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Fluoroalcohols-induced modulation and amyloid formation in conalbumin

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ABSTRACT

In this study we are reporting the conformational modulation as well as the plausible mechanism of structural perturbation in conalbumin (CA). The overall structure of CA is altered by the change in dielectric constant upon addition of fluoroalcohols (TFE and HFIP). We studied the formation of aggregates in CA at pH 7.0 by multiple structural probes in the presence of TFE and HFIP. The protein aggregates maximally in 15% (v/v) TFE and 3% (v/v) HFIP. ANS, ThT binding and transmission electron microscopy data suggest that the aggregates induced by TFE and HFIP have amyloid-like properties. Higher concentrations of TFE and HFIP causes increase in helical propensity. Far-UV circular dichroism, intrinsic fluorescence, ANS and ThT fluorescence data suggests that formation of a partially structured intermediate state precedes the onset of the aggregation process.

1. Introduction

Till now, about 20 diseases have been reported in humans caused by protein aggregation forming well defined cross β-sheet structure, without showing any sequence homology and structural similarity in the proteins. Previous reports indicate that amyloid formation is a shared property and not confined to only the disease related proteins [1–3]. Many diseases like Alzheimer's, Parkinson's, type II diabetes mellitus, and spongiform encephalopathies are direct result of normal cellular protein transformation into the amyloid fibrils [4–6]. The initiation of amyloid formation takes place from the native functional protein to non-native, non-functional, self assembled and insoluble aggregates through various conformational changes [7–9]. Several protein forms different aggregates with distinct morphologies showing structure like spherical oligomers, curly protofibrils, rod shape protofibrils and annular protofibrils [10,11]. In case of Aβ, AFM and solid state NMR studies prove the existence of amyloid fibrils of diverse morphologies because of internal structural distinction [12]. Addition of cosolvents, high temperature and extreme pH are the favourable conditions for the amyloid formation [13–15]. Besides, many more physical as well as chemical factors are also involved in amyloid formation such as UV-illumination in lysozyme [16], agitation in Aβ (1–42) [17], ultrasound sonication in Aβ (1–40) [18], vortex in insulin [19], heat in β-lactoglobulin [20], Ca^{2+} in Aβ (1–40) [21], Ca^{+2}–Zn^{+2} in bovine β-lactoglobulin [22], unsaturated fatty acid in superoxide dismutase 1 mutants [23], glycosaminoglycans in Aβ 40 and 42 [24], lysophosphatidic acid in β2-microglobulin [25]. Alcohols are reported to exert the stress on proteins and peptides by three ways: (a) destruction of the rigid native structure; (b) induction of α-helices; and (c) dissolution of peptide aggregates [26]. Sequence of protein also helps alcohols to attain native like β-hairpins by stabilizing the local interaction with β-strand in vicinity [27,28]. In comparison with other alcohols, fluorinated alcohol, i.e. 2,2,2-trifuoroethanol (TFE) and 1,1,1,3,3,3-hexafluoropropan-2-ol (HFIP) more strongly disrupts the secondary structure of proteins by lowering the solvent polarity extensively and α-helix induction by means of more intramolecular hydrogen binding. Due to presence of three extra fluorine atoms in HFIP, it is ∼34 times more acidic and much efficient than TFE [29]. The appropriate concentration of HFIP forms aggregates via significant coordination between hydrophobic and polar interaction (H-bonding) obligate for fibril formation with the suggestion that HFIP also interacts to the backbone of polypeptides [30,31]. The fluorinated alcohols TFE and HFIP are frequently used to observe the interaction of alcohols with proteins and peptides, especially HFIP by showing the dissolving property of the prions and Alzheimer's Aβ-peptide aggregates [32,33]. TFE concentration more than 50% induces very

Abbreviations: ANS, 1-anilino-8-napthalene sulfonate; CA, conalbumin; HFIP, 1,1,1,3,3,3-hexafluoro-propan-2-ol; TEM, transmission electron microscope; ThT, Thioflavin T; TFE, 2,2,2-trifuoroethanol.

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high α-helical structure meanwhile low concentration, about 20% of TFE gives aggregates containing β-sheets, recently it has been reported that low concentration (1–4%) of HFIP is found to be enough to induce amyloid fibril formation [34–36]. At physiological pH, 20–40% (v/v) TFE induces aggregation in keyhole limpet hemocyanin, a large sized multimeric protein [37] and increases α-helicity as well as aggregation in bovine serum albumin [38]. Small angle X-ray scattering reveals the presence of more dynamic but less stable micelle like alcoholic clusters at high concentration of TFE and HFIP aqueous solution [39,40]. Lengthened incubation period of Aβ with HFIP followed by drying is amyloidogenic, Aβ 40, Aβ 42–43 manages ordered conformation and form ring like structure through short fibrillar aggregation, fibrillation also occurred in Aβ 16–22 under same process [41]. For Aβ-(1–40) peptides, only 2% (v/v) HFIP induced aggregates in very short span of time (∼10 min) [42]. The study of combined effect of elevated temperature with TFE exhibited the amyloid fibrils formation in peptide LYS (11–36), a region of T4 consisting β-sheet and α-synuclein [43,44]. TFE at 25% (v/v) forms various morphological fibrils of insulin by altering the trimeric form in to monomer without entering in the soluble oligomerization state [45]. At neutral pH TFE and HFIP cause fibril formation in human β_2-microglobulin [46].

Conalbumin (CA) or ovotransferrin is a protein having antibacterial and antiviral properties with high nutritional and biological importance serving itself as a model for protein study. It is acute phase protein and its normal concentration increases significantly in diseased conditions. CA is identified as a biomarker for the chicken if it is infected by bacteria and viruses [47–49]. Recently the avian ovotransferrin under thiol-linked autocleavage has been reported with anticancer potential for human colon and breast [50]. Acid unfolding study of native CA clearly indicates that it attains various conformational states such as pre-molten globule at pH 4.0, molten globule at pH 3.0, and at pH 1.0 it is denatured. The α-helical and β-sheet contents of CA are 29% and 17% respectively [51]. Still the exact mechanism behind the effect of cosolvents is not much clear, and is explained on the consensus which is based on general observation. In this study we are investigating the effect of aqueous solution of fluorinated alcohols (TFE and HFIP) using combination of spectroscopic, dye binding and imaging methods for the determination of critical concentration which induces the amyloid formation and conformational states in CA.

2. Materials and methods

2.1. Materials

Iron-free CA from chicken egg white (C-0755), TFE (426237), HFIP (H-8508), Thioflavin T (T3516), ANS were purchased from Sigma Chemical Co. (St. Louis, Mo, USA). All other reagents and buffer compounds used were of analytical grade.

2.2. Methods

2.2.1. Determination of protein concentration

Protein stock was prepared in 20 mM sodium phosphate buffer pH 7.0, and its concentration was measured by using the extinction coefficient at $E^{1\%}_{280\,nm} = 12.0$ [52] on Perkin Elmer (Lambda 25) double beam spectrophotometer attached with Peltier temperature programmer (PTP-1).

2.2.2. Turbidity measurements

The turbidity of the protein sample with increased concentration of TFE and HFIP was monitored by measuring absorbance at 350 nm using a Perkin Elmer UV–vis spectrometer model lambda 25 in a cuvette of 1 cm path length. The measurements were carried out at 25 °C.

2.2.3. Rayleigh scattering measurements

Rayleigh scattering measurements were performed on Shimadzu spectrophotometer RF-5301 PC at 25 ± 0.1 °C with a 1 cm pathlength cell. Protein samples were excited at 350 nm and spectra were recorded in the range of 300–400 nm. Data were plotted at 350/350 nm.

2.2.4. Tryptophanyl fluorescence measurements

Fluorescence measurements were performed on a Shimadzu spectrophotometer RF-5301 PC. The fluorescence spectra were measured at 25 ± 0.1 °C with a 1 cm pathlength cell. The fluorescence was measured by exciting the protein at 295 nm and emission spectra were recorded in the range of 300–400 nm. Protein concentration was taken 5 μM.

2.2.5. Circular dichroic measurements

CD measurements were carried out with a Jasco spectropolarimeter (J-815) equipped with a Peltier-type temperature controller (PTC-424S/15). The instrument was calibrated with D-10-camphorsulphonic acid. Spectra were collected in a cell of 1 and 10 mm pathlength and protein concentrations used were 5 μM for far-UV CD. The scan speed was 100 nm/min and response time of 1 s for all of the measurements. Each spectrum was the average of 2 scans.

The raw CD data obtained in millidegrees were converted to mean residue ellipticity (MRE) in deg cm^2 dmol^{-1} which is defined as

$$\mathrm{MRE} = \frac{\Theta_{obs}(m\,deg)}{10 \times n \times C \times l} \quad (1)$$

where Θ_{obs} is the CD in millidegrees, n is the number of amino acid residues (686 − 1 = 685), l is the path length of the cell in cm and C is the molar concentration.

2.2.6. ThT binding assay

A stock solution of Thioflavin T (ThT) was prepared in double distilled water. The concentration of ThT was determined using extinction coefficient of (εM) = 36,000 M^{-1} cm^{-1} at 412 nm. Protein samples of 5 μM at different alcohols concentration (v/v) were incubated in 1:2 molar ratio of ThT for 30 min at 25 °C. The fluorescence of ThT was excited at 440 nm. The spectra were recorded from 400 nm to 600 nm.

2.2.7. ANS binding assay

A fresh stock solution of ANS was prepared in double distilled water, and its concentration was determined using molar extinction coefficient of (εM) = 5000 M^{-1} cm^{-1} at 350 nm. For ANS-binding experiments, the molar ratio of protein to ANS was 1:50. The excitation wavelength was set at 380 nm, and the emission spectra were taken in the range of 400–600 nm [53].

2.2.8. Transmission electron microscopy (TEM)

The morphology and size of CA aggregates with and without cosolvents was observed with a JEOL JEM-2100F transmission electron microscope (TEM) with an accelerating voltage of 200 kV. 10 μl of four-fold diluted samples were adsorbed onto copper 400 mesh grid, previously covered by carbon-coated film. After 2 min, excess fluid was drawn out using a paper filter, a drop of 1% uranyl acetate was added and after a few seconds and the samples were observed. A control of native protein solution was also placed on the grids. Images were viewed at 10,000×.