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Biophysical evaluation of amyloid fibril formation in bovine cytochrome *c* by sodium lauroyl sarcosinate (sarkosyl) in acidic conditions

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ABSTRACT

Surfactant-induced amyloid fibril formation in protein has a lot of association in the laboratory and industrial application. Sodium lauroyl sarcosinate (sarkosyl) is used to solubilize protein aggregates during protein purification, however, its amyloid fibril induction properties are very less understood. The aim of the current work is to examine the role of sarkosyl on the amyloid fibrillation of bovine cytochrome *c* protein at pH 3.5. We have used various spectroscopic (turbidity, RLS, ThT and far-UV CD) and microscopic (TEM) techniques to characterize the fibrillation of cytochrome *c* at low pH. We have demonstrated that sarkosyl in the concentrations range 0.5 to 10.0 mM is inducing large size aggregates in cytochrome *c* with fibrillar morphology. At concentrations of ≤0.5 mM, sarkosyl was not able to induce amyloid-like aggregates in cytochrome *c* at the same pH. The α-helical content of cytochrome *c* is transformed into β-sheet structure (single minima at 218 nm) in the presence of ≥0.5 mM of sarkosyl. The amyloid fibril formation potency is dependent on the concentrations of sarkosyl and solution pH. We believe the results of this work will not only help to explain the molecular mechanism of amyloid fibril formation, but also give us an insight on rationally designing potential anti-amyloidogenic molecules.

1. Introduction

Many neurodegenerative diseases occurred due to protein misfolding. Huntington's disease, prion disease, Alzheimer's disease, frontotemporal dementia and Parkinson's disease are some examples of neurodegenerative disorders [1,2]. The deleterious effects of protein aggregates mandate scientists worldwide to understand in depth the mechanism(s) of protein aggregation, its structure and functions [3]. Protein aggregates can form amorphous or amyloid-like aggregates depending upon surrounding conditions [4]. Many in vitro factors are known to be responsible for promoting amyloid fibril formation in various proteins [5,6]. Amyloid fibrils can be unbranched/filamentous and generally have cross-β sheet structures [7]. Partially or totally unfolded protein states under in vitro stress, or in some instances non-stressed condition, can start and propagate the aggregation process [8]. A major challenge in the field of amyloids is the identification of the mechanism of amyloid fibril formation and whether amyloid fibril formation starts with partially unfolded or totally unfolded states of proteins [9]. Available literature suggests that partially unfolded states have the tendency to form aggregates compared to natively folded states [10]. Proteins associated with neurodegenerative diseases have high tendency to form amyloid-like fibrils under in vivo conditions [11]. Moreover, several new findings indicate that amyloid fibril formation is also seen in proteins that are totally irrelevant to any neurodegenerative diseases [12,13]. The question of whether all or some proteins have the tendency to form amyloid-like aggregates is under investigation by many scientists. Recently, two dominant theories have come into existence. The first one proposes that it is a general property of all proteins and peptides to form amyloid under certain conditions that can be far from physiological conditions [14]. The second one suggest that only certain proteins have the tendency of forming amyloids, and are classified as 'amylome' [15]. As a result, we think it is of great importance to investigate the amyloid fibril formation mechanism of non-neurodegenerative disease proteins and use them as a surrogate for proteins involved in neurodegenerative disorders to further our understanding of these diseases and disorders.

Cytochrome *c* is a heme (iron) containing protein that is not involved in any types of amyloid-related disorders but has been used before by a researcher as a model protein to elucidate the molecular mechanism of aggregation process [16]. Cytochrome *c* is an essential protein, involved in various biological function such as electron transport chain and apoptosis [17]. Cytochrome *c* belongs to c-type cytochromes family and contains heme as a prosthetic group that is covalently attached to two cysteine residues (Cys14 and Cys17). Both cysteins are attached to each other through a disulfide bond. These cysteines are also located within a CXXCH motif [18]. Cytochrome *c* is composed of 104 amino acids and have four α-helices and very less β-sheet as a secondary structure and a molecular weight of 12 kDa [19]. To this

Abbreviations: Sarkosyl, sodium lauroyl sarcosinate; RLS, Rayleigh light scattering; T-ThT, thioflavin; TEM, transmission electron microscopy.

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date, studies are lacking a comprehensive understanding of the aggregation and amyloid fibrillation of cytochrome *c* protein. We think it is of great importance to delve into and analyze the structural transition in the events of fibrillation. Cytochrome *c* was taken as a model protein to study the mechanism of amyloid fibril formation by an anionic surfactant.

Surfactants are distinctive group of compounds with unique physicochemical properties manifested at interfacial surfaces as well as in bulk solutions with diverse applications in both fundamental as well as applied science fields [20–22]. Sodium lauroyl sarcosinate is an anionic surfactant has been primarily utilized to purify proteins. In addition to protein purification, protein-surfactant interaction is a well-studied area due to its wide applications in chemical, cosmetic and pharmaceutical industry [23]. In this work, we have analyzed the binding of sodium lauroyl sarcosinate (sarkosyl) to cytochrome *c* at low pH conditions at room temperature. Proteins in general become positively charged when subjected to pH environments below their isoelectric point [12]. Basic amino acids (the Lysines, Arginines, and Histidines) of cytochrome *c* (isoelectric point = 10.0) get protonated to maximum extent as we incubate them in low acidic environments resulting in a protein with an overall positive charge. Sarkosyl, on the other hand, is an anionic surfactant containing one hydrophilic carboxylate head and one-12-carbon hydrophobic chain. The negatively charged carboxylate head of sarkosyl on low pH conditions interacts electrostatically with the positively charged cytochrome *c*, neutralizaing most charges and inducing aggregation. Maximum protonation of cytochrome *c* is achieved through incubating it in such pH conditions. The critical micellar concentration (CMC) of sarkosyl was found to be around 15.0 mM. It has been reported that other anionic surfactant, such as sodium dodecyl sulphate (SDS), facilitate the amyloid fibril formation at ranges below CMC concentration in various proteins [24,25]. Low concentration of SDS promote aggregation of cationic peptide "novispirin" [26]. SDS has a high ability to stimulate elongation of various proteins including β_2-microglobulin, lysozyme and human complement receptor-1 protein [27,28]. To this date, sarkosyl was not reported to induce amyloid fibril formation for any protein at low pH conditions. Most of its applications resides in its use in the solubilization of bacterial expressed proteins [29]. Here, we explore the ability of sarkosyl in promoting amyloid-formation at low pH using cytochrome *c* as a model protein. We think the results of this work will help to further or understanding of the underlying mechanism of amyloid fibril formation.

2. Experimental

2.1. Materials

Bovine cytochrome *c* (Lot # SLBH3058V), sarkosyl surfactant and thioflavin-T were purchased from Sigma Chemical Co. (USA). Tris-HCl, hydrochloric acid (HCl) and other reagents were obtained from Sigma Chemical Co. (USA). Milli-Q water was used for solutions preparation. Milli-Q was acquired from the Millipore-RO plus 10 System (USA). All other chemicals used were purchased from analytical grade manufacturers.

2.2. Protein and surfactant stocks preparation

Bovine heart cytochrome *c* was dissolved in 20 mM Tris-HCl buffer pH 7.4. The dissolved cytochrome *c* solution was further dialyzed to remove all the stabilizers and other unwanted molecules. Bovine cytochrome *c* concentration was calculated by using an extinction coefficients of 1.06×10^5 M^{-1} cm^{-1} at 410 nm for holo cytochrome *c* on Agilent Technologies Carry 60 UV–Visible spectrophotometer [30]. The initial stock of sarkosyl was made in 20 mM Tris–HCl buffer pH 7.4. The concentration of ThT was calculated by using an extinction coefficient of 36,000 M^1 cm^1 at 412 nm [31]. All stock solutions and buffers were filtered through 0.45 μm Millipore syringe filter.

2.3. pH determination

pH of all solutions were measured by Mettler Toledo pH meter (Seven Easy S20–K) using Expert "Pro3 in 1" type electrode. The least count of the pH meter was 0.01 pH unit. All experiments were carried out in 20 mM sodium acetate, pH 3.5 buffer. Prior of the experiments, all buffers were filtered through 0.45 μm Millipore Millex-HV PVDF filter.

2.4. Turbidity measurements

The turbidity of cytochrome *c* samples alone and under different concentrations (0.0–10.0 mM) of sarkosyl at pH 3.5 were measured by measuring absorbance at 350 nm on Agilent Technologies Carry 60 double beam UV–Visible spectrophotometer. Cytochrome *c* concentration was fixed at 0.2 mg ml^{-1} for all samples. Turbidity measurements were carried out at room temperature using a 1 cm path length cuvette. All samples containing fixed concentrations of cytochrome *c* and different amounts of sarkosyl were incubated overnight before measurements. Turbidity of control samples containing different amounts (0.0–10.0 mM) of sarkosyl at pH 3.5 were also measured.

2.5. Rayleigh light scattering (RLS) measurements

The RLS studies were performed on Agilent Technologies Carry Eclipse fluorescence spectrophotometer at room temperature. Cytochrome *c* (0.2 mg ml^{-1}) with and without varying concentrations (0.0–10.0 mM) of sarkosyl were excited at 350 nm and emission was measured at 350 nm. The excitation and emission slits width were set 2.5 nm for all RLS measurements. Before RLS measurements, all samples were treated with sarkosyl and incubated for 12 h at room temperature. RLS measurements were also performed on control samples using different concentrations of sarkosyl at pH 3.5.

2.6. RLS kinetics

To examine the effects of sarkosyl on cytochrome *c* aggregation kinetics. RLS kinetics were performed on Agilent Technologies Carry Eclipse fluorescence spectrophotometer attached to a temperature Peltier and a stirrer at constant rpm. RLS kinetics reaction was carried in 20 mM sodium acetate buffer at pH 3.5 and the presence of two concentrations of sarkosyl (1.0 and 4.0 mM). The protein (cytochrome *c*) concentrations were set 0.2 mg ml^{-1} in all samples. The fluorescence intensity at 350 nm was measured as a function of time in seconds in one-centimeter path length cuvette. The excitation and emission wavelengths were fixed at 350 nm. The excitation and emission slits width were fixed at 5 nm for all samples.

2.7. ThT fluorescence spectroscopic assay

The ThT dye binding studies were performed on Agilent Technologies Carry Eclipse fluorescence spectrophotometer at room temperature. The ThT stock was made in distilled water and filtered via 0.2 μ filter. Every sample (treated with and without sarkosyl) was excited at 440 nm and emission was followed in the range of 450 to 600 nm. The excitation and emission slits width were set at 5 nm. The cytochrome *c* concentrations were fixed at 0.2 mg ml^{-1}, and sarkosyl concentrations were varied from 0 to 10.0 mM at pH 3.5. Before measurements, sarkosyl treated samples were incubated overnight and then mixed with 5.0 μM ThT and further incubated for 30 min in the dark.

2.8. Circular dichroism spectroscopy

Far-UV CD measurements were performed using Applied Photophysics, Chira-scan plus, UK spectropolarimeter at room