Guanidine Hydrochloride Denaturation of Dopamine Induced α -Synuclein Oligomers: A Small Angle X-ray Scattering Study

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ABSTRACT

Alpha-synuclein (α -syn) forms the amyloid-containing Lewy bodies found in the brain in Parkinson's disease (PD). The neurotransmitter dopamine (DA) reacts with α -syn to form SDS-resistant soluble, non-amyloid, melanin-containing oligomers. Their toxicity is debated, as is the nature of their structure and their relation to amyloid-forming conformers of α -syn. The small angle X-ray scattering technique in combination with modelling by the ensemble optimisation method showed that the un-reacted native protein populated three broad classes of conformer, while reaction with DA gave a restricted ensemble range suggesting that the rigid melanin molecule played an important part in their structure. We found that 6 M guanidine hydrochloride did not dissociate α -syn DA-reacted dimers and trimers, suggesting covalent linkages. The pathological significance of covalent association is that if they are non-toxic, the oligomers would act as a sink for toxic excess DA and α -syn; if toxic, their stability could enhance their toxicity. We argue it is essential, therefore, to resolve the question of whether they are toxic or not.

ABBREVIATIONS

AUC, analytical ultracentrifugation; α -syn, α -synuclein; β -syn, β -synuclein; CD, circular dichroism; DA, dopamine; DAQ, DA quinone; DAT, DA transporter; DHI, 5,6-dihydroxylindole; DLS, dynamic light scattering; EOM, ensemble optimisation method; γ -syn, γ -synuclein; GuHCl, guanidine hydrochloride; GWAS, genome-wide association studies; IQ, indole-5,6 quinone; PD, Parkinson's disease; ROS, reactive oxygen species; SDS, sodium dodecyl sulphate; SANS, small angle neutron scattering; SAXS, small angle X-ray scattering; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; WT, wild type.

INTRODUCTION

Parkinson's disease (PD) is marked by the selective loss of dopaminergic neurons in the *substantia nigra* of the mid-brain and by the occurrence there of Lewy bodies consisting mainly of fibrillar aggregates of α -synuclein (α -syn), a 140 residue intrinsically disordered protein associated with presynaptic vesicles^{1,2}. The role of α -syn at the molecular level in the pathogenesis of PD was inferred from the link between its mutant and amplified forms and familial PD³⁻⁶. Recent genome-wide association studies (GWAS) suggest that wild-type α -syn is a link between sporadic and familial PD⁷.

The loss of dopaminergic neurons has prompted studies on the involvement of the neurotransmitter dopamine (DA) in PD pathogenesis⁸⁻¹⁰. DA is readily oxidised to reactive DA quinone (DAQ) which cyclises and oxidises further to give dopaminochrome. The latter rearranges to give 5,6-dihydroxylindole (DHI), which readily oxidises to give indole-5,6-quinone (IQ). The indole and quinone can both polymerise to form melanin¹¹. Transition metal ions in the ground state, such as manganese and iron, catalyse the formation of quinone^{12,13} and Fe^{3+} interacts with melanin to form additional reactive oxygen species (ROS)^{14,15}, which may be implicated in cytotoxicity. Being electron deficient, the quinones are susceptible to attack by cellular nucleophiles, such as the sulfhydryls on reduced glutathione, free cysteine, cysteinyl residues, the free N-terminal and ϵ - NH_2 amino groups and histidine residues of proteins. Oxidative intermediates on the melanin pathway, dopaminochrome, DHI and especially IQ have been shown to be the reactive agents^{11,16,17}, initially oxidising the protein methionine residues^{17,18} and forming melanin that is bound to the SDS-resistant oligomers¹⁹. The role of these reactions *in vivo* remains unclear, although some studies have suggested that DA and its oxidative quinones interact with α -syn to enhance cellular toxicity²⁰⁻²². DA-induced α -syn (α -syn:DA) oligomers have been found in cultured cells²²⁻²⁴. Some studies indicate that the SDS-resistant α -syn:DA oligomers are toxic^{22,23,25} while others show

evidence of non-toxic DA-induced oligomeric species in α -syn over-expressing cells^{23,24}.

This divergence of opinion stresses the importance of understanding the structure of the oligomers and its possible roles in toxicity.

The family of synuclein proteins consists of α -syn, β -synuclein (β -syn) and γ -synuclein (γ -syn)²⁶. The α -syn and β -syn occur primarily in brain tissues being located mainly in the pre-synaptic nerve terminals, while γ -syn occurs primarily in the peripheral nervous system and the retina, although it has also been observed to be highly expressed in some tumor tissues, including breast, ovarian and bladder tissues. The β -syn lacks the non-amyloid component (NAC) of α -syn in its hydrophobic central domain and does not aggregate *in vivo*. However, it has been suggested that all three synucleins may modify the trafficking, expression and function of monoamine transporters at the cell surface²⁷, leading in PD to an increased re-uptake of high levels of DA and resultant neurotoxicity^{9,28}. Duplication of the α -syn gene has been found in patients with sporadic PD²⁹ with two instances marked by rapid cognitive decline³⁰. Although these cases support the findings of the GWAS regarding the role of the WT α -syn in sporadic PD, asymptomatic carriers of the duplication have been found. Such phenotypic heterogeneity could arise from enhanced clearance of the excess α -syn in some subjects but not in others³¹. One clearance mechanism could be the formation of DA-melanin adducts. One could envisage a negative feedback loop, where excess α -syn from gene duplication or triplication encourages increased re-uptake of DA through its effect on the DA transporter (DAT)³². Further, DA derived quinones conjugate with cysteine residues inhibiting DA uptake³³. The oxidation products of the excess DA could react with the α -syn, mitigating the former's toxicity and at the same time providing a sink for the excess α -syn. Various influences, such as a redox environment that produced overwhelming amounts of ROS, could break or by-pass this virtuous loop. The resistance to degradation of the α -syn:DA oligomers would be essential to this loop. Conversely if they were proven to be toxic their stability could enhance the pathogenic effects of the redox process.

Conway et al.³⁴ suggested that stable covalent bonds were formed by reaction of DA-derived quinone with the ϵ -amino groups of α -syn lysine and tyrosyl residues (Fig. 1). The importance of covalent linkages was supported by studies showing that DA and DA analogues inhibited α -syn fibril formation through their quinones reacting with its amino groups to form imino bonds³⁵. Other studies, however, have shown that covalent bonds were either of less importance than non-covalent interactions³⁶ or of no importance^{37,38}. Norris et al.¹⁶ showed that the DA oxidation product dopaminochrome inhibited α -syn fibrillisation by interacting with the ¹²⁵YEMPS¹²⁹ sequence in the protein C-terminal region.

In an earlier study, we found by analytical ultracentrifugation (AUC) and MALDI-TOF mass spectrometry of fractions isolated by size-exclusion chromatography (SEC) that DA melanin polymer was bound stoichiometrically to α -syn in the ratio of 22 DA units to a dimer and 42 to a trimer¹⁹. This suggested that the binding was specific. However, such stoichiometry could be due to both or either covalent or non-covalent binding. In this paper we report the results of small angle X-ray scattering (SAXS) studies on α -syn:DA dimers and trimers in the presence of the denaturing agent 6 M guanidine hydrochloride (GuHCl).

MATERIALS AND METHODS

Materials

Dopamine hydrochloride was purchased from Sigma-Aldrich (St Louis, MO, USA). SDS-PAGE analysis was performed using 12% Bis-Tris gel from Invitrogen (Carlsbad, CA, USA) and the silver stain reagents were from Bio-Rad (Hercules, CA, USA). All other reagents, solvents, buffer and media components were of analytical grade or of the highest purity available.

Sample preparation for SAXS measurements

α -syn was over expressed in *Escherichia coli* BL21 (DE3) that had been transformed with pRSETB expression plasmid containing α -syn DNA sequence, isolated and purified as described previously³⁹. The purity of the protein was determined by SDS-PAGE analysis and mass spectrometry. The protein concentration was determined using the 280 nm extinction coefficient of $5120 \text{ M}^{-1}\text{cm}^{-1}$.

DA reaction with α -syn was carried out and the Met-oxidised monomer, α -syn:DA dimer and trimer fractions were isolated by SEC in 10 mM sodium phosphate buffer pH 7.4 as described previously¹⁹. To obtain high protein concentrations for SAXS measurements, the Met-oxidised monomer, α -syn:DA dimer and trimer from SEC were concentrated using spin columns with MWCO 3,000 Da (Millipore, Billerica, MA, USA). Half of each sample was dialysed against 10mM sodium phosphate buffer pH 7.4 (native conditions) and the other half was dialysed against 6 M GuHCl in 10 mM sodium phosphate pH 7.4 (denatured conditions). The dialysate was collected for use as buffer blank in the SAXS experiments. Protein concentrations of the Met-oxidised monomer, α -syn:DA dimer and trimer were determined by bicinchoninic acid (BCA) protein assay using un-oxidised α -syn monomer as the standard protein. For the un-oxidised α -syn monomer sample, the purified lyophilised protein was dissolved in 6 M GuHCl, 10 mM Tris.HCl at pH 7.5. Buffer exchange was performed on a PD-10 desalting column (GE Healthcare, Uppsala, Sweden) equilibrated with 10 mM sodium phosphate buffer pH 7.4. The un-oxidised monomer was then subjected to dialysis in parallel with the α -syn:DA samples under native and denatured conditions. The purity of the samples was ascertained by SDS-PAGE analysis. Silver staining of SDS-PAGE was performed according to the manufacturer's protocol (Bio-Rad). Dynamic light scattering (DLS) measurements were carried out using a DynaPro Titan instrument (Wyatt Technology, Santa Barbara, CA, USA) to ensure the samples were not aggregated. AUC data from a previous study¹⁹ were processed using SOMO UltraScan 9.9 (revision 1927 for Windows[®])^{40,41}. The experimentally determined hydrodynamic data for the oligomers were

calculated and compared with those obtained from the bead models derived from their SAXS profiles using the SOMO package. The MW of the dimer (32.24 kDa) and trimer (49.71 kDa) used in this calculation were those previously derived by MALDI-TOF mass spectroscopy¹⁹.

SAXS measurements

All the samples were centrifuged at 15,000 x g for 5 min immediately prior to measurement to remove any aggregates. SAXS measurements were made at 27°C on the SAXS/WAXS beam line of the Australian Synchrotron (Clayton, Victoria, Australia). Using the high-intensity undulator source on this instrument, an energy resolution of 10^{-4} was obtained from a cryo-cooled Si (111) double-crystal monochromator, and the beam size (fwhm focused at the sample) was $250 \times 100 \mu\text{m}^2$ with a total photon current of about 2×10^{12} photons sec^{-1} . Scattering patterns were recorded with an X-ray energy of 12 keV using a Pilatus 1M detector with an active area of $170 \times 170 \text{ mm}^2$ and a sample to detector distance of 1573 mm. This corresponded to maximum q range from 0.005 to $0.6 \text{ \AA}^{-1}$ (where $q = 4\pi \sin \theta/\lambda$, 2θ is the X-ray scattering angle and λ the X-ray wavelength). Samples were slowly pumped through a 1.5 mm ID quartz capillary (Hampton Research, Aliso Viejo, CA USA) and exposures were made at 1 second intervals, the number being determined by the concentration of the sample and the background scattering of the control buffer. SAXS measurements were performed at protein concentration ranging from 0.018 mM to 0.438 mM, as listed in Table S1 in the Supporting Information. Absolute intensities were calibrated using water in the 1.5 mm ID quartz capillary.

SAXS data analysis

All two-dimensional scattering patterns obtained were reduced to one-dimensional profiles of intensity I versus q using the Saxs15id software package⁴². Radius of gyration (R_g) calculation, Kratky plots, Guinier and Porod analyses and the determination of the size and shape of the molecules in solution from the SAXS profiles were made using the ATSAS

suite of programs⁴³⁻⁴⁷, 2.5.0-2 release available from <http://www.embl-Hamburg.de/ExternalInfo/Research/Sax/index.html>. The data were processed and the overall parameters were computed following standard procedures using the program PRIMUS. R_g from Guinier plots and pair distribution functions $P(r)$ were calculated and their values from both methods were within error limits. Aside from these methods, two procedures were used to extract structural information from the scattering profiles. Since α -syn is an intrinsically disordered protein², the ensemble optimisation method (EOM) was used (Advanced EOM 2.0) for the analysis of the flexibility and size distribution of possible structures of unfolded samples⁴⁸⁻⁵⁰. Where EOM indicated a narrower range of flexibility, clustering analysis was used where experimental data smeared by GNOM were input into DAMMIF on-line⁵¹ (www.embl-hamburg.de/biosaxs/atsas-online/dammif.php). An average model for each cluster was generated and filtered for regions of high volume occupancy. Clustering is an algorithm that chooses one or several solutions which represents shapes that best fit the data; that is a set of models that are similar to each other and selecting those that best represent the cluster. Molecular dimensions were confirmed using HYPERCHEM[®] and final illustrations were prepared with the molecular renderer RasMol 2.7.4_Pere1_1. CRY SOL was used to compute scattering profiles from *.pdb files generated by coil library models and to fit them to the experimental curves.

RESULTS AND DISCUSSION

Treatment of α -syn:DA oligomers with urea

In our earlier studies¹⁹, we proposed that the formation of SDS-resistant α -syn:DA oligomers involved the covalent cross-linking of DA-melanin with α -syn, though hydrogen bonding and hydrophobic interaction could not be discounted in the native preparations. In the present study, the stability of α -syn:DA oligomers was initially investigated using urea as a denaturant. Pre-formed α -syn:DA oligomers were obtained by incubation of α -syn at 200

μM with 1 mM DA at 37°C for 16 h in 10 mM sodium phosphate buffer at pH 7.4, giving a mixture of various size species from monomer to higher MW oligomers^{15,21}. Samples of pre-formed α -syn:DA oligomers (20 μM) were incubated with various concentrations of urea (0–8 M) for 3 h at room temperature followed by SDS-PAGE/silver staining analysis (Fig. 2).

The resistance of α -syn:DA oligomers to dissociation in SDS already suggested that the oligomers were covalently linked; we found that they were also resistant to 8 M urea, giving a similar pattern to that observed in the absence of urea (Fig. 2). The resistance to 8 M urea denaturation reinforces the idea that at least to some extent the oligomers were stabilised by covalent bonds. However, SDS-PAGE analysis in the presence of denaturants can give no indication of the shape of the oligomers and the possible location and extent of covalent linkages or hydrogen bonds and hydrophobic interactions in their native state. SAXS can help answer these questions. Rather than using urea, which has been shown to destabilise proteins by forming H-bonds between side chains and backbones⁵², we used GuHCl which, as molecular dynamics simulation suggests interacts with hydrophobic regions^{52,53}. At high concentrations (6–8 M) it is a strong chaotropic agent, breaking hydrogen bonds and hydrophobic contacts^{54–57}, while leaving covalent bonds intact. Its high electron density at these concentrations reduces the contrast in SAXS and, coupled with relatively low protein concentrations, makes the use of synchrotron radiation essential, although our earlier studies on the native proteins in buffer could use a laboratory X-ray source¹⁹.

Small angle X-ray scattering of monomeric α -syn

Fig. 3 A shows scattering profiles for monomeric α -syn in 10 mM sodium phosphate buffer at pH 7.4 (native conditions) and in 6 M GuHCl + 10 mM sodium phosphate buffer at pH 7.4 (denaturing conditions). The scattering profile of the denatured monomer shows signs of aggregation/ presence of higher-order oligomers with the intensity I , increasing at low q values. Another indication of aggregation/higher order polymers is the absence of a well

defined R_g value, whether derived from a Guinier plot, which curves upwards (Fig. 3 B line (ii)), or using GNOM which gives real and reciprocal space R_g values by inverse Fourier transformation of the scattering profile. DLS of the denatured monomer showed an increase in hydrodynamic radius with increasing concentration. Denaturation may be exposing more hydrophobic residues of the monomer, thus encouraging association. The tendency to aggregate was also seen in the denatured oligomers using SEC and it was significant over 30-60 min, particularly at protein concentrations exceeding 1 mM.

Attempts were made to complement the SAXS studies with small angle neutron scattering (SANS), which would have been particularly useful in elucidating the nature of the oligomer interface. We were unable to find sample conditions where α -syn was monodisperse and stable in buffer/D₂O or in the presence of 6M GuH/D/Cl/D₂O at the concentrations and over the times required for SANS measurements. On the other hand, for the synchrotron SAXS measurements with their shorter exposure, the native monomer gave a well-defined R_g (Table I) and a linear Guinier region (Fig. 3 B, line (i)).

A number of criteria have been used to show that native α -syn in buffer solution is intrinsically unfolded⁵⁸⁻⁶⁰. However, it has been shown that relatively small changes in the peptide environment can cause significant changes in its conformation leading to a pre-oligomeric state⁶¹. Our previous AUC analysis along with CD spectra indicated that monomeric α -syn in native conditions has an extended conformation¹⁹ consistent with natively unfolded protein. To confirm that the monomeric protein prepared as described in Methods for the current study was natively unfolded, we generated a “coil library” of *.pdb files using the tool found at <http://unfolded.uchicago.edu/>^{62,63}. Using CRY SOL, 1000 of these files were converted to SAXS intensities, which were averaged and fitted to the experimental intensity curve (Fig. 3 A (t)). It can be seen that there is a close correspondence between the calculated and experimentally determined intensities. Furthermore, the Kratky

plots ($q^2 I(q)$ vs. q , Fig. 3 C) indicate that our starting material was natively unfolded protein (see Figure 3 of⁵⁰).

SAXS profiles corrected for protein concentration for native Met-oxidised monomer and denatured Met-oxidised monomers are shown in Fig. 4 A. Here the denatured Met-oxidised monomer showed no signs of aggregation, which could be due to the increased polarity induced in the molecule by oxidation of the methionine residues. The theoretical intensity derived from the “coil library” is also plotted in Fig. 4 A (t), where it can be seen that the native and denatured Met-oxidised monomer fits are less satisfactory than for the un-oxidised α -syn monomer shown in Fig. 3 A. This would be due to conversion of the four methionine residues to methionine sulfoxide. Both native and denatured Met-oxidised monomer give linear Guinier plots in the $q = 0.01$ - $0.03 \text{ \AA}^{-1}$ range (Fig. 4 B). Denaturation increased the R_g value (Table I) slightly from $36 \pm 1 \text{ \AA}$ (native) to $38 \pm 1 \text{ \AA}$ (denatured). The Kratky plots (Fig. 4 C) are consistent with unfolded conformation in both proteins. The $P(r)$ plots (Fig. 4 D) obtained by inverse Fourier transformation of the SAXS profiles indicate that the native Met-oxidised monomer (i) had an elongated shape with maximum dimension $D_{\max}$ of $145 \pm 4 \text{ \AA}$, while the denatured Met-oxidised monomer (ii) had an elongated shape with a $D_{\max}$ of $155 \pm 3 \text{ \AA}$. The R_g and $D_{\max}$ values for the native Met-oxidised monomer are similar to those reported by Rekas et al¹⁹.

Small angle X-ray scattering of the native and denatured α -syn:DA dimer

SAXS profiles corrected for protein concentration of the α -syn:DA dimer under native and denatured conditions are shown in Fig. 5 A (i) and (ii) respectively. The Guinier plots in the $q = 0.01$ - $0.03 \text{ \AA}^{-1}$ range are linear (Fig. 5 B). A globular, rather than elongated, shape would be indicated by a characteristic maximum at low q in Kratky plots, so the comparison of their Kratky plots (Fig. 5 C) with the theoretical curve (Fig. 4 C (t)) shows that both native and denatured dimers were less unfolded than the monomer. Our previous CD

study showed that dimer formation was associated with an increase in β -sheet and turns content from 21% in the monomer to 47% in the dimer¹⁹. This indicated that dimer formation had a stabilising effect on the structure.

The principal effect of denaturation is to elongate the dimer, which is evident from the increase in R_g values obtained by Guinier analysis and $P(r)$ analysis (Table I) and an increase in $D_{\max}$ from 150 ± 3 Å to 160 ± 3 Å as determined from the $P(r)$ plots (Fig. 5 D). The agreement within error of the I_0 values for native and denatured proteins obtained by extrapolating the forward scattering, corrected for concentration differences, to $q = 0$, (Table I) indicate that there was no significant dissociation. This agrees with the SDS-PAGE/silver stain analysis of pre-formed soluble α -syn:DA oligomers treated with various concentrations of urea (0-8 M), where the oligomers remained stable even in the presence of 8M urea (Fig. 2).

Small angle X-ray scattering of the native and denatured α -syn:DA trimer

SAXS profiles corrected for protein concentration for the α -syn:DA trimer under native and denaturing conditions are shown in Fig. 6 A (i) and (ii) respectively. The agreement within error of the I_0 values (Table I) indicated that, as in the case of the dimer, there was no significant dissociation. The Guinier plots in the $q = 0.01$ - 0.03 Å⁻¹ range are linear (Fig. 6 B). As in the case of the dimers, the effect of denaturation is to increase the R_g (Table I). According to the Kratky plots (Fig. 6 C), both samples were partly compact with only a slight change on treatment with 6 M GuHCl, suggesting that further reaction with DA had a stabilising effect on oligomer structure. This is consistent with previously observed globularisation of the molecules as expressed by the size of the Kratky plot maximum increasing with molecular mass¹⁹. The elongated shape observed with the monomer and dimer is also seen in the $P(r)$ plots of the trimer (Fig. 6 D). The native trimer has a $D_{\max}$ of 168 ± 2 Å and the value for the denatured trimer is 180 ± 2 Å. The length and the R_g value

for the native trimer agree with those previously published¹⁹. The nearly identical pairs of I_0 values (Table I) for the native and denatured dimer and trimer indicate that even in 6M GuHCl, the oligomers did not dissociate into monomeric species. This observation taken with that of their stability on electrophoresis under denaturing conditions suggests that the oligomers are covalently bonded. Denaturation increased the R_g of the dimer and trimer by 36% and 26 %, respectively (Table I). Such an increase is considerably greater than that expected from the effects of denaturation on a partly unfolded protein⁶⁴, and indicates greater stabilisation of the trimer by interaction with DA.

models of α -syn monomer, α -syn:DA dimer and trimer

Modelling fully or partly unfolded proteins poses distinct problems, particularly regarding the relevance of conventional *ab initio* methods such as DAMMIF and GASBOR which are valid for structured proteins, and particularly where supplementary high resolution data are available. One of the tools available for modelling of unfolded protein is EOM. It, however, needs to be used with strong caveats where modified residues are present, for example produced by the oxidation or nitration of unfolded proteins in some pathological conditions. This is because there is no single letter code for post-translationally or chemically-modified residues in the IUB/IUPAC amino acid database used for the FASTA sequence, which is a required input for EOM 2.0.

Ensemble optimisation modelling

The Kratky plot of the monomer showed that the protein was natively unfolded. There is much evidence pointing to conformational variability in such proteins that might provide a key to elucidating the misfolding and aggregation mechanisms underlying a range of pathologies, including PD^{65,66}. The existing evidence for the role of conformational flexibility comes largely from theoretical studies predicting that the apparent structural disorder results from the coexistence of a complex ensemble of conformers. The use of

SAXS provides an approach to characterising macromolecular flexibility in solution by EOM^{49,51}. We, therefore, entered the amino acid sequence of the native monomeric α -syn (Fig. 1) into the EOM 2.0 package, with the relevant parameters set to “random”, P1 symmetry, 10,000 models and 20 runs of the genetic algorithm. This gave a best fit to the experimental curve with $\chi = 0.458$ (Fig. S1 A, Supporting Information) and a three-peak R_g distribution plot for the ensemble was obtained with an average values of 37 Å (Fig. S1 B, Supporting Information).

Because of their reaction with DA, all four methionine residues in the α -syn sequence are converted to methionine sulfoxide. This is reflected in the significantly different scattering profiles of the un-oxidised and Met-oxidised protein (see Fig. 3 and 4), demonstrating that oxidation leads to significant conformational change. With the caveat imposed by the presence of the oxidised methionine residues, we ran EOM using the un-modified α -syn sequence as described above. We obtained a less good fit ($\chi = 0.784$) to the scattering profile of the Met-oxidised monomer (Fig. S1 C, Supporting Information). This less good fit was observed whether the software’s random or native option was used.

Geometrical validation studies around the $C\alpha$ carbon and the $C\beta$ variation have shown the latter to be particularly sensitive to interactions between side chains and the backbone⁶⁷. It might be expected, therefore, that the change caused in the methionine side chain by oxidation would be reflected in the conformation and scattering profile of Met-oxidised α -syn monomer. It can be seen that the fit was less good at q values > 0.35 . The ensemble R_g profile (Fig. S1 D, Supporting Information) showed a significant change from that observed for the un-oxidised native monomer, with the higher peak being skewed towards a lower value with the average R_g being 37 Å. The profile suggests a shift to lower proportions of the more extended configurations. It is of interest that the oxidised protein is less prone to fibril formation and this may be related to the lower population of extended conformations⁶⁸. The denatured Met-oxidised monomer also gave a less good fit ($\chi = 0.996$) to its scattering profile

(Fig. S1 E, Supporting Information) with an increase to 44 Å in ensemble average R_g and a shift in the maximum to higher values (Fig. S1 F, Supporting Information) compared with the non-denatured preparation.

Although our earlier CD data¹⁹ showed a small increase in folding on reaction of the α -syn with DA, the Kratky plots (Fig. 5 C and Fig. 6 C) point to the existence of considerable flexibility in both the dimer and trimer. Advanced EOM 2.0 is particularly capable of oligomeric modelling, so with the caveats regarding residue modification in mind, it was run on the native and denatured dimer and trimer. For the native dimer, a poor fit at $q > 0.4 \text{ Å}^{-1}$ was obtained with a $\chi=0.786$, while the R_g distribution has a prominent single peak with an average of 38 Å (Fig. S2 A and B, Supporting Information). The denatured dimer also showed a poor fit at $q > 0.4 \text{ Å}^{-1}$ with a $\chi=0.746$, while the ensemble R_g distribution is heterogeneous with a large peak at the higher value and the average R_g is 49 Å (Fig. S2 C and D, Supporting Information). The native trimer showed a poor fit at $q > 0.35 \text{ Å}^{-1}$ with a $\chi=0.786$, while the R_g distribution has a major peak shifted to a slightly higher value compared with that of the native dimer with an average R_g of 41 Å (Fig. S2 E, F Supporting Information). The denatured trimer showed a poor fit at $q > 0.3 \text{ Å}^{-1}$ with a $\chi=0.742$ (Fig. S2 G, Supporting Information) and the R_g distribution (Fig. S2 H, Supporting Information) shows a split peak shifted towards higher values than that for the native trimer with an average R_g of 48 Å.

Cluster analysis of the oligomers

Since the R_g distribution for the native dimer and trimer was considerably narrower than for the native and denatured monomers and the denatured oligomers, we turned to cluster analysis for added insights into changes observed consequent upon DA binding. We used DAMCLUST, which does not depend on the protein's sequence. Relative distances between clusters for the native dimer and trimer are given in Table S2, in the Supporting

Information. The closer the distance between two pairs of models the more closely they resemble each other. The models created by DAMCLUST are shown in Fig. S3 (Supporting Information). For ease of comparison, the bead models have been transformed into envelopes, using the “map resolution 5” command of Rasmol. The similarity of the four dimer and two trimer models (Fig. S3 A and B, respectively, Supporting Information) is reflected in the closer distance between the pairs of models (Table S2, Supporting Information) and their narrower R_g distribution (Fig. S2 B and F, Supporting Information).

Comparison of hydrodynamic coefficients obtained by AUC and those calculated from the models

Aside from *ab initio* modelling, EOM, the R_g , molecular mass and $P(r)$ derived values, the hydrodynamic coefficients obtained from AUC can also contribute to our understanding of the shapes of the oligomers. Although the long term aggregation of the oligomers in 6M GuHCl precluded acquisition of hydrodynamic data by sedimentation equilibrium/velocity in AUC, such data were available from a previous study for the native monomer, dimer and trimer, supported by MALDI-TOF determination of MW¹⁹. AUC showed that the monomer, dimer and oligomers prepared as described in Methods were monodisperse and were stable over the 16 hr of an AUC experiment¹⁸. The sedimentation coefficients and frictional coefficients were obtained from the raw AUC data¹⁹ and, using the SOMO package^{40,69}, compared with those calculated from the bead models obtained in the present study. The bead models were the average pdb files obtained from the DAMCLUST analysis (Fig. S3, Supporting Information). There is good agreement between the experimental and modelled coefficients (Table S3, Supporting Information) with the frictional coefficients showing an increase in the aspect ratio of the oligomers, compared with the monomer.

CONCLUSION

In drawing conclusions from our study it is important to recognise that there are two distinct pathways for α -syn oligomerisation/aggregation. One involves the selection by a poorly understood process of an aggregation-prone conformer from an ensemble of conformations of the native protein⁷⁰⁻⁷². It is generally accepted that the oligomers and fibrils so derived are toxic although there is debate as to which forms play a major role⁷³. In the other pathway, α -syn reacts with DA and is extensively modified by the oxidation of its methionine residues and the reaction with the DAQ formed as part of the accompanying redox process. Here, there is debate as to the toxicity of the oligomers and aggregates.

In the case of the native α -syn we have applied EOM to the X-ray scattering data and the sequence to show that its R_g and size distribution point to three major groups of conformers, an intriguingly similar distribution to that observed at the single molecule level by AFM⁷⁴. Oxidation and denaturation of the oxidised protein demonstrated the sensitivity of the protein's conformer distribution to chemical modification or environmental change. SAXS could be used to study the conformer profiles of α -syn mutants, known to have enhanced pathogenicity. Having established these profiles it might be possible to study the effects of promoters and inhibitors of fibrillisation.

In the case of the α -syn:DA oligomers, we conclude that denaturation resistant covalent linkages play a significant part in their structure. The molecular size of the oligomers under denaturing conditions remains unaltered as shown on SDS-PAGE (Fig. 2). The SAXS data suggest that denaturation leads to changes in conformation, as shown by the increases in R_g and $P(r)$ on GuHCl treatment. EOM showed that denaturation resulted in an increase in the proportion of more extended members of the assemblage and clustering analysis showed considerable diversity under denaturing conditions, although less for the oligomers than that found with the monomer. Subject to the caveat that the modelling could have been influenced by the inability to allow for the presence of the oxidised methionine residues, it is probable that the decrease in flexibility on oligomerisation showed by the narrower R_g distribution

compared with the monomer is due to extensive covalent linkages to the rigid DA melanin⁷⁵. The most likely candidates for forming these covalent linkages³⁴ are those amino acid side chains likely to participate in reaction with DAQ, and detailed mutational studies to determine the extent of the linkages would prove profitable. Physiologically, the covalent linkages would confer stability on the oligomers that would make them suitable sinks for excess DA and α -syn, thus playing a role in the homeostasis of these toxic molecules.

The widely-abused 'designer' cathinone analogs, such as 4-methylmethcathinone (mephedrone) and 3,4-methylenedioxymethcathinone (methyldone), along with the structurally related compounds, 3,4-methylenedioxymethamphetamine (MDMA) and methamphetamine owe their effects in part to their interaction with the monoamine transporter system⁷⁶. This leads to excess DA levels, perhaps reinforcing a tendency already to high levels of the neurotransmitter in some individuals. Conversely, if the complexes were toxic, their activity could be enhanced by their greater stability. Such an enhancement in toxicity conferred by covalent cross-linking by non-peptide bonding has been claimed for the amyloid β peptide of Alzheimer's disease^{77,78}. However, membrane association and lipid binding is also important for the functioning of α -syn. Both covalent and non-covalent linkages can introduce inflexible constraints into the α -syn:DA oligomers, and the covalent stable cross-links between DA-melanin and the lysine residues at the lipid binding region toward the N-terminal of α -syn, could prevent the interaction of α -syn with lipids to form pores that permeabilise the membrane (*unpublished*). As we pointed out in the Introduction, the toxicity, or lack of it, of the oligomers is contested. α -Syn:DA oligomers therefore may not be pathogenic but the formation of such oligomeric species can affect the actions of α -syn and disruption of cellular function. Our conclusions, therefore, stress the importance of resolving the controversy over the non-toxicity of the oligomers. As a therapeutic target, should we be discouraging or encouraging their formation?

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FIGURE LEGENDS

FIGURE 1

The α -syn amino acid sequence highlighting the distribution of nucleophilic residues that could be subject to quinone reaction.

FIGURE 2

SDS-PAGE analysis of urea treated α -syn:DA oligomers. α -syn (200 μ M) were incubated in the absence and presence of 1 mM DA at 37°C for 16 h. Pre-incubated α -syn samples (20 μ M) in the absence (**A**) and presence (**B**) of DA were treated with various concentration of urea (0-8 M) for 3 h at room temperature follow by SDS-PAGE/silver stain analysis.

FIGURE 3

Small angle X-ray scattering analysis of α -syn monomer. **A**: Experimental data corrected for protein concentration (c) of: **(i)** monomeric α -syn in 10 mM sodium phosphate buffer at pH 7.4 and **(ii)** in the 6 M GuHCl + 10 mM sodium phosphate buffer at pH 7.4. **(t)** theoretical scattering profile computed from the coil library. The log $[I]$ scale is arbitrary to offset the profiles for clarity. **B**: Guinier plot of **(i)** native and **(ii)** denatured sample. **C**: Kratky plot of native monomer. Smooth line is derived from the coil library based scattering profile. **D**: Distance distribution for the native α -syn monomer.

FIGURE 4

Small angle X-ray scattering analysis of α -syn Met-oxidised monomer. **A**: Experimental data corrected for protein concentration **(i)** in the 10 mM sodium phosphate buffer at pH 7.4 and **(ii)** in the 6 M GuHCl + 10 mM sodium phosphate buffer at pH 7.4. **(t)** theoretical scattering profile computed from the coil library. **B**: Guinier plot of **(i)** native dimer and **(ii)** denatured dimer. **C**: Kratky plots of **(i)** native and **(ii)** denatured Met-oxidised monomer with smooth

line derived from the coil library based scattering profile. **D:** Distance distribution function for the **(i)** native and **(ii)** denatured oxidised monomer.

FIGURE 5

Small angle X-ray scattering analysis of α -syn:DA dimer. **A:** Experimental data corrected for protein concentration **(i)** in the 10 mM sodium phosphate buffer at pH 7.4 and **(ii)** in the 6 M GuHCl + 10 mM sodium phosphate buffer at pH 7.4. **B:** Guinier plot of **(i)** native dimer and **(ii)** denatured dimer. **C:** Kratky plots of **(i)** native and **(ii)** denatured dimer. **D:** Distance distribution function for **(i)** native and **(ii)** denatured dimer.

FIGURE 6

Small angle X-ray scattering analysis of α -syn:DA trimer. **A:** Experimental data profiles corrected for protein concentration **(i)** in the 10 mM sodium phosphate buffer at pH 7.4 and **(ii)** in the 6 M GuHCl + 10 mM sodium phosphate buffer at pH 7.4. **B:** Guinier plot of **(i)** native trimer and **(ii)** denatured trimer. **C:** Kratky plots of **(i)** native and **(ii)** denatured trimer. **D:** Distance distribution function for **(i)** native and **(ii)** denatured trimer.

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Figure 1

MDVFMKGLSK—AKEGVVAAAE—KTKQGVAEAA—

GKTKEGVL^YV—GSKTKEGVV^H—GVATVAEKTK—

EQVTNVGGAV—VTGVTAVAQK—TVEGAGSIAA—

ATGFVKKDQL—GKNEEGAPQE—GILEDMPVDP—

DNEAYEMPSE—EGYQDYEPEA

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