 in TFE (1 mg/100 μL) was examined by transmission electron microcopy. A 5 μL sample was placed on carbon-coated grids and left there for 1 min. The sample grids were stained with 1% (w/v) phosphotungstic acid for 20 s, washed, and air dried. Grids were imaged using a Philips CM20 Transmission Electron Microscope operating at 200 kV and a magnification of 20,000×.

3. Results and discussion

The time dependent IR and VCD spectra of VP1 in TFE are shown in Fig. 1. The IR spectrum at 30 min shows a broad amide I band centered at 1674 cm^{-1} and the corresponding VCD spectrum shows a negative band at 1670 cm^{-1}, both characteristic of a β-turn structure. The IR spectrum at 6.5 h shows decrease in intensity at 1674 cm^{-1} (Fig. 2) and a new amide I band developing at 1620 cm^{-1} and this later band is characteristic of β-sheet structure. The corresponding VCD spectrum, accordingly, showed a negative couplet, negative at 1609 cm^{-1} and positive at 1624 cm^{-1}, and two additional negative VCD bands at 1682 and 1640 cm^{-1}. It should be noted that the VCD intensities at 1609 and 1624 $cm^{-1}$are significantly enhanced when compared to the initial VCD (note that IR absorption intensity at 1620 cm^{-1} is only ~0.1 at 6.5 h). The dissymmetry factor, ΔA/A, at 1624 cm^{-1} is ~5 times larger compared to that in the initial spectra. Enhanced VCD intensities were also observed previously for protein fibrils that are enriched with β-sheet structure [21]. Enhanced VCD is also characteristic of supramolecular chirality as observed in insulin fibrils [22]. Furthermore, the fibrils formed from a shorter octapeptide also showed enhanced VCD intensities in the amide I region [26]. Theoretical models for VCD intensities of amyloid-like fibrils rationalizing the different levels of intensity enhancements, starting from a factor of 2 and higher, have also been proposed recently [27]. Thus, the current time dependent VCD

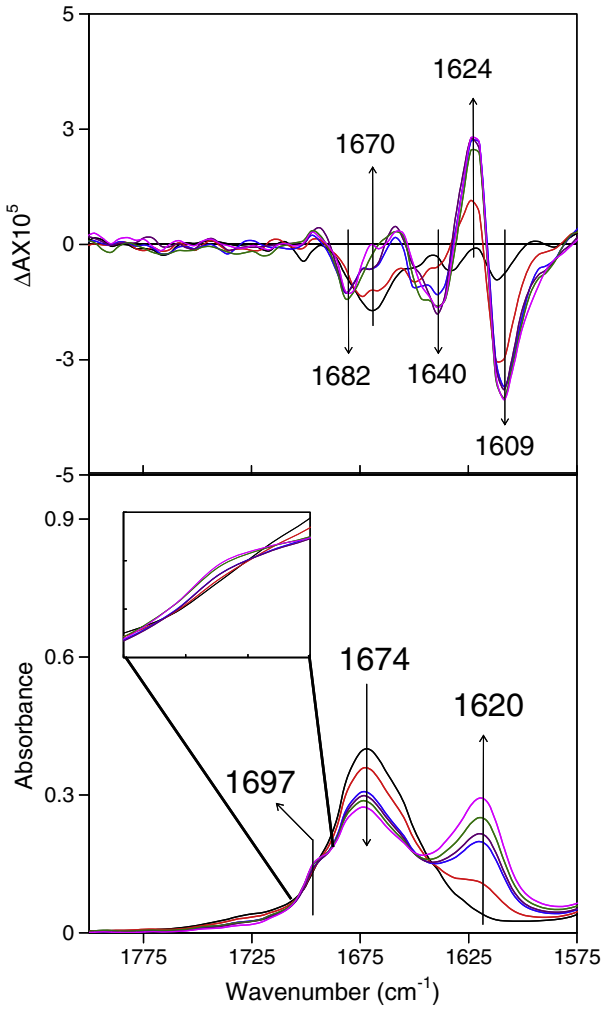

Fig. 1. Time dependent VCD (top) and IR absorption (bottom) spectra of VP1 peptide in TFE (concentration 9.2 mM, path length 100 μm, and resolution 8 cm^{-1}) measured at 0.5 h (black), 6.5 h (red), 23.5 h (blue), 29.5 h (violet), 50.5 h (green) and 96 h (pink). Inset: expanded region of 1697 cm^{-1} band.

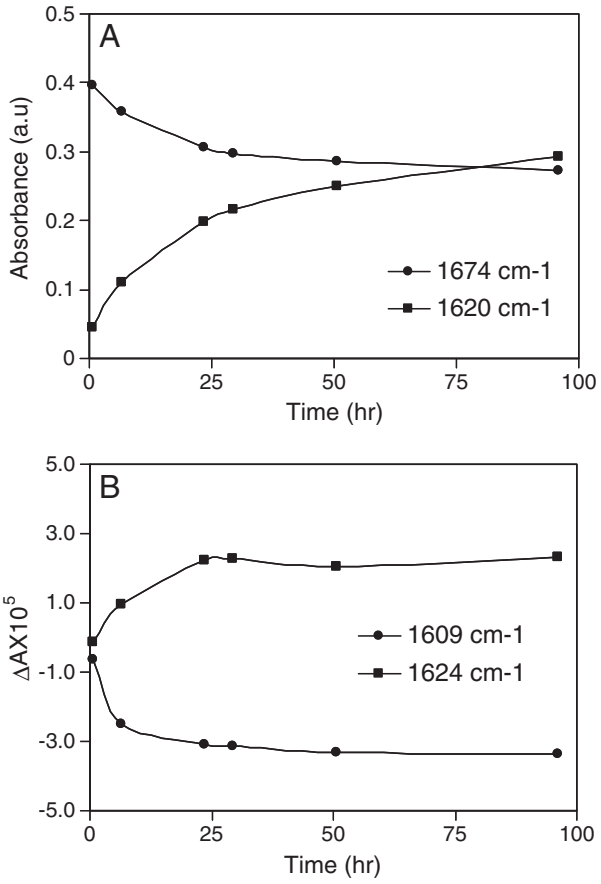

Fig. 2. Time dependent IR absorption (A) and VCD intensity (B) changes of VP1 peptide in TFE (concentration 9.2 mM, path length 100 μm, and resolution at 8 cm^{-1}).

and IR spectra suggest that VP1 in TFE solvent could be adopting fibril-like structure consisting of β-sheet aggregates. Further incubation of VP1 in TFE shows further increase in amide I band intensity at 1620 cm^{-1} and decrease in band intensity at 1674 cm^{-1}. The time dependent changes in IR and VCD intensities, associated with 1620 and 1674 cm^{-1} bands, suggested that the time span of conformational changes is longer than ~48 h (Fig. 2). It should also be noted that a new weak shoulder has emerged at 1697 cm^{-1} (Fig. 1, inset) in the IR spectrum, which is characteristic of peptides adopting anti-parallel β-sheet structure. The corresponding VCD spectrum did not show significant VCD intensity at 1697 cm^{-1}. Both IR and VCD spectra clearly reveal the unusual formation of β-sheet structure by VP1 in TFE. This result is unusual because TFE is often used to induce conformational transition to α-helix rather than β-sheet structure. The two possible visions here are that either TFE destabilizes the unstructured form of the peptide [28] or it directly associates with the folded peptide chain [29,30]. We postulate that, at higher concentration of the peptide used, the destabilization of unstructured form of VP1 peptide favors the hydrogen bonding interaction between the side chains in the adjacent strands (vide infra), thereby stabilizing intermolecular anti-parallel β-sheet structure. Although the time dependent VCD experiments were carried out at a higher concentration of VP1, a similar conformational transition might be expected at lower concentrations, but at slower rates than that at higher concentration. The concentration dependence of VP1 self-aggregation in TFE and other solvents will be investigated in the future.

Historically, ECD has been the primary spectroscopic technique for peptide and proteins structural studies [31]. The time dependent ECD spectra of VP1 in TFE are shown in Fig. 3. ECD spectra could not be measured below 200 nm due to excessive absorbance. ECD spectrum measured at 30 min (Fig. 3, black curve) shows a strong negative band

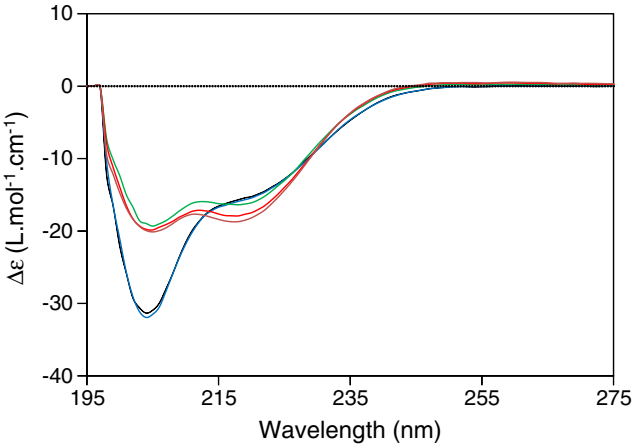

Fig. 3. Time dependent ECD spectra of VP1 in TFE measured at 0.5 h (black), 1 h (blue), 25 h (green), 49 h (red), and 73 h (brown) (concentration 9.2 mM and path length 0.1 mm).

at 204 nm and a weak shoulder band at 220 nm. The β-turn forming peptides with low β-sheet content also exhibit these characteristic negative bands at 204 nm and 220 nm [17], and β-turn structure is consistent with the VCD and IR spectra (vide supra). The ECD spectrum did not change after 1 h (Fig. 3, blue), but at 25 h, the ECD band intensity at 220 nm has increased, with slight blue shift to 218 nm, and the band intensity at 204 nm has decreased significantly (Fig. 3, green). This ECD spectrum at 25 h (negative double minima at 205 nm and 218 nm) appears similar to the ECD spectrum of a α-helix, because characteristic ECD double minima for α-helix would appear [31] at 208 nm and 222 nm. However, the current VCD and IR spectra indicated increased β-sheet structure under similar conditions. While the ECD spectrum with double minima may suggest a helical structure, in order for it to be consistent with IR and VCD spectral observations discussed above, we suspect that the ECD spectrum is dominated by the turn segments of the peptide. Similar ECD observations were reported for the peptides that have a tendency to adopt both β-sheet and β-turn structure in the same sequence [17,18].

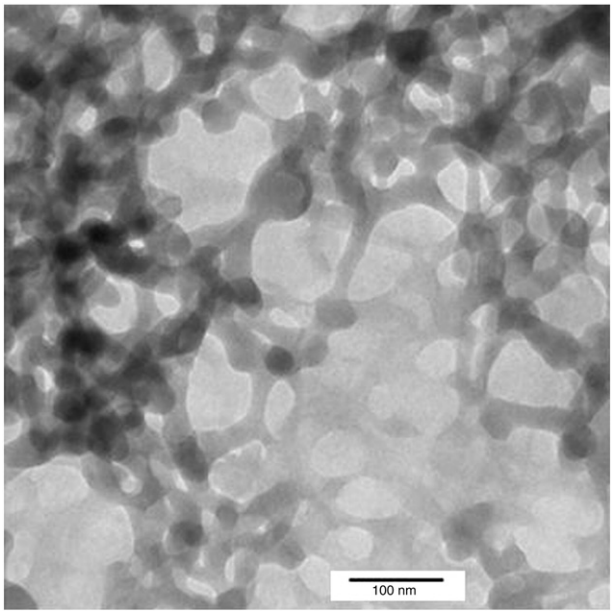

Fig. 4. Negatively stained transmission electron micrograph of VP1 aggregates harvested from VP1 peptide solution (9.2 mM) which was incubated ~96 h. The scale bar represents 100 nm.

Fig. 5. Proposed models for VP1 aggregates containing anti-parallel β-sheet structures. Amino acids are represented by a single letter code. Black and red letter color codes represent two individual strands of VP1 peptide arranged in anti-parallel direction.

Since the enhanced VCD intensities for VP1 suggested the formation of β-sheet aggregates in TFE, we sought to find if VP1 aggregates possess any amyloid-like fibrils. The transmission electron micrograph (TEM) image of VP1, obtained from the aged sample (96 h) that was used for VCD studies is shown in Fig. 4. The TEM images clearly revealed that VP1 aggregates adopt some kind of assemblages with different pore sizes. Although these assemblages are not similar to amyloid fibrils, where fibrils are long and un-branched with aperiodic twist, the TEM images showed fibril-like cross assemblages. This fibril type assemblage structure can be correlated with the enhanced VCD intensities observed for 1609 and 1624 cm^{-1} bands that are associated with the β-sheet structure of VP1.

Based on current spectroscopic and TEM results, we propose, as shown in Fig. 5, that VP1 adopts intermolecular anti-parallel β-sheet structure with a turn at the C-terminal. Since Gly is the most conformationally flexible of the amino acids, and is known to have high turn propensity [32–34], we believe that the C-terminal of VP1 adopts turn conformation while the N-terminal is involved in the β-sheet formation. The proposed arrangements (Fig. 5) also provide the possibility of hydrogen bonding interaction between the side chain arginine NH_2 and threonine OH groups in the adjacent strands which could further stabilize the anti-parallel β-sheet structure. Two relevant points might be noted here: (a). based on the studies on cyclic peptides [17] it was suggested that gly-gly segment at i + 1 and i + 2 positions is unlikely to favor a type II′ turn. Since the VP1 peptide is non-cyclic and side chain steric interactions also influence the beta turn stabilization, it may not be possible to generalize the previous observations to the present VP1 peptide and (b). theoretical VCD [35] predicted for different types of turns indicated that some discrimination between different types of turns may be discerned through VCD associated with amide II bands, but not amide I bands. VCD in the amide II region is overwhelmed, in the present case, by solvent interference. Therefore it is not possible to determine the type of turn in the VP1 structure from the current data.

The above mentioned conclusions about β-sheet aggregate formation by VP1, derived from time dependent solution phase studies in TFE, and the TEM images, differ from those deduced from IR investigations on film samples [12,13] and have several implications. (a). Brandenburg et al. [12] and Dennison et al. [13] suggested that the activity of m-calpain appeared to arise from the oblique oriented α-helical structure of VP1 fragment (domain V). The α-helical structure of VP1 was considered by Dennison et al. [13] to be favored by amphiphilic environment [13] of the interface, rather than the presence of anionic lipid. But the current observation of β-sheet structure for VP1 in amphiphilic TFE solvent warrants further investigations on the origin of m-calpain activity. Although the concentration used in the present studies is higher than biologically relevant concentrations, we suspect that similar structural transition may occur under biological conditions, but at a much slower pace. (b). The antimicrobial activity of peptides has been generally correlated to their α-helical structures. Dennison et al. suggested, based on their IR and monolayer data, that "VP1 has the ability to partition into bacterial membranes via the use of an amphiphilic oblique oriented α-helix". But some peptides with β-sheet structure are also known to have antimicrobial activities [36] and the current results suggest that VP1 may fall into this latter category. The TEM evidence (Fig. 4) for fibril formation in amphiphilic TFE solvent by VP1 peptide suggests that a possible role for peptide fibrils in antimicrobial activity may also need to be investigated. (c). The observation of peptide fibrils in TFE solvent is, in itself, unprecedented because TFE is generally considered to be a α-helix promoting solvent and we are not aware of fibril (β-sheet) formation by any peptide in TFE solvent itself.

4. Conclusions

The antimicrobial VP1 peptide, which is also an active domain of m-calpain, is found to form β-sheet aggregates in TFE solvent. TEM images indicate that these β-sheet aggregates possess fibril type assemblies. The observation of β-sheet fibril formation by a peptide in TFE solvent, in general, and by VP1, in particular, has far reaching implications in understanding the membrane interactions and antimicrobial activity.

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