Amyloid formation and disaggregation of α-synuclein and its tandem repeat (α-TR)


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Abstract

The aggregation of α-synuclein is clearly related to the pathogenesis of Parkinson’s disease. Therefore, a comprehensive understanding of the mechanism of amyloid fibril formation is highly valuable for the development of clinical treatment and also of the diagnostic tools. Here, we have investigated the interaction of α-synuclein with ionic liquids by using several biochemical techniques including Thioflavin T assays and transmission electron microscopy (TEM). Our data shows a rapid formation of α-synuclein amyloid fibrils was stimulated by 1-butyl-3-methylimidazolium bis(trifluoromethylsulfonyl)imide [BIMbF3Im], and these fibrils could be disaggregated by polyphenols such as epigallocatechin gallate (EGCG) and baicalein. Furthermore, the effect of [BIMbF3Im] on the α-synuclein tandem repeat (α-TR) in the aggregation process was studied.

1. Introduction

Parkinson’s disease (PD) is characterized by degeneration of dopaminergic neurons in the substantia nigra and by deposits of cytoplasmic inclusions (Lewy bodies). α-Synuclein, which is involved in neuronal plasticity, stabilization of lipid membranes, neurotransmitter release, and protein networks, is the major building block of the pathological fibrillar deposits within Lewy bodies [1–3]. α-Synuclein is a small (14 kDa) low-structured protein, possessing several imperfect KTKEGV repeats in the N-terminal portion (a.a. 1–95), and an highly acidic C-terminal portion (a.a. 96–140). Specifically, the central region (a.a. 61–95) is the core region that is mainly responsible for fibril formation. For example, the alterations of several hydrophobic amino acids in this region can significantly change the polymerization into amyloid [4–6]. Using several biophysical techniques, α-synuclein was shown to undergo conformational changes from its low-structured native state to the highly crossed β sheet-rich conformation of amyloid fibrils [7–9]. Additionally, the conversion of soluble α-synuclein into insoluble aggregates is believed to be a key event in the pathogenesis of PD and related diseases. Interestingly, in contrast to α-synuclein, β- and γ-synuclein, which are not related to neurodegenerative diseases, cannot readily form amyloid fibrils in vitro [6,8]. Therefore, a comprehensive understanding of the mechanism of amyloid fibril formation at the molecular level is very important to elucidate the pathogenesis of PD and for the development of clinical treatment and diagnostic tools.

Although increasing evidences suggest that a prefibrillar intermediate, not amyloid fibril, is mainly responsible for neuronal cell death, the nature of the neurotoxic species and its mode of action still remain largely unknown. To date, therapeutic strategies are mainly focused on the inhibition of the amyloid formation process, as early as its initial stage, by using organic molecules, heat shock proteins and antibodies [10–12]. Here, we suggest alternative strategies which avoid cytotoxic intermediates by using ionic liquids (ILs) that could stabilize the formation of amyloid fibrils [13,14]. As a first step, we have investigated the interaction of α-synuclein with ionic liquids, using several biochemical techniques, including Thioflavin T assays and transmission electron microscopy (TEM). We showed a rapid formation of α-synuclein amyloid fibrils was stimulated by 1-butyl-3-methylimidazolium bis(trifluoromethylsulfonyl)imide [BIMbF3Im]. Furthermore, the effect of [BIMbF3Im] on the α-synuclein tandem repeat (α-TR) in the aggregation process was studied. Our data shows that these fibrils could be disaggregated by the polyphenols such as epigallocatechin gallate (EGCG) and baicalein. Therefore, use of ionic liquids such as [BIMbF3Im] could represent an alternative strategy for attacking neurodegenerative diseases. Considering the enormous chemical variability of ionic liquids, this study will encourage further investigations on the effects of ionic liquids on the neurodegenerative diseases such as Parkinson’s disease (PD).
2. Materials and methods

2.1. Expression and purification of α-synuclein and its related proteins

Wild type α-, β-, and γ-synuclein were expressed and purified as previously described [7]. γ-synuclein plasmid was a kind gift from Dr. J. Liu (Department of Veterans Affairs, Palo Alto Health Care System, USA). For α-synuclein tandem repeat (α-TR), codon optimization of the complete ORF (840 bp) of the gene sequence was performed, which was then commercially synthesized (Gen-Script Corp., Piscataway, NJ). The synthesized α-TR gene was cloned into pET-21a using NdeI and HindIII restriction sites and it was confirmed by enzymatic digestion and DNA sequencing. Subsequent expression and purification procedures were the same as used for α-synuclein. Briefly, each plasmid was transformed into BL21 competent cells, plated onto LB-agar plates (supplemented with 100 µg/ml ampicillin), and grown overnight at 37 °C. Single colonies of BL21 (DE3) were grown, and lyophilized and stored at −80 °C until further use. The protein purities were judged to be >95% from SDS–PAGE and protein concentration was determined as described [13,15].

2.2. Fibrillization studies

The time course fibrillizations of α-synuclein and its tandem repeat (α-TR) was measured by the ThT fluorescence assay [13]. Purified proteins were incubated at room temperature in 1.5-ml sterile polypropylene tubes with continuous shaking in the absence and presence of ionic liquids at a ratio (v/v) of 1%, 2%, or 5%. For quantitative assessment of fibrillation, aliquots (10 µl) of the α-syn incubations (final protein concentration of 7 µM) were removed at various time points and added to 100 µl of 100 µM ThT and 100 µl of 10 mM Tris buffer, pH 8.5. Fluorescence measurements were conducted with a spectrophotometer (FP-6200, Jasco) at 25 °C and the fluorescence of ThT was excited at 460 nm with a slit width of 5 nm, and the emission was measured at 490 nm with a slit width of 5 nm using a FP-6200 spectrophotometer (Jasco, Japan).

2.3. Size exclusion chromatography (SEC)

The hydrodynamic sizes of α-synuclein and its tandem repeat (α-TR) were determined on an HPLC 1100 system (Agilent, USA) equipped with TSK-G4000PW column (TOSOH, Japan). A standard curve was calibrated by using well-known protein markers [16].

2.4. Circular dichroism spectroscopy

Circular dichroism spectra were obtained by using a Jasco J-715 spectropolarimeter (Jasco Corp., Tokyo, Japan.) The Far UV-CD spectra (190–250 nm) were collected at room temperature in a 1-mm path length quartz cuvette containing 0.2 mg/ml of α-synuclein and its tandem repeat (α-TR) in PBS or sodium phosphate buffer [16]. The backgrounds of buffers were subtracted for all samples, and the data were converted into mean residue ellipticity.

2.5. Transmission electron microscopy (TEM)

Transmission electron microscopy (TEM) analysis was performed using a Hitachi H–8000 TEM. Samples of each protein in [BIMBF4][m] (5%, v/v) were prepared by shaking at room temperature for 7 days. 10 µl aliquots of diluted samples were deposited on carbon-coated copper grids and stained with 2% (w/v) uranyl acetate solution for 1 min. Samples were air-dried and grids were examined in JEM1010 (JEOL, Japan) TEM at 40 kV, and digital images were acquired with a charge-coupled device camera [13].

3. Results

Abnormal aggregation of α-synuclein is clearly related to the etiology of Parkinson’s diseases (PD) and current studies are mainly focused on the suppression or inhibition of the formation of cytotoxic intermediates [2,5,8]. In a previous report, we have shown that ionic liquids could assist and stabilize the fibril formation process of α-synuclein [13]. Here, we examined the effects of two representative ionic liquids, 1-butyl-3-methylimidazolium bis(trifluoromethylsulfonyl)imide [BIMBF4][m] and 1-butyl-3-methylimidazolium tetrafluoroborate [BIMBF4], on the fibril formation of α-synuclein and its tandem repeat (α-TR). The chemical structures of [BIMBF4][m] and [BIMBF4], which have the same cationic components but different anionic parts, are shown in Fig 1A. In addition, EGCG and baicalein, which were reported to inhibit the fibril formation of α-synuclein [17,18], were used in this study (Fig. 1B). Using a thioflavin T (ThT) fluorescence assay, effects of ionic liquids on α-synuclein fibril formation were analyzed. In general, α-synuclein could form amyloid fibrils at room temperature only after several weeks [13,19]. Fig. 1C and D showed the characteristic ThT fluorescence curve of α-synuclein with [BIMBF4][m] and [BIMBF4], which is reminiscent of a nucleation-dependent polymerization model [20]. Although the ThT fluorescence of α-synuclein alone was almost unchanged during the incubation, addition of [BIMBF4][m] to the reaction mixture significantly increased the intensity in a concentration-dependent manner. Higher concentrations of [BIMBF4][m] resulted in the larger ThT values. Maximum signal intensity occurred at ~4 days even at a concentration of 1% BIMBF4[m], suggesting that amyloid fibril formation was saturated. In contrast, [BIMBF4] was found to have almost no promoting effect on α-synuclein fibrillation at 1% concentration. Moreover, the addition of 2% or 5% BIMBF4 did not change the fibril formation of α-synuclein as characterized by the similar ThT fluorescence intensity vs. its control value (Fig. 1D). These results suggest that α-synuclein fibril formation is differentially affected by the characteristics of ionic liquids such as concentration or anionic components.

The morphologies of α-synuclein and its related proteins in the presence of 5% [BIMBF4][m] were investigated using transmission electron microscopy (TEM). Numerous clusters of fibrils were observed with the α-synuclein at pH 7.5 or pH 10.0 (Fig. 2A and B). At pH 4.5, small and short fibrils were shown (Fig. 2C), while the fibrils formed at pH 7.5 or 10.0 were very similar in size and shape. Interestingly, no amyloid fibrils but amorphous aggregates were observed for β-, and γ-synuclein (Fig. 2D and E). Accordingly, ThT fluorescence did not change significantly during the incubation of β- and γ-synuclein with [BIMBF4][m] (data not shown). Actually, we tried to form amyloid fibrils for β- and γ-synuclein at several pH conditions, and using different ionic liquids, but no fibrils were detected (data not shown). A series of peptides in α-synuclein (pep 20–30, pep 30–41, pep 71–82, pep 97–108, pep 109–124, pep 122–132, and pep 122–140) were tested under the same conditions, but did not show any abilities to polymerize into amyloid fibrils, judging from ThT fluorescence or TEM analysis, even after three weeks (Fig. 2F, data not shown).

Next, we examined whether the amyloid fibrils obtained with [BIMBF4][m] could be disaggregated in vitro. Based on the previous reports concerning the disaggregating activities of polyphenols on the preformed fibrils of Aβ or α-synuclein [17,18,21], we investigated the effects of epigallocatechin gallate (EGCG) and baicalein for the disaggregation of α-synuclein fibrils. Various concentrations of EGCG and baicalein were added to the α-synuclein fibrils prepared by [BIMBF4][m], and the disaggregation kinetics was investigated. Fig. 3A and 3B show the changes in relative ThT fluorescence intensity during the fibril disaggregation. For EGCG and
Fig. 1. Compounds used in this study and the ThT assay of α-synuclein amyloid formation. Chemical structures of ionic liquids and polyphenols used in this study are shown (A, B). (A) Structure of 1-butyl-3-methylimidazolium bis(trifluoromethylsulfonyl)imide [BIMbF3Im] (top) and 1-butyl-3-methylimidazolium tetrafluoroborate [BIMBF4] (bottom). (B) Structure of epigallocatechin gallate (EGCG) and baicalein. Kinetics of α-synuclein fibril formation with [BIMbF3Im] (C) and [BIMBF4] (D) were monitored by ThT fluorescence assay. Ionic liquids with different anionic parts were added to α-synuclein (14 μM in 20 mM Tris–HCl, pH 8.0), and the final solutions were continually shaking at room temperature. ThT fluorescence of α-synuclein incubated with 0%, 1%, 2%, and 5% [BIMbF3Im] or [BIMBF4] at room temperature are shown and data are presented from at least three sets of independent experiments.

Fig. 2. The effect of [BIMbF3Im] on the morphology of α-synuclein and its related proteins monitored by transmission electron micrographs (TEM). Negative-stain TEM images were obtained after incubation with 5% [BIMbF3Im] for 6 days. α-synuclein (A, B, C) at pH 7.5 (A), 10.0 (B), 4.5 (C), β-synuclein at pH 7.5 (D), γ-synuclein at pH 7.5 (E), and pep71–82 at pH 7.5 (F) are shown. Other peptides did not show any ThT enhancements and fibril-like morphologies in ThT fluorescence or TEM images.
baicalein, time- and dose-dependent decreases in ThT signal were observed, while the absence of these molecules resulted in almost no significant loss of ThT signal. Specifically, relative ThT fluorescence values of fibrils incubated with 20 μM EGCG showed a 55% reduction (loss of fibrils) compared to the control sample (Fig. 3A). SDS-PAGE of supernatant after incubating α-synuclein fibrils in the presence of EGCG shows a time-dependent increased monomer fraction, implying the disaggregation of α-synuclein fibrils. Similarly, baicalein could disaggregate α-synuclein fibrils in a dose-dependent manner (Fig. 3B).

It has been reported that dimeric forms are key components in the fibril formation of α-synuclein as well as Aβ [22,23]. To get a full understanding of a dimer, we made a α-synuclein tandem repeat (α-TR) and investigated the effects of ionic liquids on this dimer. In size exclusion chromatography, α-synuclein and α-TR eluted with molecular weights of ~50 kDa and ~70 kDa, respectively (Fig. 4A). Because of their low structured conformations, all synucleins eluted with an apparent molecular weight larger than what would be expected [7,15]. Accordingly, the far-UV CD spectrum of α-TR showed a negative peak at ~200 nm (Fig. 4B). This indicates that α-TR has an elongated conformation, which is very similar to that of α-synuclein. Similarly, in the ThT assay and TEM analysis, [BIMbF3Im] promoted the fibril formation of α-TR, whereas [BIMBF4] did not have any effects on this process at pH 7.5 or 4.5 (Fig. 4C, 4D).

4. Discussion

Amyloid deposits of α-synuclein are clearly linked to the pathogenesis of Parkinson’s disease (PD), but little information is known concerning the nucleation steps and cytotoxic forms of α-synuclein. Recent studies suggest that α-synuclein protofibrillar intermediates, not amyloid fibrils, are the pathogenic species in neuronal cell death and neurodegeneration [23,24]. Therefore, inhibition or suppression of cytotoxic intermediates, as early as its nucleation steps, is highly valuable for the development of therapeutic compounds and diagnostic tools. Here, we showed that ionic liquids such as [BIMBF4]Im promoted the fibril formation of α-synuclein, suggesting that ionic liquids can allow water molecules to be excluded from the hydrophobic regions [25]. Since α-synuclein is intrinsically unstructured, [BIMBF4]Im could facilitate the association of α-synuclein’s hydrophobic surfaces (for example, a.a. 71–82) into fibrils. The observation that only α-synuclein, not β- and γ-synuclein, could form amyloid fibrils may reveal the specific interactions between α-synuclein and [BIMbF3Im]. Furthermore, it will be helpful to employ various functionalized ionic liquids to accelerate the fibril formation to inhibit the production of neurotoxic intermediates. Therefore, ionic liquids can be used as a novel class of inhibitors aimed at countering the production of cytotoxic α-synuclein intermediates through the decrease of lag time in fibril formation.

Previous studies have demonstrated that polyphenols such as EGCG and baicalein are potent inhibitors of α-synuclein fibril formation [17,26,27]. In a recent report, EGCG and baicalein could remodel amyloid fibrils of α-synuclein and Aβ into nontoxic amorphous aggregates [17]. EGCG and baicalein can specifically bind to α-synuclein fibrils and facilitate the fibril disassembly kinetics through hydrophobic π-π stacking interactions toward α-synuclein fibrils. Our studies indicate that EGCG and baicalein recognize and disaggregate preformed α-synuclein fibrils prepared by the incubation with [BIMBF4]Im and subsequently destabilize of α-synuclein fibrils into soluble amorphous aggregates. This suggests that treatment with ionic liquids did not change cross β-sheet rich fibril structures compared to non-treated samples, although they could greatly accelerate the fibril formation. Accumulation of dimers is believed to a decisive step in the aggregation kinetics of α-synuclein, and dimer formation is directly correlated with the appearance of the amyloid fibrils [22,28]. Stable disulfide-linked dimers with Y39C and Y125C or dityrosine cross-linked dimers are shown to enhance protein aggregation and cellular toxicity during oxidative stress. Furthermore, soluble amyloid-β dimers are the dominant contributors to Alzheimer’s disease pathophysiology [23]. Therefore, the tandem repeat of α-synuclein (α-TR) could serve as a molecular model for the investigating the mechanism of aggregation processes in α-synuclein aggregation.

In summary, we have found that α-synuclein fibril formation can be greatly controlled by the addition of [BIMBF4]Im. Specifically, in the presence of [BIMBF4]Im, the time for α-synuclein fibril formation was dramatically shorter than that of the untreated samples. Although this study has been focused on the role of [BIMBF4]Im as an accelerator toward α-synuclein fibril formation, other ionic liquids are also likely to affect the fibril formation of α-synuclein. The ability of [BIMBF4]Im to stimulate fibril formation from α-synuclein will shorten the timeframe required for screen for possible therapeutic molecules. In addition, it is interesting to note that imidazolium-based ionic liquids could be used as anti-cancer...
agents [29]. Thus, this system will be of importance for finding compounds that could prevent the formation of cytotoxic intermediates of α-synuclein.

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References


