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α-Synuclein Misfolding Versus Aggregation Relevance to Parkinson's Disease: Critical Assessment and Modeling

Ruben Berrocal · Velmarini Vasquez · Sambasiva Rao KRS · Bharathi S Gadad · Rao KS

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Abstract α-Synuclein, an abundant and conserved presynaptic brain protein, is implicated as a critical factor in Parkinson's disease (PD). The aggregation of α-synuclein is believed to be a critical event in the disease process. α-Synuclein is characterized by a remarkable conformational plasticity, adopting different conformations depending on the environment. Therefore, it is classified as an "intrinsically disordered protein." Recently, a debate has challenged the view on the intrinsically disordered behavior of α-synuclein in the cell. It has been proposed that α-synuclein is a stable tetramer with a low propensity for aggregation; however, its destabilization leads to protein misfolding and its aggregation kinetics. In our critical analysis, we discussed about major issues: (i) why α-synuclein conformational behavior does not fit into the normal secondary structural characteristics of proteins, (ii) potential amino acids involved in the complexity of misfolding in α-synuclein that leads to aggregation, and (iii) the role of metals in misfolding and aggregation. To evaluate the above critical issues, we developed bioinformatics models related to secondary and tertiary conformations, Ramachandran plot, free energy change, intrinsic disordered prediction, solvent accessibility, and FoldIndex pattern. To the best of our knowledge, this is a novel critical assessment to understand the misfolding biology of synuclein and its relevance to Parkinson's disease.

Keywords α-Synuclein · Aggregation · Parkinson's disease · Misfolding · Conformation · Metals

Introduction

The discovery of α-synuclein in 1997 provided a new dimension in understanding the significance of α-synuclein to neurodegenerative diseases particularly Parkinson's disease (PD) [1]. The discovery was followed rapidly by a report identifying full-length α-synuclein in Lewy bodies (LB) from postmortem brain tissue of sporadic Parkinson cases [2]. However, further studies revealed that α-synuclein is the predominant protein in LB in sporadic as well as in familial PD indicating that the protein is actively sequestered into these inclusions [3]. Another familial form of PD is linked to an α-synuclein gene mutation causing an alanine to proline substitution at position 30 (A30P) [4]. Also, a novel α-synuclein gene mutation causing the substitution of glutamate to lysine at residue 46 (E46K) is discovered in Spanish kindred with familial PD [5]. Further, α-synuclein gene mutation causing substitution of alanine for threonine at position 53 (A53T) presents a higher fibril formation rate which is considered to contribute to early onset PD [6]. Taken together, these findings provided strong evidence that α-synuclein plays a pivotal role in the pathogenesis of PD, despite the fact that most cases of PD cannot be traced to a mutation in the α-synuclein gene. Further, Puschmann et al. [7] hypothesized that special pediatric disease, like myoclonus epilepsy type I, familial and sporadic parkinsonism, and dementia phenotypes, are found to be associated with duplications, triplications, and possibly higher order multiplications of the α-synuclein (SNCA) gene. The

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above hypothesis is supported by the distribution of afflicted family members within the pedigree and by genealogical information [7]. Biskup et al. [8] reviewed mutations in several causative genes that are associated to monogenic forms of the disorder. Point mutations, duplications, and triplications in the α-synuclein gene are known to cause a rare dominant form of PD in families, while mutations in the parkin gene, DJ-1, PINK1, and ATP13A2 are attributed to cause autosomal recessive parkinsonism of early onset. These monogenic variants are important tools in identifying cellular pathways and also provide an insight on the molecular pathogenesis of sporadic PD. Now it is clear that these genes play a role in the etiology of the common sporadic form of PD through synuclein [8].

Besides understanding how these mutations induce α-synuclein over expression, the greatest challenge is to understand the aggregation behavior of α-synuclein. Protein folding is essential for life's many functions; it is not therefore too surprising that protein misfolding can lead to various diseases [9]. Recent advances may demonstrate that an increasing number of disorders are related to protein misfolding and aggregation. The term "misfolding" is used to describe conformational changes that result in a protein acquiring a sufficient number of persistent nonnative interactions to affect its overall architecture and/or its properties in a biologically significant manner [9]. Experimental and theoretical studies have, however, provided a resolution of this apparent paradox. It is now evident that the folding process does not involve a series of mandatory steps between specific partially folded states, but rather a stochastic search of the many conformations accessible to a polypeptide chain [10–12]. The aggregation kinetics of α-synuclein is multifaceted passing through monomer, oligomer, protofibrils, and aggregates. The folding and misfolding behavior of α-synuclein is unique and beyond protein folding and thermodynamics principles. The misfolded α-synuclein undergoes folding when bound to lipid moiety of membranes, thus leading to hypothesize the functional ability of α-synuclein in signal transduction. This provided new understanding on the functional role of α-synuclein as a function of folding concept. In the present paper, we would emphasize on the mechanisms of misfolding behavior of α-synuclein and its aggregation propensity and whether correcting misfolding can be of therapeutic relevance and an application of bioinformatics models to understand folding complexity of synuclein is suggested.

α-Synuclein Secondary Structure

α-Synuclein is a relatively small (140 amino acids), natively unfolded protein, exhibiting a random coil secondary structure in normal physiological conditions [10, 11]. At neutral pH, it is calculated to have 24 negative charges (15 of which are located in the last third of protein sequence) leading to a strong electrostatic repulsion, which contributes to the lack of folding of α-synuclein [10, 11]. Natively unfolded proteins possess "turn-out" responses to changes in the environment, as their structural complexities increase at high temperature or at extreme pH [10, 11]. They are very flexible, but might adopt relatively rigid conformations in the presence of natural ligands [10, 11]. For example, α-synuclein is shown to adopt mostly α-helical secondary structure upon association with small unilamellar vesicles or detergent micelle surfaces [10, 11].

Tertiary Structural Details of α-Synuclein

α-Synuclein being an unfolded protein has posed problems to structural biologists to understand its 3D structure. Both NMR and crystallography studies have faced problems as α-synuclein tends to aggregate quickly thus making the 3D understanding of its conformation difficult. However, structural biologists solved the partial conformation details of α-synuclein using NMR at freezing temperatures and solid-state models [13].

Recent NMR studies showed that α-synuclein fibrils from the PD-related A53T mutant of α-synuclein fibrils contain regions with increased mobility and structural elements different from beta-strand character, in addition to the rigid beta-sheet-rich core region. But in the wild-type α-synuclein, the C-terminus is found to be flexible and unfolded, whereas the main core region is highly rigid and rich in beta-sheets. These results provided insight that a disease-related mutant of α-synuclein differs in both aggregation kinetics and fibril structure [13]. Further, Heise et al. [13] showed that oligomers or protofibrils adopting the cross-beta-sheet structure is the characteristic of fibrillating proteins and these conformations are assumed to be the cytotoxic species.

Further, Bertoncini et al. [14] through NMR studies showed that the spin-labeled monomers would have a reduced shielding effect between different domains of the molecule due to the accelerated aggregation kinetics in A53T α-synuclein. This may result in a more efficient exposure of the C-terminal region leading to the more facile association of neighboring molecules. Also the accelerated aggregation kinetics itself may result in a polymorphic pattern of assembly as observed by an extended β-sheet core region. But in the fibrils of both the mutant and the wild-type α-synuclein, the C-terminus extending from at least residue 107 is flexible, whereas the N-terminus extending from residue 22 is rigid. All together the data provided important information on the effect of altered aggregation properties on the fibril morphology mutant of α-synuclein. This data is crucial in understanding protein misfolding in disease condition.

Vilar et al. [15] based on NMR data and other data from literature proposed a folding pattern in the core of α-synuclein fibrils. The proposed folding conformation has a five-layered

β1-loop–β2-loop–β3-loop–β4-loop–β5-loop-sandwich model. The five layers in parallel are generated when incorporated into a protofilament of a fibril. Besides in the straight fibril type, two protofilaments align with each other to form a fibril that can self-align again by itself. But in the twisted fibrils, two protofilaments twist along each other. All these models indicated that multiple protofilament packing is possible. Further, the comparative analysis indicated that the two packings of straight and twisted fibrils also require different filament interactions. Further, a comparative analysis between the beta-sheet secondary structural elements of the fibrils and the protein sequences indicates that the deletion of residues 74–84 in mammalian α-synuclein interferes with the fibril formation, because these residues constitute beta-strand in the fibrils. These data provided insight that amino acid sequence and layered folding pattern have influence on α-synuclein folding behavior.

Further, Wu et al. [16] characterized the conformational properties of natively unfolded human and mouse α-synuclein. Human α-synuclein is highly homologous in amino acid sequence to mouse α-synuclein. The mouse α-synuclein is known to aggregate more rapidly than human α-synuclein. They also made novel studies using NMR on both α-synuclein at supercooled temperatures (263 K). The studies indicated that both α-synuclein are natively unfolded at low temperature. Further, they have found different propensities to secondary conformation in synuclein. Mouse α-synuclein showed a higher propensity to form helical conformation around the C-terminal and also the loss of transient long-range contacts from the C- to the N-terminal end. Besides, mouse α-synuclein showed the lack of back-folding from the C-terminal end to the N-terminal region. Also the authors proposed that the restricted mobility in the N-terminal region may arise from transient interchain interactions, suggesting that the N-terminal KTK(E/Q)GV repeats may serve as initiation sites for aggregation in mouse α-synuclein [16]. These studies provided novel data on conformational dynamics of α-synuclein based on species specificity.

Very recent studies indicated that the segment residues 38 to 94 adopt mainly β-sheet conformation, with a fibrillar core and residues 15–20 from the N-terminus showed a rigid ordered β-sheet [17]. Schwalbe et al. [18] reported that polyproline II plays an important role in aggregation-nucleation sites, indicating that this region is important for aggregation.

It is still puzzling to understand the mode (antiparallel or parallel) of α-synuclein self-assembly and the role of exact alignment of the central hydrophobic region folding of α-synuclein. Further, Jiang et al. [19] studied α-synuclein assembly using with or without the incorporation of artificial leucine zippers. The studies indicated that Zips can accelerate filament assembly in both the parallel and antiparallel fashions. Further, the results also indicated that the central hydrophobic region is not essential for precise alignment. The results also suggested that α-synuclein can assemble in both parallel and antiparallel modes. These studies provide a new clue in conformation dynamics of α-synuclein.

α-Synuclein Modeling Data: Different Theories and Models

α-Synuclein consists of three major domains: the N-terminal region (residues 1–65), the non-A-beta-amyloid component (NAC) region (residues 65–95), and the C-terminal region (residues 95–140). The N-terminal region is characterized by a lipid-binding domain similar to apolipoprotein domains which forms amphipathic α-helices [20]. The NAC domain presents high propensity to undergo conformational change from random coil to β-sheet leading to amyloid fibril formation. Both the N-terminal and NAC region present six to seven imperfect repeats of the KTKEGV motif, which has lipid association property [21]. The C-terminal region has no defined conformational state and only presents a low hydrophobicity and negative net charge (due to the presence of acidic amino acids) [22].

As mentioned earlier, three familial PD mutations namely A30P, E46K, and A53T are found to be located in the N-terminal domain and influence α-synuclein misfolding, aggregation, and polymorphism in fibrillation [4, 5, 23]. Still it is not clear how these familial mutations affect the domain specific folding behavior of α-synuclein. This raises the fundamental issues on how familial PD mutations can influence domain specific folding behavior. And are they thermodynamically favorable? If we can understand the above issues, it is possible to develop a favorable conformation through small molecules which leads for future drug discovery. We used bioinformatics tools to understand the folding complexity of α-synuclein. The data on secondary conformations, solvent accessibility, disorder structure prediction, 3D predicted models, Ramachandran plot, and energy change per residue analysis are developed.

(i) The NetSurfP secondary structure prediction (Fig. 1a, b) method is used to understand the possible conformations in wild-type (WT) and α-synuclein mutants [24]. WT α-synuclein presents lower alpha helix propensity values at residues 34–52, 93–105, and 116–120. E46K mutation, which involves the substitution of glutamate (E) for lysine (K) at position 46, shows higher α-helix propensity value. This change suggests that substitution of negatively charged (E) for positively charged (K) could favor α-helix conformation around residue position 46. Further, at this site, there is a decrease in β-sheet propensity around residues (46–59) compared to WT α-synuclein. Random coil propensity increases only for E46K mutation at residues 47–54. There are no significant localized changes in other analyzed PD mutations (A30P and A53T).

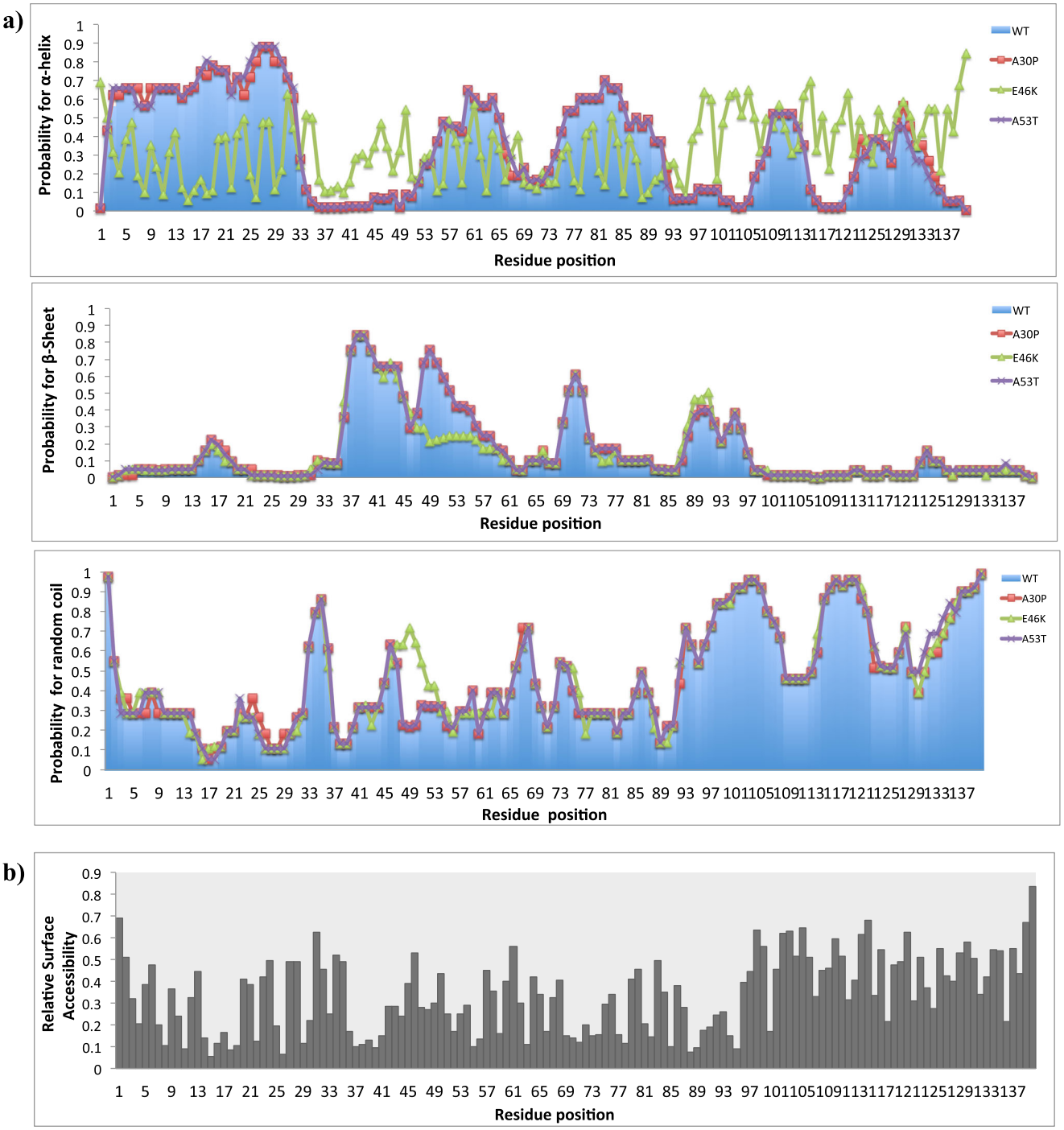

Fig. 1 **a** Secondary structure prediction for WT and mutant α-synuclein. All mutant predictions were superimposed over WT α-synuclein (*blue bars*) to understand how mutations influence α-synuclein structural conformation. **b** Relative protein surface accessibility per amino acid residue. Highest relative solvent accessibility is observed at the C-terminal domain. Secondary structure prediction and relative solvent accessibility prediction were obtained from the NetSurfP web server (http://www.cbs.dtu.dk/services/NetSurfP/)

(ii) Relative solvent accessibility (RSA) analysis (Fig. 1b) indicates the extent of exposure and burial of residues when a protein folds into a 3D structure [24]. The NetSurfP RSA analysis is used to assess which α-synuclein domains are prone to adopt a folded or misfolded state. Results indicate that the C-terminal region is highly exposed region, whereas the N- and NAC regions present the buried residue regions. These observations are in agreement with studies on α-synuclein core region (residues 31–101) which is protease resistant [25]. This raises the question if the NAC domain is

the most likely region where α-synuclein folding may start. RSA analysis is done for WT and mutant α-synuclein amino acid sequence and no changes are observed.

(iii) Predicted protein disordered regions give an estimation of residue segments of the peptide chain that do not adopt a stable conformational state [26]. These unstructured regions are essential for protein structure predictions and functionality. Intrinsic disordered segments in proteins also may be involved in functional roles such as molecular recognition, cell signaling, transcription, and translation [26]. Disordered regions in α-synuclein are determined using the DISOclust system [27]. Figure 2 demonstrates that the predicted disordered regions in α-synuclein are found at the N-terminal domain (1–7 residues) and in the C-terminal domain (residues 95–140). The NAC region does not present any disordered conformation.

The FoldIndex analysis (Fig. 3) predicts that α-synuclein presents only one disordered region in residues 97–140, with an unfoldability value of 0.053 and a phobic value of 0.455. α-Synuclein unfolded region is characterized by low hydrophobicity and high charge value contributing to the lack of folding at the C-terminal region [28]. This result is consistent with the predicted disordered region from the DISOclust disordered prediction using a different algorithm. The three mutations do not change the FoldIndex pattern of synuclein.

(iv) I-Tasser and PSIPRED automated structure modeling servers used to generate WT, lipid-bound, and α-synuclein mutants predicted 3D structural models [29–31]. Figure 4(a) demonstrates that when α-synuclein binds to lipid, then it presents two major α-helices at the N-terminal region of the protein. Furthermore, predicted lipid-associated α-synuclein presents two alpha helices joined by a turn similar to the helix-turn-helix structural motif. This structural motif is considered a transcription factor that enables DNA binding, consistent along with experimental studies reporting α-synuclein has a propensity to bind to DNA molecule [32]. Interestingly, the repeat hexamer motif (KTK(E/Q)GV) is found in the same residue region involved in α-helix formation of the predicted lipid-bound synuclein. This observation agrees with reported α-synuclein lipid

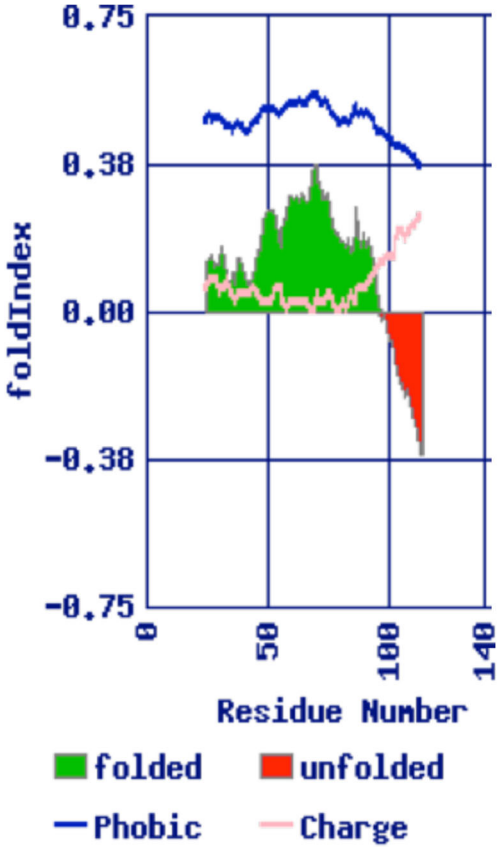

Fig. 3 α-Synuclein FoldIndex plot with window size 51. Prediction tool was used to predict if a given protein is intrinsically unfolded. α-Synuclein presents an unfolded region at the C-terminal domain (residues 97–140), with unfoldability value of 0.053 (charge 0.064, phobic 0.455)

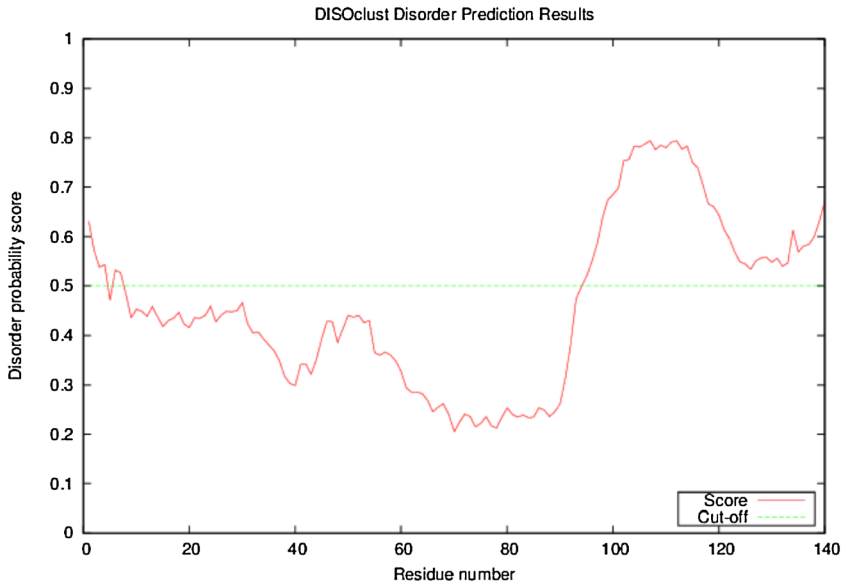

Fig. 2 DISOclust prediction of disorder regions in α-synuclein. The predictor score is plotted against the residue number. The threshold is 0.5 and residues above this value are considered disordered. Residues from 95 to 140 present a higher confidence value for the predicted disordered region

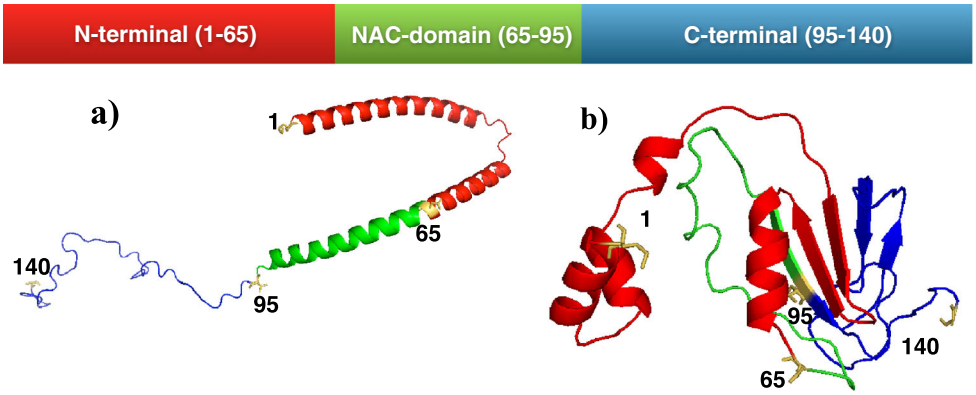

Fig. 4 *a* Lipid-associated α-synuclein obtained from I-Tasser protein structure prediction server using PDB IXQ8 as target template alignment. *b* Predicted structural model for free state α-synuclein obtained from PSIPRED workbench web server. Figures are colored according to the different protein domains: N-terminal (*red*), NAC domain (*green*), C-terminal (*blue*)

binding at the N-terminal and NAC domain [20]. Free state α-synuclein model (Fig. 4(b)) presents four helical structures at the N-terminal region and β-sheet strands at the NAC region. The predicted structural model for free state α-synuclein presents two distinct structural motifs: helix-loop-helix and beta hairpin. The helix-loop-helix and beta hairpin are also found close to the KTK(E/Q)GV sequence repeats at the N- and C-terminal regions. The NAC region presents antiparallel beta strands, which are in agreement with reported α-synuclein β-sheet-rich core region (residues 38–94) [17]. The C-terminal region remains unfolded in predicted structural models, lipid-bound, and free α-synuclein suggesting that residues at the C-terminal do not play a major role in α-synuclein folding pattern. Studies have reported that α-synuclein fibrillation is blocked when the C-terminal is removed [33, 34]. If presence of the C-terminal restrains fibrillation of α-synuclein, what residue and conditions are necessary for these interactions to remain and maintain the natively unfolded structure of α-synuclein?

(v) PROCHECK Ramachandran plot (RP) (Fig. 5) is used as tool to understand if the predicted lipid-bound, free WT, and α-synuclein mutant structures have any backbone conformation preference [35]. The RP for the lipid-bound structure indicates that there is a strong bias to adopt a helical conformation, and no nonglycine residues are found in the disallowed regions of the plot suggesting no conformational constrain. In contrast, the RP for the free state synuclein is not biased to adopt any specific conformation; a significant number of residues is found in the α-helix and β-sheet regions of the plot. Additionally, nonglycine residues (Asp98 and Thr64) are found in the disallowed regions, indicating conformational constrains at the NAC and C-terminal domain. A53T RP does not present any conformational constrain in comparison to free and mutated α-synuclein structures. On the other side, it is important to highlight that both A30P and E46K structures present conformational constrains at the N- and C-terminal domains.

Overall, the RP analysis indicates that the lipid-bound and mutant A53T α-synuclein structural model present no residues in disallowed regions, which suggests stability. This raises the question of whether formation of lipid α-synuclein complex and mutation A53T in α-synuclein enhance favorable conformations to aggregate.

(vi) Figure 6 presents the energy change per residue analysis by FoldX [36–38]. The results indicate that lipid-bound α-synuclein present the lowest energy change per residue at the NAC domain (residues 65–92). The N-terminal (residues 35–47) and C-terminal domain (residues 92–135) of lipid-bound α-synuclein presents the highest energy change per residue in comparison to the C-terminal of free state and mutant α-synuclein structure. When comparing all the studied structures, the C-terminal domain seems to be the most unstable domain since it presents the highest energy change values per residue. In contrast, the NAC domain is the most stable domain with the lowest energy change values per residue. Residue energy change values at the NAC domain indicate residue interactions at this region may lead to stable conformation. Furthermore, it seems that mutations at the N-terminal domain reflect an unfavorable thermostability effect at the C-terminal domain. Overall, α-synuclein energy change per residue pattern presents a varied energy landscape with very low energy minima peaks in the middle of the chain and very high-energy peaks at the end of the chain. The assorted distribution of high and minima energy values along the peptide chain explains the conformational plasticity of α-synuclein when adopting a specific conformation in the presence of a specific ligand (i.e., DNA, lipid) [39].

The energy change per residue value as seen in Fig. 7 is summed up to the present total energy change for lipid-bound and α-synuclein mutants against free state synuclein. For example, the total energy change of α-synuclein mutant structures is calculated from the free α-synuclein using the following formula: ($\Delta\Delta G$ (change)$=\Delta G$(MT)$-\Delta G$(WT)). Positive total energy change in A30P and E46K mutants indicates low stability. However, the lipid-bound and A53T mutant total energy change is negative. These observations indicate that the dynamic folding behavior of α-synuclein at these states is favored at low energy barriers. This may be the reason why α-synucleins have high affinity for lipid binding during fibril formation and possibly explains why A53T mutant α-synuclein fibrillates faster than WT and other two mutant structures [40, 41]. All the above

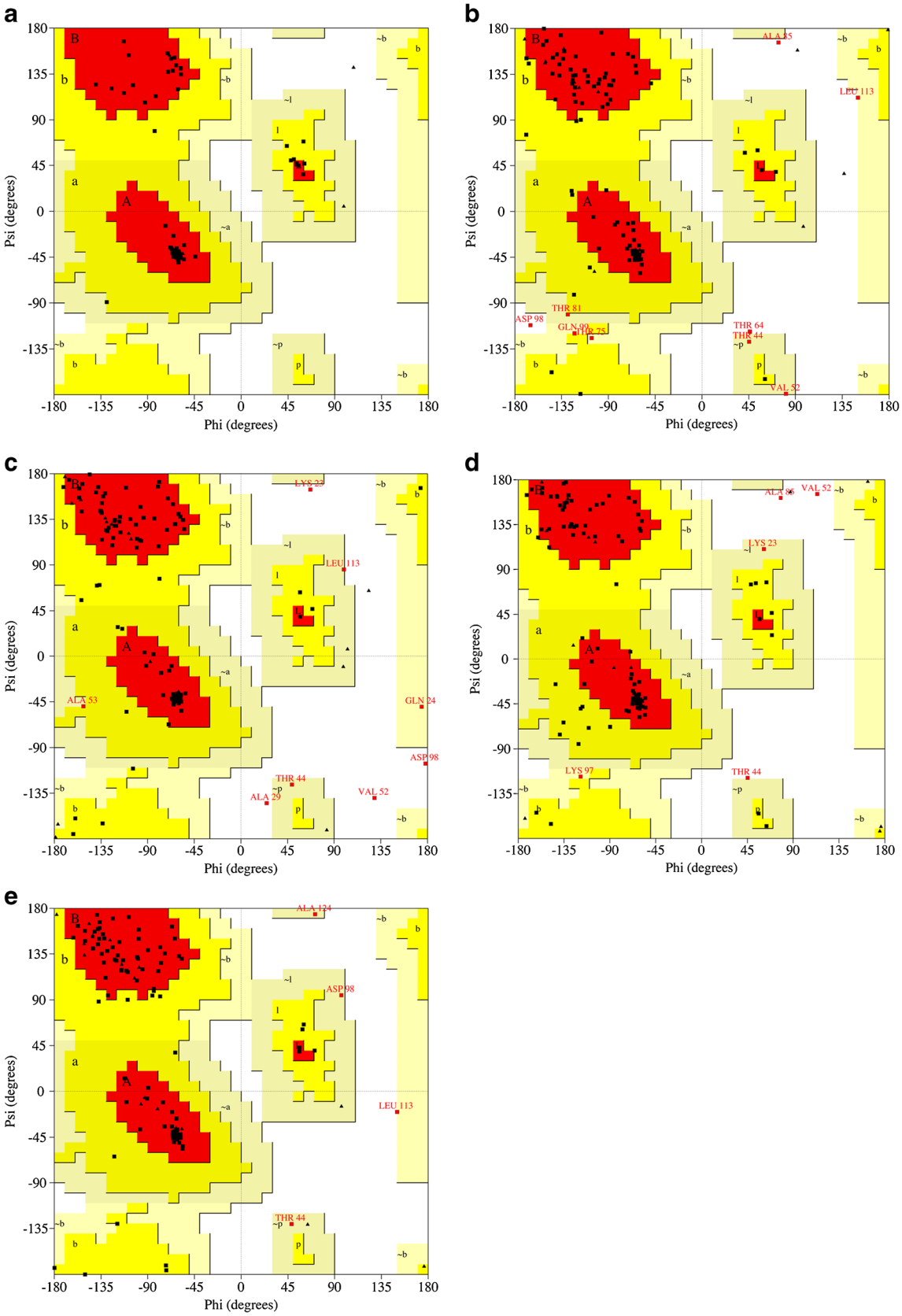

Fig. 5 Ramachandran plot for **a** lipid-associated α-synuclein, **b** wild-type α-synuclein, **c** Ala30Pro mutated α-synuclein, **d** Glu46Lys mutated α-synuclein, **e** Ala53Thr mutated α-synuclein

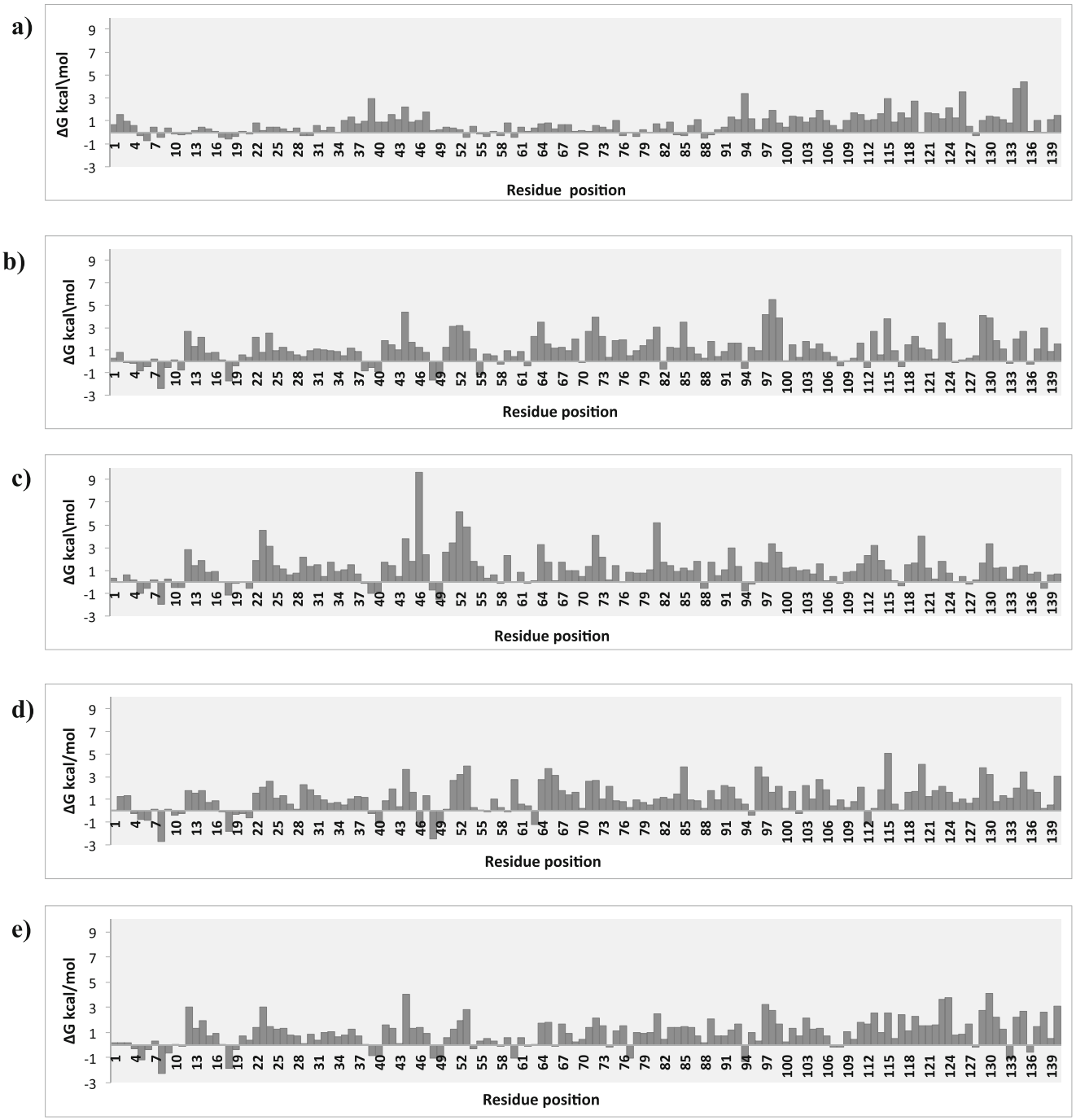

Fig. 6 α-Synuclein energy stability per residue. Representation of the energy contribution of each residue to the stability of the protein. **a** Lipid-bound α-synuclein. **b** WT α-synuclein. **c** Mutant A30P α-synuclein. **d** Mutant E46K α-synuclein. **e** Mutant A53T α-synuclein

studies indicate that lipid α-synuclein complex has a helix propensity, however with total low energy. The mutants caused disordered propensities leading to faster fibrillation process. We have to look for ligands which favor α-helicity with thermodynamically stable conformation with low aggregation pattern.

Based on the above studies, we argue that the general conformational properties of α-synuclein are low compactness, absence of globularity, low secondary structure content, and high flexibility [42]. The structural parameter is the degree of globularization that reflects the presence or absence of a tightly packed core in the α-synuclein molecule. α-Synuclein is coil-like, characterized with low intramolecular packing density. Thus, natively unfolded proteins illustrate the absence of a globular structure, as they lack tightly packed core under physiological conditions in vitro [9, 10]. The most unambiguous characteristic of the conformational state of a α-synuclein is the

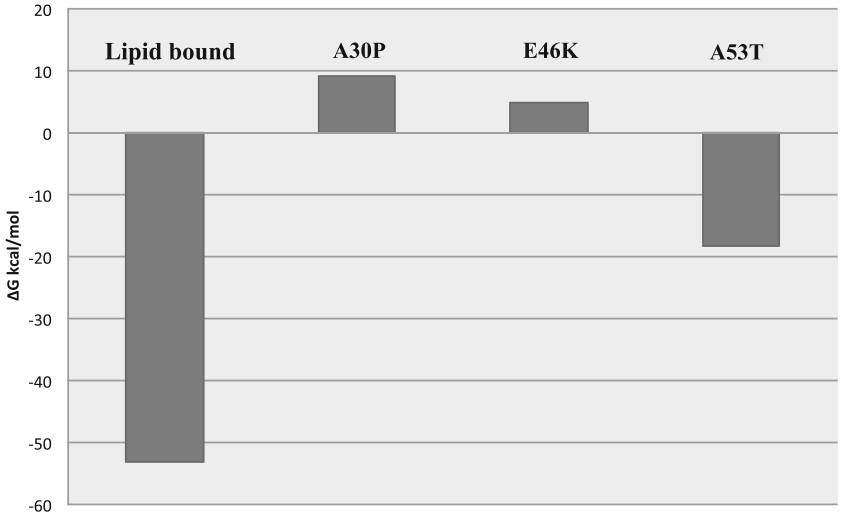

Fig. 7 Total energy change for lipid-bound and mutant α-synuclein. Lipid-bound and mutant α-synuclein total energy chance per residue value was subtracted from the total energy change per residue value of WT α-synuclein. Energy change per residue values were obtained using FoldX forcefield command

hydrodynamic dimensions. Based on the hydrodynamic dimensions, whether it is unfolded or has lost its noncovalent structure can be identified [42]. This is because an essential increase in the hydrodynamic volume is associated with the unfolding of the α-synuclein molecule. It is also known that globular proteins whether or not may exist in at least four different conformations, native, molten globule, premolten globule, and unfolded, that can discriminate the degree of compactness. α-Synuclein is characterized by an increased intramolecular flexibility derived from heteronuclear advanced NMR studies. Increased flexibility of natively unfolded proteins is indirectly confirmed based on the resistance for protease digestion [42].

Further, the amino acid sequences of these proteins indicate that these proteins have little ability to adopt a folded structure under physiologic conditions [42]. This is mainly attributed to the low mean hydrophobicity and relatively high net charge. The high net charge induces charge-charge repulsion [42]. But the low hydrophobicity causes less driving force for a compact structure. It is still puzzling on the presence of many ways in which a specific sequence can lead to the absence of a globular structure. In α-synuclein, the isolated N-terminal region is predicted to fold. But the relevance of these characters in monomeric to oligomeric and protofibrils to aggregation kinetics is still not clear.

Probable Functions of α-Synuclein

The exact role of α-synuclein in normal cell functioning is not known to date. Understanding the role of α-synuclein in normal cell life might be of critical importance since disruption of its normal function might result in neurodegeneration [43]. Several hypotheses for the normal function of α-synuclein have been proposed, based on its structure, physical properties, subcellular localization, and interacting partners [44]. The primary site of α-synuclein function is most likely presynaptic, as it can be isolated from synaptic-membrane fractions and localizes predominantly near and around the vesicles of the presynaptic terminal [45]. Interestingly, α-synuclein binds exclusively to acidic phospholipids, especially phosphatidic acid and to vesicles with small diameters. This may target the protein to specific subpopulations of membranes or vesicles [46].

Though it appears that lipid binding is a broadly distributed property of α-synuclein [47], not all cellular α-synuclein seems to be linked to membranes, since it can also be purified from the cytosol and nucleus as reported earlier [48]. The physical and functional homologies of α-synuclein with 14-3-3 chaperone proteins suggest that α-synuclein may play a role in cell signaling pathways [49]. α-Synuclein stimulates protein kinase A that phosphorylates the Ser262 and Ser356 residues of tau protein [50], which is the major constituent of insoluble paired helical filaments found in neurofibrillary tangles and plaque neuritis in AD [51]. α-Synuclein is detected in axons and developing presynaptic terminals after their formation in rat embryonic hippocampal cells in culture, suggesting a possible role in synaptic development and maintenance. α-Synuclein also seems to contribute to neuronal differentiation. The arguments in this direction are (a) in vivo, α-synuclein is localized in the cell body of neuronal precursors during early embryonic development [52], but in presynaptic terminals in postnatal and adult cortex; and (b) a sustained increase in α-synuclein levels is observed when rat pheochromocytoma cells (PC12) are treated with nerve growth factor, which induces a neuronal phenotype [53]. The involvement of α-synuclein in synaptic plasticity and neuronal differentiation may be mediated by the selective inhibition of phospholipase D2 by α-synuclein, since isoforms of phospholipase D were shown to be implicated in cell growth and differentiation [54].

However, the involvement of α-synuclein in neuronal and synaptic development could not be confirmed in mice lacking the α-synuclein gene homozygously, since these mice are behaviorally normal and showed neither macroscopic nor microscopic changes in their nervous system [55]. Hence,

inactivation of the α-synuclein gene does not lead to a significant neurological phenotype, although changes in dopaminergic electrophysiology may reflect a specific function related to neurotransmitter release. When α-synuclein expression is markedly reduced in cultured rat neurons or abolished in α-synuclein knockout mice [55], the number of vesicles in the distal pool of the presynaptic terminal is reduced indicating a role for α-synuclein in vesicular dynamics. According to Cole et al. [56], α-synuclein involvement in lipid metabolism cannot be ruled out, given its propensity to bind molecules with high hydrophobic content or exposed hydrophobic domains. The major difficulty in understanding α-synuclein function has been its inherent flexibility in native structure and its altered conformation in the presence of lipids. It is not known whether the molecules that bind to α-synuclein in vivo do so in its membrane-associated (modular) or free (natively unfolded) state, or both [56]. It also remains to be investigated whether the interactions of α-synuclein with membranes influence its self-assembly and filament formation. The functional biology of synuclein needs to be further explored.

There are limited studies on α-synuclein phylogenetic conservation regions that provide guidance for understanding the structure and function of this disordered protein. Yuan and Zhao [57] provided insight on specific evolutionary relationship across all three synuclein subfamilies (alpha, beta, and gamma). The analysis of 253 synuclein sequences from 73 organisms presented a highly conserved N-terminal, while no global conservation is observed within the C-terminal in all the synuclein subfamilies. Most of the conserved residues (42 in total) are found within the 6 of the 11 repeat motif KTK(E/Q)GV, similar to the observed motifs in apolipoproteins [20]. This finding suggests that conserved residues are likely to play a structural role, however less is known about the functional role of these repeats. Further, conservation analysis of the synuclein subfamilies showed that some residues have evolved into group-specific residues providing distinct functional properties to synuclein subfamilies. For instance, the analysis revealed that His50 is 91 % conserved only in α-synuclein subfamily. Dudzik et al. [58] studied the functional role of this residue as metal binding by substituting histidine for alanine at position 50 resulting in impaired copper binding at this position.

Levitan et al. [59] indicated that conserved C-terminal negatively charged residues play a protective role against rapid α-synuclein aggregation. The experimental results showed that α-synuclein with a truncated C-terminal exhibited a faster aggregation rate due to the absence of acidic residues, whereas α-synuclein phosphorylated at Ser129 showed a slower aggregation rate as a result of increased negative charge at the C-terminal. Alignment of 15 homologous α-synuclein sequences from different mammals demonstrated complete conservation of Ser129 and negatively charged residues at the C-terminal. This is in agreement with Sato et al. [60] who reported that Ser129 is the site where 90 % of the aggregated α-synuclein gets phosphorylated. Thus, these studies indicate the role of conserved negatively charged residues in the modulation of α-synuclein aggregation.

Aggregation of α-Synuclein

α-Synuclein is natively unfolded protein and tends to aggregate into fibrils. Though the mechanism of aggregation is not understood, few pathways are mapped. The mechanism of aggregation is multifaceted in nature and understanding aggregation has a therapeutic potential [61]. The native conformation of synuclein in the human brain is not known. A recent study indicated that synuclein exists in metastable conformers and stable monomer in the human brain [62].

α-Synuclein Monomer

α-Synuclein is located in synaptic nerve terminals in the normal brain [62, 63]. However, its functional structure at this location is less clear as it may exist in an exchangeable soluble cytosolic pool of monomeric α-synuclein and a vesicle-associated pool, where the latter may contain helical monomeric conformers as well as oligomeric species [56]. A range of posttranslational modifications of monomeric α-synuclein like C-terminal truncations, phosphorylations, ubiquitin-immunoreactive inclusions, and oxidative modifications have been detected in pathological nerve cell lesions [64]. Further, extrinsic factors affecting the aggregation of monomeric α-synuclein comprise metal ions, etc., as discussed later.

α-Synuclein Oligomers

When α-synuclein monomers start to associate, we envision the existence of an initial population of oligomers that is in equilibrium with the monomers [65–67]. In contrast to the monomers, these amyloid-type oligomers display distinct structural features that can be visualized by oligomer-specific antibodies [68]. They are enriched in cross-β structure and have been described as about 4 nm in height and 20 nm wide [69], but their morphology appears heterogeneous. The low amount of the oligomers present during α-synuclein aggregation and their transient nature have made their characterization difficult but size estimates based on gel filtration are in the range of 140–800 kDa [68], and antibody binding demonstrates that they display some epitopes exclusive for oligomers and some shared with filaments [68]. The protocols for the preparation of oligomers have allowed the generation of large amounts of oligomers that share a range of biophysical characteristics with the abovementioned oligomers [70]. These methods are based on the rapid reconstitution of lyophilized α-synuclein to a high concentration in buffer at low temperature for a relatively short time period

followed by filtration and gel filtration. It will be important to compare these so-called fast oligomers to the less abundant oligomers formed during the normal slow aggregation of recombinant α-synuclein but also to the α-synuclein in brain tissue that displays a reduced solubility. Such in vivo oligomers have previously only been indicated by the presence of α-synuclein species in the soluble fraction of brain extracts that displays resistance to depolymerization [71]. A study with atomic force microscopy investigation of glial cytoplasmic inclusions isolated from unfixed brain tissue may have revealed novel insight into the nature of brain α-synuclein oligomers [72]. The purified glial cytoplasmic inclusions containing both filamentous and granular material were solubilized in mild detergents leaving α-synuclein-positive annular particles. The sizes of the particles did depend on the detergent used as did the size of recombinant monomeric α-synuclein and they can accordingly not directly be compared with in vitro formed oligomers made without detergents. However, this provocative study suggests that annular α-synuclein oligomers are associated to the glial cytoplasmic inclusions or even that the filaments may be composed of annular oligomers joined by hydrophobic interactions that are sensitive to mild detergents.

α-Synuclein Filaments

Filaments develop during the aggregation process when the oligomers are consumed [73]. The in vitro formed filaments are 8–10 nm in height and 10 nm wide as determined by atomic force microscopy and by electron microscopy, respectively [6] They have a straight appearance [6, 69] and possess a cross-β structure as demonstrated by dye binding properties, microscopy, and spectroscopy [69]. The in vitro formed α-synuclein filaments are similar to th filaments extracted from Parkinson's disease and Lewy body dementia brains in terms of diameter [6, 2]. This contrasts those from multiple system atrophy brain tissues that are of a larger diameter [74] which may be due to coating materials including proteins [75]. However, washing of those filaments revealed a core filament of similar diameter as the in vitro formed filaments, suggesting a heavy coating by filament-associated proteins [75]. The existence of such coat proteins is corroborated by the discovery of several filament-binding proteins [76, 68]. This raises the question as to whether α-synuclein-positive filaments in brain inclusions consist of homopolymers of α-synuclein heteropolymers wherein or whereupon α-synuclein aggregate binding proteins are incorporated. The filaments formed in vitro are usually straight and unbranched and similarly structured filaments are likely to exist in vivo as judged from electron microscopic studies of LB and glial cytoplasmic inclusions [2, 77]. The electron micrographs display mesh-like α-synuclein-positive structures with frequent branching [68, 69]. The branching α-synuclein aggregates may represent heteropolymers where the α-synuclein aggregate binding proteins form the sites for elongation in more directions. Moreover, it suggests that several protein factors may be involved in the regulation of α-synuclein aggregation in vivo and represent effectors for the possible toxicity of α-synuclein aggregates.

Metals as Biofactors Modulating α-Synuclein Conformation and Aggregation

Essential metals like Cu, Fe, Mg, etc. have a pivotal role in brain function. Any elevation or decrease in these metal ions leads to neuronal dysfunction. There are lines of evidence regarding the elevation of Cu and Fe in neurodegenerative disorders and their significant role in neuropathology in the brain [78–81]. Also lines of evidence suggest that metals act as etiological factors in aggregation and thus gained central role neurodegeneration [82, 83]. Herein we summarized the data on the interaction of metals with α-synuclein. A number of metals have been shown to stimulate α-synuclein aggregation in vitro, including Al^{3+}, Fe^{3+}, Co^{3+}, Mn^{2+}, Ca^{2+}, and Cu^{2+} [10]. Al^{3+} displays the most pronounced effect on increasing the α-synuclein aggregation [10]. Interactions of metals with the acidic C-terminus have been proposed as a model for these effects [10]. Mg^{2+} inhibits spontaneous as well as iron-induced α-synuclein aggregation [84], suggesting that some metals can counteract the effect of others. Further studies showed that redox-active metal ions such as iron (Fe) and copper (Cu) are known to enhance α-synuclein fibrillogenesis in a differential pattern and hypothesized that protein oligomers have a dynamic function in neuronal cell dysfunction [65, 85]. Studies from Bharathi and Rao [86] reported the binding efficiency of Cu and Fe to α-synuclein by fluorescence studies. They found that Cu and Fe showed a differential binding pattern toward α-synuclein (wild-type and A30P, A53T, and E46K mutant forms) as revealed by intrinsic tyrosine fluorescence, thioflavin-T fluorescence, and 1-anilino-8-naphthalenesulfonate-binding studies. The experimental data may be useful in understanding the hierarchy of metals binding to α-synuclein. Further, Bharathi et al. [87] reported that Cu (II) and Fe (III) selectively and differentially induced the formation of discrete α-synuclein fibrillar species. Cu (II) has induced thin long network-like fibrils with the wild type of α-synuclein, while the mutant showed amorphous aggregates with no fibrillar forms. Fe (III) induced short and thick fibrils with both wild and mutant forms and similar to α-synuclein fibrils incubated without metal ion. These studies provided a new clue that metals induce differential polymorphic aggregates in α-synuclein. Also Bharathi and Rao [88] provided new evidence on thermodynamic imprinting of stoichiometry binding of metals to α-synuclein. α-Synuclein monomer (wild and mutant forms) titrated by Cu (II) showed two binding sites, with an apparent and with Fe (III) single binding

site. Altogether, the data prove conclusively that Cu (II) has preferentially two sites of binding to α-synuclein. Fe (III) possesses a single binding site and has high binding efficiency. And Cu (II) is enthalpically driven with large entropy than Fe (III). Presumably, these findings might lead to a new conceptual scheme to understand the hierarchy of metal-α-synuclein interactions, which based on the principles of thermodynamics, thus reflects a better mode to understand the binding properties of α-synuclein with metals [88]. The study of Bharathi et al. [85] reported an independent Cu binding site in both the lipid-binding N-terminal domain and the highly acidic C-terminal domain of α-synuclein. The NMR studies clearly showed that Cu binds at the N-terminal and the histidine sites, as well as the sites in the C-terminal tail. Binolfi et al. [90] reported the binding features of the divalent metal ions Fe (II), Mn (II), Co (II), and Ni (II) and their effects on α-synuclein fibril formation. The results indicated that the divalent metal ions bind preferentially and with low affinity (millimolar) to the C-terminus of α-synuclein. These findings indicated a strong link between the specificity of α-synuclein-metal (II) interactions and the enhancement of aggregation of α-synuclein. Further, Drew et al. [91] studied the Cu (II) binding to recombinant human α-synuclein using electron paramagnetic resonance (EPR) spectroscopy. The study clearly showed that the wild-type (wt) α-synuclein has a binding stoichiometric Cu (II) via two N-terminal binding modes at pH 7.0. Further studies using electron spin-echo envelope modulation (ESEEM) indicated that α-synuclein confirmed the second binding mode at pH 7.4 through the coordination of His50. However, at acidic pH 5.0, His50-anchored Cu (II) binding is diminished, but the {NH2, N (−), beta-COO (−), H_2O} binding still persisted in conjunction with another two binding modes. In total, the studies showed that there are four Cu (II) binding modes within the pH range of 5.0–7.4. Lee et al. [92] studied the Trp4 fluorescence decay kinetics and reported that Cu (II) binds tightly (K_d~100 nM) near the N-terminus at pH 7. Further studies with histidine mutant showed that a histidine imidazole is not a ligand in this high-affinity site. Kostka et al. [93] studied the role of Fe (III) at low micromolar concentrations and found that it increases aggregation and also favors the formation of larger oligomers ("intermediate II"). These oligomer species will seed fibril formation. Interesting findings from this study are that Fe (III)-induced oligomers are SDS resistant and could form ion-permeable pores in a planar lipid bilayer. These results provide insight on the role of ferric iron and of toxic oligomer species in PD. A study from Liu and Franz [94] tried to understand the phosphorylation-dependent metal binding by α-synuclein peptide fragments. They have studied the metal-binding properties of synthetic peptides and phosphopeptides of residues 119–132 of the C-terminal, polyacidic stretch of human α-synuclein, with the sequence Ac-Asp-Pro-Asp-Asn-Glu-Ala-Tyr-Glu-Met-Pro-Ser-Glu-Glu-Gly (α-synuclein 119–132). The peptide pY125, which is a tyrosine, is replaced with phosphotyrosine. The interaction of other metal ions is studied by electrospray ionization mass spectrometry, which confirmed that pY125 (phosphorylated tyrosine-125) is selective for trivalent metal ions over divalent metal ions. The study also indicated that Fe (III) and Al (III) induce peptide dimerization through metal ion cross-links. The circular dichroism studied provided insight that Fe (III) causes a partially folded conformation for pY125, with no change for pS129, the unphosphorylated analog. The studies indicated that the phosphorylated amino acid influences a peptide's metal-binding specificity and its influence on conformation. Recent studies indicated that the phosphorylation at sites Y125 and S129 influences the metal-binding properties on synuclein [94]. These studies provide lines of evidence on the role of metals in modulating synuclein aggregation.

Conclusions

Assuming that protein misfolding is triggered by conformational changes stabilized by protein oligomerization, an interesting strategy would be to preclude the stabilization of the misfolding or better to destabilize the monomeric intermediate and the early oligomers. We have postulated that if small molecules or molecular chaperones destabilize the abnormal conformation, this might be useful to correct protein misfolding. Considering that in most neurodegenerative disorders the misfolded protein is rich in beta-sheet structure, our focus should be mainly on the design of small molecules to prevent and reverse beta-sheet formation (beta-sheet breakers). The principles to manipulate protein conformation may provide a general platform technology to design drugs for the treatment of PD.

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