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# Role of Different Regions of α-synuclein in the Interaction with the Brain Fatty Acid DHA

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## Abstract

α-Synuclein is a protein involved in Parkinson's disease. Its interaction with brain polyunsaturated fatty acids is attracting a strong interest. Previously, we investigated the interaction of α-syn with docosahexaenoic acid (DHA), and a mutual effect between the monomeric protein and the fatty acid was observed. α-Syn acquires an α-helical secondary structure with a rapid equilibrium between its free and the lipid bound form. In the α-helical form the protein segment 73-102 is flexible and is not characterized by a persistent structure. DHA forms oil droplets in the presence of the protein. To investigate at molecular level the involvement of different regions of the protein in the interaction with DHA, truncated protein species were prepared (syn1-99, syn1-52, syn57-102 and syn108-140) and their ability to interact with DHA was analyzed by circular dichroism and proteolytic mapping. CD data showed that all the peptides, except that corresponding to the C-terminal region, are able to acquire alpha-helical structure in the presence of DHA. The different molar ratio polypeptide/DHA necessary to reach the maximum folding and the different final helix content between the two N-terminal peptides (syn1-99, syn1-52) and the NAC peptide (syn57-102) suggest that the sequence repeats plays an important role in the α-helix transition and thereby in the interaction with DHA. The proteolysis experiments showed that all the truncated species are resistant to proteolysis in the presence of saturating concentrations of DHA, indicating that almost all the residues are engaged in the interaction with the lipid. The C-terminal region, at variance, seems only to modulate the portion of the molecule buried into the lipid compartment. Moreover the ability of the peptides to affect the self-assembly of the fatty acid was also analyzed showing that the peptides that interact also change the physical state of DHA, by the positive charged N-terminus.

**Keywords:** α-Synuclein; Protein mapping; Docosahexaenoic acid; Circular dichroism; Pyrene fluorescence; Transmission electron microscopy

## Introduction

α-Synuclein (α-syn) is a 140 amino acid, natively unfolded protein. Its structure can be divided into three regions (Figure 1a): the N-terminus (residues 1-60), characterized by seven amphipathic repeats (KTKEGV), the hydrophobic central region, formed by residues 61-95 (Non Amyloid-b Component, NAC, residues 61-95), and the C-terminus (residues 96-140), which is rich in acidic residues and prolines. The protein is highly expressed in the presynaptic nerve terminals in central nervous system. It is the major fibrillar component of Lewy bodies, proteinaceous inclusions typical of Parkinson's disease (PD) [1]. Despite the evidence for a key function of α-syn in the onset of PD, there is little information about its physiological function in the brain, that is correlated with lipids and membranes, since this protein seems to modulate presynaptic vesicle pool size and vesicle recycling and to work as a SNARE-complex chaperone [2]. Indeed α-syn is principally described as a soluble cytosolic protein, but it was also found reversibly associated with membranes [3]. In the presence of membrane mimetic systems (SDS micelles, liposomes rich in acidic phospholipids), α-syn acquires an α-helical structure [3,4]. The interaction appears to be mediated by 6-7 imperfect repeats in its N-terminal region that are homologous to the α-helical region of apolipoproteins [5]. It was observed that the protein can interact also with fatty acids and that this interaction is favored with unsaturated and polyunsaturated fatty acids (PUFA) [6-8]. Also in this case the N-terminal region of α-syn seems to have a major role in the binding to PUFA [9]. Docosahexaenoic acid (DHA) is one of the main brain fatty acid and it has been found in high levels in brain areas containing α-syn inclusions in patients affected by PD [10,11].

Previously, we have studied the interaction between α-syn and DHA showing that there is a mutual effect between the protein and the fatty acid *in vitro* [12]. Upon binding to DHA, α-syn acquires an α-helical conformation and significantly modifies the physical state of DHA, reducing the size of the droplets and lowering its critical aggregative concentration. Proteolysis mapping of the protein-fatty acid complex was performed to identify the region(s) of α-syn directly involved in the interaction, demonstrating that the region 1-72 is strongly involved in the binding while the segment 73-102 is flexible and is not characterized by a persistent structure. We also found that DHA readily promotes α-syn aggregation and that the morphology of these aggregates is dependent on the ratio between the protein and DHA (De Franceschi et al. [13]). In the presence of a molar ratio protein/DHA of 1:10, amyloid-like fibrils were formed. At a protein/DHA molar ratio of 1:50, we observed the formation of stable oligomers that resulted toxic to dopaminergic cell line [13] and able to permeabilize model membranes [14].

Here, we have investigated the interaction of fragments of α-syn with DHA in order to analyze the involvement and the role of the different regions of the protein in the interaction with the fatty acid. To this aim fragments corresponding to different regions of the protein were studied: the truncated species syn1-99 that lacks the C-terminus but retains all the seven imperfect repeats; syn1-52 that lacks the NAC

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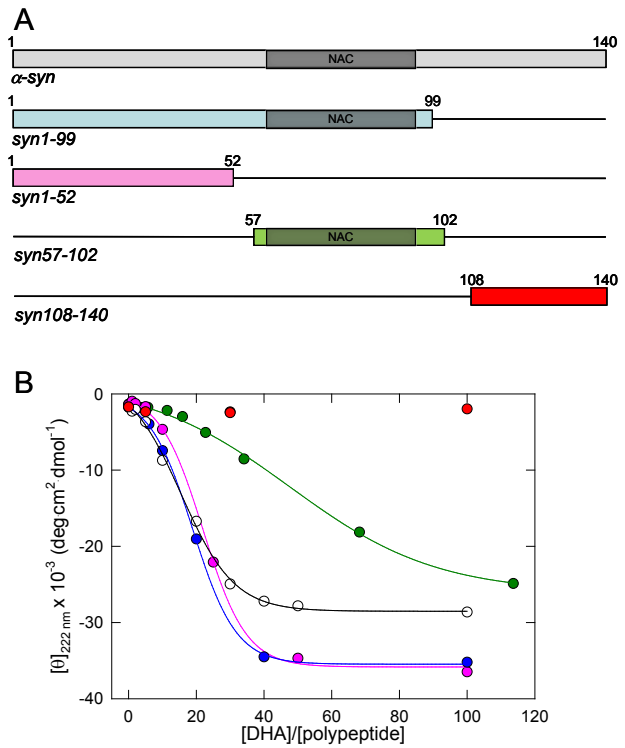


**Figure 1:** (A) Schematic representation of α-syn peptide species. The α-syn peptides herewith studied correspond to 1-99 (syn1-99, blue), 1-52 (syn1-52, pink), 57-102 (syn57-102, green) and 108-140 (syn108-140, red). At the N-terminal syn1-52 and syn57-102 have extra-sequence GSH and GSHM residues, respectively. (B) DHA-induced structural changes monitored by far-UV CD spectroscopy. (B) Ellipticity at 222 nm are plotted against the molar ratio between DHA and polypeptides. The analysis was conducted in PBS pH 7.4, using a peptide concentration of 5 μM. The titration curve of α-syn was also reported as reference.

region (61-95) and includes four imperfect repeats and the central polypeptide; syn57-102 that includes the NAC region and the last three imperfect repeats. The peptide syn108-140 that spans almost the whole acidic tail was also analyzed. These species were analyzed by circular dichroism to verify their ability to bind DHA. The complex peptide-fatty acid was mapped by proteolysis, considering that the peptide bonds hidden by fatty acids or engaged in the interaction are expected to be less prone to proteolysis. Moreover, we also analyzed the effect of the peptides on the aggregative state of DHA. It has confirmed that only the peptides corresponding to the N-terminal and the central part of α-syn are able to affect the physical state of the fatty acid.

## Materials and Methods

### Materials

Proteinase K from Tritirachium album and porcine trypsin were purchased from the Sigma Chem. Co. (St. Louis, MO). All other chemicals were of analytical reagent grade and were obtained from Sigma or Fluka (Buchs, Switzerland).

**Expression and purification of syn1-99, syn1-52 and syn57-102:** Syn1-99 cDNA was amplified by PCR with synthetic oligonucleotides (Sigma-Genosys) containing NcoI and XhoI restriction sites and designed to obtain the region coding for the first 99 amino acids of human α-synuclein (syn1-99). After digestion with restriction enzymes, the PCR product was subcloned into the NcoI-XhoI-linearized pET28b expression plasmid (Novagen) and introduced into an E. coli BL21(DE3) strain. Histidine-tagged syn1-52 and syn57-102 was produced as described in Bisaglia et al. [15]. The peptides were expressed in *E. coli* C41 cell line transfected with pET28b/syn1-52 and syn57-102 plasmids. Overexpression of the peptides was achieved by growing cells in LB medium (1% Bacto tryptone, 0.5% yeast extract, 0.5% NaCl) at 37°C to an OD600 of 0.6-0.8 followed by induction with 0.5 mM isopropyl β-thiogalactopyranoside for 4 h. For the purification of syn1-99, the cells were harvested by centrifugation and the pellet was resuspended in 30 ml 20 mM Hepes pH 7.0 per liter of LB medium. After adding 100 mM PMSF and protease inhibitors cocktails (Sigma), to have a final dilution of 1:100, the cells were lysated by sonication. After centrifugation, the soluble fraction was treated with 20-50% ammonium sulphate, the pellet was resuspended in 20 mM Hepes pH 7.0 and dialyzed overnight against water. The solution was centrifuged and filtered before loading it into a Resource S column (Amersham Biosciences) and then eluted using a NaCl gradient (from 0 to 300 mM). The peptides syn1-52 and syn57-102 were purified on a Cobalt-agarose resin (Clontech) using the manufacturer's recommended protocol. To eliminate the His-tag, the peptides were digested with thrombin (Amersham Pharmacia), using the manufacturer's protocol. The final products contain extra G-S-H and G-S-H-M sequence at the N terminus of syn1-52 and syn57-102, respectively. After cleavage, the His-tag was separated from the protein by HPLC purification using a Juppiter-$C_4$ column (4.6 × 150 mm) (Phenomenex, CA, USA) eluted with a linear gradient of acetonitrile (0.085 % TFA) versus water (0.1 % TFA), from 5% to 50% in 40 min at a flow rate of 0.6 ml/min. The identity of the eluted material were assessed by RP-HPLC-mass spectrometry.

**Chemical synthesis and purification of peptide syn108-140:** The peptide corresponding to residues 108-140 of α-syn was synthesized by the solid-phase Fmoc method [16] using an Applied Biosystems (Palo Alto, CA) peptide synthesizer (model 431A). Fmoc-protected amino acids were used with the following side chain protection: *tert*-butyl ether (tBu) for Tyr, *tert*-butyl ester (OtBu) for Glu and Asp, and trityl (Trt) for Asn and Gln. Deprotection of the Fmoc group, at every cycle, was obtained by a 10 min treatment with 20% piperidine in *N*-methylpyrrolidone. Chain elongation was performed using a 10-fold excess (0.5 mmol) of Fmoc- protected amino acid, 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate, and 1-hydroxybenzotriazole (1:1:1) in the presence of a 20-fold excess of *N,N*-diisopropylethylamine. After completion of the last cycle, the resin was washed with N-methylpyrrolidone and a dichloromethane/methanol mixture (1:1, v/v) and then dried *in vacuum*. The synthetic peptide was cleaved from the resin and deprotected by treatment of the peptide-resin with a 95:5 (v/v) mixture of TFA and 1,2-ethanedithiol for 2 h at 4°C. The resin was filtered, and cold diethyl ether was added to the solution to precipitate the crude peptide, which was recovered by centrifugation and purified by RP-HPLC using a Vydac C18 column eluted with a gradient of acetonitrile (0.085% TFA) versus water (0.1% TFA) (from 5 to 25% over 5 min and from 25 to 29% over 17 min).

### Circular dichroism

Peptide concentrations were determined by absorption measurements at 280 nm using a double-beam Lambda-20 spectrophotometer from Perkin Elmer (Norwalk, CT). The extinction coefficients at 280 nm were 1490 $M^{-1}$ (syn1-52, syn1-99), 4470 M-1 (syn57-102) as evaluated from its amino acid composition by the method of Gill and von Hippel [17]. The concentration of syn108-140 was calculated by monitoring sample absorbance at 205 nm [18]. Circular dichroism spectra were recorded on a Jasco J-710 (Tokyo, Japan) spectropolarimeter. Far-UV CD spectra were recorded using a 1-mm path-length quartz cell and a protein concentration of 5-15

μM. The mean residue ellipticity [$\theta$] (deg·cm$^2$·dmol$^{-1}$) was calculated from the formula [$\theta$]=($\theta_{obs}$/10) (MRW/$lc$), where $\theta_{obs}$ is the observed ellipticity in deg, MRW is the mean residue molecular weight of the protein, $l$ the optical pathlength in cm and $c$ the protein concentration in g/mL. The spectra were recorded in PBS (8 mM $Na_2HPO_4$, 137 mM NaCl, 2 mM $KH_2PO_4$, 2.7 mM KCl), pH 7.4, in the absence or in the presence of DHA ranging from 5-500 μM

**Proteolysis of the peptide-lipid complex:** Proteolysis experiments of the complex formed by DHA and the peptides were carried out at room temperature using proteinase K [19] at E/S ratio of 1:1000 (by weight). The reactions were conducted in PBS, pH 7.4, in the absence or in the presence of different concentrations of DHA. The peptide concentrations were always 5 μM. The reactions were quenched at specified times by acidification with TFA in water (4%, v/v). The proteolysis mixtures were analyzed by RP-HPLC. A Vydac $C_{18}$ column was used to analyze the proteolytic patter of syn108-140 using a gradient of acetonitrile/0.085% TFA *vs*. water/0.1% TFA from 5% to 25% in 5 min, from 25% to 28.5% in 24 min, at a flow rate of 1 ml/min. For syn1-99, syn1-52 and syn57-102 the same gradient and the same columns utilized for the purification were used. Each column was provided by a guard column SAX (Phenomenex, USA). The sites of cleavage along the polypeptide chains were identified by mass spectrometry analyses of the protein fragments purified by RP-HPLC. Mass determinations were obtained with an electrospray ionization (ESI) mass spectrometer with a Q-Tof analyzer (Micro) from Waters (Manchester, UK). The measurements were conducted at a capillary voltage of 2.5-3 kV and a cone voltage of 30-35 V. The molecular masses of protein samples were estimated using the Mass-Lynx software 4.1 (Micromass).

**Determination of DHA critical aggregate concentration based on turbidity measurements and pyrene fluorescence:** Aliquots of DHA were stored at a concentration of 76 mM in 100% ethanol at -80°C. Air was evacuated with helium gas in order to prevent oxidation. The aggregation of DHA, in the absence or in the presence of α-syn peptides (2.5-5 μM), was analyzed by turbidity measurement at 400 nm of different samples containing increasing amounts of DHA (0-500 μM) in PBS, pH 7.4 [20]. The analyses were carried out with a Perkin-Elmer model Lambda 25 UV-VIS spectrometer (Norwalk, CT, USA) using 10 mm path length quartz cuvette. The critical concentration for aggregate formation of DHA was determined by the pyrene 1:3 ratio methods [21-23]. Two series of pyrene fluorescence emission measurements (1 μM) were obtained increasing the fatty acid concentration from 0 to 500 μM in the absence or in the presence of the peptides (5 μM). The fluorescence emission spectra were recorded in PBS (pH 7.4) using an excitation wavelength of 335 nm and the intensity of the maxima corresponding to the first ($I_1$) and third ($I_3$) vibronic band, located near 373 and 384 nm respectively, was measured. A spectrofluorimeter FP6500 Jasco (Tokyo, Japan) was employed and a 2×10 mm path length quartz cuvette was used. The critical aggregate concentration was obtained from the inflection point of the pyrene 1:3 ratio plots, that can be described by a decreasing sigmoid of the Boltzmann type [21]. The fluorescence emission of pyrene was recorded also up to 600 nm to monitor the increase of the signal near 470 nm (band $I_{ex}$), ascribed to the pyrene excimer emission [24] and the ratio $I_{ex}/I_1$ was plotted against the DHA concentration.

### Transmission electron microscopy

In order to evaluate the morphology and the size of the species deriving from the self-assembly of DHA aliquots of the samples were examined by transmission electron microscopy (TEM). A drop of the samples solution was placed on a Butvar-coated copper grid (400-square mesh) (TAAB-Laboratories Equipment Ltd, Berks, UK), dried and negatively stained with a drop of uranyl acetate solution (1%, w/v). TEM pictures were taken on a Tecnai G2 12 Twin instrument (FEI Company, Hillsboro, OR, USA), operating at an excitation voltage of 100 kV.

## Results

### Circular dichroism

Far UV CD spectroscopy was used to evaluate the secondary structure content of the different fragments of α-syn. All species do not acquire regular secondary structure in solution at pH 7.5 in analogy of the whole protein [25]. Titration experiments of the peptides in the presence of increasing concentration of DHA were performed. In Figure 1B, the ellipticity at 222 nm calculated from the CD spectra in the far UV of the polypeptides is shown as a function of the molar ratio between DHA and the peptides. Increasing the amount of DHA, syn108-140 remains unfolded, while a corresponding increment of the ellipticity is observed for syn1-99, syn1-52 and syn57-102. The trends of the curves for syn1-99 and syn1-52 are quite similar to that of α-syn [12]. The conformational transition involves the whole polypeptide chain; indeed syn1-99 has a helix content of ~87% and syn1-52 of ~90% [26]. Moreover, from the plots it can be estimated that ~40 and 38 DHA molecules are required per syn1-99 and syn1-52 for a complete folding. On the contrary, syn57-102 needs a higher amount of DHA to complete the transition and to reach the saturation of in the α-helix content that is estimated to be ~67%.

### Proteolytic mapping

In order to verify the accessibility of the polypeptide chains of the different truncated form of α-syn bound to DHA, peptide mapping experiments were performed. Proteinase K was used as protease, since it displays substrate aspecificity, consequently the peptide cleavages are dictated by the stereochemistry and flexibility of the protein substrate and not by the specificity of the protease [27]. The RP-HPLC analyses of the proteolytic mixture of syn1-99, syn1-52 and syn57-102, in the absence (top) and in the presence (bottom) of 250 μM DHA after 1 hour of incubation were reported (Figure 2). In the absence of DHA, the peptides are cleaved quite rapidly and proteinase K cleavage produces a large number of peptides as indicated by the presence of numerous peaks in the chromatograms. The identification of the peptide materials was obtained by mass spectrometry analysis (Table 1) that shows that obtained fragments span all the sequence of the peptide chains. In the presence of the fatty acid, syn1-99, syn1-52 and syn57-102 show to be resistant to proteolytic attack and the peak relative to the intact peptide is the main species in the chromatogram after 1 hour of incubation.

At variance from the other species, syn108-140 results resistant to proteolytic attack in the absence as well as in the presence of the fatty acid. The unusual rigidity of the C-terminal tail of α-syn to proteases was already seen for the interaction between α-syn and SDS micelles [28]. This fact cannot be attributed to possible structural transitions of the C-terminal of the molecule, but rather to a protection deriving from the unusual sequence (14 negative charges and 5 Pro residues) that confers unusual rigidity to the peptide.

### Effects on DHA aggregates

To verify if the peptides are able to affect the aggregative state of DHA, as the full length protein [12], turbidity measurements and pyrene fluorescence assay were performed. Turbidity measurements show that the presence of the peptides (5 μM), with the exception of syn108-140, prevents the formation of large fatty acid aggregates

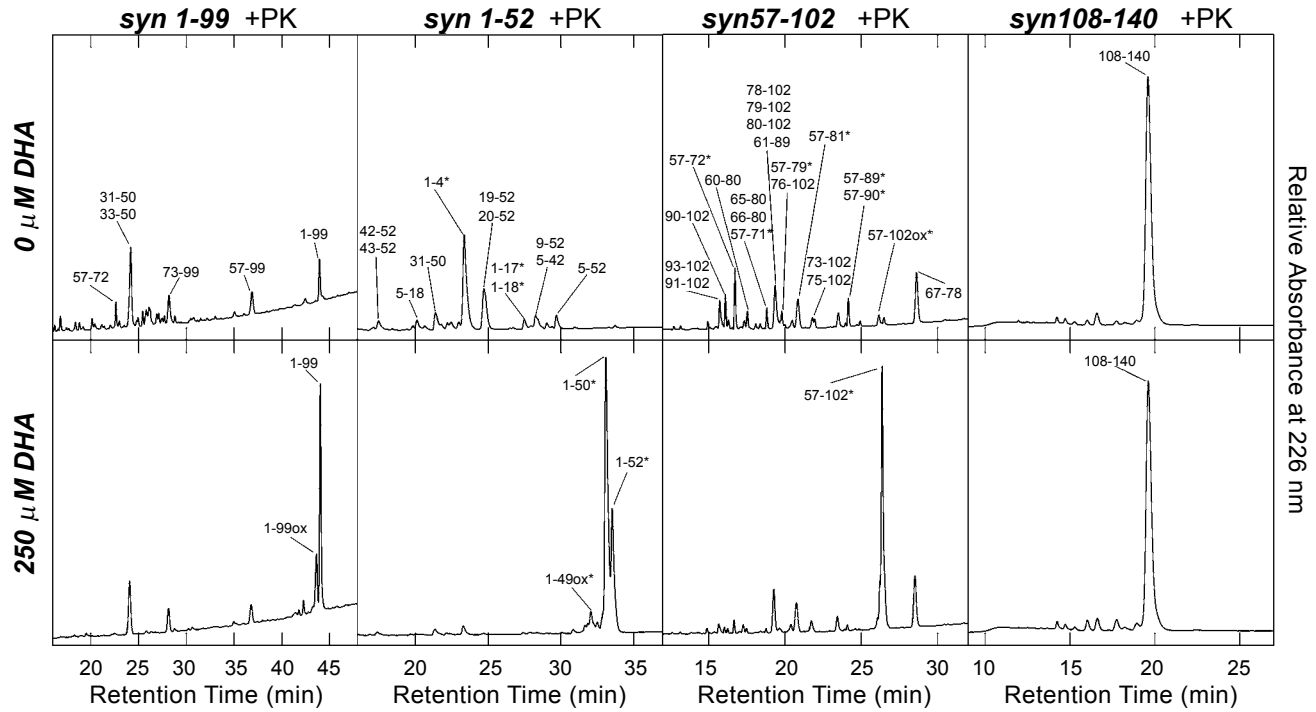


**Figure 2:** Proteolysis of syn1-99, syn1-52, syn57-102 and syn108-140 by proteinase K analyzed by RP-HPLC. The reactions were conducted at a protein concentration of 5 μM in PBS buffer in the absence (top) or in the presence (bottom) of 250 μM DHA, using an E/S ratio of 1:1000. RP-HPLC chromatograms correspond to the analysis of aliquots taken from the proteolysis mixtures after 1-hr of incubation with the protease.

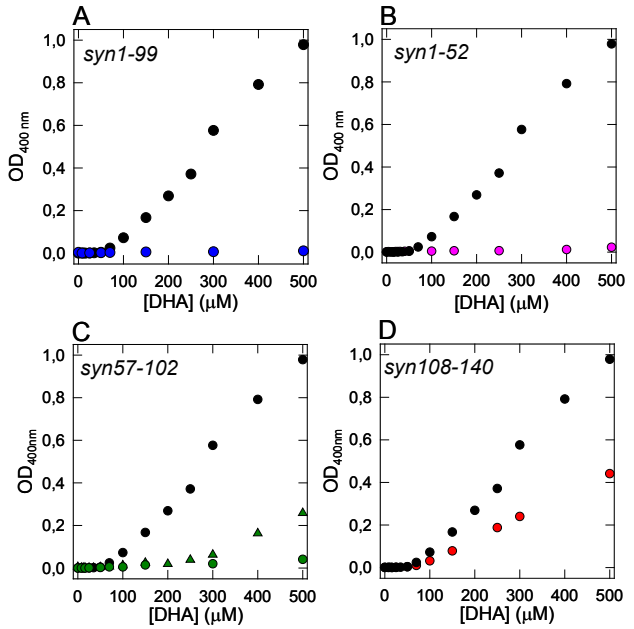


**Figure 3:** Turbidity analysis at 400 nm of samples containing DHA up to 500 μM, in the absence (black circles) and in the presence of syn1-99 (A, 5 μM blue circles), syn1-52 (B, 5 μM, pink circles), syn57-102 (C, 2.5 μM, green triangles, 5 μM green circles) and syn108-140 (D, red circles).

(Figure 3), since there isn't an increase in OD at 400 nm. The turbidity of DHA samples was also measured in the presence of 2.5 μM syn57-102 (Figure 3C, green triangles): there is no variation of the OD up to 242 ± 49 μM DHA. This value is very similar to that obtained with 2.5 μM α-syn [12]. Above this concentration, the turbidity starts to increase as a function of DHA concentration, showing that the amount of syn57-102 is not sufficient to prevent the formation of large lipid aggregates. In the presence of syn108-140 (5 μM) there is no appreciable variation of turbidity at 400 nm up to 75 ± 9 μM DHA. Increasing the concentration above this value, an increment of turbidity is observed, but the OD values are lower than those obtained for DHA samples alone (Figure 3D).

The critical concentration for aggregate formation (CAC) of DHA was also evaluated by pyrene fluorescence assay. In Figure 4 the $I_1/I_3$ pyrene ratio was plotted as a function of DHA concentration in the presence of syn1-99 (A, blue circles), syn1-52 (B, pink circles), syn57-102 (C, green circles) and syn108-140 (D, red circles). In the plots $I_1/I_3$ ratio for DHA alone was reported as a control (black circles and line). For all the samples, increasing the DHA amount the $I_1/I_3$ ratio decreases. This fact suggests that a more hydrophobic micro-environment is induced. The experimental data points fit with a sigmoidal curve so the CACs of the fatty acid in the presence of the several peptides can be calculated at the inflection point of the plots [21]. In the presence of 5 μM syn1-99 and syn1-52 , the $I_1/I_3$ ratio shows a steeper decrease with an inflection point at 71 ± 7 μM and 69 ± 22 μM DHA, respectively (Figure 4A, blue dash line and B, pink dash line). This indicates that syn1-99 and syn1-52 exert a similar effect in reducing DHA concentration for aggregate formation, comparable to that of α-syn (47 ± 13 μM). On the contrary, syn57-102 affects in minor extent the aggregative properties of DHA, since in the presence of this polypeptide CAC of DHA is 103 ± 11 μM (Figure 4C, green dash line). The addition of syn108-140 does not interfere with pyrene fluorescence and it is possible to calculate a CAC for DHA of 140 ± 8 μM (Figure 4D, red dash line).

| | | Molecular mass (Da) | |
|---|---|---|---|
| **Peptide** | **Fragment[a]** | **Found[b]** | **Calculated[c]** |
| syn1-99 | 57-72 | 1658.8 | 1659.8 |
| | 33-50 | 1930.3 | 1931.2 |
| | 31-50 | 2116.4 | 2116.4 |
| | 73-99 | 2604.2 | 2604.9 |
| | 57-99 | 4247.3 | 4246.7 |
| | 1-99 | 9892.6 | 9891.4 |
| syn1-52 | 1-4* | 792.4 | 791.8 |
| | 43-52 | 1053.7 | 1052.6 |
| | 42-52 | 1140.8 | 1139.6 |
| | 5-18 | 1388.0 | 1387.8 |
| | 1-17* | 2090.4 | 2091.5 |
| | 31-50 | 2117.0 | 2116.5 |
| | 1-18* | 2162.5 | 2162.5 |
| | 20-52 | 3385.8 | 3385.8 |
| | 19-52 | 3456.8 | 3456.9 |
| | 5-42 | 3792.6 | 3792.4 |
| | 9-52 | 4397.9 | 4398.0 |
| | 5-52 | 4827.6 | 4827.6 |
| | 1-49ox* | 5323.9 | 5324.1 |
| | 1-50* | 5445.6 | 5445.3 |
| | 1-52* | 5601.4 | 5601.4 |
| syn57-102 | 67-78 | 1000.9 | 1001.1 |
| | 93-102 | 1118.7 | 1119.3 |
| | 91-102 | 1290.8 | 1291.5 |
| | 66-80 | 1355.7 | 1356.5 |
| | 90-102 | 1361.8 | 1362.6 |
| | 65-80 | 1468.9 | 1470.6 |
| | 57-71* | 1971.2 | 1971.2 |
| | 60-80 | 2053.6 | 2056.3 |
| | 57-72* | 2071.5 | 2072.3 |
| | 80-102 | 2276.0 | 2276.6 |
| | 79-102 | 2404.4 | 2404.7 |
| | 78-102 | 2476.3 | 2475.8 |
| | 76-102 | 2645.8 | 2646.0 |
| | 57-79* | 2699.3 | 2699.0 |
| | 61-89 | 2713.0 | 2714.0 |
| | 75-102 | 2747.1 | 2747.1 |
| | 73-102 | 2903.6 | 2903.3 |
| | 57-81* | 2928.4 | 2928.3 |
| | 57-89* | 3612.3 | 3613.0 |
| | 57-90* | 3684.5 | 3684.1 |
| | 57-102* | 4957.5 | 4957.6 |
| | 57-102ox* | 4973.22 | 4973.6 |
| syn108-140 | 108-140 | 3787.8 | 3787.9 |

[a]Peptides obtained by proteolysis by proteinase K, purified by RP-HPLC (Figure 2).
bExperimental molecular masses determined by ESI-MS.
cMolecular masses calculated from the amino acid sequence.

**Table 1:** Molecular masses of fragments obtained by proteolysis of α-syn by proteinase K.

A morphological analysis of DHA aggregate species was performed by TEM. In (Figure 5) TEM images relative to DHA in the presence of the different peptides are reported. As controls, in panels two representative pictures of DHA sample in PBS pH 7.4 are shown. They indicate the presence of DHA aggregates with a heterogeneous distribution of sizes. It is possible to see collapsed small spherical particles (red arrow) and large particles with different degree of contrast due to a different exclusion of the stain (green arrows). In panel B smaller spherical aggregates are formed in the presence of α-syn as already seen [12]. Also the peptides with the exception of syn108-140 cause a resizing of

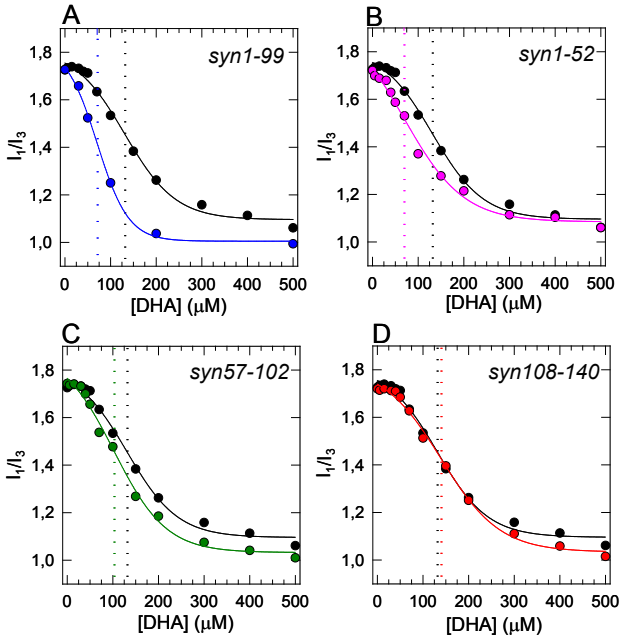


**Figure 4:** Pyrene fluorescence assay. Ratio between the first and the third vibronic band (I1/I3) of the pyrene emission spectrum as a function of DHA concentration (0-500 μM) in the absence (black circles) and in the presence of μ-syn fragments. The analyses were conducted using 5 μM peptide in PBS pH 7.4 and are reported as follows: A, syn1-99, B, syn1-52, C, syn57-102 and D, syn108-140.

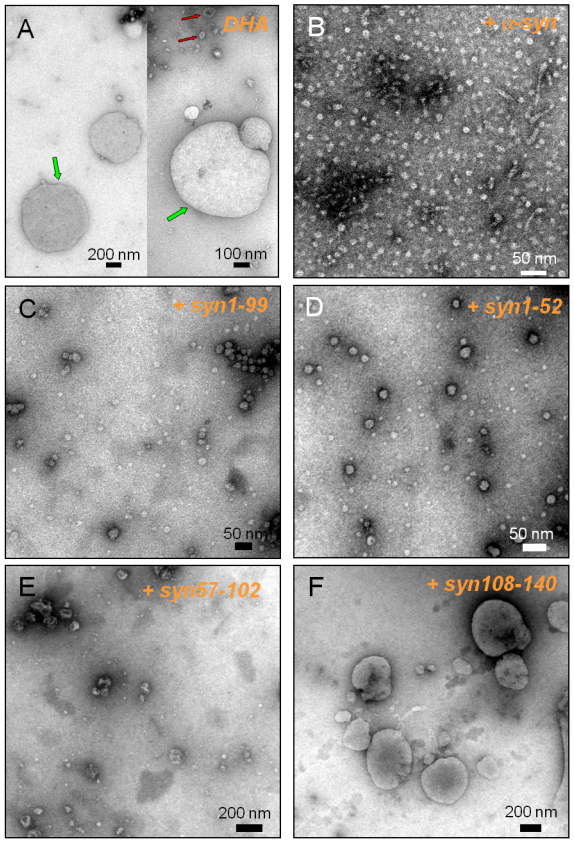


**Figure 5:** TEM pictures of DHA samples in the absence (A) and in the presence of α-syn (B) or its truncated forms (C-F).

DHA assemblies (panel C, D, E, F). In the presence of syn1-99 (panel C) and syn1-52 (panel D) DHA molecules form high contrast spherical aggregates with diameters of ~20 and ~24 nm, respectively. On the other hand, DHA assembles in smaller species also in the presence of syn57-102, but morphologically these aggregates can be described as collapsed spheres (panel E). Syn108-140 does not modify DHA aggregates morphologies (panel F), TEM picture shows large spherical particles comparable to those observed for DHA samples (Panel A).

## Discussion and Conclusion

The data obtained in this study suggest that the N-terminal region of α-syn exerts a main role in the interaction of the protein with DHA and in the alteration of the aggregative properties of the fatty acid. The conformational analyses of the truncated species of α-syn suggest that there is an important action of the 7 imperfect repeats in the random-helix transition of the protein induced by DHA. The 11-residue repeats of apolipoproteins are known to assume the conformation of amphipathic α-helix, which is essential for the binding to lipids. As predicted by the structural similarity to apolipoproteins, the N-terminal region of α-syn is shown to form an amphipathic α-helix that can interact with acidic phospholipids [3]. Here we demonstrate that this N-terminal amphipathic α-helix of α-syn is also required for its binding to DHA. Syn1-99, syn1-52 and syn57-102 have a similar isoelectric point (9.4, 9.4 and 9.31) and they contain 7, 4 and 3 imperfect repeats of the entire molecule, respectively. However, syn1-99 and syn1-52 exhibit a similar conformational behavior in the presence of DHA. They completely undergo helical structural transition and their maximum folding is achieved at a lipid/peptide molar ratio (~40 and ~38, respectively) comparable to that required for α-syn (~35) [12]. At variance, syn57-102 reaches the maximum folding at a higher DHA/peptide molar ratio (>100) and shows lower helix content (67%). Since this last peptide lacks the first 4 structural repeats, these results may suggest that these segments in the N-terminal region of α-syn are important to trigger the conformational transition of α-syn in the presence of DHA. There are no evidences that competitive phenomena such as aggregation are taking place in the time of experiments, but this could give another possible explanation of the inability of syn57-102 to completely convert its conformation in α-helical structure. The C-terminal species syn108-140 remains unfolded in the presence of any kind or amount of lipid [28-30]. However, proteolysis data shows that also the C-terminal region has a role in the α-syn lipid-bound conformation, since the truncated forms exhibit a different susceptibility to protease attack in comparison to the intact molecule. The fragment species herewith investigated are resistant to proteolysis in the presence of saturating concentrations of DHA. This gives an indication that, at variance from α-syn [12], all the peptide sequence is probably involved into the interaction and no particular region is available to the protease. Indeed, the proteolysis of syn1-99 and syn57-102 in the presence of DHA does not produce resistant peptides, as in the case of α-syn in which the fragment 1-72 is formed and accumulates even after prolonged incubation of the protein with the protease [12]. These results suggest that the C-terminal tail could only modulate the portion of α-syn buried into the lipid compartment. Electrostatic repulsion between the acidic residues of region 108-140 and the deprotonated head groups of the fatty acid molecules could be involved in this process.

Under the conditions used in our experiments, DHA aggregates span a wide range of diameters from 50 to more than 600 nm and exhibit features compatible with the formation of lipid droplets, with a CAC in the 100-130 μM range [12]. The addition of α-syn causes a double effect, depressing DHA CAC and favouring the formation of nano lipid droplets. A similar effect is observed adding to DHA the peptides corresponding to the N-terminal and central part of α-syn. The depression of the critical micelle concentration for anionic detergents was regularly observed in the presence of cationic electrolytes including proteins such as tau [31] and results from the electrostatic interactions between positively charged amino acid side chains and negatively charged fatty acids or detergent. Necula et al. [32] suggested that for amphoteric polyelectrolytes such as proteins, the depression of anionic surfactant critical concentration is mediated by clustered charges rather than a net charge of the whole protein. This is the case of α-syn that is negatively charged at pH 7.4, but displays three structural regions with different charge properties. Indeed the acidic C-terminus syn108-140 does not alter the physical properties of DHA, while the syn1-99 and syn1-52 with a net charge of +4.0 and +3.2, respectively exert a effect similar to α-syn in the reduction of the fatty acid concentration for aggregate formation. Syn57-102 (net charge of +2.1) has an ambiguous and weaker effect on DHA self-assembly properties, indeed it prevents the turbidity of DHA samples but it depresses the DHA CAC in minor extent. Probably both the short sequence and the low positive net charge contribute to this behaviour.

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