The Mysterious C-Terminal Tail of Alpha-Synuclein: Nanobody's Guess

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The protein alpha-synuclein (aS) is well known for several reasons. First, aS is the primary (but not the only) protein component of the Lewy body and Lewy neurite deposits that are found in the brains of the victims of Parkinson's disease (PD). Second, genetic lesions resulting in either increased levels of wild-type aS or point mutations in aS cause Parkinsonism. Third, aS is one of the earliest identified and best characterized members of the recently recognized class of intrinsically disordered proteins. Despite immense interest in aS, many questions remain regarding both the precise pathogenic role of aS in PD and the normal physiological functions of the protein, which appear to include a role at the synapse, likely involving binding to synaptic vesicles and somehow modulating their biogenesis, trafficking, or exocytosis, and possibly also an uncharacterized role at the nuclear envelope membrane. A key aspect of aS function appears to be membrane binding, and the primary sequence of the protein reflects this through the presence of seven imperfect 11-residue tandem repeats, with a central consensus motif of KTKEGV, that are characteristic of several classes of proteins that bind to membranes in a reversible fashion, including the apolipoproteins. These repeats are contained within the ~100 N-terminal residues of the protein, which constitute the lipid-binding domain (LBD) of aS and thereby manifest a known aspect of aS function. Interestingly, the primary site responsible for aS aggregation into amyloid fibrils, the so-called NAC region (residues 61–95), is also located within the LBD.

C-terminal to its LBD, aS contains an additional ~40 residues that are commonly referred to as its C-terminal tail (CTT). The most remarkable physicochemical characteristic of the CTT is its negative charge content, which at neutral pH arises from 14 negatively charged side chain as well as the C-terminal carboxyl group. Interestingly, the CTT also features two 16-residue tandem pseudo-repeats.[1] While the LBD is known to mediate both membrane binding and aggregation of aS, the functional and pathogenic roles of the CTT have remained poorly understood and highly controversial. One early functional attribute assigned to the CTT was a chaperone activity, based on observations that aS could prevent the aggregation of other proteins in a manner that required the CTT.[2,3] While occasional reports of aS chaperone function *in vitro* continue,[4] it is not generally accepted as a likely function of the protein *in vivo*.

Another proposed role of the CTT is metal binding, including calcium, copper, iron, and possibly other metals. The presence of many negatively charged side chains in the CTT indeed leads to binding of a variety of metal or other cations. Roles for asyn as a copper- or iron-binding metalloreductase have been proposed[5] but have not gained widespread traction. Calcium binding by aS may be more interesting given the proposed role of aS in synaptic vesicle exocytosis (a strongly calcium-dependent process) as well as reported interactions of aS with the calcium-binding protein calmodulin.[6,7] Nevertheless, it appears that metal binding by the CTT is largely nonspecific and recapitulates what would be expected for any polypeptide sequence of a similar amino acid composition.[8] This does not rule out a functional role for metal binding but does make the notion less compelling. From the viewpoint of aS pathology, metal binding to the CTT neutralizes its highly negative charge and thereby enhances aggregation.

The CTT has also been observed to form transient long-range interactions with other regions of aS, including the positively charged N-terminal region,[9] and these have been proposed to be protective against aggregation.[10] Indeed, truncation[11,12] or neutralization[13] of the CTT results in forms of aS that are much more prone to aggregate. However, the most probable explanation for this effect is that

the highly negative charge of the CTT leads to intermolecular electrostatic repulsion between synuclein molecules.[14,15] The intramolecular interactions of the CTT are likely too transient to provide any significant occlusion of aggregation sites, and in fact, an increase in such interactions is associated with increased, rather than decreased, aggregation.[14,16]

Interestingly, despite general agreement that the CTT does not interact with membranes, various reports have suggested that this may not be entirely true. Oxidative modifications in the CTT exert a measurable effect on the affinity of aS for membranes,[17] and titration of aS with high concentrations of detergents or lipids leads to small but measurable changes in NMR parameters, including both chemical shifts and resonance intensities.[18,19] Even phosphorylation in the CTT can influence the membrane interactions of some aS variants.[20] Some of these effects may be explained by changes in intramolecular interactions upon lipid binding by the N-terminal LBD, but a potential role for the CTT in modulating aS membrane interactions remains possible.

The most compelling putative function of the CTT is to mediate protein–protein interactions. A number of potential aS-interacting proteins have been identified, and many of them have been reported to interact with the CTT. A few examples include a reported interaction with the enzyme phospholipase D (PLD), which not only required LBD-mediated membrane binding but also requires the CTT,[21] presumably for direct interactions with PLD, perhaps at its active site. This interaction has been called into question,[22] but the matter remains to be conclusively resolved. Other individual proteins, including tau[23] and MAP-1B,[24] have been reported to bind to aS via the CTT and larger-scale efforts have identified larger numbers of proteins that bind to the CTT in a phosphorylation-dependent manner.[25] Indeed, the CTT contains several sites for posttranslational modifications of aS, including phosphorylation sites at Ser129 and Tyr125, which are likely to modulate protein–protein interactions. Most recently, aS has been reported to interact with glucocerebrosidase A,[26] a protein that, when mutated, confers an increased risk of PD, and synaptobrevin-2,[27] a SNARE protein required for synaptic vesicle exocytosis, through its CTT.

The CTT of aS is not part of the β-sheet-rich structured core of the amyloid fibril aggregates associated with PD,[28–33] and indeed its accessibility to antibodies was key to the discovery that Lewy bodies are enriched in aS.[34] Nevertheless, it is clear that the CTT can profoundly influence the process of aS aggregation into fibrils, as its removal greatly increases the rate of fibril formation, as well as influences the morphology of the resultant fibrils.[11,35] Notably, phosphorylation at Ser129 in the CTT is considered one of the clearest markers of pathological forms of aS,[36] although the role of this modification in the aggregation and toxicity of aS remains controversial. Furthermore, despite not being involved in the core structure of aS amyloids, there have been some hints that the CTT is sensitive to the proximity of that core structure. For example, the accessibility of epitopes in the CTT is modified in some fibrillar aS aggregates compared with others,[37] or compared with unaggregated protein.[38]

A collaboration between the Dobson and Wyns groups at University of Cambridge and Vrije Universiteit Brussel has now resulted in the development of small single-domain antibody fragments, known as nanobodies, directed against different regions of the aS CTT. Interestingly and rather unexpectedly, it appears that the affinity and binding modes of these nanobodies to aS fibrils formed *in vitro* depend on both their age and origin. These differences imply both changes in the accessibility of the CTT as fibrils mature and heterogeneous populations of CTT structural ensembles in at least some types of fibril assemblies. While detailed information is available regarding the conformational ensemble of the CTT of aS in both mature fibrils and in its monomeric state, much less information is available regarding its behavior in any of the potentially numerous intermediates that likely occur (whether off or on pathway) during fibril formation. The initial studies using nanobodies reveal that even relatively mature fibrillar aggregates are likely to undergo significant structural rearrangements, consistent with, for example, reports of β-strand register changes in fibrillar species.[39] Thus, the reported nanobody reagents are likely to prove powerful tools for probing the structural and thermodynamic properties of the CTT in many different contexts, both *in vitro* and potentially *in vivo*, and both in the context of pathological aS aggregation and in the context of normal aS function.

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