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Compact conformations of α-synuclein induced by alcohols and copper

Antonino Natalello,[1] Federico Benetti,[2,3] Silvia Maria Doglia,[1] Giuseppe Legname,[2,3] and Rita Grandori[1]*

[1] Department of Biotechnology and Biosciences, University of Milano-Bicocca, 20126 Milan, Italy
[2] Laboratory of Prion Biology, Neurobiology Sector, Scuola Internazionale Superiore di Studi Avanzati (SISSA), 34136 Trieste, Italy
[3] Italian Institute of Technology—SISSA Unit, 34136 Trieste, Italy

ABSTRACT

The intrinsically disordered protein α-synuclein aggregates into amyloid fibrils, a process known to be implicated in several neurodegenerative states. Partially folded forms of the protein are thought to trigger the aggregation process. Here, α-synuclein conformers are characterized by analysis of the charge-state distributions observed in electrospray-ionization mass spectrometry under negative-ion mode. It is found that, even at neutral pH, a small fraction of the molecular population is in a compact conformation. Several distinct partially folded forms are then identified under conditions that promote α-synuclein aggregation, such as solutions of simple and fluorinated alcohols. Specific intermediates accumulate at increasing concentrations of ethanol, hexafluoro-2-propanol, and trifluoroethanol. Finally, extensive folding induced by Cu^{2+} binding is revealed by titrations in the presence of Cu^{2+}–glycine. The data confirm the existence of a single, high-affinity binding site for Cu^{2+}. Because accumulation of this partially folded form correlates with enhancement of fibrillation kinetics, it is likely to represent an amyloidogenic intermediate in α-synuclein conformational transitions.

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Key words: intrinsically disordered proteins; induced folding; protein aggregation; amyloidogenic intermediate; electrospray-ionization mass spectrometry; Parkinson's disease; charge-state distributions; ethanol; trifluoroethanol; hexafluoro-2-propanol.

INTRODUCTION

The α-synuclein protein is highly expressed in the brain and is the primary component of the Lewy body deposits found in dopaminergic neurons that characterize Parkinson's disease (PD) and other neurodegenerative states.[1] The protein accumulates into these insoluble aggregates through the formation of amyloid fibrils, a process that can be reproduced *in vitro*.[2,3] Mutations in the α-synuclein gene, leading to the single amino acid substitutions A53T, A30P, and E46K, are associated to early disease onset in familial PD. Understanding the molecular mechanism of α-synuclein fibril formation and the environmental factors affecting its aggregation propensity is crucial for the development of novel therapeutic strategies.

The α-synuclein protein is composed of 140 amino acids. The isotopically averaged molecular weight is 14460.1 Da, and the isoelectric point is 4.67, as calculated based on its primary structure. Three regions have been recognized in the polypeptide chain[4,5]: the N-terminal region (residues 1–67) containing imperfect tandem repeats of the sequence motif XKTKEGVXXXA (Swissprot: syua_human) is prone to form amphipathic helical structures upon interaction with membranes[6–8]; a central hydrophobic region usually referred to as "non-Aβ component" (NAC, identified in amyloid preparations taken from the brains of patients with Alzheimer's disease) thought to mediate aggregation[9]; and a highly acidic C-terminal region rich in Glu residues.

The α-synuclein protein belongs to the class of intrinsically disordered proteins (IDPs). These proteins lack an ordered three-dimensional structure and exist in solution as highly dynamic polypeptide chains and highly heterogeneous molecular ensembles.[10,11] Interactions with other proteins, membranes, or small ligands can stabilize folded or partially folded states, establishing novel biochemical and biophysical properties.

Additional Supporting Information may be found in the online version of this article.

Abbreviations: ANS, 8-anilino-1-naphthalenesulfonate; CD, circular dichroism; CSD, charge-state distribution; ESI-MS, electrospray-ionization mass spectrometry; HFiP, hexafluoro-2-propanol; IDP, intrinsically disordered protein; NAC, non-Aβ component; NMR, nuclear magnetic resonance; PD, Parkinson's disease; TFE, trifluoroethanol.

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*Correspondence to: Rita Grandori, Department of Biotechnology and Biosciences, University of Milano-Bicocca, Piazza della Scienza 2, 20126 Milan, Italy. E-mail: rita.grandori@unimib.it.
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The aggregation of α-synuclein into fibrils seems to be triggered by partially folded forms of the protein with intrinsic amyloidogenic potential.[12,13] The fibrillation kinetics *in vitro* is characterized by a lag phase, typical of a nucleation-dependent mechanism. A partially folded state of α-synuclein seems to be the obligate intermediate for the formation of the soluble aggregates constituting fibril nuclei.[3]

A strong correlation has been observed between conditions that promote fibrillation and accumulation of partially folded forms of α-synuclein. Low pH has been shown to accelerate the fibrillation kinetics relative to neutral pH.[13] The protein conformation at low pH has been investigated by an array of different techniques. Small-angle X-ray scattering[13] electrospray-ionization mass spectrometry (ESI-MS)[14,15] and ion-mobility (IM)-ESI-MS[14] revealed stabilization of partially folded conformations under acidic conditions. Nuclear magnetic resonance (NMR) measurements identified the major structural change at low pH as a compaction of the acidic C-terminal region.[16,17] This conformational transition is accompanied by small changes in the far-UV circular dichroism (CD) and 8-anilino-1-naphthalenesulfonate (ANS) fluorescence spectra.[13] Elevated temperature, another condition known to promote aggregation, promotes the production of partially folded dimers that can be identified by gel filtration, again along with minor changes in CD and ANS fluorescence spectra.[12]

The effects of simple and fluorinated alcohols on α-synuclein secondary structure and fibrillation kinetics have been investigated in the attempt to model the effects of membranes.[18] Based on far-UV CD measurements, it is suggested that low concentrations of such organic solvents lead to an accumulation of the same partially folded intermediate, which is, in turn, undistinguishable from that found at low pH or high temperature by the same technique. The effects of alcohols diverge at high concentrations. Methanol, ethanol, and propanol stabilize β structures. Hexafluoro-2-propanol (HFiP) stabilizes helical structures. Trifluoroethanol (TFE) stabilizes β structures up to a concentration of 15% and helical structures at higher concentrations. All the tested alcohols enhance fibrillation only at low concentrations and redirect the pathway toward amorphous aggregates or soluble monomers at high concentrations. A partially folded form accumulating at high ethanol concentrations could be detected by ESI-MS and distinguished from the low-pH intermediate.[15]

As generally observed for amyloid protein aggregation,[19,20] α-synuclein fibrillation is strongly affected by metal ions.[21–25] The copper ion Cu^{2+} is highly effective inducing α-synuclein oligomerization[26] and fibrillation[23] and has been shown to bind to the protein with high affinity, although large discrepancies exist among the reported values for equilibrium binding constants and stoichiometry.[24–29] The conformational effects of Cu^{2+} binding to α-synuclein are not known in detail. Although no global structural rearrangement could be detected by fluorescence in α-synuclein tryptophan mutants[29] nor by ESI-MS,[28] an increase in helix content has been suggested based on CD data.[23] That Cu^{2+} might induce α-synuclein folding has also been shown by ^{1}H-^{15}N heteronuclear single-quantum correlation NMR measurements, that indicated how resonances assigned to residues far apart in the sequence, particularly H50 and D121, were affected during titrations.[24]

In this work, the conformational transitions of α-synuclein induced by alcohols and Cu^{2+} have been investigated by nano-ESI-MS. Protein ionization by this technique is highly sensitive to the structural compactness at the moment of transfer to the gas phase, offering a global probe for protein tertiary structure, which is nicely complementary to far-UV CD and IR spectroscopy.[30] Furthermore, protein–protein and protein–ligand interactions can be investigated by this method because most noncovalent complexes are preserved during the flight of ions through the mass spectrometer.[31] Interactions of proteins with metal ions, in particular, have been widely studied by MS.[32–35] Thus, as charge reports on structure and mass reports on association, combined information on folding and binding can be retrieved. Finally, all MS methods provide species-specific signals free from averaging over the molecular ensemble, leading to direct detection of the distinct components populating heterogeneous samples.[36] These features make nano-ESI-MS a particularly valuable tool for the study of highly dynamic systems such as IDPs.[37]

MATERIALS AND METHODS

Materials and sample preparation

Glycine, $CuSO_4 \cdot 5(H_2O)$, guanidinium chloride, alcohols, ammonium acetate, ammonium hydroxide, and formic acid were purchased from Sigma Aldrich (St. Louis, MO).

Wild-type human α-synuclein was expressed as a recombinant protein and purified as previously described.[38] Protein concentration was determined by the absorption in 6*M* guanidinium chloride, using the calculated extinction coefficient $\epsilon_{280\ nm} = 5120\ M^{-1}\ cm^{-1}$. The protein was stored as a dry pellet at −20°C. A stock protein solution was prepared by dissolving the lyophilized powder in Milli-Q water at a concentration of 100 μ*M*. The protein solution was then diluted at a final concentration of 12 μ*M* in 10 m*M* ammonium acetate at the required pH, adjusted by the addition of ammonium hydroxide or formic acid. The addition of alcohols or $CuSO_4$ at the highest tested concentrations does not alter the pH of the solution. Before MS measurements, α-synuclein was incubated for 30 min with the indicated alcohol or Cu^{2+} concentrations, with the exception of methanol 40% and Cu^{2+} 80 μ*M*. In these cases, MS measurements were performed immediately after sample preparation to avoid signal reduction and spray instability, probably due to protein aggregation induced by these conditions.

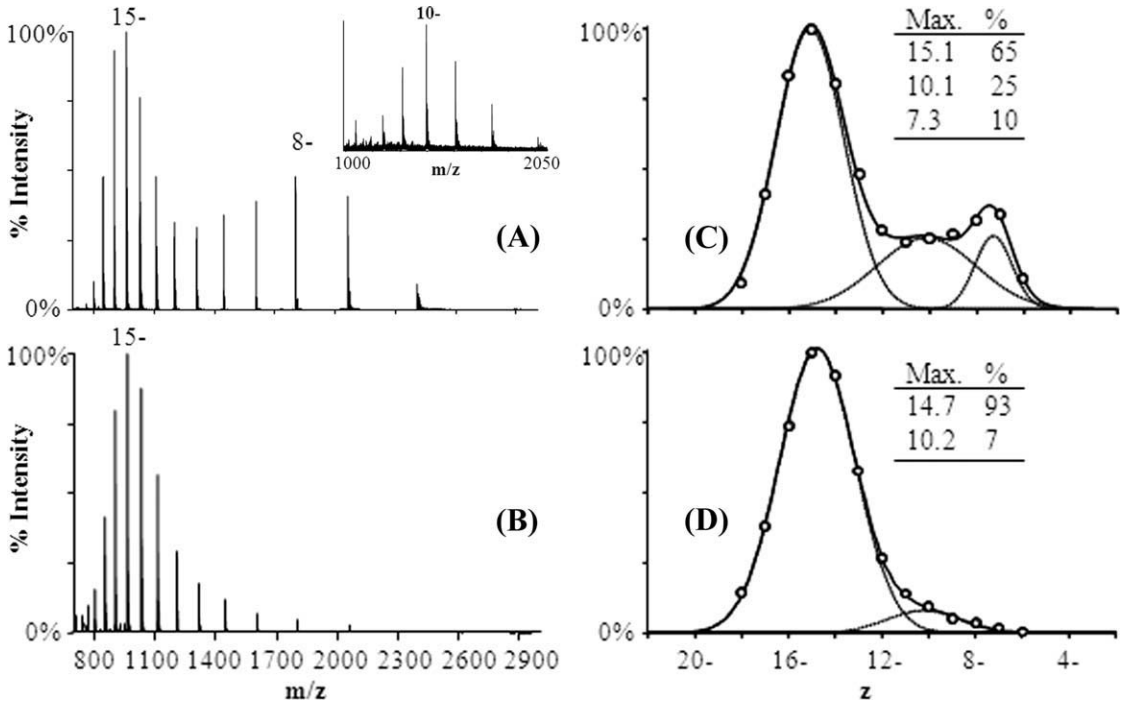

Figure 1

Effect of pH. Nano-ESI-MS spectra in negative-ion mode of 12 μ*M* α-synuclein in 10 m*M* ammonium acetate, pH 7.4 (**A**) and pH 11 (**B**). The main charge state of each peak envelope is labeled by the corresponding number of charges. The inset of panel (A) shows the spectrum in 10 m*M* ammonium acetate, pH 2.5. Panels (**C**) and (**D**) show the results of Gaussian fitting of the spectra in (A) and (B), respectively.

Mass spectrometry

Mass spectra were collected in negative-ion mode on a QSTAR-Elite instrument (Applied Biosystems, Foster City, CA) equipped with a nano-ESI sample source. Metal-coated borosilicate capillaries (Proxeon, Odense, Denmark) with medium-length emitter tip of 1-μm internal diameter were used for electrospray. The following conditions were used: −1150 V ion-spray voltage, −80 V declustering potential, 20 PSI curtain-gas pressure, and room-temperature interface. These values were selected within ranges in which none of the parameters had significant effect on the qualitative features of the spectra. The spectra were averaged over 3-min acquisitions. The different components of the CSDs were quantified by Gaussian fitting of the spectra reporting the ion relative intensity versus charge.[36,39] These data were fitted by the minimal number of Gaussian functions leading to a stable fit. Their center and width were allowed to change to optimize the final fitting (final $R^2 > 0.99$). The Gaussian fitting was performed by the software Origin 8 (Originlab, Northampton, MA).

RESULTS

Heterogeneity at neutral pH

Conformational analysis of α-synuclein by ESI-MS has been reported both in positive-[15] and negative-[14,40] ion mode. With the instrumentation used in this work, data acquisition in negative-ion mode has proven more effective in monitoring conformational changes of this acidic protein. Figure 1(A) shows the spectrum of 12 μ*M* α-synuclein in 10 m*M* ammonium acetate, pH 7.4. The charge-state distribution (CSD) is sharply bimodal, with one envelope centered on the 8−/7− peaks and another one centered on the signals of the 15−/14− ions. Such a result indicates the coexistence of two major conformers of different compactness. The compact form (8−/7−) is of surprising compactness, approximating the limit charge predicted on the basis of the empiric charge-to-mass relation for a normal globular protein of the same size ($z = 9$).[41] The compact form persists in spectra acquired at higher ionic strength, up to 50 m*M* ammonium acetate, although with lower spectrum quality (data not shown). Above such buffer concentration, the protein signal was lost completely. The less compact form (15−/14−) does not further shift significantly under a wide array of different solvent conditions, including, besides those shown below, 50% acetonitrile (data not shown). Therefore, we assign the high-charge component in the spectrum of Figure 1(A) to the fully unfolded form of the protein. These data confirm the widely documented notion that α-synuclein at neutral pH is highly disordered but also indicate existence of a minor population in a compact state. This form is

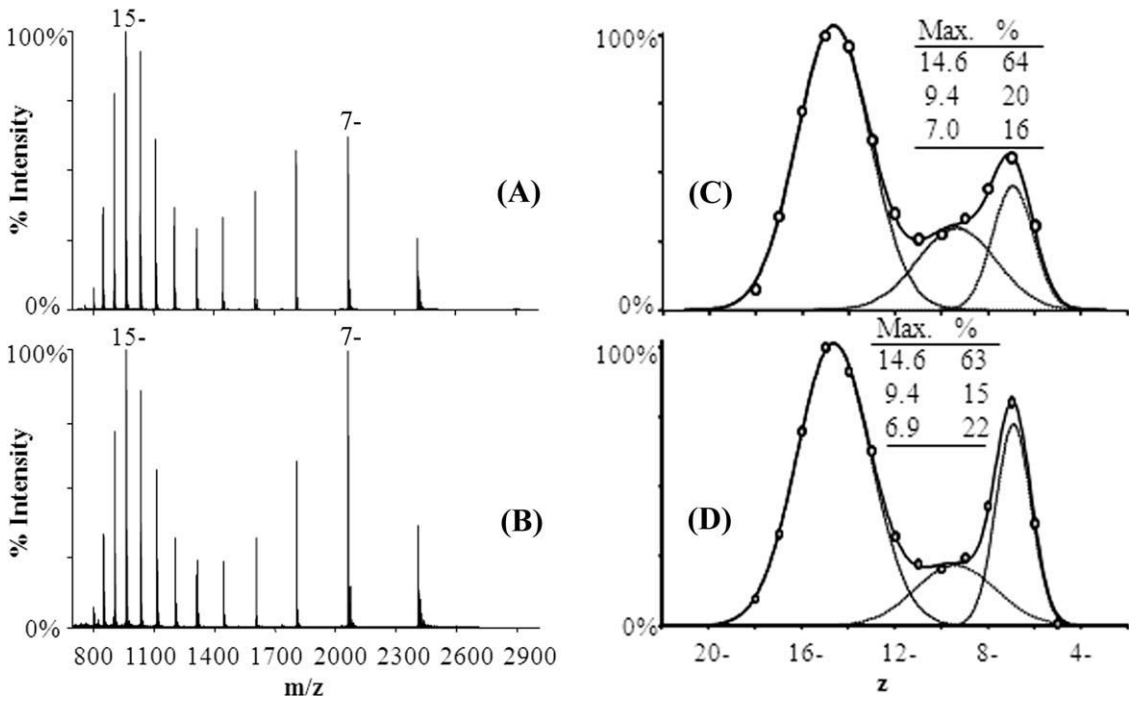

Figure 2

Effect of methanol. Nano-ESI-MS spectra in negative-ion mode of 12 μ*M* α-synuclein in 10 m*M* ammonium acetate, pH 7.4 plus 10% (**A**) and 40% (**B**) methanol. The main charge state of each peak envelope is labeled by the corresponding number of charges. Panels (**C**) and (**D**) show the results of Gaussian fitting of the spectra in (A) and (B), respectively.

distinct from the partially folded form that accumulates at low pH, which is characterized by a unimodal CSD with main charge state 10−, under our experimental conditions [inset Fig. 1(A)]. Deconvolution of the spectra over the full *m/z* range yields a mass value of 14460.9 ± 0.4, in close agreement to the theoretical one. The same result (within the standard deviation) is obtained by deconvolution over the *m/z* range 900–1100 or 1600–2100, indicating that the two components are homogeneous in terms of protein mass.

The different components of the CSD can be better identified and quantified by Gaussian fitting, after transformation to $x = z$ as abscissa axis.[36,39] The results are shown in Figure 1(C). This analysis reveals that it is not possible to fit the spectrum with only two Gaussian functions and that a third component is required to achieve convergence of the fitting algorithm. One component is centered at $z \simeq 15-$ and corresponds to the fully unfolded form. Another component is centered at $z \simeq 7-$ and corresponds to the compact form. The third component, which is not resolved in the raw spectrum, is centered on the 10− charge state, as the partially folded form that accumulates at low pH. Therefore, two distinct collapsed states are detected for α-synuclein at neutral pH. One (∼25%) is similar to the low-pH intermediate and the other one (∼10%) represents a highly compact form that is not detectable at low pH. These results are in general good agreement with previous analyses in positive-ion mode, although, in that case, an additional, partially folded form is detected at neutral pH and the compact form does not disappear at low pH.[15]

Searching for conditions that might destabilize the compact form, we have tested the effect of basic pH, as this is a condition known to dissolve effectively α-synuclein aggregation seeds[18] and, therefore, likely destabilizes partially folded conformations. The results are shown in Figure 1(B,D). By raising the pH to 11, both collapsed forms decrease, with accumulation of the highly disordered component to ∼93%. Although it could be argued that solution pH might affect the ESI process, numerous studies have shown that this parameter does not significantly alter CSDs in nano-ESI-MS, unless it changes protein conformation.[30,42–45] Thus, the results reported in Figure 1 suggest that the distinct components observed in the spectrum reflect conformational properties of α-synuclein in solution.

Effect of methanol

Previous studies have indicated that simple alcohols stabilize a partially folded intermediate of α-synuclein rich in β structures.[15,18] Figure 2 shows the effect of increasing methanol concentrations on α-synuclein at

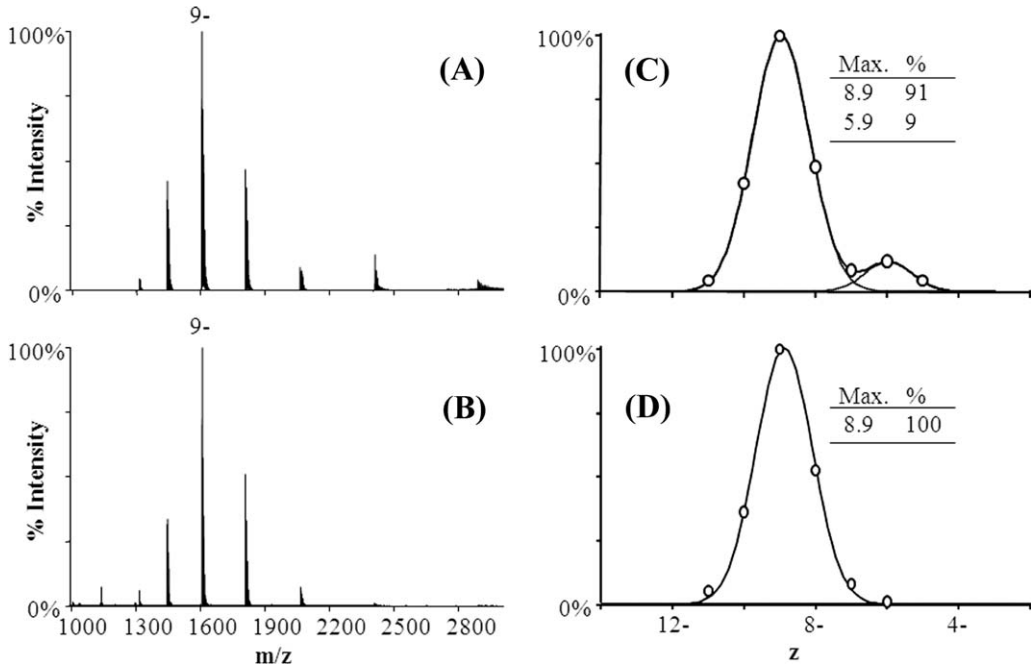

Figure 3

Effect of HFiP. Nano-ESI-MS spectra in negative-ion mode of 12 μ*M* α-synuclein in 10 m*M* ammonium acetate, pH 7.4 plus 2.5% (**A**) and 15% (**B**) HFiP. The main charge state is labeled by the corresponding number of charges. Panels (**C**) and (**D**) show the results of Gaussian fitting of the spectra in (A) and (B), respectively.

pH 7.4. The results show a progressive accumulation of the compact form centered on the 8−/7− ion. Its relative amount approximately doubles, going from 0 to 40% methanol. It could be argued that methanol might simply affect the relative signal yield of folded versus unfolded protein ions,[46] and that the observed trend might, therefore, reflect unspecific solvent effects on the electrospray mechanism. However, no evidence of such an effect emerges from the analysis of other proteins, such as cytochrome *c*,[47] ubiquitin, and lysozyme.[44] Therefore, although solvent effects cannot be completely ruled out, they do not likely account for the dramatic spectral changes observed in this case. In conclusion, methanol seems to stabilize the compact form of α-synuclein at neutral pH. This result is in general agreement with the finding that simple alcohols stabilize partially folded α-synuclein conformations.[15,18] However, the methanol-induced intermediate identified here seems to be characterized by slightly higher compactness than the previously described intermediate in the presence of ethanol (main charge state 10+).[15] At 40% methanol, the spray becomes unstable (data not shown), indicating rapid progression of protein aggregation. This observation is in agreement with light scattering results, indicating a high aggregation propensity of the protein under this condition.[18]

The results of Gaussian fitting [Fig. 2(C,D)] indicate that the compact form accumulates at the expenses of the 10− intermediate, whereas the highly disordered component remains approximately unchanged (∼65%). Therefore, methanol stabilizes the compact form relative to the 10− intermediate, leaving constant the total fraction of protein in collapsed states (∼35%).

Although detailed α-synuclein characterization by CD has already been reported, we have repeated analyses under all the here tested conditions to acquire CD data at identical solvent composition as used for ESI-MS. The results are consistent with those in the literature and are reported as Supporting Information.

Effect of HFiP

Fluorinated alcohols are frequently used as cosolvents to induce protein conformational transitions and to modulate protein aggregation. In particular, they are thought to mimic the effect of biological membranes on α-synuclein structural properties.[18] HFiP enhances α-synuclein fibrillation at very low concentrations (1–5%), while exerting an inhibitory effect at higher concentrations. Based on far-UV CD, it has been suggested that low concentrations of HFiP (e.g., 2.5%) stabilize the same intermediate as do low concentrations of simple alcohols (e.g., 10% methanol).[18] Figure 3 shows the effect of increasing HFiP concentrations on α-synuclein CSD at pH 7.4. Already at 2.5% alcohol, almost all the protein is found as an intermediate, with main charge

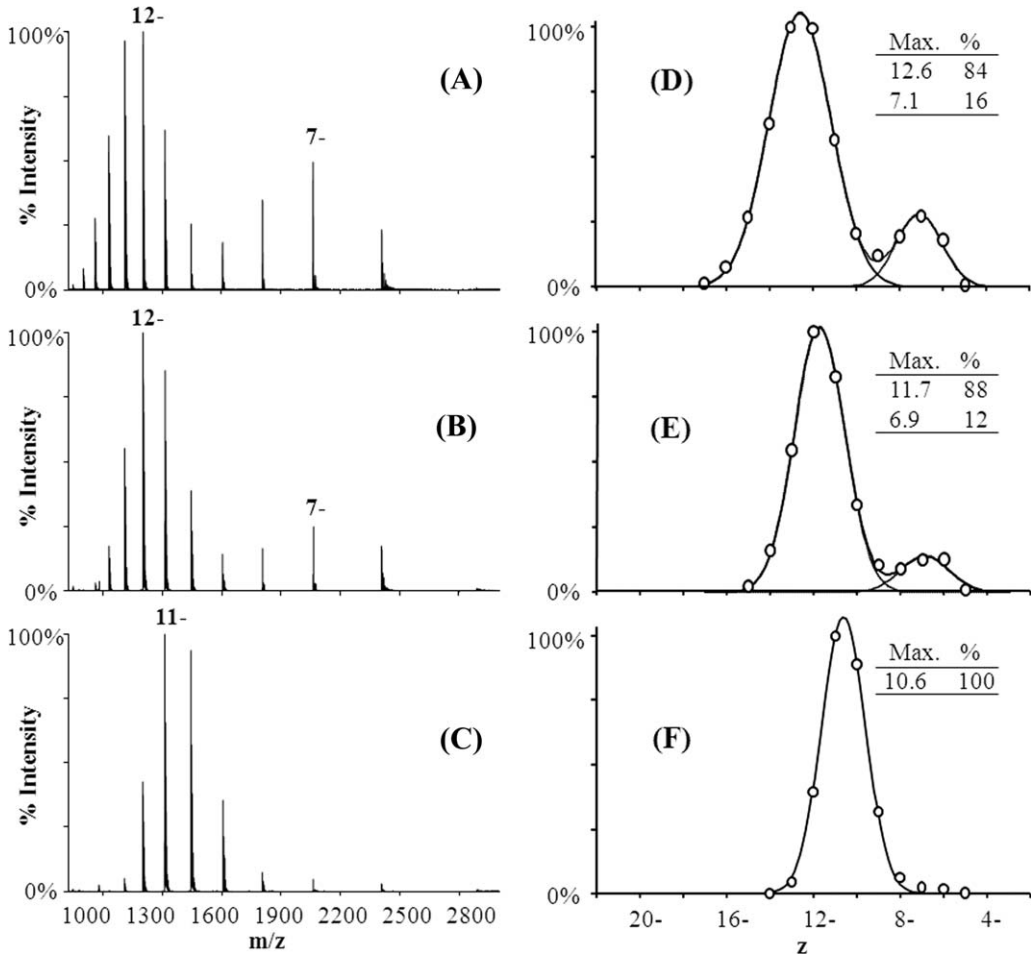

Figure 4

Effect of TFE. Nano-ESI-MS spectra in negative-ion mode of 12 μ*M* α-synuclein in 10 m*M* ammonium acetate, pH 7.4 plus 5% (**A**), 10% (**B**), and 40% (**C**) TFE. The main charge state of each peak envelope is labeled by the corresponding number of charges. Panels (**D**–**F**) show the results of Gaussian fitting of the spectra in (A–C), respectively.

state 9−. This partially folded form is distinguishable from both the disordered and compact states that represent the two main components of the protein at pH 7.4 in the absence of cosolvents. In addition, a small fraction of the population [∼9%, Fig. 3(A,C)] is found in a highly compact state, similar to the compact form described above. Further increase in HFiP concentration does not shift the CSD of the intermediate but makes the distribution definitely unimodal and highly symmetric, suggesting that soluble protein monomers populate a quite homogeneous conformational state [Fig. 3(B,D)]. The main partially folded form stabilized by HFiP is distinguishable from the methanol-induced intermediate being characterized by lower compactness.

Effect of TFE

The α-synuclein protein responds to titrations by TFE in a peculiar way. Far-UV CD data indicate that the protein accumulates β structures up to ∼15% TFE and then converts to a highly helical form at higher concentrations of the cosolvent.[18] No effect of TFE could be observed by ESI-MS in positive-ion mode.[15] However, titrations in negative-ion mode at pH 7.4 reveal conformational changes induced by TFE and give striking agreement with the discontinuity at ∼15% TFE observed by CD (Fig. 4). Up to this concentration, the spectra reveal progressive accumulation of a partially folded form centered on the 12− ion. Nevertheless, the sample at 15% TFE is characterized by signal instability and very low spectrum quality (data not shown), suggesting that the protein aggregates immediately upon transfer to such condition. At higher TFE concentrations, the protein is well soluble again and populates a quite homogeneous intermediate state centered on the 11− ion.

As indicated by the results of Gaussian fitting [Fig. 4(D–F)], TFE causes complete loss of the 10− intermediate already at a concentration of 5% and

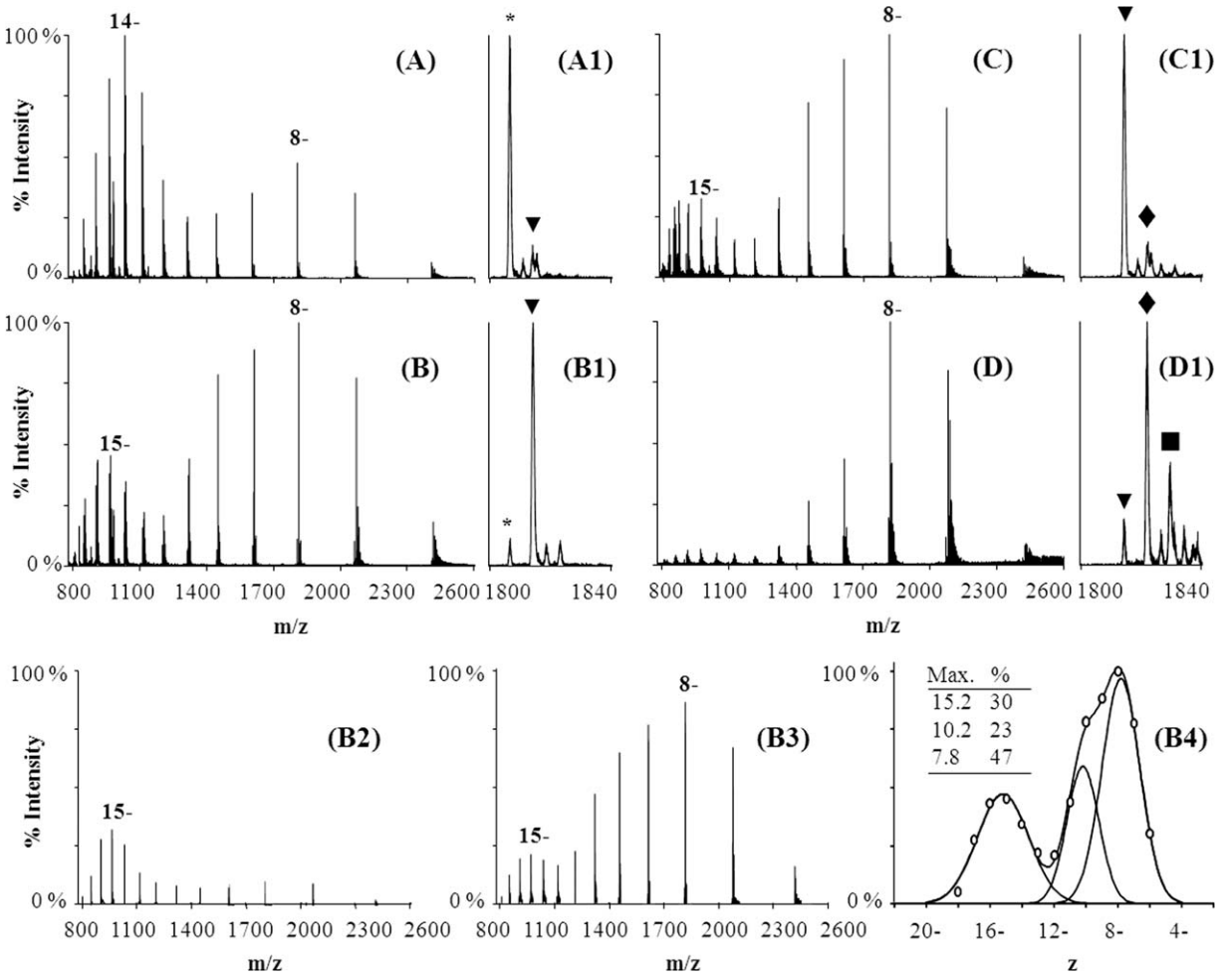

Figure 5

Effect of copper. Nano-ESI-MS spectra in negative-ion mode of 12 μ*M* α-synuclein in 10 m*M* ammonium acetate, pH 7.4, and variable concentrations of Cu^{2+} and glycine: Cu^{2+} 1 μ*M*, glycine 4 μ*M* (**A**); Cu^{2+} 5 μ*M*, glycine 20 μ*M* (**B**); Cu^{2+} 80 μ*M*, glycine 320 μ*M* (**C**); and Cu^{2+} 80 μ*M*, glycine 0 μ*M* (**D**). The peak of the 8− ion is displayed in an enlarged *m/z* scale in (**A1**), (**B1**), (**C1**), and (**D1**) to better show the signals of the free α-synuclein (star), the 1:1 α-synuclein:Cu^{2+} complex (triangle), the 1:2 α-synuclein:Cu^{2+} complex (diamond), and the 1:3 α-synuclein:Cu^{2+} complex (square). Data for (A1), (B1), (C1), and (D1) panels are taken from (A), (B), (C), and (D), respectively. The peaks of free α-synuclein and the 1:1 complex extracted from (B) are shown separately in (**B2**) and (**B3**), respectively. Panel (**B4**) shows the results of Gaussian fitting of the CSD of the bound protein reported in (B3).

progressive disappearance of the compact form as the concentration is raised from 5 to 40%.

Effect of copper

The above results indicate that the experimental approach used in this work allows accurate monitoring of α-synuclein conformational transitions. Thus, titrations of 12 μ*M* protein were performed by increasing concentrations of Cu^{2+} (0–80 μ*M*) to investigate the structural effects of complex formation with this known strong ligand. The experiment was performed in the presence or absence of glycine (four-molar excess to Cu^{2+}) in the attempt to discriminate specific from unspecific binding.[28] As shown in Figure 5, metal binding is revealed by the appearance of new peaks in the spectra, corresponding to α-synuclein/Cu^{2+} adducts. The mass shift is by multiples of 61.5 Da, indicating that ligation of each Cu^{2+} ion (63.5 Da) displaces two protons from the protein. No significant effect of the declustering potential is observed by varying the setting from −80 to −150 V (data not shown). Although up to three Cu^{2+} ions bind in the titration performed in the absence of glycine, only the 1:1 complex accumulates in the presence of glycine. The 2:1 complex (Cu^{2+} to protein) becomes barely detectable at the highest tested Cu^{2+} concentration in the presence of glycine. These data confirm that α-synuclein has a single, high-affinity

binding site for Cu^{2+} ions. Quantitation of the free and bound forms of the protein from the deconvoluted spectra (data not shown) indicates that almost all the metal is bound at the intermediate points of the titration under these conditions. Therefore, it can be concluded that the used concentrations are well above the equilibrium dissociation constant. According to previous reports,[22,24,26] α-synuclein is able to bind Cu^{2+} ions also via a second site with dissociation constant in the micromolar range, likely because of nonspecific electrostatic interactions with charged amino acids. This is in agreement with the different patterns observed in the presence or absence of glycine [Fig. 5(C,D)].

Figure 5 also shows that Cu^{2+} binding induces extensive structural rearrangements. A progressive conversion of the highly charged component into the low-charge component takes place during the titration. At 80 μ*M* Cu^{2+}, almost all the protein is found in a highly compact state with main charge state 8−. The conformational effect is even more evident if only the peaks of the 1:1 complex are plot and compared to the CSD of the free protein [Fig. 5(B2,B3)]. No significant difference can be detected between the CSDs of the 1:1, 1:2, and 1:3 complexes in the absence of glycine (data not shown), indicating that only the first binding event induces a significant conformational change in the protein. The conformation induced by Cu^{2+} has comparable compactness to that of the compact form detectable at pH 7.4 in the absence of ligand. However, further structural characterization will be needed to establish structural similarity between these two forms. Differently from the effect of methanol, the accumulation of the compact form induced by Cu^{2+} takes place at the expenses of the highly disordered component (Fig. 5). As indicated by the Gaussian fitting reported in Figures 1(C) and 5(B4), the 15− component decreases from ∼65 to 30% comparing the free and bound form, whereas the 7−/8− component increases from ∼15 to 45%. The 10− intermediate, instead, remains around 25% in the 1:1 complex. The results described here indicate that the protein undergoes folding induced by Cu^{2+} binding, leading to extensive formation of tertiary structure. The intermediate that accumulates during the titration is, therefore, a good candidate as the amyloidogenic conformer that triggers α-synuclein fibrillation in the presence of Cu^{2+} ions.

DISCUSSION

Compact state at neutral pH

The IDP α-synuclein is known to undergo folding induced by acids.[14,15] At neutral pH, the protein exists in a highly disordered conformational state.[3] The results reported here show that such a state is, indeed, characterized by at least three distinct populations of different structural compactness, in agreement with another report by ESI-MS in positive-ion mode.[15] The predominant form is highly disordered and does not contain significant amounts of tertiary structure. This form coexists with a partially folded form similar to the low-pH intermediate and a minor component of remarkable compactness. This conclusion is also supported by results of single-molecule force spectroscopy. This methodology has revealed a class of conformations in monomeric α-synuclein at neutral pH that offer a high mechanical resistance to pulling, consistent with a folded state rich in antiparallel β-strands.[48] Thus, evidence from different kinds of experiments consistently hints to the existence of compact conformations of α-synuclein, even at neutral pH. The results described in this work suggest that the compact conformation at neutral pH is more structured and less stable than the partially folded form at pH 2.5, as indicated, respectively, by its lower charge state and its lower relative amount.

The highly dynamic and heterogeneous molecular ensemble characterizing IDPs in solution represents a major obstacle to biophysical investigation. Short-lived ordered conformations are easily missed by technologies that monitor average properties of the molecular population. The ion-sorting mechanism inherent to MS measurements helps detecting labile, metastable states, free from the background of predominant states. Growing evidence indicates that IDPs in the absence of interactors transiently visit compact and/or ordered states.[15,49–54] Some recently described examples belong to the class of the cyclin-dependent protein kinase inhibitors. In these cases as well, a certain degree of secondary and tertiary structure characterizes the IDP in the unbound state.[49,50,52,55] It is likely that such intrinsic structure is of physiological relevance, offering seeds for the development of interaction surfaces and/or protecting the protein from proteolytic degradation *in vivo*.

Distinct intermediates induced by alcohols

This work has led to the identification of distinct, partially folded forms that accumulate in response to different alcohols. The results indicate that the predominant species induced by methanol, TFE, and HFiP can be discriminated in terms of structural compactness. These data complement previous CD analyses.[18] In particular, 2.5% HFiP, 15% TFE, and 40% methanol seem to correspond to different conformational states, even if they all give similar CD spectra. ESI-MS data also provide information on the structural heterogeneity of the molecular population. It is shown that only high concentrations of fluorinated alcohols give rise to apparently homogeneous conformational states, while at least two main conformers coexist under all the other tested conditions.

When more than one species are present, assessing contributions of the distinct components to the average

CD signal is not straightforward. However, by comparing the transitions recorded by MS and CD[18] (Supporting Information), a tentative assignment can be made. For instance, the β-rich intermediate induced by methanol seems to correspond to the peak envelope centered on the 7− ion, as it is a compact species that accumulates at increasing methanol concentrations. This intermediate coexists, even at high methanol concentrations, with a highly extended and a partially folded species.

In the case of HFiP, previous results by CD and IR spectroscopy indicate progressive formation of helical structure during titrations between 3 and 20% cosolvent.[18] The helical intermediate that accumulates at high HFiP concentrations can be identified in the component centered on the 9− ion in the ESI-MS spectra. The β-structural intermediate at low HFiP concentrations could correspond to the minor compact form with main charge state 6−.

Although no TFE effect could be monitored by ESI-MS in positive-ion mode,[15] the conditions used in this work revealed formation of TFE-induced intermediates. Titrations by TFE monitored by CD and ESI-MS consistently indicate a discontinuity at around 15% of cosolvent. Such a condition likely corresponds to maximal accumulation of the β-rich intermediate that causes formation of soluble aggregates. The results presented here suggest that such an intermediate corresponds to the component centered on the 12− ion, which progressively accumulates in the 0–15% TFE range. At higher TFE concentrations, a distinct, highly soluble intermediate centered on the 11− ion accumulates instead. This form can be assigned to the helical state identified by CD at high TFE concentrations.[18]

All the conformational effects induced by alcohols described in this work were found to be reversible (data not shown). Reversibility was tested by incubating 12 μ*M* α-synuclein for 30 min under the relevant solvent conditions (40% methanol, 2.5% HFiP, or 15% TFE), diluting the samples to final 10% methanol, 0.5% HFiP, or 5% TFE, respectively, and comparing their ESI-MS spectra to that of fresh protein prepared at the same final conditions. However, only soluble species are detected by ESI-MS. Therefore, reversibility refers only to the conformational state of soluble monomers and not to the whole system, where part of the protein might aggregate irreversibly.

In conclusion, we have found that the predominant conformational state of α-synuclein in the presence of simple and fluorinated alcohols varies, depending on the nature and the concentration of the cosolvent, even for conditions resulting in similar CD spectra. This conclusion does not rule out that similar conformers promote protein aggregation under different conditions. More studies will be needed to identify the actual amyloidogenic intermediate in the presence of different alcohols. Furthermore, it would be interesting to establish whether there is a relation between the various conformational states of the soluble monomers and the final fibril structure.

Folding induced by copper

Only one ESI-MS report appeared so far in the literature on α-synuclein interaction with Cu^{2+}.[28] In that case, the analyses were performed in positive-ion mode. The results indicate formation of a 1:1 complex in the presence of glycine, but no conformational effect is revealed by the CSDs. The results presented here, instead, indicate a dramatic effect of Cu^{2+} binding on protein tertiary structure. One possible interpretation is that the experimental conditions in the previous study were too aggressive to preserve protein conformation, although mild enough to maintain the protein–metal complex. Alternatively, the difference may be ascribed to the polarity used for electrospray, because conformational effects of an acidic protein might be more evident in negative-ion mode.[42,56]

The results presented in this work indicate that Cu^{2+} binding to α-synuclein stabilizes a highly compact, partially folded conformation of the protein. The effect is remarkable, both in terms of the extent of the conformational change (shift in the main charge state from 15− to 8−) and in terms of stabilization (around 45% of the bound protein is found in the compact state). The Cu^{2+}-induced folding observed here is consistent with previous tyrosine fluorescence studies, suggesting an effect of Cu^{2+} ions on α-synuclein tertiary structure.[23] Moreover, an NMR study[24] reported that the Cu^{2+}-binding site includes amino acid residues located far apart in the α-synuclein primary sequence (His50 and Asp121), suggesting that a partial folding of the protein occurs upon Cu^{2+} interaction, even if these effects do not alter appreciably the secondary structures of the protein. Indeed, no important changes in the chemical shifts of backbone amide groups have been observed upon Cu^{2+} binding.[24] Moreover, no[57] or minor[23] differences were observed in the far-UV CD spectra of α-synuclein in the absence and in the presence of Cu^{2+}. Under the conditions used in this work, no appreciable changes in protein secondary structure were detected by Fourier-transform infrared spectroscopy, and very minor reduction in random-coil content was observed by CD (Supporting Information).

The ion Cu^{2+} is known to promote α-synuclein fibrillation. Effects on the fibrillation kinetics[23] and effects on the lag phase of the reaction[24] have been reported, based on ThT-fluorescence assays. In either case, this evidence strongly suggests that the compact state identified here in the presence of Cu^{2+} may represent the amyloidogenic intermediate that leads to protein aggregation.

Disaccording stoichiometries have been reported in the literature for the formation of α-synuclein–Cu^{2+} complex (from 1:1 to 1:10 protein:metal). Quantitation from titration experiments can be complicated by solubility problems and unspecific interactions. As previously reported by Hong and Simon,[28] the addition of glycine as a weak Cu^{2+} binder protects against both complica-

tions. By using the same conditions, we have found good agreement with their data. Only one high-affinity site for Cu^{2+} seems to be present in α-synuclein. Because all the added ligand is bound at the intermediate points of the titration, attempting an estimation of the affinity is not feasible. However, the K_d may be assumed to be well below the micromolar range, consistent with the previously reported value of 0.2 n*M* at pH 7.4, based on ITC measurements.[28] Such an affinity would be consistent with a physiological relevance of the effect of Cu^{2+} reported here, because the estimated Cu^{2+} concentration in the brain is in the 15–80 μ*M* range.[58–60]

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