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Structure and Properties of a Complex of α-Synuclein and a Single-Domain Camelid Antibody

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The aggregation of the intrinsically disordered protein α-synuclein to form fibrillar amyloid structures is intimately associated with a variety of neurological disorders, most notably Parkinson's disease. The molecular mechanism of α-synuclein aggregation and toxicity is not yet understood in any detail, not least because of the paucity of structural probes through which to study the behavior of such a disordered system. Here, we describe an investigation involving a single-domain camelid antibody, NbSyn2, selected by phage display techniques to bind to α-synuclein, including the exploration of its effects on the *in vitro* aggregation of the protein under a variety of conditions. We show using isothermal calorimetric methods that NbSyn2 binds specifically to monomeric α-synuclein with nanomolar affinity and by means of NMR spectroscopy that it interacts with the four C-terminal residues of the protein. This latter finding is confirmed by the determination of a crystal structure of NbSyn2 bound to a peptide encompassing the nine C-terminal residues of α-synuclein. The NbSyn2:α-synuclein interaction is mediated mainly by side-chain interactions while water molecules cross-link the main-chain atoms of α-synuclein to atoms of NbSyn2, a feature we believe could be important in intrinsically disordered protein interactions more generally. The aggregation behavior of α-synuclein at physiological pH, including the morphology of the resulting fibrillar structures, is remarkably unaffected by the presence of NbSyn2 and indeed we show that NbSyn2 binds strongly to the aggregated as well as to the soluble forms of α-synuclein. These results give strong support to the conjecture that the C-terminal region of the protein is not directly involved in the mechanism of aggregation and suggest that

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Abbreviations used: IDP, intrinsically disordered protein; ITC, isothermal titration calorimetry; ANS, 1-anilino-8-naphthalene sulfonate; ThT, thioflavin T; TEM, transmission electron microscopy; ASA, solvent-accessible surface area; HSQC, heteronuclear single quantum coherence; CDR, complementarity-determining region; EM, electron microscopy; PBS, phosphate-buffered saline; PEG, polyethylene glycol; BSA, bovine serum albumin.

binding of NbSyn2 could be a useful probe for the identification of α-synuclein aggregation *in vitro* and possibly *in vivo*.

Keywords: α-synuclein; amyloid; nanobody; nuclear magnetic resonance; X-ray crystallography

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Introduction

Numerous studies have indicated that α-synuclein plays a crucial role in the development of Parkinson's disease and a number of related neurological disorders including dementia with Lewy bodies, multiple system atrophy, and the Lewy body variant of Alzheimer's disease.[1–6] It is very widely believed that the aggregation of α-synuclein to its alternative 'amyloid' state is a crucial aspect of all these disorders.[2–6] Moreover, and in common with a range of other neurological diseases, it is thought that pathogenesis results from the oligomeric precursors or fragments of the fibrillar amyloid state that are toxic and able to disrupt the function in particular of dopaminergic neurons.[1]

Human α-synuclein is a 140-residue intrinsically disordered protein (IDP) of unknown function, although there is increasing evidence that the protein is involved in vesicular axonal transport.[7] The aggregation behavior of α-synuclein has been widely studied *in vitro* and it is thought that self-association occurs initially to give a range of oligomeric species.[8–11] These oligomers can then aggregate further to generate protofibrils that eventually transform into mature amyloid fibrils. As discussed above, recent studies have suggested that the oligomeric species are highly toxic, perhaps through their ability to interact with and to disrupt membranes.[12–19]

The natively unfolded character of α-synuclein in its monomeric form, as well as the existence of an ensemble of oligomeric intermediates that are transiently populated during the aggregation process leading to fibril formation, makes it challenging to obtain structural details about individual species and to elucidate the molecular mechanisms of the process that lead to toxicity.[20] The use of specific molecular probes, such as antibodies, has, however, the potential to obtain such information in exquisite detail, because these can be raised to be selective not just for proteins in their native state but also for non-native states populated in the process of aggregation, including transient species such as small oligomers.[21] Of particular interest in this regard are camelid heavy-chain antibodies that lack the light chains of conventional antibodies and enable single-domain binding fragments to be obtained, species known as nanobodies.[22–26] Such nanobodies are small, highly stable and soluble, and easily expressed in large quantities but retain the high specificity and high affinity typical of conventional antibodies binding to their targets.[23–26] These qualities mean that nanobodies are extremely powerful as molecular probes of fibril formation using biophysical methods such as NMR and X-ray crystallography[27–29] and potentially as diagnostic and therapeutic reagents for amyloid diseases.[30,31]

As a result of the link between the aggregation of α-synuclein and Parkinson's disease, the ability of molecules and ions to enhance or inhibit the aggregation propensity of α-synuclein has been widely studied. Thus, for example, the binding of divalent cations has been shown to increase dramatically the rate of fibril formation by this protein,[32–35] as has the binding of polyamines.[36–40] In both cases, it is believed that the positively charged ions and molecules interact with the highly negatively charged C-terminal region of the α-synuclein sequence, reducing electrostatic repulsion and disrupting long-range interactions, hence leading to an increased ability to self-associate.[32–43] However, the disruption of long-range contacts might not be the most important factor that leads to enhanced fibril formation, as the disease-related variants do not show a significant reduction in long-range contacts, with the E46K variant actually enhancing the C-terminal to N-terminal contacts in α-synuclein.[44] The ability of α-synuclein to interact with larger molecules, including proteins, remains, however, largely unexplored and mainly limited to interactions with molecular chaperones.[45–47] One example, however, where detailed studies have been carried out involves the chaperone Hsp70, which has been shown to have the ability strongly to inhibit fibril formation by α-synuclein, through a mechanism that appears to involve binding to oligomeric species, thereby preventing them from undergoing further self-association.[46]

In this article, we describe detailed studies of the interaction of a nanobody, NbSyn2, with α-synuclein and of its effect *in vitro* on the aggregation properties of the latter. NMR spectroscopy and X-ray crystallography reveal that NbSyn2 binds to residues in the C-terminal region of α-synuclein. A range of biophysical studies shows that, although NbSyn2 binds tightly to α-synuclein, no significant structural changes can be detected within the α-synuclein molecule associated with its binding. Finally, the aggregation behavior of α-synuclein *in vitro* was found to be unaffected by the binding of the nanobody, a result that sheds light on the likely