doi:10.1016/j.jmb.2009.10.066 J. Mol. Biol. (2010) 395, 627–642

JMB

Available online at www.sciencedirect.com

ScienceDirect

ELSEVIER

Towards Multiparametric Fluorescent Imaging of Amyloid Formation: Studies of a YFP Model of α-Synuclein Aggregation

Tjakko J. van Ham[1], Alessandro Esposito[2], Janet R. Kumita[3], Shang-Te D. Hsu[3], Gabriele S. Kaminski Schierle[2], Clemens F. Kaminski[2], Christopher M. Dobson[3], Ellen A. A. Nollen[1]* and Carlos W. Bertoncini[3]*

[1]*Department of Genetics, University of Groningen, Groningen, The Netherlands*

[2]*Department of Chemical Engineering and Biotechnology, University of Cambridge, Cambridge, UK*

[3]*Department of Chemistry, University of Cambridge, Cambridge, UK*

Received 17 July 2009; received in revised form 4 October 2009; accepted 27 October 2009
Available online 3 November 2009

Misfolding and aggregation of proteins are characteristics of a range of increasingly prevalent neurodegenerative disorders including Alzheimer's and Parkinson's diseases. In Parkinson's disease and several closely related syndromes, the protein α-synuclein (AS) aggregates and forms amyloid-like deposits in specific regions of the brain. Fluorescence microscopy using fluorescent proteins, for instance the yellow fluorescent protein (YFP), is the method of choice to image molecular events such as protein aggregation in living organisms. The presence of a bulky fluorescent protein tag, however, may potentially affect significantly the properties of the protein of interest; for AS in particular, its relative small size and, as an intrinsically unfolded protein, its lack of defined secondary structure could challenge the usefulness of fluorescent-protein-based derivatives. Here, we subject a YFP fusion of AS to exhaustive studies *in vitro* designed to determine its potential as a means of probing amyloid formation *in vivo*. By employing a combination of biophysical and biochemical studies, we demonstrate that the conjugation of YFP does not significantly perturb the structure of AS in solution and find that the AS-YFP protein forms amyloid deposits *in vitro* that are essentially identical with those observed for wild-type AS, except that they are fluorescent. Of the several fluorescent properties of the YFP chimera that were assayed, we find that fluorescence anisotropy is a particularly useful parameter to follow the aggregation of AS-YFP, because of energy migration Förster resonance energy transfer (emFRET or homoFRET) between closely positioned YFP moieties occurring as a result of the high density of the fluorophore within the amyloid species. Fluorescence anisotropy imaging microscopy further demonstrates the ability of homoFRET to distinguish between soluble, pre-fibrillar aggregates and amyloid fibrils of AS-YFP. Our results validate the use of fluorescent protein chimeras of AS as representative models for

**Corresponding authors.* C. W. Bertoncini is to be contacted at the Department of Chemistry, University of Cambridge, Lensfield Road, Cambridge CB2 1EW, UK. E. A. A. Nollen, Department of Genetics, University of Groningen, Oostersingel entrance 47, 9700 RB Groningen, The Netherlands. E-mail addresses: e.a.a.nollen@medgen.umcg.nl; cwb32@cam.ac.uk.

Present address: T. J. van Ham, Cardiovascular Research Center, Massachusetts General Hospital, Harvard Medical School, 149 13th Street, Charlestown, MA 02129, USA.

Abbreviations used: AS, α-synuclein; YFP, yellow fluorescent protein; FAIM, fluorescence anisotropy imaging microscopy; ThT, thioflavin T; TEM, transmission electron microscopy; CR, Congo red; HSQC, heteronuclear single quantum coherence; BS, β-synuclein; GFP, green fluorescent protein; emFRET or homoFRET, energy migration Förster resonance energy transfer.

studying protein aggregation and offer new opportunities for the investigation of amyloid aggregation *in vivo* using YFP-tagged proteins.

Keywords: protein misfolding; protein aggregation; fluorescence anisotropy imaging microscopy; Parkinson's disease; fluorescence protein

Edited by S. Radford

Introduction

Parkinson's disease and dementia with Lewy bodies are characterized by the deposition of cytoplasmic, protein-rich inclusions in certain regions of the brain, known as Lewy bodies.[1] The major constituent of such deposits is the 140-residue protein α-synuclein (AS), together with several minor components such as chaperones and ubiquitin.[2] Direct evidence for the involvement of AS in disease arises from the identification of point mutations and duplication of the AS locus linked with the early onset of neurodegeneration.[3–7] The biological function of AS appears to be related to synaptic plasticity by enhancing the priming step in the formation of synaptic vesicles, directly acting on the folding of SNARE proteins, possibly as a chaperone.[8] Gene knock-out studies have shown, however, that AS is not an essential protein, at least in rodents, which strongly suggest that the link to disease arises from a toxic gain of function.[9] AS is intrinsically unfolded in the cytoplasm but acquires substantial α-helical structure in its first 100 residues upon binding to lipid membranes.[10,11] Although it lacks defined secondary structure, long-range tertiary interactions exist and may help to prevent it from misfolding and aggregation to form amyloid deposits.[12–15] Interestingly, however, amyloid fibrils may not represent the species most toxic to neurons; instead, soluble oligomeric species and insoluble pre-fibrillar precursors to such fibrils appear to generate toxicity in cells.[16–20]

The vast majority of studies on amyloidogenic proteins have been carried out *in vitro* or *ex vivo* with material extracted from living organisms, as a consequence of the lack of suitable means of addressing the process of protein oligomerization and conformational changes in intact living cells. Recent technical advances in fluorescence microscopy, however, provide a wealth of tools that are uniquely suited for probing heterogeneous and transient species in the complex environment of a cell.[21] Fluorescence imaging offers many advantages for the study of misfolding and amyloid formation, in particular as observations can be made both *in vitro* and *in vivo*, facilitating direct comparison between both environments. Indeed, several approaches, such as tetracysteine FlaSH-ReaSH tagging, fluorescent protein tagging, split-fluorescent protein reconstitution (BiFC), and PDZ conjugation, have been used successfully to image AS misfolding and fibrillization.[18,22–25] Of these techniques, fluorescent protein tagging is of special interest as fluorescent protein derivatives come in a variety of spectral and photoswitchable variants that allow multicolor and high-resolution imaging.[21,26] Furthermore, fluorescent proteins are a very valuable tool for live imaging in whole organisms such as the nematode worm *Caenorhabditis elegans*, the fruit fly *Drosophila melanogaster*, and even mice. Indeed, many animal models of disease have been established, employing fluorescent protein tagging to study misfolding and accumulation of AS,[27–31] along with other misfolding disease-related proteins.[32–35]

Conjugation of proteins by fluorescent proteins may, however, perturb the native structure and function of the tagged protein given the bulkiness of the fluorescent moiety (~28 kDa). In the case of AS, this may be of particular concern, not only because of its relatively small size (~14 kDa) but also because of its lack of persistent structure in solution. It is possible, therefore, that fluorescent protein tagging may affect the intrinsic propensity of AS to form fibrils and thus render the conjugate less valuable than one might hope for studying the behavior of native AS. Not surprisingly, some controversy has been raised from studies of AS misfolding and aggregation using fluorescent proteins *in vivo*, and the validation of this system by biophysical techniques is essential. Here, we conduct a detailed biophysical characterization of AS tagged C-terminally with the yellow fluorescent protein variant Venus [36] (termed here AS-YFP) and investigate its use as a reporter of AS misfolding and aggregation. We further examine the fluorescence properties of aggregated AS-YFP and demonstrate the applicability of fluorescence anisotropy imaging microscopy (FAIM) in the characterization of amyloid aggregation of proteins.

Results

AS-YFP forms amyloid fibrils *in vitro* in a similar manner to wild-type AS

A fluorescent protein chimera of AS was constructed by fusing the YFP gene (named Venus) to the C-terminus of AS, separated by a short linker peptide (AlaProValAlaThr), designed to minimize any effects of the fusion process on both proteins.[35] To investigate the amyloid aggregation behavior of AS-YFP, we performed protein aggregation assays on the purified chimeric protein; as a control, wild-type AS was assayed in parallel. Both proteins were subjected to standard *in vitro* aggregation procedures, incubating the purified proteins at 37 °C with constant agitation. Aliquots were taken from both