Stabilization of Partially Folded Conformation during α-Synuclein Oligomerization in Both Purified and Cytosolic Preparations*

Received for publication, September 24, 2001, and in revised form, October 3, 2001
Published, JBC Papers in Press, October 5, 2001, DOI 10.1074/jbc.C100551200

Vladimir N. Uversky†,†,†, He-Jin Lee¶, Jie Li‡, and Seung-Jae Lee**

From the †Department of Chemistry and Biochemistry, University of California, Santa Cruz, California 95064, the §Institute for Biological Instrumentation, Russian Academy of Sciences, 142292 Pushchino, Moscow Region, Russia, and The Parkinson’s Institute, Sunnyvale, California 94089

Aggregation of α-synuclein is tightly associated with many neurodegenerative diseases, such as Parkinson’s disease, dementia with Lewy body, Lewy body variant of Alzheimer’s disease, multiple system atrophy, and Hallervorden-Spatz disease, implicating a crucial role of agerelated diseases, including Parkinson’s disease (PD),1 demen-
tia with Lewy body (DBL), Lewy body variant of Alzheimer’s disease, and multiple system atrophy (1, 2). Importantly, two

Deposition of aggregated forms of α-synuclein in neuronal or glial cytoplasm is a pathological hallmark of many neurodegen-
erative diseases, including Parkinson’s disease (PD),1 demen-
tia with Lewy body (DBL), Lewy body variant of Alzheimer’s disease, and multiple system atrophy (1, 2). Importantly, two

mutations of the α-synuclein gene with autosomal dominant inheritance were linked to early-onset familial PD (3, 4), and the mutant forms of the protein tend to form aggregates more rapidly than the wild type (5–8). The transgenic flies that express human α-synuclein in various levels produced behavioral and pathological phenotypes that resemble human PD, including an age-dependent onset of dopaminergic neuronal loss, fibrous Lewy body (LB)-like inclusions, and a progress decline of locomotor activity (9). Transgenic mice that express human α-synuclein also produced neuronal inclusions and dopaminergic presynaptic degeneration in striatum (10). Together, these findings strongly suggest that α-synuclein, in its aggregated forms, is involved in the pathogenesis of PD. α-Synuclein spontaneously forms fibrils that are rich in cross-β structure by a nucleation-dependent mechanism (11, 12). Interestingly, monomeric α-synuclein does not contain any stable structure, hence the term “natively unfolded” has been proposed to describe this protein (13), whereas fibrillar and some pre-fibrillar oligomeric forms of this protein are highly structured (8, 12, 14). Therefore, the transition from monomer to fibril is a process of acquiring structure. The fibrillization process appears to be a multistep process, because various forms of pre-fibrillar oligomeric species have been identified during fibril formation in vitro (6, 15). However, structural changes in the early stages of oligomerization are poorly understood. It has been reported previously that either an increase in temperature or a decrease in pH level rapidly transformed natively unfolded α-synuclein into a partially folded conformation and that this structural transition was fully reversible (16). Moreover, the same study showed a tight correlation between the increase of this intermediate and the enhanced formation of α-synuclein fibrils (16). Here, we investigated the oligomerization and concomitant structural changes of α-synuclein in prolonged incubations at high temperature conditions, both in vivo and in vitro, to obtain insights into the molecular mechanism of the rate-limiting, early stage of oligomerization.

MATERIALS AND METHODS

Purification of Recombinant α-Synuclein—Expression in Escherichia coli and purification of α-synuclein were performed as described previously (16).

Expression of α-Synuclein in COS-7 Cells and Preparation of Cyto-
sol—COS-7 cells were maintained in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) with 10% fetal bovine serum (HyClone Laboratories, Inc., Logan, UT) in a 37 °C 5% CO2 incubator. Construction of an adeno vector for human wild type α-synuclein, named adenoα-syn, and the procedure for the expression of α-synuclein in COS-7 cells were described elsewhere.2 For the preparation of cytosolic fraction, cells were harvested in phosphate-buffered saline with protease inhibitor mixture (Sigma) and disrupted with cell cracker (17). The cell homogenate was centrifuged at 16,000 × g for 5 min. The supernatant was further centrifuged at 200,000 × g for 30 min to obtain the cytosol.

Oligomerization Assay—Purified recombinant α-synuclein (1 μg) or COS-7 cytosol preparation (total protein 10 μg) was incubated in phosphate-buffered saline with protease inhibitor mixture (Sigma) in a final volume of 200 μl at different temperatures for up to 3 days. Aliquots were removed at given time points and subjected to Western blot analysis according to the procedure of Lee et al. (18). The primary
antibody against α-synuclein used in this study was LB509 (Zymed Laboratories Inc., South San Francisco, CA).

**CD Measurements**—CD spectra were obtained on an Aviv 60DS spectrophotometer (Lakewood, NJ) using an α-synuclein concentration of ~1.0 mg/ml at 23 °C as described previously (16).

1-Anilino-8-naphthalene-3-sulfonic Acid (ANS) Binding—A stock solution of 1.0 μM ANS (Sigma) was prepared in distilled, deionized water. The concentration of the dye was determined by its absorption at 350 nm. The [ANS]/[protein] ratio in all experiments was kept equal to 5. Fluorescence measurements were performed at 23 °C in 1 ml of semi-micro quartz cuvettes (Hellma) with a 1-cm excitation light path. Emission spectra were recorded from 400 to 600 nm with excitation at 350 nm in increments of 1 nm, an integration time of 1 s, and a slit-width of 1 nm for both excitation and emission. Data were processed using DataMax software.

**Size Exclusion Chromatography**—Size-exclusion measurements were performed on a Sephadex 200 column calibrated with a set of native globular proteins of known molecular masses as described elsewhere (6). The hydrodynamic dimensions (Stokes radii, R_s) of α-synuclein in different associated forms were recalculated from apparent molecular masses, M_app, determined by gel-filtration chromatography using Equation 1 (19, 20).

\[
\log(R_s) = 0.369 \cdot \log(M_{app}) - 0.254
\]

(Eq. 1)

The hydrodynamic dimensions of a natively unfolded protein with a molecular mass of 14,460 (or 28,920) Da and of a pre-molten globule-like partially folded protein with a molecular mass of 28,920 (or 14,460) Da were calculated from the empirical Equation 2 and Equation 3 (21),

\[
\log(R_s^{NU}) = -0.551 \pm 0.032 + 0.493 \pm 0.008 \cdot \log(M)
\]

(Eq. 2)

\[
\log(R_s^{PMG}) = -0.239 \pm 0.055 + 0.403 \pm 0.012 \cdot \log(M)
\]

(Eq. 3)

where M is the molecular mass, and R_s^{NU} and R_s^{PMG} are the Stokes radii of natively unfolded (NU) and pre-molten globule-like (PMG) protein, respectively.

**RESULTS**

**Temperature-induced Self-assembly of α-Synuclein**—Incubation of α-synuclein at high temperature induces a partially folded conformation (16). This structural transformation was completely reversible when the heat treatment was transient. If the partially folded conformation serves as an intermediate of the fibril assembly, populating this structure for a longer period of time should induce the self-assembly and trap the structure in oligomeric forms. Here, we tested whether sustained heat treatment, thereby sustaining partially folded conformation, can stabilize the structure and initiate the oligomerization. Incubation of purified wild type human recombinant α-synuclein at different temperatures for up to 3 days resulted in a temperature-dependent, progressive aggregation. The incubation at 65 °C showed small oligomers at day 1, and at day 3 larger aggregates were detected with increased amounts of small oligomers (Fig. 1A). Although oligomerization at 50 °C was slower but apparent at day 3 no oligomer of any size was detectable at 37 °C or room temperature for incubations up to 3 days. To test whether the self-assembly of α-synuclein occurs in complex cytosolic milieu, cytosolic preparations were obtained from COS-7 cells that express α-synuclein from recombinant adenoviral vector and incubated at different temperatures for up to 3 days. Oligomerization of the cytosolic α-synuclein showed similar kinetics and temperature dependence to the purified protein (Fig. 1B), suggesting that the source of the protein does not affect the overall conformational behavior of the protein. Moreover, the fact that the pattern of small oligomeric species in the cytosolic preparation was almost identical to that of the purified protein confirms that the oligomeric species of α-synuclein formed in the cytosolic preparation were the result of the selective self-assembly rather than heteromeric association with other cytosolic proteins.

**Stabilization of the Partially Folded Structure during the Oligomerization of α-Synuclein**—To analyze the conformational properties of the oligomeric forms, structural changes induced in α-synuclein during the incubation of this protein at different temperatures were studied by far-UV CD spectroscopy and ANS fluorescence. To this end, aliquots of 1.0 mg/ml α-synuclein solution were incubated at the given temperature for the desired amount of time, cooled to room temperature (23 °C), and then subjected to far-UV CD or fluorescence analysis.

**Secondary Structure Changes Monitored by Far-UV CD**—Far-UV CD spectra of human recombinant α-synuclein were measured at 23 °C after the different durations of incubation at 65 °C. At early time points (up to 1 day), α-synuclein shows a far-UV CD spectrum typical of an essentially unfolded polypeptide chain (Fig. 2A). This includes the characteristic minimum in the vicinity of 198 nm and low amplitude in the 210–230-nm region. In agreement with earlier studies (16), this spectrum was absolutely super-imposable with that measured for the non-heated protein, reflecting complete reversibility of the temperature-induced structural changes. However, when α-synuclein was incubated at 65 °C for longer time, irreversible changes in the shape of its far-UV CD spectrum were observed (Fig. 2A). A decrease in the minimum at 198 nm was accompanied by an increase in negative intensity around 222 nm, reflecting formation of a small amount of ordered secondary structure. This process was completed in 6 days, and longer incubation of α-synuclein at 65 °C was not accompanied by further structural changes (Fig. 2B). The efficiency of accumulation of this trapped conformation was temperature-dependent. This was demonstrated in Fig. 3 by a temperature-sensitive loss of reversibility in heat-induced structural changes, measured after prolonged incubations at different temperatures. Here the protein was incubated at desired temperatures for 6 days, cooled down to room temperature, and then analyzed by far-UV CD spectra at 23 °C. Larger changes in the secondary structure were observed when the protein was incubated at higher temperature (Fig. 3A), suggesting that the partially folded conformation is more effectively trapped at...
high temperature. Close correlation between the structural changes and the oligomerization suggests that as a result of prolonged incubation at elevated temperatures, a small amount of ordered secondary structure was stabilized by the self-assembly of partially folded -synuclein. Interestingly, the CD spectrum of this trapped conformation was similar to that of the partially folded monomeric conformer induced by low pH levels or high temperature (16).

Changes in ANS Fluorescence—Non-native partially folded conformations of proteins are characterized by the presence of solvent-exposed hydrophobic clusters to which ANS binds. This interaction results in a considerable increase in the ANS fluorescence intensity and in a pronounced blue shift of the ANS fluorescence maximum (22). Therefore, we measured the change of ANS fluorescence to monitor the gain of structure in -synuclein as a function of incubation temperature (Fig. 3B). The characteristic blue shift of the ANS fluorescence maximum from 475 nm indicative of binding was also observed after incubation at 65 °C. This reflects the temperature-induced transformation of -synuclein from the natively unfolded state to the partially folded conformation.

Hydrodynamic Properties of the Monomer and Dimer of -Synuclein—Because the unfolding of a protein is associated with an increase in the hydrodynamic volume, hydrodynamic parameters are very useful in predicting the degree of folding.

It is well known that the hydrodynamic radius of globular proteins increases by ~15–20% as they transform into the molten globule state (19, 20, 23). The hydrodynamic volume of the pre-molten globule conformation is even larger (23, 24). Moreover, it has been established that native, molten globule, pre-molten globule, and unfolded conformations of globular proteins possess very different molecular mass dependences of their hydrodynamic radii, R S (19, 21, 25, 26). Thus, equilibrium conformations can easily be discriminated by the degree of compactness of the polypeptide chain.

Hydrodynamic properties of monomeric and dimeric forms of -synuclein were studied by gel-filtration chromatography (Table I). In the case of monomeric -synuclein, the experimentally determined value (R S = 31.3 Å) perfectly matched the value calculated for a natively unfolded protein of 14,460 Da (R S = 31.6 Å) (see "Materials and Methods"). R S measured for the dimer (R S = 36.3 Å) coincided with expected dimension of a pre-molten globule protein with a molecular mass of 28,920 Da (R S = 36.2 Å). Therefore, the partially folded pre-molten globule-like conformation of -synuclein seems to be stabilized as the protein undergoes a highly selective self-assembly process during prolonged incubation at elevated temperatures.

DISCUSSION

In the previous study, it was shown that a partially folded state of -synuclein might be induced immediately in elevated temperatures and low pH conditions and that these structural changes were fully reversible (16). In this report, we provide evidence that the sustained presence of the partially folded state by prolonged incubation at elevated temperatures induces oli-
Serum albumin (66 kDa), carbonic anhydrase (29 kDa), and cytochrome P450 (56 kDa), which was calibrated with blue dextran (2,000 kDa), β-amylase (200 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), carbonic anhydrase (29 kDa), and cytochrome c (12.4 kDa).

These results are consistent with the model depicted in Fig. 4. Under normal conditions, the occurrence of the partially folded monomeric form of α-synuclein is very rare, because the equilibrium between the unfolded monomer and the partially folded monomer strongly favors the natively unfolded form. However, in the event of prolonged elevation of the concentration of the partially folded form, which in the monomeric form is in rapid equilibrium with the unfolded state, some of the partially folded molecules undergo self-assembly to form dimers and small oligomers. This self-assembly stabilizes the pre-molten globule conformation because of the slow rate of dissociation of the oligomers. The dynamic relationship between the pre-molten globule-like dimer (or oligomers) and the fibril nuclei is still hypothetical. However, because fibril formation was enhanced under conditions that increase the concentration of the partially folded conformation, it is reasonable to predict that the small oligomers with pre-molten globule structure are the earliest oligomeric intermediates for the fibrillization process and evolve into fibril nuclei. This model predicts that one of the earliest events in the pathogenesis of LB-related diseases is the shift of the equilibrium between the unfolded monomer and partially folded monomer in favor of the latter and that the identification of physiological factors that influence this event will increase our understanding on these diseases.

Our data show that the heat treatment induced oligomerization not only from the purified α-synuclein but also from the crude cytosolic preparation from COS-7 cells that expressed α-synuclein. It is important to note that the Western blot profile of small oligomers formed in cytosolic preparation appears identical to that formed from the purified protein. These data suggest that the oligomerization was initiated by selective self-association between the partially folded α-synuclein conformers rather than by heteromeric association of α-synuclein with other proteins in a complex cytosolic milieu. This model is supported by the biochemical analyses of brain extracts from PD, DLB, or multiple system atrophy. Western blotting showed that brains from the individuals suffered from these diseases contained the 36- to 52-kDa α-synuclein immunoreactive protein, as in our in vitro oligomerization study, but age-matched controls did not (27–29). Moreover, the pattern of oligomers on the Western analysis with LBs isolated from DLB was strikingly similar to the one obtained in the present study. Both the isolated LB and the oligomers produced in our study contained 36- to 52-kDa immunoreactive proteins, as well as much larger aggregated proteins (30). The fact that our in vitro oligomerization study produced the same oligomeric species found in human LB preparations suggests that our experimental observations may reflect the in vivo mechanism of α-synuclein aggregation in the early stage of pathogenesis.

Recent studies also suggest the role of pre-fibrillar oligomeric α-synuclein as a pathogenic species. For example, pre-fibrillar oligomers bind to lipid membranes more readily than does monomer or fibril, and a subset of them possesses channel-forming activity in bilayer membranes, implicating a potential biological activity that might confer toxicity (14). Therefore, elucidation of the structural details of early stage, oligomeric intermediates may increase our understanding on the biological activities of these pre-fibrillar species, as well as the fibril-forming process.

**REFERENCES**

1. Lucking, C. B., and Brice, A. (2000) Cell. Mol. Life Sci. 57, 1894–1908
2. Trojanowski, J. Q., Goedert, M., Iwatsubo, T., and Lee, V. M. (1998) Cell Death Differ. 5, 832–835
3. Polymeropoulos, M. H., Lavedan, C., Leroy, E., Ide, S. E., Dehejia, A., Dutra, A., Pike, B., Root, H., Rubenstein, J., Boyer, R., Stenroos, E. S., Chandrasekharappa, S., Athanassiadou, A., Papapetropoulos, T., Johnson, W. G., Lazzarini, A. M., Davison, R. C., Di Torio, G., Golbe, L. I., and Nussbaum, R. L. (1997) Science 276, 2045–2047
4. Kruger, R., Kuhn, W., Muller, T., Weitalla, D., Graeber, M., Kosel, S., Przuntecki, H., Epplen, J. T., Schols, L., and Riess, O. (1998) Nat. Genet. 18, 106–108
5. Conway, K. A., Harper, J. D., and Lansbury, P. T. (1998) Nat. Med. 4, 1318–1320
6. Conway, K. A., Lee, S.-J., Rochet, J. C., Ding, T. T., Williamson, R. E., and Lansbury, P. T., Jr. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 571–576
7. Narhi, L., Wold, S. J., Stevensson, S., Jiang, Y., Wu, G. M., Anafi, D., Kaufman, S. A., Martin, F., Sitzmen, K., Deniz, P., Louis, J. C., Wyprych, J., Bierle, A. L., and Citron, M. (1999) J. Biochem. 274, 9843–9846
8. Li, J., Uversky, V. N., and Fink, A. L. (2001) Biochemistry 40, 11604–11613
9. Feany, M. B., and Bender, W. W. (2000) Nature 404, 394–398
10. Maslow, E., Rockenstein, E., Weiner, G., Mallory, M., Hashimoto, M., Takeda, A., Sagara, Y., Sisk, A., and Mucke, L. (2000) Science 287, 1256–1269
11. Serpell, L. C., Berriman, J., Jakes, R., Goedert, M., and Crowther, R. A. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 4897–4902
12. Conway, K. A., Harper, J. D., and Lansbury, P. T., Jr. (2000) Biochemistry 39, 2555–2563
13. Weinreb, P. H., Zhen, W., Poon, A. W., Conway, K. A., and Lansbury, P. T., Jr. (1996) Biochemistry 35, 13709–13715
14. Villegas, M. J., Lee, S.-J., Rochet, J. C., Shiltelman, M. D., Ding, T. T., Kessler, J. C., and Lansbury, P. T., Jr. (2001) Biochemistry 40, 7812–7819
15. Goldberg, M. S., and Lansbury, P. T., Jr. (2000) Nat. Cell Biol. 2, E115–E119
16. Uversky, V. N., Li, J., and Fink, A. L. (2001) J. Biochem. 276, 10737–10744
17. Schmidt, A., and Huttner, W. B. (1998) Methods 16, 160–169
18. Lee, S.-J., Liyange, U., Bickel, P. E., Xia, W., Lansbury, P. T., Jr., and Kosik, K. S. (1998) Nat. Med. 4, 730–734
19. Uversky, V. N. (1998) Biochemistry 37, 13288–13298
20. Uversky, V. N. (1994) Int. J. Biochromatogr. 1, 103–114
21. Uversky, V. N. (2001) Eur. J. Biochem., in press
22. Semisotnov, G. V., Rodionova, N. A., Razgulyaev, U. N., Uversky, V. N., Giproas, A. F., and Gilmanshin, R. I. (1991) Biopolymers 31, 119–128
23. Pitsyn, O. B. (1995) Adv. Protein Chem. 47, 83–229
24. Uversky, V. N., and Pitsyn, O. B. (1994) Biochemistry 33, 2782–2791
25. Tanford, C. (1968) Adv. Protein Chem. 23, 121–292
26. Tcherkeskaaya, O., and Uversky, V. N. (2001) Proteins 44, 244–254
27. Campbell, B. C., Li, Q. X., Culvenor, J. G., Jakala, P., Cappai, R., Beyreuther, K., Masters, C. L., and McLean, C. A. (2000) Neurobiol. Dis. 7, 192–200
28. Langston, J. W., Sastri, S., Chan, P., Forno, L. S., Bolin, L. M., and Di Monte, D. A. (1998) Exp. Neurol. 154, 684–690
29. Campbell, B. C., McLean, C. A., Culvenor, J. G., Gai, W. P., Blumbergs, P. C., Jakala, P., Beyreuther, K., Masters, C. L., and Li, Q. X. (2001) J. Neurochem. 76, 87–96
30. Baba, M., Nakajo, S., Su, T. H., Tomita, T., Nakaya, K., Lee, V. M., Trojanowski, J. Q., and Iwatsubo, T. (1998) Am. J. Pathol. 152, 879–884
Stabilization of Partially Folded Conformation during α-Synuclein Oligomerization in Both Purified and Cytosolic Preparations
Vladimir N. Uversky, He-Jin Lee, Jie Li, Anthony L. Fink and Seung-Jae Lee

J. Biol. Chem. 2001, 276:43495-43498. doi: 10.1074/jbc.C100551200 originally published online October 5, 2001

Access the most updated version of this article at doi: 10.1074/jbc.C100551200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

This article cites 29 references, 6 of which can be accessed free at http://www.jbc.org/content/276/47/43495.full.html#ref-list-1