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Detection and analysis of protofibrils and fibrils of hemoglobin: Implications for the pathogenesis and cure of heme loss related maladies

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ABSTRACT

TFE induces structural alterations of proteins similar to the lipid environment of biological membranes, implicating these studies worthy of analyzing protein conformation in membranes such as red blood cells (RBCs). Heme loss occurs on rupturing of RBCs as found in diseases namely haemophilia, haemolytic anaemia, diabetes mellitus. TFE can be implied in discovering therapeutic targets, as it mimics the biological membrane environment. A global transition of hemoglobin (Hb) in presence of TFE was studied by using multi-methodological approach. The presence of partially folded state of Hb at 15% v/v TFE was confirmed by altered tryptophan environment, and retention of native-like secondary and tertiary structure. Molten globule state was observed at 20% v/v TFE as detected by increase tryptophan and high ANS fluorescence, slight alterations in Soret band relative to native. TFE on increasing concentration induced protofibrils at 25% v/v and fibrils at 45% v/v as depicted by altered tryptophan environment, heme loss, increase in non-native β-sheet secondary and tertiary structure, large hydrodynamic radii of heme-protein, high ANS, thioflavin T fluorescence and shift in Congo Red absorbance. Comet assay showed that protofibrils are cytotoxic to lymphocytes. SEM and XRD confirmed these aggregates to be fibrillar in nature.

Introduction

Protein aggregation is a generic property of polypeptide chains and does not depend on specific amino acid sequences [1]. These aggregates are cytotoxic in nature and result in considerable loss of cellular machinery or even cell death via apoptosis or necrosis. Protein aggregation can induce amyloid like aggregates in vitro after a long incubation period. Human diseases associated with amyloid fibrils include Alzheimer's, Parkinson's, Creutzfeldt–Jakob diseases and type II diabetes [2,3]. Previous studies reported that amyloid formation is the root cause of protein aggregation disorders [4]. There is an issue whether mature amyloid fibrils or quickly formed aggregates that lead their formation, is the main key players responsible for the diseases to occur [5,6]. Though toxic nature of mature fibrils is reported in some amyloid diseases however in most of the cases, rapidly formed aggregates are found to be the primary toxic species [5]. This toxic nature of protofibrils results from common structural attributes rather than from specific sequence of peptide backbone. It is believed that for a globular protein to aggregate, it must convert from its folded configuration into a structure that is appreciably unfolded relative to the native state [7]. In some cases, however, the initial aggregation pathway consists of protein molecules that are primarily in native or native-like states preceding the formation of protofibrils [8]. So a major objective always lies to characterize these partially folded native-like states that lead to protofibrils and fibrils formation. Organic solvents such as 2, 2, 2-trifluoroethahnol (TFE) are commonly used to investigate these aggregation prone intermediates [9]. It decreases hydrophobic interactions and increases polar interactions, as well as its action in forming amyloid fibrils appears to depend on how it affects the balance between these interactions for any particular protein [10]. Four human diseases namely haemophilia, haemolytic anaemia, diabetes mellitus etc. are known to occur due to loss of heme. Therefore, TFE can be considered in revealing therapeutic approaches to cure these blood related diseases. TFE provokes the conformational changes of proteins by mimicking the lipid milieu of cell membranes [11], hence these studies can be used in evaluating conformation of cell membrane proteins such as in RBCs[1]. Moreover, characterizing the TFE-induced intermediate states of the proteins may unravel the mechanism of their folding [12]. TFE induces protofibrils and fibrils in several mod-

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[1] *Abbreviations used:* ANS, 8-anilino-1-naphthalene-sulphonic acid; ATR-FTIR, attenuated total reflection fourier transform infra red spectroscopy; CD, circular dichroism; CR, congo red; DNA, deoxyribonucleic acid; DLS, dynamic Light Scattering; Hb, hemoglobin; MRE, mean residual ellipticity; RBCs, red blood cells; ROS, reactive oxygen species; SEM, scanning electron microscopy; ThT, thioflavin T; XRD, X-ray diffraction.

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el proteins [10,13]. Small globular proteins like Hb represent important model systems for investigating the general characteristics of the mechanism of amyloid formation as well as for understanding the impact of protein aggregation in vivo [14,15]. Hb is a well-known allosteric heme-protein composed of two α and two β subunits having 141 and 146 amino acids, respectively. It can transport molecular oxygen in red blood cells. It can be an important model system to study helix/sheet transitions, as it possesses 70% α-helix and 30% β-sheet. Besides, it also has bioinformatics applications such as evolving new proteins of heme family by using BLAST and CLUSTALW tools. The transition of α-helix to β-sheet can be a reliable strategy to discover new therapies to cure severe ailments.

We have demonstrated from our experiments that the partially folded and molten globule states of Hb are observed at low concentration of TFE i.e. 15% v/v[2] and 20%, respectively. Various proteins not linked to amyloid diseases tend to aggregate into fibrils in vitro studies that are similar to aggregates formed by disease-associated proteins [16]. In our studies, Hb is forced to form protofibrils and fibrils on increasing concentration of TFE at 25% and 45%, respectively that differ from each other in their external structures.

Materials and methods

Materials

Hb (bovine blood), fluorescent probes, viz., ANS, CR, ThT and 2, 2, 2-trifluoroethanol were purchased from Sigma (St. Louis). Sodium phosphate monobasic and sodium phosphate dibasic were purchased from SRL (Mumbai, India) to prepare buffer.

Hb was solubilised in 20 mM phosphate buffer of pH 7 to make a stock solution of 5 mg/ml and subsequently dialyzed in the same buffer to eradicate impurities. Protein concentration was found out employing molar extinction coefficient (at 405 nm) of 179 mM^{-1} per heme (Hb) on Hitachi single beam spectrophotometer of 1 cm path length [17].

Effect of TFE on Hb

Hb samples in increasing concentration, i.e. 0–50% of TFE were prepared in 20 mM phosphate buffer, pH 7 then incubated for 4 h at 37 °C before operating spectroscopic techniques. All the measurements were taken at room temperature. Three replicates for all the samples were analyzed for results.

Intrinsic fluorescence measurements

The fluorescence spectra were recorded on a Shimadzu RF-5301 spectrofluorometer (Tokyo, Japan) using 1 cm path length quartz cell. The excitation wavelength was 280 nm and the emission was recorded in the range of 300–400 nm [18]. Final concentration of Hb was 3 μM.

Acrylamide quenching

In the acrylamide-quenching experiments, aliquots of 5 M acrylamide stock solution were added to a protein stock solution (3 μM) to achieve the desired acrylamide concentration. Excitation was set at 295 nm in order to excite tryptophan fluorescence only, and the emission spectrum was recorded in the range 300–400 nm. The slit width was set at 10 nm for both excitation and emission. The decrease in fluorescence intensity at λ_{max} was analyzed according to the Stern–Volmer equation [19]:

$$F_0/F = 1 + K_{sv}[Q]$$

where F_0 and F are the fluorescence intensities at an appropriate wavelength in the absence and presence of a quencher (acrylamide), respectively, K_{sv} is the Stern–Volmer constant for the collisional quenching process, and [Q] is the concentration of the quencher.

ANS fluorescence measurements

ANS binding was detected by fluorescence emission spectra with excitation at 380 nm and emission was recorded from 400 nm to 600 nm [20]. ANS concentration was 100 M surplus of the protein concentration and protein concentration was around 3 μM.

Soret absorbance spectroscopy

The soret absorption of heme moeity was monitored by a Shimadzu UV-1700 Spectrophotometer, using a 1 cm path length cell. The final concentration of protein in each sample was 3 μM and observations were taken in the range of 350–700 nm [21].

CD spectroscopy

CD spectra of Hb samples were recorded on a J-810 Jasco CD spectropolarimeter calibrated with ammonium D-10-camphorsulfonate. Cells of path lengths 0.1 and 1 cm were used for scanning between 250–200 nm and 300–250 nm, respectively. Each spectrum was the average of four scans [22]. The final concentration of protein in each sample was 8 μM for far-UV CD studies and 15 μM for near-UV CD studies. The results were expressed as the mean residue ellipticity (MRE in ° cm^2 $dmol^{-1}$), which was defined as:

$$\mathrm{MRE} = \frac{\theta_{obs}(m^\circ)}{10 \times n \times \mathrm{Cp} \times l}$$

where θ_{obs}, was the observed ellipticity in degrees (°), n the number of residues, Cp the molar fraction, and l the length of light path in cm.

ATR-FTIR spectroscopy

FTIR spectra were recorded with Interspec 2020 FTIR spectrometer in deuterated buffer of pH 7. The samples for FTIR studies were prepared just by dissolving the protein and aggregates from Sigma. Solutions are placed between two CaF_2 windows with a spacer. Since the D_2O bend vibration absorbs strongly below 1500 cm^{-1} the path length must be kept to a minimum, therefore sample concentrations must be relatively high. Protein concentration was 80 μM. The scanning wave number was from 1000 to 4000 cm^{-1} [23]. The samples for FTIR studies were prepared just by dissolving the protein and aggregates from Sigma in deuterated buffer.

Rayleigh scattering measurements

Rayleigh scattering at 350 nm was performed on a Shimadzu RF-5301 spectrofluorometer (Tokyo, Japan) in a 1 cm path length quartz cell. Both excitation and emission wavelength were set at 350 nm. The final concentration of Hb was 3 μM.

DLS measurements

DLS measurements were carried out at 830 nm by using DynaPro-TC-04 dynamic light scattering equipment (Protein Solutions, Wyatt Technology, Santa Barbara, CA) equipped with a temperature-controlled micro sampler. Hb (30 μM) with TFE was incubated overnight at room temperature. The samples were spun at

[2] Unless otherwise indicated all TFE concentrations are present in v/v.