Conformational Properties of the SDS-Bound State of α-Synuclein Probed by Limited Proteolysis: Unexpected Rigidity of the Acidic C-Terminal Tail†

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ABSTRACT: α-Synuclein (α-syn) is a "natively unfolded" protein constituting the major component of intracellular inclusions in several neurodegenerative disorders. Here, we describe proteolysis experiments conducted on human α-syn in the presence of SDS micelles. Our aim was to unravel molecular features of micelle-bound α-syn using the limited proteolysis approach. The nonspecific proteases thermolysin and proteinase K, as well as the Glu-specific V8-protease, were used as proteolytic probes. While α-syn at neutral pH is easily degraded to a variety of relatively small fragments, in the presence of 10 mM SDS the proteolysis of the protein is rather selective. Complementary fragments 1−111 and 112−140, 1−113 and 114−140, and 1−123 and 124−140 are obtained when thermolysin, proteinase K, and V8 protease, respectively, are used. These results are in line with a conformational model of α-syn in which it acquires a folded helical structure in the N-terminal region in its membrane-bound state. At the same time, they indicate that the C-terminal portion of the molecule is rather rigid, as seen in its relative resistance to extensive proteolytic degradation. It is likely that, under the specific experimental conditions of proteolysis in the presence of SDS, the negatively charged C-terminal region can be rigidified by binding a calcium ion, as shown before with intact α-syn. In this study, some evidence of calcium binding properties of isolated C-terminal fragments 112−140, 114−140, and 124−140 was obtained by mass spectrometry measurements, since molecular masses for calcium-loaded fragments were obtained. Our results indicate that the C-terminal portion of the membrane-bound α-syn is quite rigid and structured, at variance from current models of the membrane-bound protein deduced mostly from NMR. Considering that the aggregation process of α-syn is modulated by its C-terminal tail, the results of this study may provide useful insights into the behavior of α-syn in a membrane-mimetic environment.

α-Synuclein (α-syn)[1] is a highly conserved protein that is mostly represented in the neuronal presynaptic pool, displaying a pathological role in neurodegenerative diseases, especially in Parkinson's disease (*1*−*3*). Structurally, α-syn belongs to the class of "natively unfolded" proteins which do not have stable structure under physiological conditions (*4*−*7*) and appears to be constituted by three distinct regions. The N-terminal region of residues 1−60 contains four KTKEGV imperfect repeat motifs (Figure 1) and the A30P, A53T, and E46K mutation sites associated with familial Parkinson's disease (*8*−*10*). The middle region of residues 61−95, termed NAC (non-A*β*-amyloid component), is highly hydrophobic and appears to be responsible for the fibrillation properties of α-syn (*11*, *12*). The C-terminal region of residues 96−140 is very acidic and contains up to 15 negatively charged residues (10 Glu and 5 Asp residues) that can bind metal ions or cationic compounds (*13*−*17*). There is strong evidence that α-syn can exert biological activities upon binding to membranes (*18*). In several studies, mostly involving NMR, it has been concluded that, upon association of the unfolded protein with a membrane, α-syn adopts a bipartite structure given by a largely helical N-terminal region of residues 1−95, while the negatively charged C-terminal region remains unfolded and available for potential interaction with other proteins (*19*).

The detailed molecular features of α-syn bound to membrane-mimetic environments, such as sodium dodecyl sulfate (SDS) micelles or small unilamellar vesicles, were studied in several laboratories by high-resolution NMR (*19*−*24*). Evidence is provided that in the micelle-bound state the N-terminal 1−95 region of the protein forms two long helical stretches interrupted by a break at residues 42−44, thus allowing the two helices to adopt a 11/3-helical conformation (*20*), a structural feature evidenced also in apo-

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[1] Abbreviations: α-syn, α-synuclein; CD, circular dichroism; E:S, enzyme:substrate ratio; ESI-MS, electrospray ionization mass spectrometry; PAGE, polyacrylamide gel electrophoresis; RP, reverse-phase; HPLC, high-performance liquid chromatography; t_R, retention time; [θ], mean residue ellipticity; TFA, trifluoroacetic acid; Tris, tris(hydroxymethyl)aminomethane; SDS, sodium dodecyl sulfate; UV, ultraviolet.

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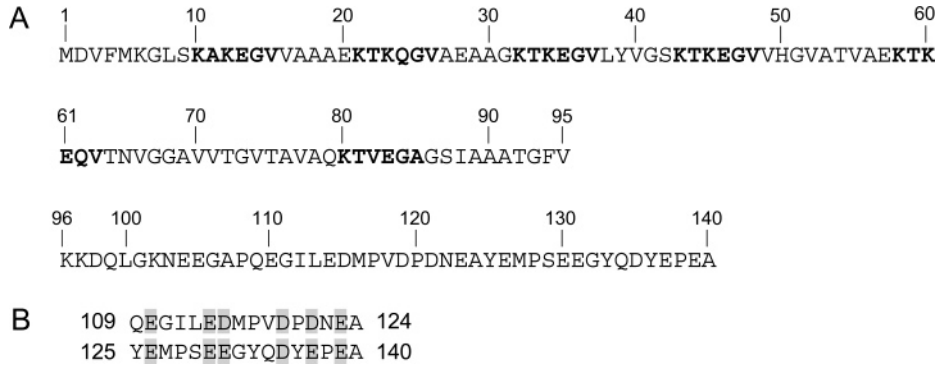

FIGURE 1: Amino acid sequence of human α-synuclein (A). The 140-residue polypeptide chain of α-syn can be subdivided into an N-terminal region (1−60) containing the imperfect KTKEGV repeats (bold), a central NAC region (61−95), and a C-terminal tail (96−140). The acidic residues in chain segments 109−124 and 125−140 are aligned and highlighted in gray in panel B.

lipoproteins (*25*). Recently, further analyses of the micelle-bound α-syn were conducted using paramagnetic spin-labels, and the existence of the 11/3-helical periodicity was confirmed (*23*). Of interest, although from NMR measurements and NOE effects it was concluded that the ∼40-residue C-terminal region of the protein is largely unfolded, Bussell et al. (*23*) observed an unexpected and unexplained protection from solvent for this region.

Even if the C-terminal tail of α-syn does not seem to acquire a regular secondary structure under different experimental conditions, it seems to be involved in several functions of the protein. The peculiar distribution of negatively charged residues (Figure 1B) makes it able to bind metal ions (*14*, *15*, *17*), polycations (*26*), and positively charged polyamine compounds (*16*). Considering the fundamental regulatory role of calcium in cellular districts, the interaction between α-syn and this metal ion was studied in more detail (*14*, *15*, *17*). Despite the fact that the calcium-binding motif of α-syn does not belong to a canonical EF-hand model or to other known calcium-binding motifs, it has been shown that binding of calcium to α-syn occurs with an IC_{50} of ∼300 μM Ca^{2+} (*14*). The interaction of metal ions with α-syn, as well as the formation of a polyamine complex, induces shielding of the negatively charged residues and affects several properties of α-syn, such as its solubility (*15*), its aggregation kinetics and fibril formation (*27*), and the morphology of the fibrils (*17*). In particular, the charge shielding effect induced by calcium binding promotes the aggregation of α-syn, in analogy to the removal of the C-terminal tail from the protein (*13*, *28*).

In this work, we have examined the conformational features of α-syn under membrane-mimetic conditions provided by SDS micelles by means of proteolysis experiments. Indeed, proteolytic probes can be used as probes of protein structure and dynamics, since the sites of proteolytic cleavage along the polypeptide chain of a protein are characterized by enhanced chain flexibility and do not occur at the level of chain regions embedded in hydrogen-bonded regular secondary structure (*29*−*31*). The use of proteases for unravelling molecular features of free or aggregated α-syn has also been exploited by others (*12*, *13*, *20*, *21*, *32*−*34*). The key result of this study is that the C-terminal tail of the micelle-bound α-syn is rather resistant to proteolysis, and thus, it appears to be rather rigid and structured under the specific experimental conditions of proteolysis, at variance from the general models so far developed for the micelle-bound state of α-syn (*19*−*24*).

EXPERIMENTAL PROCEDURES

Materials. Human wild-type α-syn was expressed in the *Escherichia coli* BL21(DE3) cell line transfected with the pRK172/α-syn plasmid. Purification of the recombinant protein was conducted as previously described (*35*), and it was further purified by RP-HPLC to remove some truncated species of the protein. Thermolysin from *Bacillus thermoproteolyticus*, proteinase K from *Tritirachium album*, and endoproteinase Glu-C from *Staphylococcus aureus* V8 were purchased from Sigma Chemical Co. (St. Louis, MO). All other chemicals of analytical reagent grade were obtained from Sigma or Fluka (Basel, Switzerland).

Spectroscopic Measurements. Protein concentrations were determined by absorption measurements at 280 nm using a double-beam Lambda-20 spectrophotometer from Perkin-Elmer (Norwalk, CT). The extinction coefficient (ϵ in milligrams per milliliter) at 280 nm for α-syn was 0.354, as evaluated from its amino acid composition by the method of Gill and von Hippel (*36*). Circular dichroism (CD) spectra were recorded on a Jasco (Tokyo, Japan) J-710 spectropolarimeter. Far-UV CD spectra were recorded using a 1 mm path length quartz cell and a protein concentration of 0.05−0.1 mg/mL. The mean residue ellipticity, [θ] (degress square centimeter per decimole), was calculated from the formula $[\theta] = (\theta_{obs}/10)(MRW/lc)$, where θ_{obs} is the observed ellipticity in degrees, MRW is the mean residue molecular weight of the protein, l is the optical path length in centimeters, and c is the protein concentration in grams per milliliter. The spectra were recorded in 10 mM Tris-HCl (pH 7.5) in the presence of 1, 10, and 100 mM SDS or without detergent.

Chemical Synthesis and Purification of Peptide 108−*140.* The peptide corresponding to residues 108−140 of human α-syn was synthesized by the solid-phase Fmoc method (*37*) using an Applied Biosystems (Palo Alto, CA) peptide synthesizer (model 431A). Fmoc-protected amino acids were used with the following side chain protection: *tert*-butyl ether (tBu) for Tyr, *tert*-butyl ester (OtBu) for Glu and Asp, and trityl (Trt) for Asn and Gln. Deprotection of the Fmoc group, at every cycle, was obtained by a 10 min treatment with 20% piperidine in *N*-methylpyrrolidone. Chain elongation was performed using a 10-fold excess (0.5 mmol) of Fmoc-

protected amino acid, 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate, and 1-hydroxybenzotriazole (1:1:1) in the presence of a 20-fold excess of *N*,*N*-diisopropylethylamine. After completion of the last cycle, the resin was washed with *N*-methylpyrrolidone and a dichloromethane/methanol mixture (1:1, v/v) and then dried in vacuo. The synthetic peptide was cleaved from the resin and deprotected by treatment of the peptide-resin with a 95:5 (v/v) mixture of TFA and 1,2-ethanedithiol for 2 h at 4 °C. The resin was filtered, and cold diethyl ether was added to the solution to precipitate the crude peptide, which was recovered by centrifugation and purified by RP-HPLC (see below for the experimental conditions).

Proteolysis of α-Syn and Synthetic Peptide 108−140. Proteolysis of α-syn was carried out at room temperature using thermolysin (*38*), proteinase K (*39*), and endoproteinase Glu-C (*40*) at E:S ratios of 1:250, 1:1000, and 1:50 (by weight), respectively. Proteolysis reactions were conducted in 10 mM Tris-HCl (pH 7.5) in the presence or absence of 10 mM SDS. Stock solutions of the proteases were prepared in Tris buffer (pH 7.5) containing 1 mM $CaCl_2$. The α-syn concentration in the proteolysis experiments was 0.6 mg/mL. The reactions were quenched at specified times by acidification with TFA in water (4%, v/v). The proteolysis mixtures were analyzed by RP-HPLC and SDS−PAGE according to the method of Schägger and von Jagow (*41*). The HPLC analyses were conducted using a Vydac C_{18} column (4.6 mm × 250 mm; The Separations Group, Hesperia, CA), eluted with a gradient of acetonitrile and 0.085% TFA versus water and 0.1% TFA: from 5 to 25% over 5 min, from 25 to 28% over 13 min, from 28 to 39% over 3 min, and from 39 to 45% over 21 min. The effluent was monitored by recording the absorbance at 226 nm. Proteolysis of α-syn synthetic peptide 108−140 by thermolysin was carried out at room temperature using an E:S ratio of 1:250 (by weight) in 10 mM Tris-HCl (pH 7.5) in the presence or absence of 10 mM SDS, at a peptide concentration of 0.6 mg/mL. In this case, the RP-HPLC analysis of the reaction mixture was conducted using the Vydac C18 column eluted with a gradient of acetonitrile and 0.085% TFA versus water and 0.1% TFA: from 5 to 25% over 5 min and from 25 to 29% over 17 min.

The sites of proteolytic cleavage along the 140-residue chain of α-syn or fragment 108−140 were identified by mass spectrometry and N-terminal sequence analyses of the protein fragments purified by RP-HPLC. Mass determinations were obtained with an electrospray ionization (ESI) mass spectrometer with a Q-Tof analyzer (Micro) from Micromass (Manchester, U.K.). The measurements were conducted at a capillary voltage of 2.5−3 kV and a cone voltage of 40−45 V. The molecular masses of protein samples were estimated using Mass-Lynx version 4.0 (Micromass). N-Terminal amino acid sequences were determined by automated Edman degradation with an Applied Biosystems protein sequencer (model Procise).

RESULTS

Effect of SDS on the Secondary Structure of α-Syn and Its C-Terminal Synthetic Fragment 108−140. The conformational properties of α-syn and its C-terminal fragment 108−140 in solution were analyzed by circular dichroism (CD) measurements. Figure 2A shows the CD spectra in far-UV region (190−250 nm) of α-syn recorded at room temperature (20−22 °C) at pH 7.5 in the absence or presence of 1, 10, and 100 mM SDS. The far-UV CD spectrum of α-syn is characterized by a minimum of ellipticity near 196 nm, which is indicative of a largely unfolded polypeptide chain (*42*−*44*). In the presence of SDS micelles, α-syn acquires α-helical secondary structure, as shown by the two characteristic minima near 208 and 220 nm, as shown previously (*20*, *21*, *23*, *24*). The conformational transition of α-syn from a random coil to an α-helical secondary structure appears to be completed in the presence of 10 mM SDS, since the shape of the CD spectrum and the intensity of the dichroic signals do not change in the presence of higher concentrations of SDS (up to 100 mM). Conversely, the synthetic peptide corresponding to the C-terminal portion of α-syn (residues 108−140) appears to be completely unfolded in aqueous solution at pH 7.5 (Figure 2B), and it does not acquire any regular secondary structure in the presence of SDS or in the presence of both calcium ions and SDS.

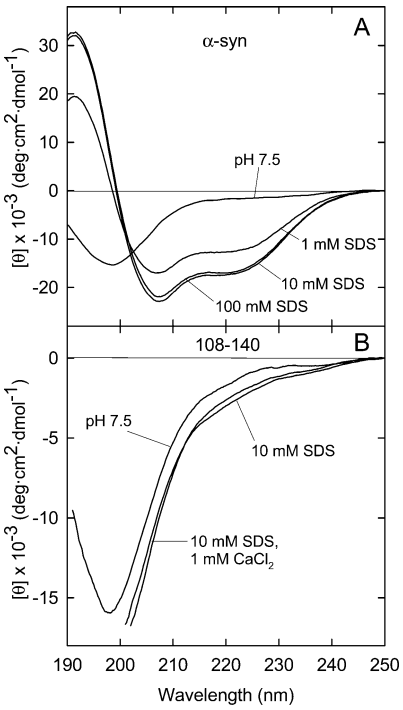

FIGURE 2: Far-UV CD spectra of human α-syn (A) and of the synthetic peptide corresponding to residues 108−140 of the protein (B). The spectra of α-syn were obtained at a protein concentration of 70 μM in 10 mM Tris-HCl (pH 7.5) containing 0, 1, 10, or 100 mM SDS. The spectra of synthetic peptide 108−140 were recorded at a concentration of 100 μM in 10 mM Tris-HCl (pH 7.5) or in the same buffer containing 10 mM SDS or 10 mM SDS and 1 mM $CaCl_2$. All measurements were taken 20−22 °C.

Proteolysis of α-Syn in Its SDS-Bound State. Proteolysis experiments have been conducted on α-syn in the presence of 10 mM SDS, which is a concentration above the CMC under the solvent conditions herewith employed (*45*). We have used three different proteases with differing substrate specificities and hydrolytic powers. Here, we report the results of the most representative reactions conducted by using thermolysin (*38*), proteinase K (*39*), and endoproteinase Glu-C (V8 protease) (*40*), since these proteases are known to retain proteolytic activity in the presence of SDS (*46*). Figure 3A shows the RP-HPLC and SDS−PAGE analyses

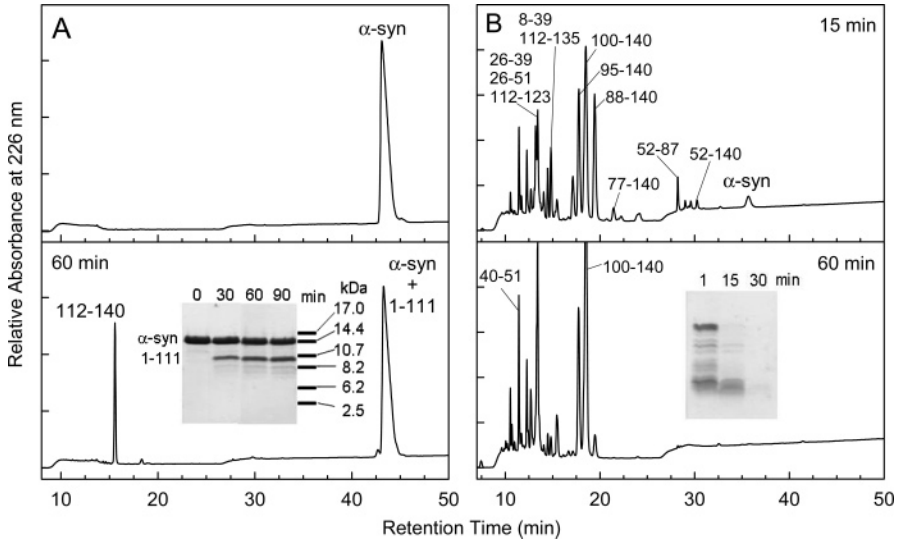

FIGURE 3: Proteolysis of α-syn with thermolysin in the presence (A) or absence (B) of SDS. Proteolysis was performed at 20−22 °C in 10 mM Tris-HCl (pH 7.5) in the presence or absence of 10 mM SDS using an E:S ratio of 1:250 (by weight) at a protein concentration of 0.6 mg/mL. Aliquots were taken from the reaction mixture at intervals and analyzed by RP-HPLC using a Vydac C_{18} column (see Experimental Procedures). The identity of the various α-syn fragments was established by ESI-MS (see Table S1 of the Supporting Information). The inset shows SDS−PAGE analysis of samples taken from the reaction mixture of α-syn digested by thermolysin in the presence of SDS (A) or without detergent (B). A partial BrCN digest of apomyoglobin was loaded onto the gel as a marker of molecular mass.

Table 1: Analytical Characterization of Fragments Obtained by Proteolysis of α-Synuclein[a]

protease	t_R (min)	molecular mass (Da) observed[b]	calcd[c]	fragment
thermolysin	15.6	3375.8	3376.5	112−140
		3413.83		112−140 with Ca^{2+}
	43.0	11103.7	11101.6	1−111
		14461.7	14460.1	1−140
proteinase K	14.3	3150.5	3150.2	114−140
		3166.5		114−140(ox)
		3187.6		114−140 with Ca^{2+}
		3204.1		114−140(ox) with Ca^{2+}
	43.4	11329.0	11327.9	1−113
		14462.1	14460.1	1−140
endoproteinase Glu-C	13.6	2006.8	2008.1	124−140
		2044.7		124−140 with Ca^{2+}
		2060.7		124−140(ox) with Ca^{2+}
	43.5	12467.4	12470.1	1−123
		14460.7	14460.1	1−140[d]

[a] The α-syn fragment species have been isolated by RP-HPLC of proteolysis mixtures of α-syn by thermolysin (Figure 3, left panels), proteinase K (Figure 4A), and endoproteinase Glu-C (Figure 4B). ox indicates an oxidized species. [b] Determined by ESI-MS. [c] Molecular masses calculated on the basis of the amino acid sequence of human α-syn. [d] The ESI-MS analyses of intact α-syn (residues 1−140) recovered by RP-HPLC of an aliquot of the proteolysis mixture provided evidence also of the existence of small amounts of a calcium-loaded protein species, but the experimental mass of this species is not given in this table.

of the proteolysis of α-syn by thermolysin. Proteolysis has been carried out at pH 7.5 using an E:S ratio of 1:250 (by weight) at 20−22 °C. Figure 3 shows that thermolysin cleaved α-syn in its SDS-associated state at only one site, namely, at the Gly111−Ile112 peptide bond, producing two complementary fragments, i.e., the N-terminal 1−111 fragment and the C-terminal 112−140 fragment (Table 1). Since thermolysin is slowly inactivated in the presence of 10 mM SDS, we have found that higher yields of selective fragmentation of α-syn can be obtained by repeated addition of protease to the reaction mixture (see Figure S1 of the Supporting Information). As shown by SDS−PAGE (Figure 3A, inset), the proteolytic digestion of α-syn leads to one major electrophoretic band in the stained gel, corresponding to the N-terminal 1−111 fragment. No other bands are seen in the Coomassie blue-stained gel, even after a prolonged time of incubation of α-syn with thermolysin. Conversely, fragment 112−140 was not visible in the gel, since this fragment contains numerous acidic and thus negatively charged residues (11 Asp/Glu residues) (Figure 1) that disfavor binding of SDS and lead to an aberrant electrophoretic mobility (*47*). Although the RP-HPLC analysis was conducted using an anion exchange guard column connected on-line with the column to remove SDS from the sample solution (*48*), the broadness of the chromatographic peak corresponding to α-syn and its retention time (t_R) of ∼43 min revealed that SDS was still bound to the protein molecule during the chromatographic run. In fact, the t_R of α-syn under the same RP-HPLC conditions, but without SDS, was ∼34 min (Figure 3B). Indeed, ESI-MS analyses revealed that up to six SDS molecules can be bound to the protein (data not shown). As expected for a largely unfolded protein, in the absence of SDS, α-syn is quickly and easily degraded to many fragments. Within 20−25 min of incubation with thermolysin, no intact protein remains in the proteolysis mixture. The peptides corresponding to most of the chromatographic peaks of the HPLC chromatogram, shown in Figure 3B, were analyzed by ESI-MS (see Table S1 of the Supporting Information), and the identities of several peptide fragments are given by the numbers near the chromatographic peaks in Figure 3B.

Figure 4 shows the RP-HPLC chromatograms of α-syn reacted with proteinase K (E:S ratio of 1:1000) and endoproteinase Glu-C (E:S ratio of 1:50) at pH 7.5 in the presence of 10 mM SDS after incubation for 1 h. While α-syn is readily cleaved by the two proteases under native conditions at pH 7.5 (not shown), in the presence of SDS micelles, only two main complementary fragments are obtained (see Figure 4 and Table 1). In particular, N-terminal fragment 1−113 and C-terminal fragment 114−140 are produced by protein-

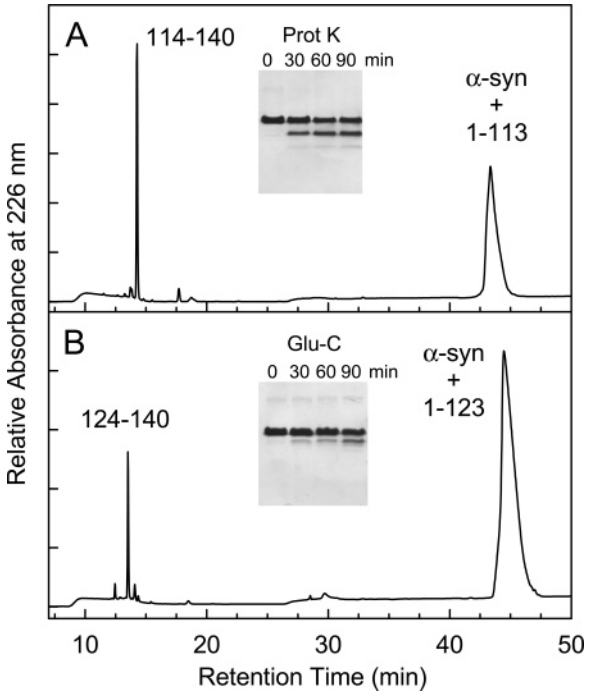

FIGURE 4: Proteolysis of α-syn by proteinase K (A) and endoproteinase Glu-C (V8 protease) (B) analyzed by RP-HPLC after incubation for 1 h with the protease and by SDS−PAGE (inset). Proteolysis was conducted in 10 mM Tris-HCl (pH 7.5) in the presence of 10 mM SDS, using an E:S ratio of 1:1000 or 1:50 for proteinase K or endoproteinase Glu-C, respectively.

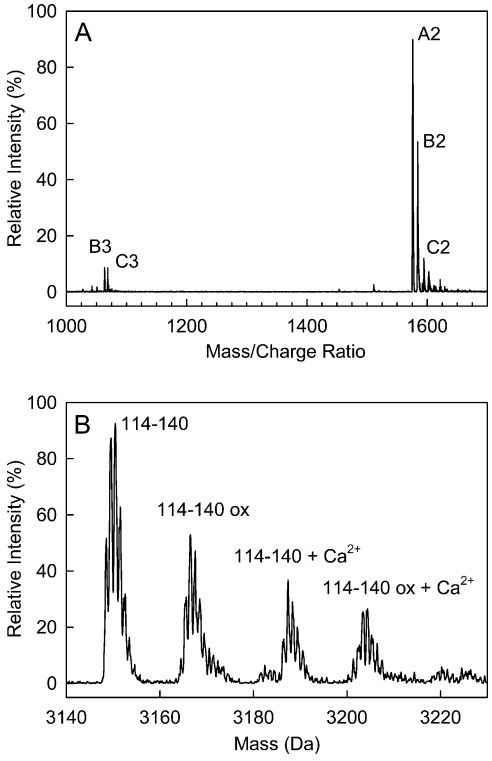

FIGURE 5: ESI-MS spectrum (A) and deconvoluted ESI spectrum (B) of C-terminal fragment 114−140 obtained by digestion of α-syn with proteinase K. Mass measurements were conducted on the peptide material eluted from the RP-HPLC column at a retention time of 14.3 min (see Figure 4A).

ase K, whereas endoproteinase Glu-C yields fragments 1−123 and 124−140. Also, in these two cases, the electrophoretic bands corresponding to N-terminal species 1−113 and 1−123 are seen in the stained SDS−PAGE gel (Figure 4, insets), whereas the complementary acidic C-terminal species are not detected in the gel.

Proteolysis of α-syn with thermolysin in Tris-HCl buffer (pH 7.5) was also performed in the presence of 1 mM SDS, i.e., under solvent conditions that do not induce a fully helical state of the protein (see Figure 1A). It was found that the rate of proteolysis was reduced, but numerous peptides were formed (not shown). Therefore, it can be concluded that in 1 mM SDS (below the CMC of SDS) equilibrium between free and SDS-bound α-syn exists and that the free, largely unfolded protein species is easily attacked at numerous sites along its 140-residue chain.

Mass Spectrometry Analysis of Proteolytic Fragments. The identity of the fragments produced by proteolysis of α-syn was established by analyses of their exact masses by ESI-MS (Table 1) and in some cases by automated Edman degradation. The analyses were conducted on the peptide material separated by RP-HPLC. Of note, intact α-syn and the N-terminal fragments produced by the three proteases, i.e., fragments 1−111, 1−113, and 1−123, eluted from a RP-HPLC column under the same chromatographic peak at ∼43 min, and all of them retained SDS bound even after chromatography, as shown by mass spectrometric analysis (not shown).

Figure 5 shows the MS spectrum of C-terminal peptide 114−140 obtained by proteolysis of α-syn by proteinase K (see Figure 4). It is seen that there are several species, the first corresponding to the free peptide (3150.04 Da), the second to the oxidized form of peptide 114−140 (3166.50 Da), the third to a calcium-bound form of the fragment (3187.62 Da), and the last one to an oxidized peptide containing calcium (3204.09 Da). Similar ESI-MS data were obtained also with the other C-terminal peptides, 112−140 and 124−140 (see Table 1), implying that the C-terminal proteolytic fragments of α-syn are able to bind calcium.

Proteolysis of Synthetic Peptide 108−140. To show if the resistance of the C-terminal region of α-syn to proteases could be a result of its interaction with the remaining part of its polypeptide chain, we analyzed by RP-HPLC the proteolysis of C-terminal synthetic peptide 108−140 by thermolysin at pH 7.5 in the absence or presence of 10 mM SDS. In the absence of detergent, the peptide was quickly hydrolyzed by the protease to several fragments, as expected for an unfolded polypeptide (Figure 6, middle panel, and Table 2). At variance, when the reaction was conducted in the presence of 10 mM SDS, proteolysis is much slower, and after incubation for 1 h with the protease, only fragment 112−140 is formed (Figure 6, bottom panel). Higher yields of fragment 112−140 can be obtained by repeated addition of thermolysin to the proteolysis mixture (see Figure S2A of the Supporting Information). The identity of fragment 112−140 was further established by fingerprinting analysis using V8 protease (Figure S2B of the Supporting Information). Moreover, its calcium binding properties were established by ESI-MS of a calcium-loaded sample of the fragment (Figure S3 of the Supporting Information).

DISCUSSION

The key result of this study is that, by using proteases with broad substrate specificity such as proteinase K and

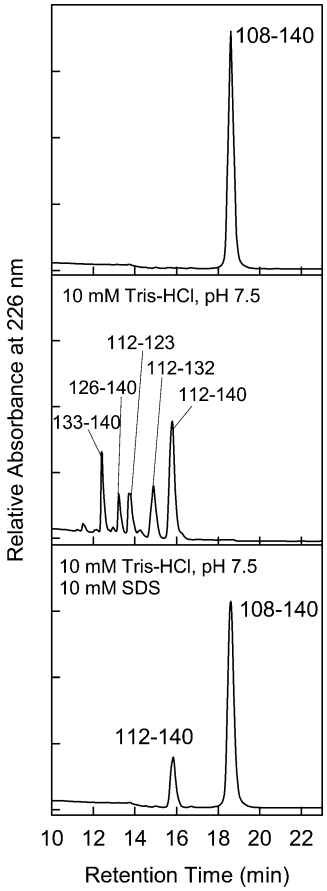

FIGURE 6: Proteolysis of synthetic C-terminal peptide 108−140 by thermolysin conducted in 10 mM Tris-HCl (pH 7.5) (middle panel) or in the presence of 10 mM SDS (bottom panel) after a 1 h incubation of the peptide with the protease. The E:S ratio was 1:250 (by weight). Analysis was performed by RP-HPLC using a Vydac C_{18} column (see Experimental Procedures). In the top panel, the elution position from the C_{18} column of the intact fragment is shown.

thermolysin, α-syn in its SDS-bound state is selectively cleaved at a single peptide bond along its 140-residue chain, leading to the two complementary N- and C-terminal fragments covering the entire protein chain. This observation is quite striking, if we consider that proteinase K is a most voracious protease displaying no substrate specificity (*39*) and that thermolysin shows only a moderate preference for attacking at hydrophobic or neutral residues (*38*, *49*). These two proteases have been used here since it is clear that the most suitable proteases for analyzing structural features of proteins are those displaying little or no substrate specificity, so that the proteolytic events are dictated by the stereochemistry and flexibility of the polypeptide substrate and not by the specificity of the attacking protease. However, even by using the Glu-specific endoproteinase (*40*), only the Glu123−Ala124 peptide bond is cleaved, despite the fact that there are many Glu residues along the 140-residue chain of α-syn, especially in its C-terminal region (see Figure 1).

It has been demonstrated that α-syn in the presence of SDS micelles adopts a helical structure given by two extended helices (residues 1−41 and 45−94), with a break at chain segment 42−44 (*20*−*24*). These two helices appear to be characterized by a periodicity of 3.67 residues per helical turn, instead of the value of 3.6 observed with regular helices, thus resulting in α-11/3-helices (*20*). Therefore, since

Table 2: Analytical Characterization of Fragments Obtained by Proteolysis of Synthetic Peptide 108−140 by Thermolysin[a]

	molecular mass (Da)		
t_R (min)	observed[b]	calcd[c]	fragment
11.5	608.2	607.6	136−140
12.7	1013.3	1014.0	133−140
	1051.3		133−140 with Ca^{2+}
13.2	1772.6	1773.8	126−140
	1789.7		126−140(ox)
	1812.6		126−140 with Ca^{2+}
13.7	1385.5	1386.4	112−123
			112−123(ox)
			112−123 with Ca^{2+}
14.9	1619.7	1620.7	112−125
	2378.9	2380.5	112−132
	2396.9		112−132(ox)
	2416.8		112−132 with Ca^{2+}
15.6	3376.4	3376.5	112−140
	3397.2		112−140(ox)
	3415.3		112−140 with Ca^{2+}
18.6	3787.8	3787.9	108−140
	3808.1		108−140(ox)
	3824.7		108−140 with Ca^{2+}

[a] The molecular masses of the α-syn fragments have been determined by ESI-MS analysis of fragments isolated after RP-HPLC (see Figure 6). ox indicates an oxidized species. [b] Determined by ESI-MS. [c] Molecular masses calculated on the basis of the amino acid sequence of human α-syn.

it has been demonstrated that helices are not targets of proteolysis (*29*−*31*, *50*), the helical secondary structure of SDS-bound α-syn should hamper proteolytic events at the level of the N-terminal region of α-syn covering approximately two-thirds of the 140-residue chain of the protein. Indeed, thermolysin and proteinase K cleave the nearby Gly111−Ile112 and Leu113−Glu114 peptide bonds, respectively, leading to protease-resistant N-terminal fragments 1−111 and 1−113, respectively. Therefore, by the criteria of the limited proteolysis approach, the N-terminal region of α-syn made rigid by the hydrogen bonding of the helical secondary structure is somewhat longer than that observed by NMR measurements, approximately up to residue 100 (*20*−*24*).

It has been proposed previously that proteases effectively cleave globular proteins mostly at flexible loops connecting chain segments in regular secondary structure such as helices (*29*). Therefore, if we accept the current view that the two N-terminal helices of α-syn in its micelle-associated state are interrupted by a short break at residues 42−44, we should explain why in this study proteolysis is not observed at this chain region. First, the lack of cleavage can be explained by the fact that the chain segment of the break is perhaps too short to allow the polypeptide substrate to be accommodated at the specific stereochemistry of the protease's active site, since a flexible chain segment of up to 12 residues is required for peptide bond fission to occur (*29*−*31*, *50*). The lack of hydrolysis at the break could be also due to the fact that this region appears to be buried inside the SDS micelle (*24*), and therefore, it is not available to the protease's attack. Moreover, since chain segment 42−44 does not exhibit enhanced chain flexibility relative to the rest of the N-terminal ∼100 residues of α-syn (*23*) and is a well-ordered extended linker (*22*), the break does not appear to be a flexible loop. However, Chandra et al. (*21*) reported that α-syn dissolved in Tris buffer (pH 7.4) containing 1 mM

SDS can be cleaved by trypsin to N-terminal fragments of ~4 and ~6 kDa. The authors concluded that, on the basis of the substrate specificity of trypsin, perhaps Lys43 or Lys45 could be the target site for tryptic hydrolysis. However, considering that proteolysis was performed in the presence of only 1 mM SDS and not in 10 mM SDS as used here, likely both free and micelle-bound α-syn do exist under these solvent conditions. Indeed, far-UV CD measurements clearly indicate that the transition of α-syn from a random coil to a helical conformation is not completed in 1 mM SDS (see Figure 2), and therefore, the peptide bond fissions of α-syn observed by Chandra et al. (*21*) could derive from proteolysis of a free, relatively unfolded protein species.

The Glu-specific endoproteinase cleaves the SDS-bound α-syn selectively at Glu123, even if at the C-terminus there are five more Glu residues in positions 126, 130, 131, 137, and 139 (see Figure 1). We have no clear-cut explanation for this, but it seems relevant to consider that the binding of Ca^{2+} ions with high affinity to α-syn depends critically on the C-terminus of the protein (residues 126−140). Indeed, with a truncated protein comprising residues 1−125, calcium binding is hampered, while C-terminal fragment 109−140 binds Ca^{2+} with an affinity equal to that displayed by the full-length protein (*14*). Thus, the Glu-specific protease appears to cleave outside chain segment 126−140 which is involved in strong ion binding.

The isolation of proteolytic fragments 112−140 and 114−140, as well as fragment 124−140, and thus the proteolytic resistance and consequent rigidity of the C-terminal region of α-syn, came as a surprise. In fact, current models of SDS-bound α-syn indicate that the C-terminal tail is largely disordered by NMR criteria (see ref *22* for references), and one would expect that this region could be easily attacked by proteases. Instead, proteolytic probes indicate a structured and sufficiently rigid C-terminal region in α-syn (Figures 3 and 4) or fragment 108−140 (Figure 6) in the presence of SDS micelles. Since it has been amply demonstrated that the C-terminal region of α-syn binds calcium ions (*14*−*17*), it seems reasonable to suggest that under the conditions of proteolysis Ca^{2+} binding is occurring with both α-syn and the fragment. Likely, in the proteolysis mixture, there is sufficient calcium for binding to the protein/peptide substrate. Since the proteases herewith employed are stabilized by calcium ions, their commercial samples all contain calcium as a stabilizer. For example, thermolysin (Sigma) is a lyophilized product from a 30% calcium acetate protein solution. Finally, in this study it was found convenient to use stock solutions of the proteases dissolved in Tris buffer (pH 7.5) containing 1 mM $CaCl_2$.

We have considered conducting proteolysis experiments in the presence of EDTA or EGTA to remove calcium ions from the proteolysis mixture. However, this experiment cannot be performed with thermolysin, since this protease is strongly and quickly inhibited by EDTA (*38*). Also, proteinase K is stabilized by calcium ions, and in the presence of chelating agents, it is slowly inactivated mostly by autolysis (*39*). Nevertheless, we have digested α-syn with proteinase K in the presence of 10 mM SDS and 2 or 5 mM EGTA. The digestion pattern was not much different from that obtained in the absence of the ion chelating agents (not shown). Therefore, this control of the proposed role of calcium ions in the limited proteolysis phenomenon herewith observed is negative. We can speculate that EGTA is unable to remove calcium bound to the polypeptide substrate under the specific experimental conditions of proteolysis. Evidence of this possibility is provided by the fact that calcium ion cannot be removed by EDTA from calmodulin (*52*).

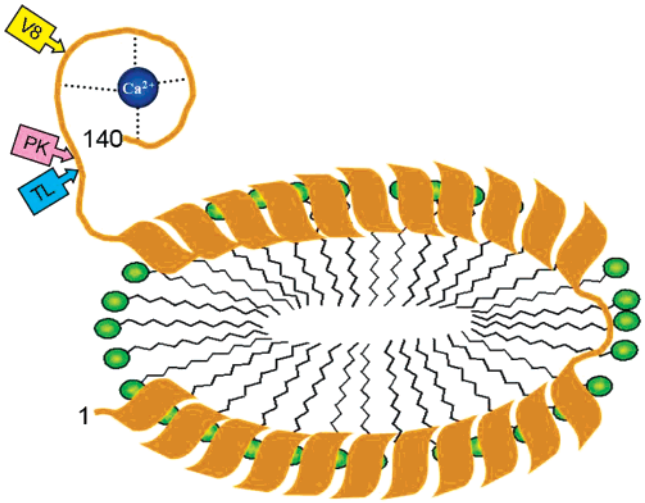

FIGURE 7: Schematic view of the SDS-bound α-syn. The N-terminal region becomes helical and forms two long helices with a break at segment 42−44 of the 140-residue polypeptide chain, while the highly negatively charged C-terminus does not acquire a regular secondary structure. Nevertheless, the tail appears to bind calcium in the presence of SDS micelles and becomes sufficiently rigid and structured to resist extensive proteolysis. The sites of limited proteolytic cleavage by thermolysin (TH), proteinase K (PK), and endoproteinase Glu-C (V8) are denoted with arrows.

Selective proteolysis of fragment 108−140 is observed only in the presence of 10 mM SDS, while in its absence, several peptide fragments are produced (see Figure 6). Therefore, SDS appears to be required to confer rigidity to the fragment, even if its far-UV CD spectrum in the presence of SDS is that of a random polypeptide (Figure 2). The lack of helix induction by SDS in fragment 108−140 can be understood by considering that Pro is a strong helix breaker residue and that the fragment contains five Pro residues evenly distributed along its polypeptide chain (see Figure 1). We may speculate that SDS may increase the affinity of Ca^{2+} for fragment 108−140, thus leading to a rather strong SDS−calcium complex, in analogy to the 1000-fold increase in the Ca^{2+} affinity observed with synaptotagmin upon phospholipid binding (*14*, *51*). Perhaps this calcium−SDS complex of the fragment is the actual substrate for limited proteolysis, leading to the selective removal of a short N-terminal tetrapeptide from its chain (see Figure 5).

In this study, ESI-MS measurements provided evidence that the C-terminal fragments of α-syn can bind calcium, at least under the special conditions of the MS analyses (Figure 5 and Tables 1 and 2). It is well-known that the ionization processes in the MS instrument are difficult to rationalize and that very often peptide moieties display MS peaks corresponding to complexes of metal ions, despite the fact that usually peptides are analyzed by MS in acidic solutions with aqueous trifluoroacetic acid (TFA). We may observe that even short peptide 133−140 appears to bind calcium by ESI-MS (see Table 2). The amino acid sequence of this octapeptide contains four nearby carboxylate groups, reminiscent of the most classical ion chelating agent EDTA. Therefore, we can understand why this relatively short

peptide could bind calcium, at least in the gas phase of the MS instrument. Recently, also binding of Tb^{3+} to short C-terminal synthetic peptides of α-syn has been analyzed by ESI-MS (*53*).

The schematic model of α-syn in the presence of SDS micelles, shown in Figure 7, summarizes the main conformational features of the protein in a membrane-mimetic environment. The main features of the model are the two long helices of the N-terminus with a break at chain region 42−44, as deduced from NMR studies (*21*−*24*), and the highly acidic and negatively charged C-terminal tail displaying Ca^{2+} binding (*14*, *17*). Even if this tail in the presence of SDS micelles does not acquire a regular secondary structure, it nevertheless adopts a quite rigid structure that hinders proteolysis. It is clear that the analysis of the conformational equilibrium attained by a natively unfolded protein such as α-syn (*4*−*7*) in the presence of detergents is quite difficult and requires the contribution of different techniques and approaches. Here, it is shown that the simple biochemical technique of limited proteolysis can provide useful protein structural informations, complementing those that can be obtained by means of other spectroscopic techniques.

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SUPPORTING INFORMATION AVAILABLE

Molecular masses of peptide fragments of α-syn digested with thermolysin in the absence of SDS (Table S1), RP-HPLC analysis of α-syn digested in the presence of SDS by repeated additions of thermolysin (Figure S1), RP-HPLC analysis of the proteolysis mixture of fragment 108−140 digested with thermolysin and of the fingerprinting mixture of fragment 112−140 digested with V8 protease (Figure S2), molecular masses of peptides obtained by digesting fragment 112−140 with V8 protease (Table S2), and ESI-MS of fragment 112−140 in the absence or presence of calcium ions (Figure S3). This material is available free of charge via the Internet at http://pubs.acs.org.

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