One-pot total chemical synthesis of human α-synuclein†

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Post-translational modifications (PTMs) regulate key aspects of the physiological and pathogenic properties of Parkinson’s disease-associated presynaptic protein α-synuclein. We herein describe a one-pot total chemical synthesis that should enable site-specific introduction of single or multiple PTMs or small molecule probes essentially at any site within the protein.

The misfolding and aggregation of the 140-residue neuronal protein α-synuclein (α-syn) play central roles in the pathogenesis of Parkinson’s disease (PD). Histologically, PD is defined post-mortem by the presence of intracellular proteinaceous inclusions known as Lewy bodies (LBs), composed mainly of insoluble α-syn amyloid fibrils. Several post-translational modifications (PTMs) have been identified in α-syn within LBs and under physiological conditions, including phosphorylation, mono- and poly-ubiquitination at multiple lysine residues, as well as truncations and nitration, suggesting that these modifications play a role in α-syn aggregation, LB formation. Increasing evidence also suggests that α-syn PTMs play important roles in regulating α-syn degradation by the proteasome or autophagy, subcellular localization and physiological functions. Thus, a better understanding of how α-syn PTMs regulate its functions in health and disease is crucial for understanding the biology of α-syn. Achieving this goal requires the ability to obtain homogenous preparations of chemically well-defined α-syn PTMs, which are typically difficult to obtain using conventional biochemical methods that often result in heterogeneous mixtures of products. This is due to the fact that the enzymes responsible for many α-syn PTMs are not yet known, thus making it difficult to achieve selective modifications and elucidate the relative contribution of each modification or cross-talk between different PTMs.

In order to overcome these limitations, our group has recently developed semisynthetic approaches based on expressed protein ligation (EPL) that allow the introduction of single or multiple PTMs at the N- or C-terminal regions of α-syn, including N-terminal acetylation, mono-ubiquitination at K6 and K12 and phosphorylation at S129 and Y125. Using these methods, we were able to produce homogenously modified proteins in milligram quantities and elucidate the role of each modification in regulating α-syn structure, membrane binding, aggregation, subcellular localization and/or degradation. Subsequently, other groups recently used similar strategies to prepare semisynthetic α-syn, for example containing FRET probes. Protein semisynthesis however restricts the introduction of PTMs or unnatural amino acids to the termini of a protein, making some of α-syn PTMs located within the middle of the protein (such as pS87) difficult to access via semisynthesis. Moreover, α-syn can be modified at multiple sites at the same time, suggesting cross-talk between different PTMs. Investigating such cross-talk requires methodologies that allow the simultaneous introduction of multiple modifications in different regions of the protein. Total chemical synthesis of proteins offers unlimited versatility regarding the type, number, and localization of unnatural amino acids to be introduced.

Here, we present the first report of an efficient one-pot total chemical synthesis of α-syn and demonstrate that synthetic α-syn exhibits similar biophysical, membrane binding and aggregation properties to recombinant α-syn obtained from E. coli.

Given that the chemical synthesis of polypeptide chains of 40–60 residues are typically feasible by Fmoc-based SPPS in sufficient yields, our initial fragment design for the total synthesis of α-syn was based on dividing the protein into four segments, α-syn(1–29)SR, Thz-α-syn(31–68)SR, Thz-α-syn(70–106)SR, and α-syn(A107C-140)OH, of sequence length in the range of 29–38 residues (Fig. 1A and Fig. S1, ESI†). Ligation sites were chosen at Ala residues which could be replaced by alkyl thioesters using sodium 2-mercaptoethanesulfonate (MESNa) before purification. The preparation of the fragments Thz-α-syn(31–68)SR and Thz-α-syn(70–106)SR was very challenging, with...
initial unacceptably low yields. However, when two Lys-Thr pseudo-proline dipeptide residues were incorporated at positions 43–44 and 58–59 and the more efficient HATU coupling agent was used for the coupling of N-terminal residues 30–44, the fragment Thz-a-syn(31–68)SR was obtained in a reasonable 10% yield. However, the overall very low yield of Thz-a-syn(70–106)SR could not be improved. This is not surprising, given that the 70–106 fragment encompassing the highly hydrophobic and aggregation-prone 61–95 region, also known as the NAC region,16 which plays a critical role in the initiation of a-syn aggregation and amyloid formation.

Numerous studies have shown that the highly acidic and proline-rich disordered C-terminal (96–140) domain enhances the solubility and thermostability of the full-length protein.17 Deletion of this domain or the last 20 a.a. accelerates a-syn aggregation.18 Given that the C-terminal fragment a-syn(A69C–140) posed the least synthetic difficulties, we sought to take advantage of the increased solubility of this domain in the synthesis of a longer fragment (69–140) that encompasses the NAC region during SPPS. Indeed, this strategy was very successful and the synthesis of the 72 residue fragment a-syn(A69C–140) was achieved with a very good 14% yield (Fig. S3, ESI†). These results demonstrate the utility of unstructured portions of the native a-syn sequence, which can fortuitously enhance access to more synthetically challenging regions.

With sufficient quantities of purified fragments a-syn(A69C–140), 2 Thz-a-syn(31–68)SR, and 3 a-syn(1–29)SR in hand, we were prepared to assemble full-length wild-type a-syn by applying two sequential NCL steps in a one-pot synthesis scheme, which takes advantage of the pH-dependence of NCL (pH 7–8) and the conversion of the Thz to Cys (pH 3.8–4) to orchestrate the desired transformations by controlled pH switching (Fig. 1B and Fig. S2, ESI†). The first ligation between peptides 1 and 2 was carried out at pH 7.5 in 6.0 M GdmHCl, 0.2 M sodium phosphate in the presence of 5.4 mM VA-044, 37°C, 1 h.

Fig. 1 One-pot synthesis of wt a-syn. (A) Schematic depiction of the a-syn sequence and the peptides chosen for the three-fragment synthetic strategy. (B) Scheme depicting the successive reactions to obtain full-length wt a-syn using the three-fragment strategy. (C) RP-UHPLC (left panel) and SDS-PAGE (right panel) analyses of the ligation between a-syn(A69C–140) and Thz-a-syn(31–68)SR. (D) RP-UHPLC (left panel) and SDS-PAGE (right panel) analyses of the second ligation reaction, between a-syn(30–140) A30C A69C and a-syn(1–29)SR. In the SDS-PAGE, lane ‘C’ denotes recombinant full-length a-syn control. (E) Purity analyses of the final desulfurized product by RP-UHPLC (top left), SDS-PAGE (top right, ‘R’ lanes denote recombinant wt a-syn; and ‘S’ lanes indicate synthetic wt a-syn), and ESI-MS.
MALDI-TOF MS analysis confirmed the identity of 6 with an observed mass of 14 525 Da (calc.: 14 525 Da, M + H+). Excess MPAA from the ligation was then removed by desalting with a PD-10 column. Free-radical desulfurization21 was then applied (see ESI†). The conversion of both cysteines at positions 30 and 69 occurred within 1 h of reaction as seen using MALDI-TOF MS with an observed mass of 14 464 Da (calc.: 14 461 Da, M + H+). The protein was then purified on a C8 semipreparative column and recovered with >95% purity with a 21% yield (0.21 mol, 3 mg, Fig. 1D). Because α-syn is a natively unfolded protein,22 it does not require a refolding step after ligation and desulfurization.

We then sought to verify that the synthetic protein behaves similarly to the most commonly used form of α-syn, recombinant α-syn produced in E. coli (not N-terminally acetylated) and purified using the same procedure. Freshly-dissolved synthetic wt α-syn was indistinguishable from the recombinant protein using circular dichroism spectroscopy, showing that both proteins exist predominantly in random coil conformations (Fig. 2A), and showed similar affinities for 100 nm diameter large unilamellar vesicles composed of phosphatidylglycerol (POPG) (Fig. 2A). Multi-angle light scattering confirmed that both freshly-prepared proteins behaved as monomers, as expected (Fig. 2B). Fibrillization studies using ThT fluorescence (Fig. 2C) did not reveal a significant difference between the end-point amyloid formation propensity of recombinant and synthetic wt α-syn. Furthermore, the loss of soluble protein as a result of aggregation was similar between both forms as assessed by SDS-PAGE analysis of supernatants after high-speed centrifugation (Fig. 2D), although synthetic α-syn seemed to aggregate slightly slower than the recombinant protein. Transmission electron microscopy (TEM) showed that recombinant and synthetic wt α-syn form similar fibrillar structures upon aggregation (Fig. 2E).

In conclusion, we have described a strategy enabling the one-pot total chemical synthesis of α-syn using three peptide fragments. This method will enable the preparation of modified forms of α-syn (such as pS87) that have not been accessible so far using semi-synthetic methods because of their location in the center of the protein; as well as combinations of different PTMs in order to explore the possibility of cross-talk between them.

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Fig. 2 Biophysical and biochemical characterization of synthetic wt α-syn in comparison with the recombinant protein. (A) Circular dichroism spectra of recombinant and synthetic wt α-syn. (B) Size-exclusion chromatography/multi-angle light scattering analysis. The left ordinate axis shows the absorbance profiles (275 nm); the right ordinate axis shows the calculated molecular weights. (C) ThT fluorescence analysis. (D) SDS-PAGE analysis of the soluble protein content during the aggregation of recombinant and synthetic wt α-syn. The lower panel shows quantification (three independent experiments) of the SDS-PAGE gels. A representative gel is shown in the upper panel. (E) TEM micrographs taken after 5 days of aggregation. Scale bars: 100 nm.

Notes and references