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Heme stabilization of α -Synuclein oligomers during amyloid fibril formation

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Abstract

Alpha-Synuclein (α Syn), which forms amyloid fibrils, is linked to the neuronal pathology of Parkinson's disease, as it is the major fibrillar component of Lewy bodies, the inclusions that are characteristic of the disease. Oligomeric structures, common to many neurodegenerative disease-related proteins, may in fact be the primary toxic species, while the amyloid fibrils exist as either a less toxic dead-end species, or even as a beneficial mechanism to clear damaged proteins. In order to alter the progression of the aggregation and gain insights into the pre-fibrillar structures, the effect of heme on α Syn oligomerization was determined by several different techniques including native (non-denaturing) polyacrylamide gel electrophoresis, thioflavin T fluorescence, transmission electron microscopy, atomic force microscopy, circular dichroism and membrane permeation using a calcein release assay. During aggregation, heme is able to bind the α Syn in a specific fashion, stabilizing distinct oligomeric conformations and promoting the formation of α Syn into annular structures, thereby delaying and/or inhibiting the fibrillation process. These results indicate that heme may play a regulatory role in the progression of Parkinson's disease; in addition, they provide insights of how the aggregation process may be altered, which may be applicable to the understanding of many neurodegenerative diseases.

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Author Contributions

EYH performed the experiments, PK and EYH performed the AFM measurements, HM provided guidance on AFM. TLW performed the calcein release assay. EYH, SRY and DLR planned the experiments and wrote the manuscript. All authors have given approval to the final version of the manuscript.

SUPPLEMENTAL INFORMATION

Supplemental methods describing the calcein release assay, and supplemental Fig. S1: CD component analysis, and Fig. S2: calcein release assay are available. Supporting materials may be accessed free of charge online at <http://pubs.acs.org>.

Keywords

Alpha-Synuclein; Parkinson's disease; oligomer; heme; inhibitor

Introduction

In neurodegenerative diseases, including Alzheimer's disease (AD), Parkinson's disease (PD), and prion diseases, proteinaceous deposits, comprised of fibrils with a common β -sheet amyloid structure, have been found in the brain¹. These deposits result from misfolding of the disease-related protein, which undergoes a conformational change from its initial structure to a cross- β sheet amyloid fold². In patients with PD, and to a lesser extent AD, the deposits contain aggregates of the protein α -Synuclein (α Syn)³. Conformational changes of α Syn from random coil to a β -pleated sheet structure lead to the formation of insoluble α Syn fibrils that are the building blocks of the pathological inclusions^{4, 5}.

Recent evidence implicates pre-fibrillar structures common to many disease-related proteins as the most toxic forms of these proteins, supporting the notion that the insoluble amyloid deposits may be either a less toxic dead-end species, or a beneficial storage mechanism to quarantine damaged proteins⁶⁻¹¹. It has been observed that a collection of amyloid forming proteins, including α Syn, can undergo a supramolecular assembly, and in reconstituted membranes they can form morphologically comparable ion-channel-like structures that elicit ion-channel currents¹¹. It is plausible that these ion channels could destabilize cellular ionic homeostasis and thus be the common structure of these proteins that induces cell pathophysiology and degeneration in amyloid diseases. Further, the link between α Syn fibrillization and PD neurotoxicity may involve inappropriate membrane permeabilization by a prefibrillar species^{12, 13}. In aqueous solution, α Syn is a natively unstructured protein. Recent studies suggest that it may exist as a tetramer physiologically^{14, 15} but these conclusions have been called into question¹⁶. Upon interaction with membranes or micelles, two regions of α -helical structure form, while the C-terminal region remains unstructured¹⁷⁻²⁰.

It has been reported that heme (Fig. 1A), and porphyrin, interactions with α Syn may play a role in its pathophysiology and can be inhibitors of fibril formation with IC₅₀ values in the low micromolar range²¹⁻²³. Biochemical analysis revealed the formation of soluble oligomeric α Syn when incubated with heme, suggesting that this may be the mechanism by which filament formation is inhibited²¹. Unlike α Syn filaments and protofibrils, these soluble oligomeric species did not reduce the viability of SH-SY5Y cells. These findings suggest that the soluble oligomers formed in the presence of various inhibitory compounds may not be toxic to neuronal cells and that heme and related compounds may therefore have therapeutic potential for α -synucleinopathies and other brain amyloidoses. A study of the generic inhibitory properties of hemin on protein misfolding found that hemin could prevent fibril formation for kappa-casein, amyloid β -protein and α Syn, but could not disaggregate preformed fibrils²⁴. Lastly, it was found that heme did not inhibit filament formation of α Syn truncated by 20 amino acids (1-120) indicating that the C-terminal region is critical for the inhibitory interaction²¹.

In this work, we examined the structural and kinetic nature of the interaction of α Syn and heme (heme B) to determine the mechanisms by which heme alters oligomerization and aggregation. We found that heme interaction with α Syn leads to the formation of unique low molecular weight structures that could play a protective role in neurodegenerative disease.

Experimental Procedures

Purification of α Syn

The cDNA of Human α Syn (NACP140, Clone ID IOH14008 from Invitrogen) was inserted into a Glutathione S-transferase (GST) pGEX-4T-1 vector. The protein is expressed as a GST fusion protein and includes a Thrombin cleavage site that allows for purification of human α Syn. This plasmid was transformed into BL21 (DE3) *Escherichia coli* (*E.coli*). The protein was over-expressed by induction with isopropyl-beta-D-thiogalactopyranoside (IPTG, Sigma) After induction with IPTG, the cells were incubated at 30 °C for 14–16 hours before sonication in the presence of a protease inhibitor cocktail and centrifuged twice to remove cell debris. The remaining supernatant was subjected to ammonium sulfate precipitation up to 40%, over a one-hour period. After centrifugation, the resulting pellet was re-suspended and centrifuged again to remove any small particles. The supernatant was then filtered and applied to a Glutathione Sepharose column (GE Healthcare) with a flow rate of ≈ 0.2 ml/min. The bound fusion protein was then washed with ten column-volumes sodium phosphate buffer, pH 7.6. α Syn was cleaved from the column-bound GST with Thrombin overnight at 4 °C. This produced wild-type α Syn, except for the addition of Gly-Ser at the amino terminal end, which remains from the Thrombin cleavage site. The Thrombin was removed from the mixture by directly connecting a Benzamidine Sepharose column to the Glutathione sepharose column, which binds trypsin-like serine proteases. The purified α Syn was collected, and then concentrated and immediately stored in liquid Nitrogen.

It has been observed that $\approx 20\%$ of the bacterially expressed human α Syn can be mistranslated in *E.coli* and that a cysteine residue is incorporated at position 136 instead of a tyrosine²⁵. There are no native cysteine residues in α Syn, and it was observed that this misincorporation resulted in higher levels of dimeric α Syn due to disulfide bond formation. To avoid potential artifacts resulting from this misincorporation, using the Stratagene Quick-change site-directed mutagenesis kit we made this corrective mutation to the codon for tyrosine 136 from TAC to TAT. This mutation has been shown to result in reliable translation of tyrosine 136²⁵. All α Syn purification is from the Y136-TAT construct, and is also referred to as α Syn. All experiments were carried out in phosphate buffered saline (PBS), with 30 mM sodium phosphate and 150 mM NaCl and pH 7.6, unless otherwise specified.

Fibril formation

90 μ M samples of α Syn were incubated with or without 90 μ M heme at 37 °C with 250 rpm shaking for 115 hours. In one experiment 0.02% NaN_3 was added to samples to ensure that there was no bacterial growth over the course of the experiment, and was confirmed not to alter the results. Heme B (Frontier Scientific, Logan, UT) was prepared as a 1 mM stock in

10 mM NaOH, and diluted in PBS pH 7.6 to the appropriate concentration before each experiment. An equivalent amount (final concentration 900 μ M) of NaOH was added to the sample without heme to ensure identical conditions with and without heme. Aliquots were removed at various times and stored immediately in liquid nitrogen.

Thioflavin T Fluorescence

Immediately before measuring the ThT spectrum, 7 μ l of 90 μ M α Syn was added into 1.793 ml of 50 mM Tris-HCl at pH 8.2 and finally 200 μ L of 100 μ M ThT was added for a final volume of 2mL. The final concentrations were 315 nM α Syn, and 10 μ M for ThT. Spectra were acquired with an excitation of 446 nm (5 nm slits widths) from 460 nm to 700 nm at intervals of 1 nm with an integration time of 2 seconds. The fluorescence intensity of the emission spectrum was plotted at 490 nm.

Atomic Force Microscopy—AFM images were obtained with a Nanoscope IIIa (Digital Instruments, Santa Barbara, CA) in tapping mode on freshly cleaved mica substrates at a resonance frequency of about 280 kHz. The scan rate was kept in the range of 0.8–2.0 Hz with 512 lines. A detailed structural analysis was performed using atomic force microscopy in non-contact “tapping” mode (AFM) and forces on the sample were limited to < 2.8 N/m as dictated by the spring constant of the tip (PPP-FMR tips from Nanosensors). The typical tip radius is less than 7 nm. The samples for AFM investigation were identical to those described under “fibril formation” above. One drop of sample was placed on a freshly cleaved mica surface, and dried at room temperature and subsequently washed two times with water, and finally wicked off with filter paper. Analysis of structures was performed using WSxM software²⁶ by measuring the average height of the cross-section of the structure of interest.

Transmission Electron Microscopy—Negative stain images were obtained with a JEOL 100CXII, or 1200EX at 80 KV. The samples for TEM investigation were identical to those described under “fibril formation” above. Samples were adsorbed onto carbon / formvar coated 300 mesh copper grids after glow discharge, and stained with 1% Uranyl Acetate.

Native Gel PAGE Western Blot—Samples were mixed with native sample buffer and loaded onto a 10% Tris-HCl poly-acrylamide gel (Bio-Rad), run at 140 V followed by overnight transfer to PVDF membrane at 40 V. Mouse Monoclonal antibody against α Syn from Zymed Laboratories was used (Syn211) in conjunction with HRP-anti-Mouse IgG (eBioscience) at 1:1000 for detection.

Circular Dichroism measurements

α Syn was prepared as described above with a 90 μ M final protein concentration. Circular dichroism spectra were collected on a JASCO J-815 CD spectrometer. Data were collected at room temperature in a 0.1 cm semimicro quartz cuvette (Hellma). Spectra were measured from 250 to 190 nm with a step size of 0.1 nm, a bandwidth of 1 nm, a response time of 4 sec, a 20 nm/min scan speed. For all spectra, an average of 2 scans was obtained. CD spectra of the appropriate buffers were measured, and found to contribute negligibly. Component

analysis was carried out using CDPro based on CONTINLL and CONTIN3, from Provencher and Glockner (1981)²⁷.

Results

Heme modifies amyloid fibril formation of α Syn

Thioflavin T (ThT) was used to quantify the amyloid fibril formation of α Syn and to determine the impact of heme on the rate of fibril formation. α Syn was incubated with or without equimolar heme, 90 μ M each, at 37 °C with agitation for 115 hours. ThT fluorescence at 490 nm, as a function of incubation time, is plotted in Fig. 1B. In the absence of heme, aggregation follows the expected nucleation dependent fibril formation kinetics with a lag-phase of 4 hours followed by rapid growth of amyloid fibrils²⁸. In the presence of heme the duration of the lag phase increased to ~15 hrs and the plateau level was reduced. The final intensity of the ThT fluorescence in the presence of heme is less than 10% of the intensity in the absence of heme. As reported by others, the ThT emission spectrum was not affected by the presence of heme (data not shown)²⁹.

Distinct α Syn intermediates are identified by Native-PAGE in the presence of heme

To determine the distribution of oligomer sizes formed during aggregation of α Syn, native (non-denaturing) polyacrylamide gel electrophoresis (native-PAGE) analyses were performed on samples with and without heme. α Syn was detected by western blot with the antibody Syn211. We observed that Syn211 was able to detect α Syn in native, oligomeric and fibril conformations. When α Syn (14kDa) was examined by SDS-PAGE we observe a single band that migrated to approximately 17 kDa (Fig. 2A), as reported by others³⁰.

In contrast to the SDS-PAGE analysis, native-PAGE results showed that prior to incubation ($t = 0$) α Syn appears as a doublet, both with and without heme, displaying an apparent molecular weight of ~60 kDa as shown in Fig. 3. We propose that the doublet represents two related conformations³¹, or two similar conformations that differ in their charge. It is also noteworthy that both in the absence and presence of heme, the higher migrating species diminished faster than the lower component. When the protein is run on SDS-PAGE (Fig. 2A) a doublet is not observed, suggesting that these species have the same molecular weight. The apparent molecular weight of ~60 is very interesting. Recent sedimentation equilibrium analytical ultracentrifugation (SE-AUC) and nuclear magnetic resonance (NMR) data both suggest that α Syn exists as a physiological tetramer of ~57 kDa, and directly correlate with a gentle purification scheme when produced recombinantly^{14, 15}. Our purification scheme indeed avoided boiling and denaturation; thus the predominant band we observe at 60 kDa may be a stable tetramer. However, the assignment of a band ~60 kDa as a tetramer has been called into question³². It has been reported that monomeric α Syn appears substantially larger when eluted from a gel-filtration column, equivalent to a molecular mass of 51 kDa³³. More recently, Fauvet et al. pointed out that molecular weight estimations from native gels are unreliable³². They reported that α Syn from the brains of several species eluted at the same apparent molecular weight as the unfolded monomeric α Syn. On this basis, we refrain from assigning the 60 kDa band as a monomer or a tetramer and just label it as the “60kDa” band.

At all times subsequent to the initial measurement, there are significant differences between α Syn incubated with or without heme as may be seen in Fig. 3. The α Syn without heme displayed a band at the bottom of the sample-well after 4 hours incubation. This is consistent with the 4 hour lag phase which was determined by ThT fluorescence and thus may correspond to aggregated protein fibrils too large to enter into the gel matrix. Concurrently, diffuse staining at the stacking and running gel interface is observed, indicating that specific oligomers are not preferentially formed. At aggregation times longer than 65 hours, the upper band of the initial species doublet appears to decrease in intensity earlier than the lower band, and finally at 136 hours the upper band of the two is no longer observed.

In contrast, in the presence of heme after 2 hours, in addition to the initial 60 kDa band, a band at approximately 80 kDa appears, which we attribute to a stable oligomer of α Syn (Fig. 3). At each increasing time-point examined in the presence of heme, distinct bands of increasing size are populated, displaying a clear ladder of oligomers. Based on the MW marker, stable distinct oligomeric intermediates in the presence of heme are roughly 40, 60, 80, 125, 170, and 220 kDa, in addition to protofibrillar and fibrillar structures eventually formed. These sizes suggest a range of oligomers varying in size from a few to several monomeric units. We hypothesize that the disperse bands in the range below 60 kDa may represent heme bound species, with an altered migration due to the charged heme. Interestingly, we observe the disappearance of the upper band of the 60 kDa doublet after the 28 hour time-point, and the eventual decrease in intensity of the lower band, although it persists through 136 hours. The overall intensity of the remaining lanes appears less intense after the 28 hour lane, presumably due to blotting defects, aggregates which adhere to the sample tube, or chemiluminescent detection artifacts.

The large differences observed between the stable species populated with heme, compared to those without heme, could result from a kinetic difference in which similar oligomeric states are transiently populated during the early steps of aggregation without heme. To address this possibility, the aggregation of α Syn at times less than 4 hours, in the absence of heme, was examined by native-PAGE (Fig. 2B). The samples were taken at 15 or 30 minute intervals between 0 and 4 hours. The predominant doublet at 60 kDa, was detected at each time-point through 4 hours of aggregation without the presence of any higher oligomeric state of α Syn. We observed a band at 40 kDa present throughout 4 hours of aggregation, which can also be seen in Fig. 2 in the presence of heme. Based on this data we conclude that the ladder of increasing larger oligomeric species is not populated in the absence of heme, or alternately, not stabilized long enough for detection.

Transmission electron microscopy determination of structure of α Syn aggregates

Negative stain TEM was performed to determine the morphology of the aggregates. After 1 h of aggregation in the absence of heme, the primary species observed were 10–20 nm diameter spherical oligomeric aggregates along with some larger structures ranging from 30 to 80 nm (Fig. 4A). At incubation times 6, 12, 20, and 54 hours, increasing amounts of typical amyloid fibrils were observed, (Fig. 4B – E.) These amyloid fibril structures of α Syn had lengths up to 1 μ m, and were between 15–40 nm wide, some with twisted morphology. In contrast, the aggregation process in the presence of heme is very different. Even at 12

hours of incubation no fibril structures are observed. Instead, as shown in Fig. 5A, the sample is composed of small non-fibrillar structures of varying sizes ranging from 10 nm to 30 nm, likely corresponding to oligomeric structures, consistent with the presence of a ladder of oligomeric structures in the native-PAGE experiments. At 54 hours (Fig. 5B) round oligomeric morphologies are observed in addition to fibrils. These data are representative of two different experiments, and indicate that the presence of heme slows and inhibits α Syn fibril formation and concomitantly stabilizes pre-fibrillar oligomeric intermediates.

Atomic force microscopy of pre-fibrillar α Syn intermediates reveals annular structures

In order to further characterize the structures observed from TEM and native-PAGE experiments, structural analysis using atomic force microscopy (AFM) was performed. Aliquots from incubating samples were removed at the indicated times and then adsorbed onto freshly cleaved mica. As shown in Fig. 6A, at 2 hours incubation, the samples are comprised of prefibrillar oligomeric structures and a small population of annular protofibrils (arrows), with typical annular structures displaying a central pore. Two examples of annuli are shown enlarged in Fig. 6B. The dimensions of the annular structures were individually analyzed using the WSxM software²⁶. The average height of the annular structures was 18 nm, based on images from multiple areas of the 2 hours sample. The height ranged from 10 to 25 nm. A histogram of the individual annular heights is shown in Fig. 6C (n= 16). Due to sample-tip convolution during AFM, lateral distances cannot be measured as accurately as height³⁴; however, the apparent diameter is on the order of 60 nm. The ability of the AFM tip to resolve a distinct central porelike feature may depend on experimental conditions such as tip morphology, or tip contamination from sample. At later aggregation times a large number of typical amyloid fibrils are present, but there are no observable annular structures (Data not shown).

In comparison, a 2-hour incubation with heme results in the formation of annular structures and amorphous structures (Fig. 6D). Two examples of annular structures are shown in Fig. 6E. In the presence of heme, annular structures are observed through all times measured (102 hours). A histogram of the heights of annular structures thus includes annular structures from 2 – 102 hours. The average height of these structures was measured and calculated to be 0.9 nm, ranging from 0.5 to 3 nm, with an apparent diameter of 10 nm, Fig. 6F, (n= 87). The difference in height between annular structures formed without heme and with heme is nearly a factor of 20, (0.9 nm with heme and 18 nm without), while the apparent diameter is approximately 6 times smaller (10 nm with heme and 60 nm without).

Heme alters the progression of secondary structure changes during aggregation

To determine the secondary structures formed prior to amyloid fibril formation, we measured the circular dichroism (CD) spectrum of α Syn in the absence and presence of heme during the aggregation time-course. CD spectroscopy is a valuable tool for characterizing the secondary structures present, although large amyloid fibrils are likely to scatter light rather than contribute to the spectral signal³⁵. At the start of the time-course in the absence of heme, the CD spectrum of α Syn displayed the expected shape for unstructured or random coil protein with a minimum in the ellipticity at 202 nm (Fig. 7A).

Between 0 and 8 hours there was a gradual increase in the ellipticity at this wavelength, with the concomitant development of a minimum at about 222 nm, suggesting that there may be some contribution of α -helical content prior to the formation of the predominant β -sheet structures. This observation is consistent with a mechanism whereby prefibrillar structures of many types form prior to the rearrangement into classical amyloid anti-parallel β -sheet conformations^{36, 37}. By 28 hours the ellipticity below 200 nm became positive and the feature at 222 nm shifted to 218 nm corresponding to an increasing contribution from β -sheet secondary structures. From 52 hours until 136 hours the secondary structure indicated by the CD spectrum showed little change. These spectra had a minimum at 218 nm, a shoulder close to 210 nm, and a positive peak below 200 nm. This overall shape is consistent with a dominant β -sheet secondary structure, with a minor contribution of α -helical content.

In comparison, in the presence of heme, we observed the expected shape for an unstructured or random coil protein with a minimum at 202 nm (Fig. 7B) This spectral shape remained nearly unchanged, except for a minor increase in ellipticity of the 202 nm feature, through 8-hours of aggregation. By 28 hours the intensity below 200 nm increased to zero, while a double minimum in ellipticity at 210 and 220 nm appeared. This overall spectral shape, which was stable until the end of the time-course (136 hours), is consistent with a dominant α -helical secondary structure, with a minor β -sheet contribution. Thus, in the presence of heme, α Syn remained unstructured for a longer period of time, and displayed more α -helical content and less β -sheet by 136 hours in comparison to α Syn without heme.

To approximate the secondary structure composition of each CD spectra, we carried out component analysis using CDPro³⁸ (Supplemental Fig. S1). We used the CONTIN/LL and CONTIN3 basis spectra, from Provencher and Glockner (1981)²⁷. While the values for α -helical, β -sheet and statistical coil structure may not be accurate in the absolute sense, one can see that the relative content of these forms of secondary structure are consistent with that observed above. Particularly, in the presence of heme, more unordered structure, 37% at 136 h, remains as compared to 32% without heme. The most striking difference is that the dominant secondary structure remains “unordered” at all aggregation times in the presence of heme, whereas α Syn converts to β -sheet as the predominant structure after 52 h.

Permeation of large unilamellar vesicles

Based on the structural differences of the oligomers formed by α Syn compared to the smaller structures we observed in the presence of heme, and the potential physiological implications of these findings, we sought to determine if there were differences in the ability of these structures to disrupt the membrane of vesicles. To do so we prepared calcein containing large unilamellar vesicles (LUV) of 100 nm diameter, comprised of 1,2-dioleoyl-sn-glycero-3-phospho-(1'-rac-glycerol) (DOPG) using a slightly modified previously published protocol³⁹. The calcein contained inside the vesicle self quenches, and when released, by disruption of the containing membrane, can be detected by fluorescence using an excitation wavelength of 490 nm, and an emission wavelength of 520 nm. 10 μ M α Syn or α Syn with equimolar heme at T= 24 h was added to the LUVs in a 96-well plate. Triton X-100 was used to determine 100% calcein release, and the results were normalized to this

value. After 2 hours incubation α Syn caused 22% calcein release, while α Syn +heme caused 25% calcein release. Buffer, or heme alone, caused a nearly identical calcein release of 8.5% (Supplemental Figure S2).

Discussion

The experiments reported here demonstrate that the addition of heme to α Syn stabilizes structures which we do not observe in the absence of heme, and has a dramatic effect on the oligomerization/aggregation as summarized in Table 1. Specifically, the presence of heme extends the lag phase for fibril formation and reduces the total amount of fibril formation by an order of magnitude. In addition to the decrease in amyloid fibril formation, in the presence of heme, the morphology of the oligomers of α Syn that are formed is dramatically different consisting of short non-fibrillar structures. Multimeric prefibrillar structures can be detected only in the presence of heme, demonstrating a greater degree of stability for low molecular weight oligomeric species. Also noteworthy is that the secondary structure analysis revealed a greater α -helical content of the small oligomers in the presence of heme, in contrast to the dominant β -sheet conformation seen in its absence. In summary, the presence of heme appears to stabilize relatively small oligomeric α -helical structures that do not readily convert to the typical fibrillar structures associated with the pathology of α Syn. It is interesting to consider the unique insight provided by the techniques used herein, and piece together the information they provide to develop a complete picture of the system under study. Native-PAGE indicated that a series of oligomers of increasing size are present during aggregation with heme, however by AFM we observed relatively uniform annular structures. The various oligomers observed by native-PAGE could account for the large amount of small amorphous material seen by AFM. Similarly, TEM and AFM suggest annular structures are formed in the absence of heme, which could account for the high molecular weight material caught at the well-bottom as seen by native PAGE.

Many different forms of α Syn oligomers have been reported in the past with vastly different morphologies ranging from small spherical structures, to annular structures of varying sizes, to long protofibril chains to fibrous tangles⁴⁰, each dependent on the conditions under which the oligomerization studies were carried out as well as the incubation time. Annular structures with diameters of 18–27 nm and 3 nm height have been observed upon interaction with vesicles and annular structures ranging in size from 30–180 nm in diameter and 3–10 nm in height have been observed under a variety of conditions^{6, 11, 13, 40–42}. Our observations of large size annular structures (~18 by ~60 nm in height and apparent diameter, respectively) in the absence of heme are consistent with prior observations, but the smaller structures in the presence of heme (~1 by 10 nm in height and apparent diameter, respectively) indicate the strong effect of the heme on the structure. On the other hand, it is noteworthy that the A30P and A53T mutants which are associated with early onset Parkinson's disease also formed small annular structures 10–12 nm in diameter⁴³. Annular structures have been examined for their ability to interact with membranes and form channels, and indeed, exhibit ion currents^{11, 44}.

The size and morphology of the α Syn oligomers have been postulated to be a critical factor in its pathophysiology. An “amyloid pore” hypothesis has been proposed, in which an

oligomeric ring structure of an amyloid forming protein forms a toxic pore by incorporation into neuronal membranes. This mechanism is similar to bacterial pore-forming toxins⁴⁵, and allows the flow of molecules or ions across the membrane^{11, 46}. The unregulated movement of ions or other molecules across the neuronal membrane would disrupt their critical levels on both the inside and outside of the cell, and in turn trigger neuronal cell death. The formation of pores by A β and α Syn was found to be accelerated by mutations associated with familial Alzheimer and Parkinson diseases, respectively, suggesting that their formation is related to pathogenic activity⁶. In contrast, recent work attempting to understand the mechanism of toxicity of the α Syn pore came to the conclusion that leakage effects may in fact be caused by bilayer defects due to membrane instability, and not a pore-type mechanism⁴⁷. Our data shows that structures formed after 24 h by α Syn or α Syn with heme, each disrupt vesicles to a similar extent, suggesting that the size of the structure formed may not be the crucial factor in membrane disruption. There are a number of reasons only a slight difference in ability to disrupt the vesicle membrane between α Syn structures with and without heme was observed. Firstly, the membrane composition may not serve as a fair representation of the physiological membrane, or even a membrane favorable for α Syn interactions. Others have shown α Syn interacts with small unilamellar vesicles comprised of POPC/POPA or DOPC:DOPS:DOPE²³. The size of the vesicles used in our study may also have been a factor. Further, the ability of α Syn structures to interact with a membrane surface may depend on structure formation in the presence of that membrane, and structures formed *in vitro*, and added later may behave differently.

On the basis of the new evidence reported here, we propose a model (Fig. 8) for the altered aggregation structure and dynamics of α Syn induced by the presence of heme. In this model the low molecular weight oligomers are stabilized by interaction with heme, and the formation of larger fibril nuclei that lead to protofibril formation is effectively blocked. Consequently, the formation of the large amyloid fibril aggregates is inhibited or at least greatly retarded. Although we observed a similar level of membrane disruption for the structures formed in the absence or presence of heme, the structures may behave differently *in vivo*.

This model is strongly supported by our AFM data, which reveal that in the presence of heme, annular structures of α Syn were preferentially formed, showing that the presence of heme diverts the aggregation to form oligomeric structures (annular and amorphous), and not fibrils. Moreover, the size of the annular structures stabilized by heme is significantly smaller in width and height (≈ 10 nm by 1 nm) than the annular structures formed in the absence of heme (≈ 60 nm by 18 nm). Annular structures were infrequent in the absence of heme, observed only at 2 hours, and were not observed by AFM past 2 hours, likely because of their low relative abundance in the sample, or differential adsorption onto the mica surface. Furthermore, consistent with native-PAGE data showing oligomers throughout the time-course in the presence of heme, AFM data also confirm the presence of annular structures through the longest time point measured, 102 hours. We found that their size is constant, as shown by analysis of 87 individual annular structures in Fig. 6F.

Using a sphere as a model for a monomeric unit, one can arrange 5 units into a “pentamer” of roughly pentagonal shape, or 6 units into a “hexamer” of roughly hexagonal shape. With

these shape scaffolds, we can estimate that a monomer with a postulated size of ~3.3 nm, would form a pentamer configuration with an outer diameter of ~8 nm, and inner diameter of ~1 nm, whereas a hexamer configuration would have an outer diameter of ~10 nm, and inner diameter of ~1.5 nm. Based on the annular structures' size as measured by AFM, the structures may be composed of six monomeric subunits. These postulated structures are consistent with molecular modeling studies by Masliah and coworkers, who reported that pentameric and hexameric conformations of the α Syn multimers could form on the membrane^{13, 48}.

In addition to the effect of heme on α Syn, the pathophysiology of AD may also be affected by the interaction of heme (Fe^{2+}) and/or hemin (Fe^{3+}) with the amyloid β -protein ($\text{A}\beta$)^{29, 49, 50}. After incubation with heme, monomers and small $\text{A}\beta$ aggregates (e.g. tetramers and trimers) were stabilized according to SDS-PAGE, whereas in the absence of heme only high-molecular-weight $\text{A}\beta$ aggregates were detected⁵⁰. Furthermore, the binding of $\text{A}\beta$ to heme is believed to cause heme deficiency in cells, resulting in many of the key cytopathologic characteristics of AD, including selective loss of mitochondrial complex IV, mitochondrial dysfunction, loss of iron homeostasis, and increased production of hydrogen peroxide^{51, 52}. It has been observed that the $\text{A}\beta$ -heme complex exhibits peroxidase activity, potentially catalyzing the oxidation and depletion of various neurotransmitters⁵⁰.

The impact of the α Syn interaction with heme described in this work opens exciting prospects for future experiments. Indeed, there is great interest in inhibitors of amyloid toxicity. Previous reports have examined small molecule inhibitors, and also found inhibition of fibril formation, stabilization of oligomers, and reduction in toxicity (baicalein⁵³, EGCG³³, gallic acid⁵⁴). Our study, combined with the results of previous work, suggests that heme may be able to slow fibril formation and dismantle amyloid aggregates^{21, 50, 55}. If, in fact, *in vivo* annular structures are cytotoxic, the ability of heme to stabilize different annular structures that are much smaller, could prove beneficial for PD, and other neurodegenerative diseases.

Conclusions

The data reported here demonstrate that heme can alter the progression of α Syn aggregation, apparently by stabilizing distinct small oligomeric conformations, thereby inhibiting the aggregation process. The presence of heme promotes the formation of annular structures, similar to those that are formed most rapidly by pathogenic mutations of α Syn⁶. As heme is available under physiological conditions with cellular levels approximately 100 nM⁵⁶, the possibility exists that it could interact with α Syn. However, whether the small oligomeric structures and the annular structures formed in the presence of heme also form *in vivo*, are cytotoxic or whether retardation of the formation of larger oligomeric species is protective against PD, all remains to be determined.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

PD	Parkinson's disease
AD	Alzheimer's disease
αSyn	α -Synuclein
Aβ	Amyloid β
ThT	thioflavin T
AFM	atomic force microscopy
TEM	transmission electron microscopy
CD	circular dichroism
LUV	large unilamellar vesicle
DOPG	1,2-dioleoyl-sn-glycero-3-phospho-(1'-rac-glycerol)

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Figure 1.

A) Molecular structure of heme B. B) Time course Thioflavin T (ThT) fluorescence, excited at 446 nm, in the absence and presence of heme with α Syn. 90 μ M α Syn was incubated at 37 $^{\circ}$ C with 250 rpm shaking over a period of 115 hours in PBS pH 7.4. In the absence of 90 μ M heme there is an increase in the fluorescence at 490 nm with increasing incubation time, corresponding to the formation of amyloid structures. In the presence of heme, there is an extension of the lag phase, as well as an overall decrease in ThT fluorescence intensity.

Figure 2. PAGE of α Syn. A, SDS-PAGE of α Syn at time 0; the protein runs as a single band at ~17kD. B, Native PAGE of α Syn at time 0 to 4 hours; no change is observed in the size or oligomer distribution of α Syn.

Figure 3.

Native-PAGE of α Syn from 0 to 136 hours of incubation in the absence and presence of heme. During the aggregation in the absence of heme the initial species of α Syn forms protofibrillar and fibrillar structures after 6 hours of aggregation as shown on the left of the figure. In contrast, in the presence of heme, a ladder of increasing sized oligomeric structures is resolved on the gel. The results presented are representative of three different experiments. On the left of the figure is the position of marker proteins, while the right shows MW calculated based on the relative migration compared to the marker.

Figure 4.

Transmission electron micrograph of negatively stained α Syn at various stages of incubation. A) Small spherical oligomers of 10–15 nm are observed to be the primary species at this T=1 h, scale bar =100 nm, arrows indicate individual oligomers, inset scale bar =30 nm. B) 6 hours, C) 12 hours, D) 20 hours, E) 54 hours. Small spherical oligomers are observed during the initial stages of aggregation. Primarily fibrils exist after 30 hours. Large scale bar = 200 nm, inset scale bar = 30 nm.

Figure 5. Transmission electron micrograph of negatively stained α Syn in the presence of heme at A) 12 hours and B) 54 hours. Small spherical oligomers, which are ≈ 15 nm in width, are observed throughout the aggregation time-course, before the formation of fibrillar structures. Large scale bar = 200 nm, inset scale bar = 30 nm.

Figure 6.

Atomic Force Microscopy of α Syn aggregates. A) Two hour incubation of α Syn showing prefibrillar oligomeric structures and annular protofibrils (arrow heads), B) Typical annular structures, note the scale bar is 59 nm. C) Histogram displaying the average height of 18 nm for annular structures formed at 2 hours of aggregation, (n=16). D) Two hour incubation of α Syn in the presence of heme, showing prefibrillar oligomeric structures and annular protofibrils (arrow heads), E) Typical annular structures, note the scale bar is 10 nm. F) Histogram of the heights of annular structures formed in the presence of heme throughout the aggregation time-course, showing an average height of 0.9 nm (n=87).

Figure 7.

Circular Dichroism spectra showing the secondary structure changes of α Syn during the time-course of aggregation. A) Spectral changes at the following times: 0, 4, 8, 28, 52, 100, 136 hours, displaying a transition from unstructured to primarily β -sheet structure with a minimum at 218 nm. B) Spectral changes of α Syn in the presence of heme, at the identical time-points as in A, showing a secondary structure transition from unstructured to partially structured combination of α -helical and β -sheet structure at the end of the aggregation time-course.

Figure 8.

Postulated model for amyloid fibril formation in the presence of heme. Heme (red squares) directly interacts with α Syn stabilizing oligomeric structures (red box) and inhibits the further aggregation into fibrillar structures. Low molecular weight oligomers are particularly stabilized by interaction with heme, reducing the chance of nuclei formation, and shifts the equilibrium away from fibril formation. The annular structures stabilized by heme are 10 nm in apparent diameter, whereas the annular structures typically formed are on the order of 60 nm.

Table 1

Comparison of α Syn structures observed by ThT Fluorescence, native PAGE, TEM, AFM, and CD spectroscopy in the presence and absence of heme.

Technique	α Syn	α Syn + Heme	Comments
ThT Fluorescence	4 hrs: lag phase 4-120 hrs: Strong fluorescence	15 hrs: lag phase 15-120 hrs: Weak fluorescence	Heme extends lag phase and has reduced aggregate formation
Native PAGE	0 hrs: Doublet near 60 kDa. 4 hrs: Aggregation; No defined oligomers. 136 hrs: high MW component of doublet disappears	0 hrs: Doublet near 60 kDa. 2 hrs: Stable oligomer (80kDa) detected. 4-100 hrs: Multiple oligomers detected. 28 hrs: High MW component of doublet disappears	Heme results in the formation of stable low molecular weight oligomers, which have long time stability.
TEM	1 hr: 10-80 nm structures detected. 6 to 54 hrs: Amyloid fibrils grow in.	12 hrs: 10-30 non-fibrillar structures detected; no fibrils. 54 hrs: Round oligomeric structures and fibrils detected.	Heme leads to the formation of stable round oligomer structures.
AFM	2 hrs: Prefibrillar oligomers and annular structures 18 nm in height. 90 hrs: Amyloid fibrils and no annular structures.	2 hrs: Amorphous structures and annular structures 0.9 nm in height. 102 hrs: Annular structures observed at all times measured.	Annular structures are formed in both the presence and absence of heme but they have very different sizes.
CD	0 hrs: Random coil 0 – 8 hrs: α -helical development 28 hrs: Conversion to β -sheet	0 hrs: Random coil 0 – 8 hrs: No changes 28 hrs: α -helix emerges	In the absence of heme β -sheet structure develops but in the presence of heme α -helical structure is formed.