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Self-oligomerization and protein aggregation of α-synuclein in the presence of Coomassie Brilliant Blue

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α-Synuclein has been implicated in various neurodegenerative disorders, including Parkinson's and Alzheimer's diseases, by its participation in abnormal protein depositions. As the protein has been suggested to play a significant role in the formation of the deposits which might be responsible for neurodegeneration, there is a strong demand to screen for α-synuclein-interactive small molecules. In this report, Coomassie Brilliant Blue (CBB) interaction of α-synuclein has been investigated with respect to induction of protein self-oligomerization in the presence of the chemical coupling reagent *N*-(ethoxycarbonyl)-2-ethoxy-1,2-dihydroquinoline. Both CBB-G and CBB-R, which differ by only two methyl groups, induced the self-oligomerization of α-synuclein in a biphasic manner with optimal dye concentrations of 250 μM and 150 μM, respectively. The protein aggregates of α-synuclein induced by the dyes in the absence of the coupling reagent were analysed by electron microscopy. Whereas CBB-G induced formation of protein aggregates with a worm-like structure, CBB-R induced clear fibrilization of α-synuclein on a background of granular structures. CBB-R interacted with α-synuclein approximately twice as effectively as CBB-G (dissociation constants 0.63 μM and 1.37 μM, respectively). These dye interactions were independent from the acidic C-terminus of α-synuclein, which was reminiscent of the Aβ25–35 interaction of α-synuclein. However, the metal-catalysed oxidative self-oligomerization of α-synuclein in the presence of Cu^{2+}/H_2O_2, which was augmented synergistically by Aβ25–35, was not affected by the dyes. This indicates that the dye binding site is also distinctive from the Aβ25–35 interaction site on α-synuclein. These biochemically specific interactions between α-synuclein and the dyes indicate that α-synuclein-interactive small molecules could provide a tool with which to approach development of diagnostic, preventive, or therapeutic strategies for various α-synuclein-related neurodegenerative disorders.

Keywords: Coomassie Brilliant Blue; neurodegenerative disease; protein aggregation; self-oligomerization; α-synuclein.

Parkinson's disease (PD) is a debilitating brain disorder which disrupts the normal motor activity of the patient, thus causing resting tremor, bradykinesia, muscle rigidity, and postural instability [1]. These behavioural deficits have been attributed to selective degeneration of dopaminergic neurons in the substantia nigra pars compacta [1,2]. Inside the degenerating neurons, eosinophilic protein deposits called Lewy bodies have been found as a pathological hallmark of the disease [3]. Although any causative role of Lewy bodies in neurodegeneration remains undetermined, the cytoplasmic inclusion has been recognized as a means of accessing PD in molecular terms. The major constituent of the inclusion from sporadic cases of PD was identified as α-synuclein; also identified were ubiquitin and neurofilaments [4]. In addition, α-synuclein was the first protein to be linked genetically to a few pedigrees of autosomal dominant familial PD. Two independent point mutations of the gene were identified that resulted in the substitution of alanine at position 30 with proline (Ala30Pro) or alanine at position 53 with threonine (Ala53Thr) [5,6]. α-Synuclein has been implicated in other neurodegenerative disorders too, such as Alzheimer's disease (AD), Dementia with Lewy bodies (DLB), and multiple system atrophy (MSA) by participating in abnormal protein depositions [7,8–11]. In fact, a hydrophobic 35-amino acid peptide fragment derived from α-synuclein was isolated from the senile plaque of an AD patient's brain as the second major constituent after the primary amyloid β/A4 protein (Aβ) [12]; this is why α-synuclein was once named NACP, the precursor protein of the non-Aβ component of AD amyloid [13]. Therefore, α-synuclein has been suspected of having the ability to form protein aggregates which might play a pathologically significant role during neurodegeneration. Several *in vitro* studies have shown that the protein can undergo self-aggregation and the aggregates formed developed into fibrils like those observed in the Lewy bodies [14–16]. Although it was questioned whether the fibrous form of the aggregate was responsible for cytotoxicity or if it was just a consequence of the detoxification mechanism

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Abbreviations: Aβ, amyloid β/A4 protein; AD, Alzheimer's disease; BCA, bicinchoninic acid; CBB-G, Coomassie Brilliant Blue G; CBB-R, Coomassie Brilliant Blue R-250; DLB, Dementia with Lewy bodies; EEDQ, *N*-(ethoxycarbonyl)-2-ethoxy-1,2-dihydroquinoline; MSA, multiple system atrophy; NAC, non-Aβ component of AD amyloid; NACP, precursor protein of NAC; PD, Parkinson's disease.

**Note*: D. Lee and E.-K. Lee contributed equally to this work.

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of the toxic oligomeric intermediate produced from soluble monomeric α-synuclein [17,18], transgenic mice and *Drosophila* were developed by overexpressing α-synuclein *in vivo* [19,20]. These two animal models of PD are therefore expected to provide valuable information about the mechanism of pathogenesis and also to contribute to the eventual development of a therapeutic drug for the disease. In this respect, there is a strong demand for screening for α-synuclein-interactive small molecules as possible drug candidates regardless of whether or not they induce the protein to form aggregates.

We have demonstrated previously that α-synuclein can undergo self-oligomerization via specific molecular interactions in the presence of various effectors; for example, Aβ25–35, a peptide fragment derived from Aβ, metals such as copper and zinc, and eosin dye [21–23]. Because it is known that the native form of α-synuclein is unfolded [24,25], this biochemically specific disorder-to-order transition of α-synuclein leading to protein self-oligomerization was analysed with chemical coupling reagents such as *N*-(ethoxycarbonyl)-2-ethoxy-1,2-dihydroquinoline (EEDQ) by observing discrete ladder formation on silver-stained 10–20% Tricine/SDS/PAGE [26]. Even in the absence of the coupling reagent, these α-synuclein self-oligomers were also observed *in vitro* under the more physiologically relevant conditions of metal-catalysed oxidation in the presence of copper and hydrogen peroxide [27]. In this report, the interaction of Coomassie Brilliant Blue (CBB) with α-synuclein has been examined by utilizing the phenomenon of self-oligomerization in the presence of EEDQ for the purpose of screening α-synuclein-interactive molecules.

EXPERIMENTAL PROCEDURES

Materials

Recombinant α-synuclein gene cloned in pRK172 (from R. Jakes, MRC-LMB Cambridge) was overexpressed in *Escherichia coli* BL21(DE3) and the protein was purified according to the procedure described previously [28]. The purified α-synuclein was quantified with a protein assay [29] using bicinchoninic acid (BCA) and stored at a concentration of 1.6 mg·mL^{-1} in 20 mM Mes buffer, pH 6.5, at −30 °C. The α-syn97, a C-terminally truncated α-synuclein containing residues 1–97, was prepared via an endoproteinase Asp-N digestion according to the method reported previously [22]. The MicroBCA assay kit and BSA were from Pierce. Protein quantification by the method of Bradford [30] was performed using an assay kit from Bio-Rad. CBB-G (dye content: 92%), CBB-R-250 (dye content: 95%), coupling reagent of EEDQ, endoproteinase Asp-N from *Pseudomonas fragi*, non-Aβ component of AD amyloid (NAC), SDS, silver nitrate, sodium carbonate, sodium citrate, and Sephadex G-25 (coarse) were from Sigma. Acetonitrile, ethanol, and methanol were from Fisher Scientific. Glycerol and formaldehyde were from Junsei Chemical Co. Glutaraldehyde (50% in water) and dimethylsulfoxide were from Fluka, Japan. Uranyl acetate was from Electron Microscopy Science (Fort Washington, PA, USA). The precast gels for 10–20% Tricine/SDS/PAGE were from NOVEX (San Diego, CA, USA).

Self-oligomerization of α-synuclein in the presence of CBB-G and CBB-R

Self-oligomerization of α-synuclein was examined in the presence of CBB-G and CBB-R by using the chemical coupling reagent EEDQ. The oligomers were visualized as a discrete ladder on silver-stained 10–20% Tricine/SDS/PAGE. α-Synuclein (100 pmol) was preincubated with the dyes at various molar ratios in 20 mM Mes, pH 6.5, for 30 min at 37 °C. Following the addition of 0.3 mM EEDQ prepared in dimethylsulfoxide, the reactions were incubated for a further 1 h at 37 °C. The chemical crosslinking reaction was stopped by mixing with a Tricine/SDS/PAGE sample buffer consisting of 8% (w/v) SDS, 24% (v/v) glycerol, 0.015% CBB-G, and 0.005% phenol red in 0.9 M Tris/HCl, pH 8.45 at a 1 : 1 (v/v) ratio. The samples were boiled for 5 min and analysed by precast 10–20% Tricine/SDS/PAGE according to the manufacturer's instructions. Ladder formation was visualized with a silver staining procedure by Morrisey [31].

Dissociation constants between α-synuclein and CBB

α-Synuclein (1 nmol) was incubated with various concentrations of CBB-G or CBB-R in 20 mM Mes, pH 6.5, for 40 min at 37 °C in a total volume of 200 μL. Protein-bound dye was separated from unbound dye by using the centrifuge column step with Sephadex G-25 (coarse) accoding to Penefsky [32]. Pre-swollen gel in Mes buffer was packed into a 3-mL syringe and dehydrated by centrifugation at 500 ***g*** for 1 min. The sample mixture was transferred to the top of the compressed gel and centrifuged for another 1.5 min at the same speed. The effluent was collected and its protein and dye contents were determined. The protein was quantified with MicroBCA assay and the amounts of CBB-G and CBB-R were

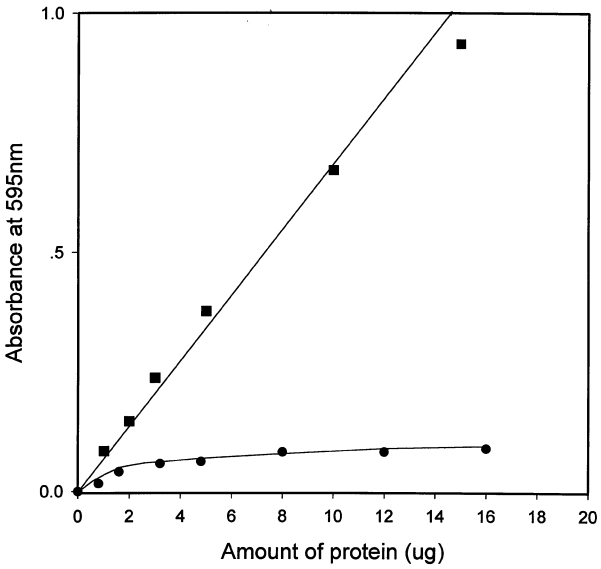

Fig. 1. Dye binding responses of α-synuclein and BSA. Various amounts of either α-synuclein (●) or BSA (■) were monitored by observing the absorption maximum of protein-bound CBB-G at 595 nm according to the method of Bradford [30].

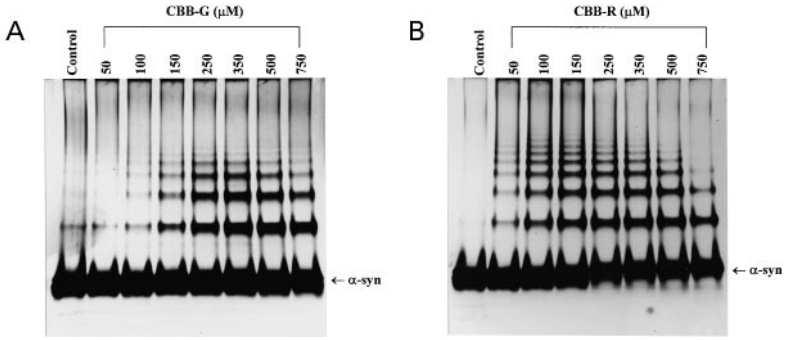

Fig. 2. Self-oligomerization of α-synuclein in the presence of CBB-G and CBB-R. The self-oligomers of α-synuclein in the presence of either CBB-G (A) or CBB-R (B) were obtained with 0.3 mM EEDQ and visualized by silver-stained 10–20% Tricine/SDS/PAGE as described. The dye concentrations used to induce the self-oligomerization are shown on the top of each panel. The lanes labelled 'Control' show the results of crosslinking reactions carried out with α-synuclein and 0.3 mM EEDQ in the absence of the dyes.

analysed spectrophotometrically at 580 and 560 nm, respectively. After correcting the amounts of eluted dye according to a calibration curve obtained by processing various concentrations of the dyes in the absence of α-synuclein, the amounts of protein-bound dye were plotted against the initially treated total dye concentrations. Dissociation constants between α-synuclein and the dyes were obtained from double reciprocal plots of the saturation curves.

Localization of the dye binding sites on α-synuclein

To elucidate C-terminal dependence of the dye-induced self-oligomerization, the α-syn97 was subjected to oligomerization by preincubating the peptide (100 pmol) with either CBB-G (2 nmol) or CBB-R (1 nmol) for 40 min at 37 °C. Following the addition of 0.3 mM EEDQ, the reaction was incubated for a further 1 h at 37 °C. Ladder formation was analyzed by silver-stained 10–20% Tricine/SDS/PAGE. To examine the effect of CBB-G or CBB-R on the copper/H_2O_2-mediated metal-catalysed oxidation of α-synuclein, the protein (5 μM) was incubated with 0.5 mM $CuCl_2$ and 0.01 mM H_2O_2 in 20 mM Mes, pH 6.5, for 4 h at 37 °C in the presence of various concentrations of the dyes. The self-oligomers of α-synuclein − due to dityrosine crosslinks − were analysed and visualized by silver-stained 10–20% Tricine/SDS/PAGE. For analysis of NAC competition during dye-induced self-oligomerization, α-synuclein (100 pmol) was preincubated with either CBB-G (2 nmol) or CBB-R (1 nmol) for 40 min at 37 °C in the presence of various concentrations of NAC at molar ratios from 1 : 1 to 1 : 100 (α-synuclein/NAC). The chemical crosslinking was done with 0.3 mM EEDQ and the oligomers were visualized on the silver-stained gel.

Electron microscopic analysis of α-synuclein aggregations induced by CBB-G and CBB-R

The aggregation of α-synuclein was induced by incubating the protein (100 μM) with either CBB-G or CBB-R at a molar ratio of 1 : 10 in 20 mM Mes, pH 6.5, for 48 h in a 37 °C water bath without shaking. An aliquot (5 μL) of the sample was adsorbed onto a carbon-coated copper grid (200 mesh) and air-dried for 1 min. The aggregates on the grid were negatively stained with 2% aqueous uranyl acetate for another 1 min and analysed with a transmission electron microscope (Hitachi, H7100).

RESULTS

It was reported previously that α-synuclein could not be quantified by the method of Bradford using CBB-G [28] because absorbance of the dye bound to α-synuclein lost its linearity as the amount of protein increased. This loss of linearity became apparent when the curve was compared with a straight line of the absorbances obtained with various amounts of BSA (Fig. 1). This unusual interaction between α-synuclein and CBB-G made us suspect that the dye interacted specifically with α-synuclein and caused protein aggregation which might preclude additional dye binding. Therefore it was decided to examine this possibility by using the self-oligomerization assay in the presence of a chemical coupling reagent for the ultimate purpose of identifying an α-synuclein-interactive small molecule. When α-synuclein was treated with various concentrations of CBB-G in the presence of 0.3 mM EEDQ, the protein

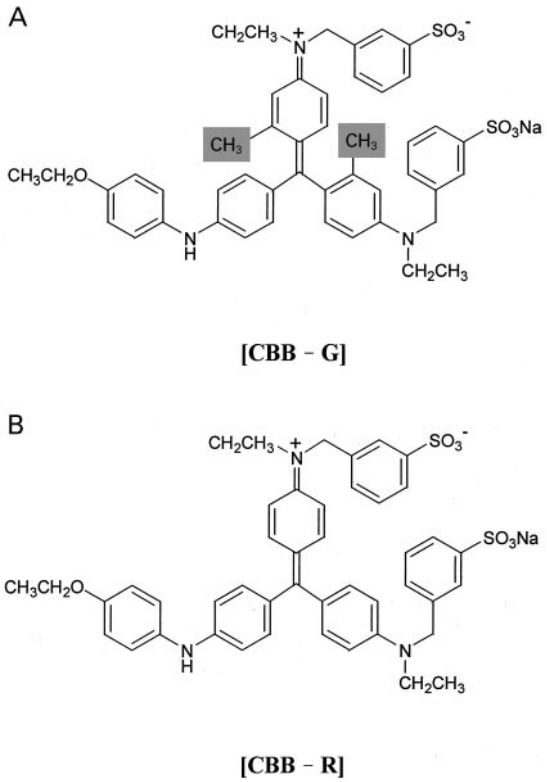

Fig. 3. Structures of CBB-G (A) and CBB-R (B).

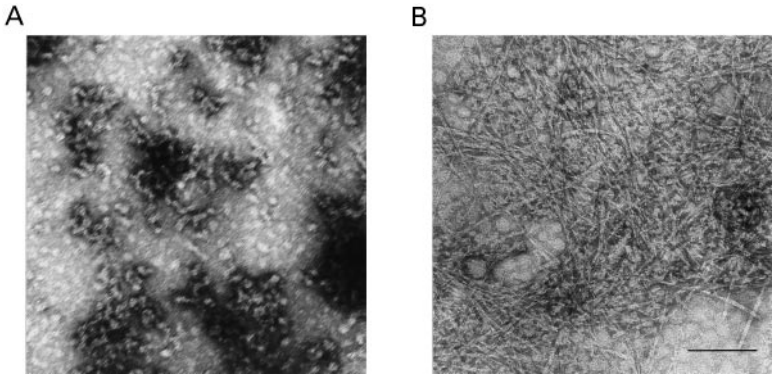

Fig. 4. Protein aggregates of α-synuclein obtained with CBB-G (A) and CBB-R (B). The protein aggregations were induced by incubating α-synuclein (100 μM) and the dyes at a molar ratio of 1 : 10 in 20 mM Mes, pH 6.5, for 48 h at 37 °C. The aggregates adsorbed onto the carbon-coated copper grid (200 mesh) were negatively stained with 2% uranyl acetate and analysed by electron microscopy. Bar = 400 nm.

was shown to experience the self-oligomerization which appeared as a ladder on silver-stained 10–20% Tricine/SDS/PAGE (Fig. 2A). This CBB-G induced self-oligomerization of α-synuclein increased slowly until the dye concentration reached 250–350 μM and then decreased. To investigate the molecular details of this selective dye interaction, a congener of the dye, Coomassie Brilliant Blue R-250 (CBB-R), that lacks two methyl groups of CBB-G (Fig. 3) was used to investigate its involvement in self-oligomerization. CBB-R also induced the oligomerization more effectively than did CBB-G (Fig. 2B). Oligomerization increased rapidly up to a dye concentration of 150 μM and then decreased gradually thereafter. This biphasic phenomenon could be due to the formation of SDS-resistant protein aggregations at higher concentrations of dye as the band intensity of monomeric α-synuclein decreased along with that of oligomers formed in the presence of dye concentrations above 150 μM. In addition, the appearance of the ladders induced by CBB-G and CBB-R were different from each other: those induced by CBB-R were more discrete than those induced by CBB-G with respect to the clear visualization of higher molecular mass oligomers (Fig. 2). These facts indicate that molecular interactions of α-synuclein between the two dyes are distinct from each other.

Because the dyes were shown to interact with α-synuclein and cause the protein to be self-interactive, the protein aggregates obtained with the dyes in the absence of the chemical coupling reagent were subjected to electron microscope analysis. The protein aggregations were induced by incubating α-synuclein (100 μM) and the dyes (1 mM) in 20 mM Mes, pH 6.5, for 48 h in a 37 °C water bath without shaking. Impressively, the α-synuclein aggregates generated in the presence of CBB-R clearly revealed the existence of fibres in addition to the bakground of granular structures (Fig. 4B) whereas the CBB-G-induced protein aggregates appeared as worm-like structures (Fig. 4A). This morphological difference is another indication that the dyes interact with the protein by somewhat different molecular mechanisms.

The dissociation constants for complexes of α-synuclein and the dyes were determined by using the centrifuge column procedure [32]. The saturation curves showed that the protein (5 μM) began to be saturated from either 1 μM CBB-R or 2 μM CBB-G (Fig. 5A). This substoichiometric saturation phenomenon could be considered to be another indication of the dye-mediated protein self-interaction. The double reciprocal plots (Fig. 5B) indicated that the CBB-R interaction with the protein was approximately twofold more effective than the CBB-G binding as the dissociation constants were estimated as 0.63 μM and 1.37 μM, respectively. In addition, the actual dye binding sites on the protein were examined. First of all, implication of the acidic C terminus of α-synuclein in the dye-mediated self-oligomerization was investigated with the C-terminally truncated α-synuclein, α-syn97. As shown in Fig. 6A, the dye interaction sites were shown to be independent from the

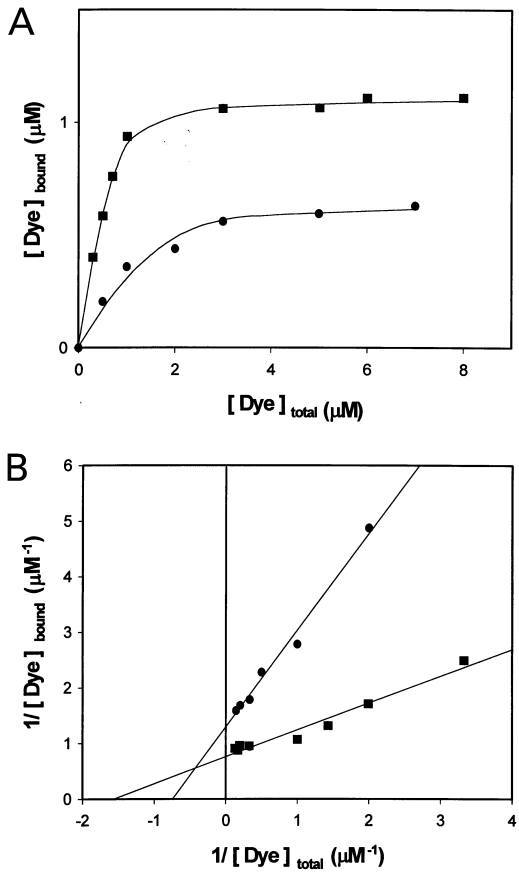

Fig. 5. Binding between α-synuclein and the dyes. (A) Dye binding to α-synuclein was examined with the centrifuge column procedure as described. The amount of CBB-G (●) or CBB-R (■) bound to the protein (1 nmol) is plotted against the dye concentration initially incubated. (B) Double reciprocal plots of the saturation curves of CBB-G (●) and CBB-R (■) are also shown.

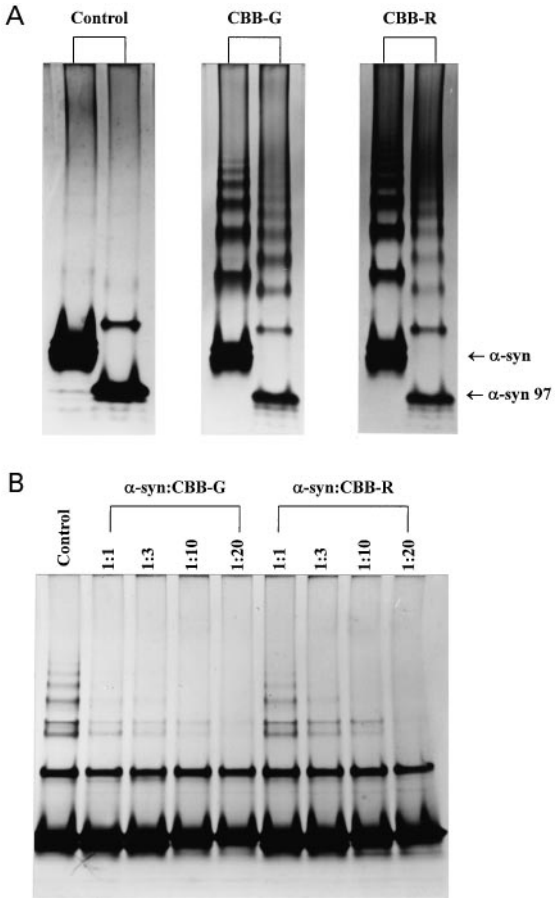

Fig. 6. Localization of the dye binding sites on α-synuclein. (A) Self-oligomerization of a C-terminally truncated α-synuclein, α-syn97 was examined with 0.3 mM EEDQ in the presence of either CBB-G or CBB-R as described. Lanes labelled 'Control' correspond to the samples of either α-synuclein or α-syn97 separately treated with the coupling reagent in the absence of the dyes. Lanes labelled CBB-G and CBB-R show the results of the dye-induced self-oligomerization of α-synuclein and α-syn97. (B) Effect of the dye on the copper/H_2O_2-mediated metal-catalysed oxidative self-oligomerization of α-synuclein. The lane labelled 'Control' corresponds to the oxidative self-oligomerization of α-synuclein observed in the presence of 0.5 mM $CuCl_2$ and 0.01 mM H_2O_2. The metal-catalysed self-oligomerizations were examined in the presence of either CBB-G or CBB-R at various molar ratios of α-synuclein and the dyes as indicated.

acidic C-terminus because the dyes still induced self-oligomerization of α-syn97, which was reminiscent of the Aβ25–35 induced self-oligomerization of α-synuclein [22]. Because it had been shown already that the Aβ25–35 interaction with α-synuclein enhanced synergistically the copper/H_2O_2-mediated oxidative self-oligomerization of the protein [27], the effect of dye on the metal-catalysed oxidation was examined. Interestingly, however, the dye did not cause α-synuclein to be more susceptible to oxidation. The dyes actually suppressed metal-catalysed self-oligomerization (Fig. 6B). In addition, dye-induced self-oligomerization of α-synuclein was not affected by the presence of NAC (data not shown) although the Aβ25–35 and the eosin-mediated self-oligomerizations were shown to be inhibited by the peptide [21–23]. These data indicate that the dye interaction sites are not only independent of the C terminus, but are also distinct from the Aβ25–35 binding site(s) which has been suggested to be closely related to the hydrophobic NAC region in α-synuclein [13].

DISCUSSION

α-synuclein is a common component of various abnormal protein depositions observed in neurodegenerative disorders such as PD, DLB, AD and MSA [9]. It has been hypothesized that this protein plays a significant role in the nucleation process that leads to the formation of abnormal deposits such as Lewy bodies, senile plaques, or neuronal and cytoplasmic inclusions. Recently, transgenic mice and flies overexpressing α-synuclein have been developed [19,20]. The mice showed that α-synuclein, which was expressed in all neurons, accumulated and formed cytoplasmic inclusions; also produced were nuclear inclusions which were not present in the brains of PD patients. The protein co-localized with ubiquitin in the cytoplasmic inclusions inside the dopaminergic neurons, reminiscent of the human PD, but it was not fibrillized [19]. Fibre formation has been considered to be a characteristic feature of the amyloidosis thought to be critical for cytotoxicity [33,34]. The mice still exhibited impaired motor activity even though the dopaminergic cells were not degenerated, but damaged only. On the other hand, the *Drosophila* model of PD with overexpression of α-synuclein, showed dopaminergic neurons that were selectively degenerated and contained cytoplasmic inclusions in which dense cores with radiating filaments were observed [20]. In addition, age-dependent locomotor dysfunction of the transgenic flies was confirmed by measuring their climbing abilities as a negative geotactic response. Now that the animal models are available there is strong demand to exploit α-synuclein interactive small molecules which could be used for developing detective, preventative, or even therapeutic strategies against the α-synucleinopathies.

In this study, the phenomenon of self-oligomerization of α-synuclein in the presence of EEDQ has been used to monitor the effectiveness of CBB-G and CBB-R interactions with the protein. The ladder formation of α-synuclein visualized by silver-stained 10–20% Tricine/SDS/PAGE proved to be a biochemically specific phenomenon in terms of its underlying molecular mechanism [21–23,26,27]. Among various Aβ-derived peptides such as Aβ25–35, its reverse peptide Aβ35–25, Aβ31–35, Aβ1–28, Aβ1–40, and Aβ1–42, α-synuclein was self-oligomerized only in the presence of Aβ25–35, indicating that the oligomerization was dependent upon not only the peptide sequence but also its orientation [21,26]. When various metals were tested in the self-oligomerization process, copper was the most effective [22]. Interestingly, this specific copper interaction was dependent upon the acidic C-terminus of α-synuclein whereas the Aβ25–35 interaction was independent of the acidic region. These facts clearly suggest that the common phenomenon of self-oligomerization of α-synuclein could be induced via totally different molecular interactions between the protein and effectors. When various dyes such as Congo red, thioflavin S & T, eosin, rhodamine 6G, and phenol red were used to observe their specific interactions with α-synuclein, eosin

was shown to be the only dye which induced the oligomerization [23]. When various eosin congeners including ethyl eosin, eosin B, phloxine B, erythrosin B, and rose bengal were examined for their effects on self-oligomerization, only erythrosin B was as effective as eosin for the specific interaction [23]. Together, these facts have led to the conclusion that the intactness of the benzoate moiety as well as localized negative charge on the xanthene moiety of eosin are critical for the interaction that leads to self-oligomerization. Based on these observations, we suggest that the phenomenon of self-oligomerization is the only *in vitro* assay system currently available to screen α-synuclein interactive molecules. Identification of α-synuclein interactive small molecules may have its own value: not only will it provide various means of determining a basic mechanism for the formation of protein aggregates but it will also allow the dissection of the physiological function of α-synuclein. This becomes possible because a list of various proteins reported to interact with α-synuclein has been formulated [35–38], which actually limits the number of physiological functions of the protein to be investigated. For instance, the small molecules could be used to monitor altered physiological responses, if any, following selective disruption of the specific interactions between α-synuclein and its natural partners.

Our results indicated that both CBB-G and CBB-R induced the common self-oligomerization of α-synuclein through fairly high affinity interactions. The mode of dye binding, however, seems to be unique for each dye because the appearances of the dye-induced ladders are different from each other. The ladders induced in the presence of CBB-R revealed higher molecular mass oligomers more clearly than those induced by CBB-G. This different mode of dye interaction with α-synuclein was also confirmed by electron microscopy of the protein aggregates generated in the presence of the dyes. The specific interaction of CBB-R with α-synuclein revealed the fibrillar structures of protein aggregates in addition to the background of granular structures. On the other hand, the protein aggregates induced by CBB-G exhibited worm-like structures. In other words, induction of the common phenomenon of self-oligomerization of α-synuclein in the presence of different effectors may lead to the formation of protein aggregates with different morphologies. Hence, α-synuclein could be considered to be a versatile molecule leading to various forms of protein aggregates. By the same token, it could be speculated that the cytotoxicities of the protein aggregates could vary according to morphology, and that the various intermediates in the transition from monomeric protein to the aggregate might also exhibit different cytotoxicities. It has been suggested recently that the fibrous form of protein aggregate might not be a true toxin for cells. Instead, intermediates including protein oligomers may be more effective in affecting cell survival [17,18].

In our aggregation study, we also observed granular forms of α-synuclein aggregates. This granular structure is physiologically relevant because the transgenic *Drosophila* have been shown to contain granular protein aggregates with radiating fibrous structures in the cytoplasmic inclusions. Although the granular protein aggregates have not been reported in Lewy bodies of human PD patients, the fibrillized protein aggregates might be an end product of the granular intermediates of α-synuclein aggregation. Under our aggregating condition of 48 h incubation at 37 °C in 20 mM Mes, pH 6.5, α-synuclein aggregated into predominantly granular structures, which subsequently transformed into fibrous aggregates following a prolonged incubation (S.R. Paik & D. Lee, unpublished observation).

In conclusion, we have reported two chemicals – CBB-G and CBB-R, which are active in inducing self-oligomerization of α-synuclein and their resulting protein aggregates appeared by electron microscopy to be different. These α-synuclein interactive small molecules might be very useful as they have the potential to be modified selectively to provide eventual diagnostic, preventive, or therapeutic strategies against the α-synuclein-related neurodegenerative disorders.

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