FEBS Letters 580 (2006) 1775–1779

Cysteine misincorporation in bacterially expressed human α-synuclein

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Received 27 January 2006; accepted 14 February 2006

Available online 24 February 2006

Edited by Jesus Avila

Abstract **Bacterially expressed human α-synuclein (α-syn) has been widely used in structural and functional studies. Here we show that ~20% of human α-syn expressed in *Escherichia coli* is mistranslated and that a Cys residue is incorporated at position 136 instead of a Tyr. Site-directed mutagenesis of codon 136 (TAC to TAT) resulted in the expression of α-syn lacking Cys. Although wild-type (Y136-TAC and Y136-TAT) and mutant (C136-TGC) α-syn had similar propensities to assemble into filaments, the levels of dimeric α-syn were increased by misincorporation. To avoid potential artefacts, we recommend use of the Y136-TAT construct for the expression of human α-syn.**

Keywords: α-Synuclein; Mistranslation; Cysteine; Dimerization; Aggregation

1. Introduction

Filamentous α-synuclein (α-syn) inclusions in nerve cells or glial cells are the defining neuropathological feature of a group of neurodegenerative diseases which includes Parkinson's disease (PD), dementia with Lewy bodies (DLB) and multiple system atrophy (MSA) [1]. In these so-called "α-synucleinopathies", α-syn is deposited in a hyperphosphorylated and partially ubiquitinated form [2,3]. Missense mutations (A30P, E46K and A53T) in the α-syn gene cause familial forms of PD and DLB [4–6]. Furthermore, multiplications (duplication and triplication) of a region on the long arm of chromosome 4 that encompasses the α-syn gene cause an inherited form of PD-dementia [7], indicating that the simple overproduction of wild-type α-syn is sufficient to cause disease.

Recombinant α-syn readily assembles into filaments that share many of the morphological and biochemical characteristics of the filaments present in human brain [8]. Mutations E46K and A53T in α-syn have been found to accelerate the rate of filament assembly [9,10]. Mutation A30P has been reported to increase the total aggregation of α-syn, but to slow the rate of mature filament formation [10].

The precise chain of events leading from soluble, monomeric to insoluble, filamentous α-syn is not known. Biophysical studies have suggested that dimerization of α-syn is a rate-limiting step in aggregation [11]. Human α-syn does not contain any Cys residues and hence, covalent aggregate formation caused by oxidation is restricted to dityrosine cross-links [12].

Here we report the unexpected finding that bacterially expressed human α-syn contains a significant amount of Cys. Analysis of dimeric α-syn showed that ~20% of the recombinant protein was mistranslated, with Cys at position 136 instead of Tyr. Mutagenesis of codon 136 from TAC to TAT resulted in the expression of α-syn lacking Cys. The ability of Y136C α-syn to assemble into filaments was not changed significantly. However, dimerization of the mutated protein was significantly enhanced.

2. Experimental procedures

2.1. Expression and purification of wild-type and mutant human α-syn

Human α-syn cDNA in bacterial expression plasmid pRK172 was used [13]. Codon 136 was changed from TAC to TAT or TGC by site-directed mutagenesis (Stratagene) (Fig. 1). All constructs were verified by DNA sequencing. Wild-type and mutant proteins were expressed in *Escherichia coli* BL21 (DE3) cells and purified using boiling, Q-Sepharose ion exchange chromatography and ammonium sulfate precipitation. α-Syn proteins were dialyzed against 30 mM Tris–HCl, pH 7.5, and cleared using a 20 min centrifugation at 113000 × *g*. Following separation by reverse phase high-performance liquid chromatography (RP-HPLC, Aquapore RP300 column), the absorbance at 215 nm was measured and compared with that of α-syn of known concentration, to give the concentration of the freshly purified protein.

2.2. Analysis of monomeric and dimeric α-syn by mass spectrometry

α-Syn (6 mg/ml) was incubated for 7 days at 37 °C in 30 mM Tris–HCl, pH 7.5, containing 0.02% sodium azide. Following a 20 min centrifugation at 113000 × *g*, the supernatant was fractionated on a Superdex 200 gel filtration column (10 × 300 mm, Amersham Bioscience) in 10 mM Tris–HCl, pH 7.5, containing

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Abbreviations: α-syn, α-synuclein; PD, Parkinson's disease; DLB, dementia with Lewy bodies; MSA, multiple system atrophy; ThS, thioflavin S; RP-HPLC, reverse phase high-performance liquid chromatography; MALDI-TOF/MS, matrix-assisted laser desorption ionization-time of flight/mass spectrometry

0014-5793/$32.00
doi:10.1016/j.febslet.2006.02.032

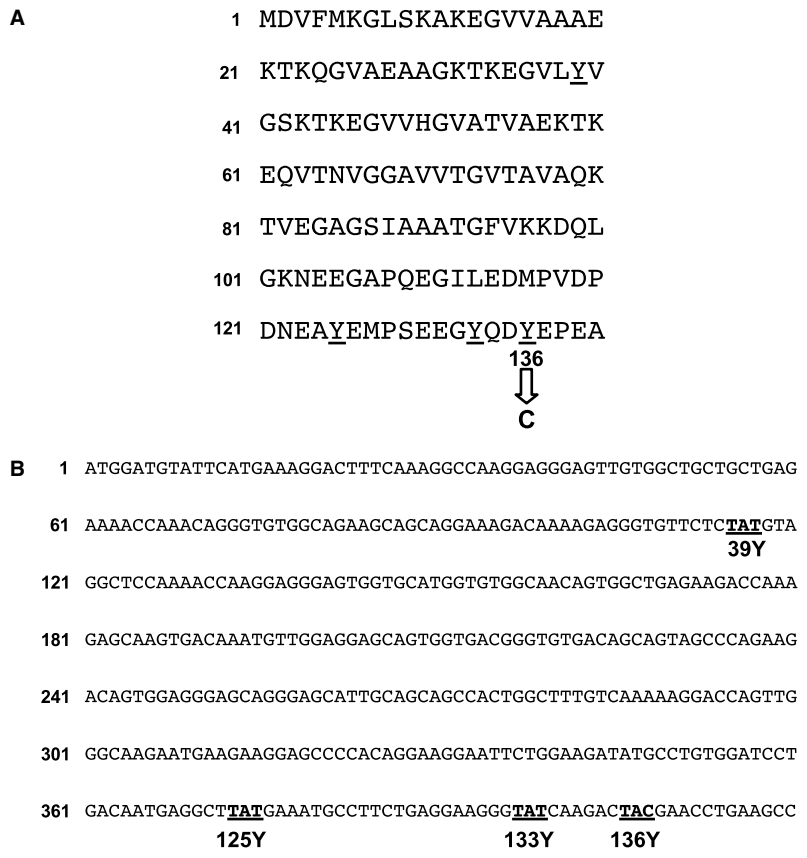

Fig. 1. Amino acid sequence (A) and cDNA sequence (B) of human α-syn. The four Tyr residues at positions 39, 125, 133 and 136 are highlighted. TAT codes for 39Y, 125Y and 133Y, whereas TAC encodes 136Y. During bacterial expression, a Cys residue is misincorporated at position 136 in a substantial fraction of α-syn.

0.15 M NaCl. The fractions were analyzed by SDS–PAGE with or without 2-mercaptoethanol (2ME) and analyzed by matrix-assisted laser desorption ionization-time of flight/mass spectrometry (MALDI-TOF/MS) using a Voyager-DE Pro mass spectrometer (PerSeptive Biosystems). Monomeric and dimeric α-syn was digested with TPCK-trypsin (Worthington Biochemical Company) for 18 h at 37 °C at an enzyme to substrate ratio of 1:20, separated by RP-HPLC on a Superspher Select B column (2.1 × 125 mm, Merck) and analyzed by mass spectrometry. The carboxy-terminal α-syn peptide was further digested for 18 h at 37 °C with V8 protease (Sigma) at an enzyme to substrate ratio of 1:10, followed by RP-HPLC. The separated tryptic-V8 peptide was then reduced for 1 h at 37 °C with 0.5% dithiothreitol (DTT) in 0.5 M Tris–HCl, pH 8.5, desalted using ZipTipC18 and analyzed by MALDI-TOF/TOF mass spectrometry using an Ultraflex (Bruker Daltonik GmbH) in a LIFT mode [14], with α-cyano hydroxyl cinnamic acid as a matrix.

2.3. Reduction and S-carboxymethylation of α-syn

Recombinant Y136-TAC and Y136-TAT α-syn (100 μg) was reduced for 1.5 h at room temperature with 1 mg DTT in 50 μl of 0.5 M Tris–HCl, pH 8.5, containing 7 M guanidine hydrochloride. Iodoacetic acid (2.5 mg) was added, followed by a 30 min incubation in the dark. The reaction mixture was then dialyzed against 30 mM Tris–HCl, pH 7.5, digested for 2 h at 37 °C with TPCK-trypsin at an enzyme to substrate ratio of 1:20 and separated by RP-HPLC.

2.4. Filament assembly of Cys-containing α-syn

Recombinant human α-syn (1 mg/ml) was incubated with shaking (200 rpm) for 96 h at 37 °C in 50 μl of 30 mM Tris–HCl, pH 7.5, containing 0.02% sodium azide. For a quantitative assessment of filament formation, the amounts of sarkosyl-insoluble α-syn and the levels of thioflavin S (ThS) fluorescence were measured as described [15].

3. Results

3.1. Identification of Cys-containing α-syn

Recombinant α-syn was incubated for 7 days at 37 °C, and fractioned by gel filtration chromatography. Two protein peaks were observed, which corresponded to monomeric and dimeric α-syn, respectively. Dimer formation was not observed in the presence of 2ME (Fig. 2A). Analysis of the monomer fraction (peak 1) by MALDI-TOF/MS gave one major signal corresponding to a molecular mass of 14460 (identical to the calculated molecular mass of monomeric human α-syn) (Fig. 2B). Analysis of the dimer fraction (peak 2) gave a signal of molecular mass 28799 (identical to the calculated molecular mass of dimeric α-syn minus 121) (Fig. 2C). To analyze this difference further, we compared the peptide maps of the tryptic digests of monomeric and dimeric α-syn. We found that the carboxy-terminal region of α-syn was modified in the dimer (see supplementary material). To identify the site of dimerization, the carboxy-terminal tryptic peptide was digested with V8 protease,

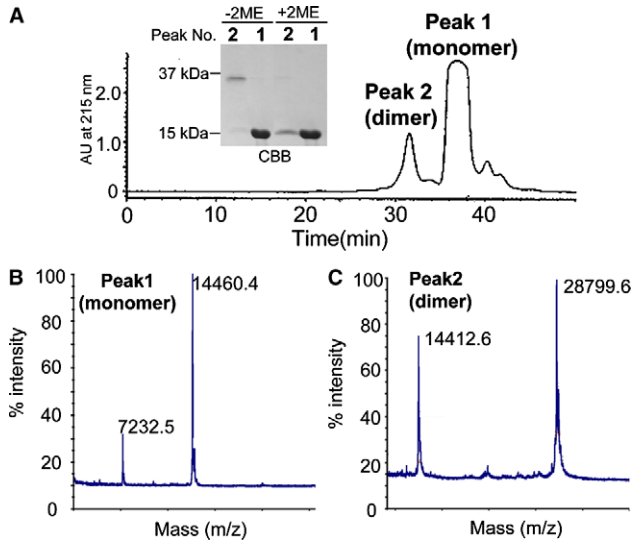

Fig. 2. Analysis of monomeric and dimeric α-syn. (A), Bacterially expressed α-syn was incubated for 7 days at 37 °C. Following ultracentrifugation, the supernatant was fractionated by gel filtration chromatography and peak 1 (monomer) and peak 2 (dimer) were run on SDS–PAGE in the absence and the presence of 2ME and visualized with Coomassie brilliant blue (CBB). (B,C), MALDI-TOF mass spectrometry analysis of peaks 1 and 2.

purified by RP-HPLC, reduced with DTT and analyzed by MALDI-TOF/TOF mass spectrometry. The product ion spectra derived from fragmentation of the charged ion at *m/z* 1011.4 resulted in a series of b and y sequence ions corresponding to the peptide GYQDCEPEA (Fig. 3). This peptide is identical to residues 132–140 of α-syn, with the exception of residue 136, which is Cys instead of Tyr. The difference between predicted and true mass of the dimer (121) could be accounted for by the mass difference between Tyr (163) and Cys (103).

3.2. Mutagenesis of codon 136 (TAC to TAT) of α-syn prevents the misincorporation of cysteine

Human α-syn contains four Tyr residues (at positions 39, 125, 133 and 136) (Fig. 1A). Tyr 136 is encoded by TAC, whereas the other three are encoded by TAT (Fig. 1B). We therefore mutated codon 136 from TAC to TAT, and found that Y136-TAC, but not Y136-TAT α-syn dimerised in the absence of 2ME (Fig. 4A). MALDI-TOF/MS analysis gave a major signal at 14460 and no signal corresponding to Cys-containing α-syn. Carboxymethylation of α-syn with codons TAC and TAT at position 136 was then used to identify the presence of Cys (Fig. 4B and C). The peptide maps of α-syn with codon TAC showed that carboxymethylation generated an extra peak at 16.7 min (arrowed in Fig. 4B). MALDI-TOF/MS and LC/MS analyses confirmed that this peak corresponded to the peptide with carboxymethylated Cys. The ratio of the peak area of carboxymethylated (16.7 min) to non-carboxymethylated (16.9 min) peptide was 1:4–5, indicating that 20–25% of recombinant α-syn carried the Y136C substitution (Fig. 4B). By contrast, when the peptide maps of α-syn with codon TAT before and after carboxymethylation were compared, no significant differences were observed (Fig. 4C). MALDI-TOF/MS and LC/MS analyses confirmed the absence of Cys and carboxymethylated Cys-containing peptides.

3.3. Filament formation of Y136-TAC, Y136-TAT and C136-TGC α-syn

Purified Y136 (TAC or TAT) and C136 α-syn were incubated for 96 h at 37 °C with agitation. Aliquots were removed daily and filament formation assessed by quantifying the level of sarkosyl-insoluble α-syn. All three proteins showed a similar

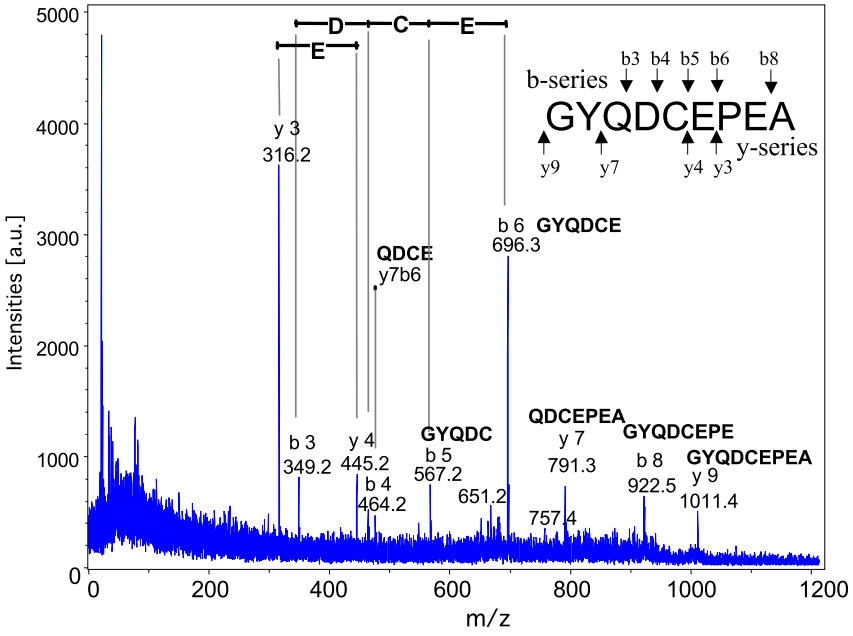

Fig. 3. MALDI-TOF/TOF mass spectrometry analysis of the dimeric peptide following digestion with V8 protease. The dimeric carboxy-terminal peptide (residues 103–140) was digested with V8 protease, purified by RP-HPLC, reduced with DTT and analyzed by MALDI-TOF/TOF mass spectrometry. The product ion spectra derived from fragmentation of the charged ion at *m/z* 1011.4 resulted in a series of b and y sequence ions, which corresponded to the peptide GYQDCEPEA (residues 132–140 of α-syn, with a Y136C mutation).

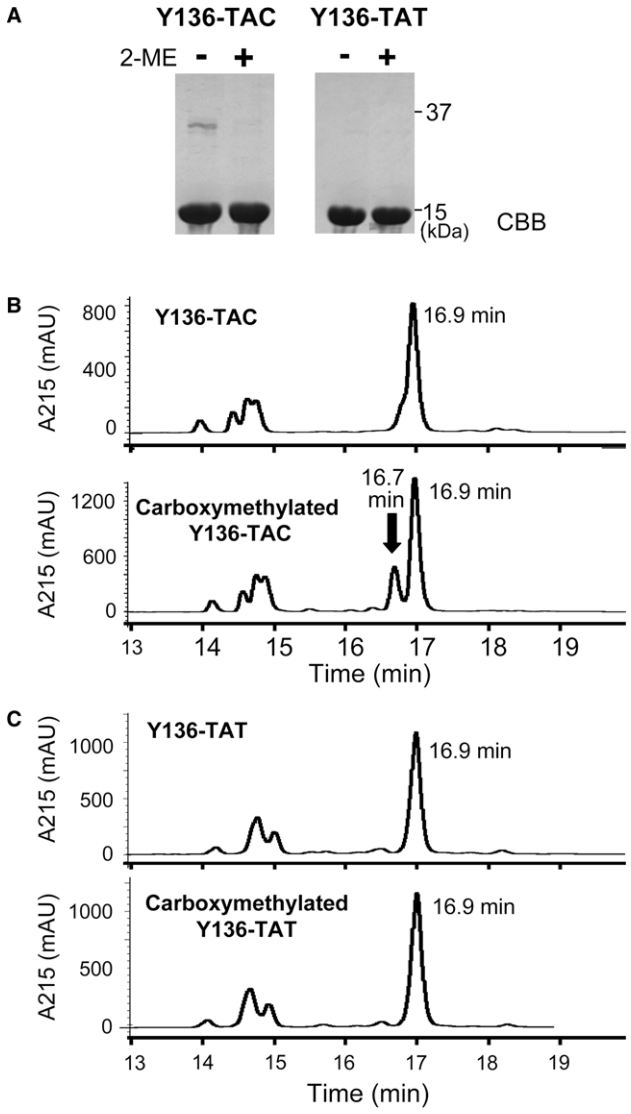

Fig. 4. Mutagenesis of codon Y136 of α-syn prevents the misincorporation of Cys. (A) α-Syn (Y136-TAC and Y136-TAT) was run on SDS–PAGE in the absence (−) and the presence (+) of 2ME and stained with CBB. (B,C), Wild-type α-syn (Y136-TAC and Y136-TAT) was carboxymethylated, digested with trypsin and separated by RP-HPLC. Separation profiles of the tryptic digests Y136-TAC (B) and Y136-TAT (C) α-syn before and after carboxymethylation. Cys-containing peptide was detected in Y136-TAC, but not Y136-TAT, α-syn following carboxymethylation (arrow at 16.7 min). These results were confirmed by MALDI-TOF/MS and LC/MS/MS analysis (data not shown).

propensity to assemble into filaments (Fig. 5). Similar results were obtained when filament formation was measured by ThS fluorescence (not shown).

4. Discussion

We report here that bacterial expression of human α-syn results in the misincorporation of Cys instead of Tyr at position 136 in 20–25% of the material. Cys-containing α-syn was found to dimerize through disulfide bond formation. Tyr 136 is encoded by TAC, unlike the other three Tyr residues in human α-syn (at positions 39, 125 and 133), which are encoded by TAT. Mutagenesis of codon 136 from TAC to TAT resulted in the expression of α-syn lacking Cys. These findings are reminiscent of protein 0.3 of bacteriophage T7, for which misincorporation of Cys instead of Tyr (one out of four Tyr residues) was observed upon bacterial expression [16]. Like codon 136 of human α-syn, Tyr 15 of protein 0.3 is encoded by TAC, which is followed by GAA in the sequence. This suggests that the misincorporation of Cys in protein 0.3 and in α-syn may have resulted from a combination of codon usage and sequence context. It remains to be seen whether the same is true of other recombinantly expressed proteins.

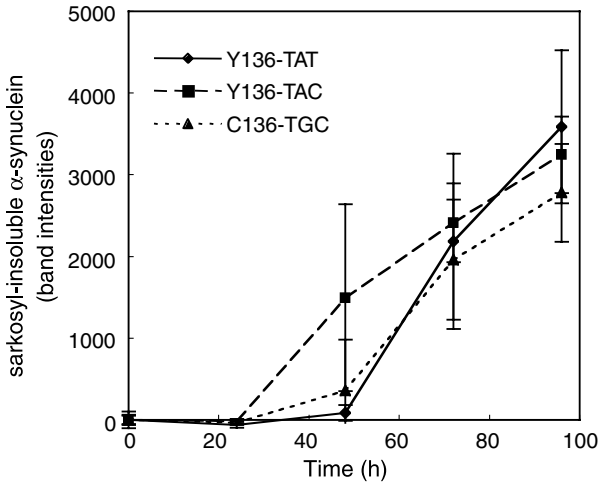

Fig. 5. Filament formation of Y136-TAC, Y136-TAT and C136-TGC α-syn. Recombinant α-syn (70 μM) was incubated with shaking. Filament formation was monitored by measuring the levels of sarkosyl-insoluble α-syn. The results are expressed as means ± S.E.M. ($n = 3$).

Recombinant α-syn is widely used for studying the protein's normal function and its abnormal assembly into filaments. Multimerization is believed to be an early step in the assembly of α-syn. We compared the ability of α-syn with Y136, C136 and a mixture of Y136 and C136 to assemble into filaments in vitro. All three protein preparations formed similar numbers of filaments with similar kinetics, as assessed by the amounts of sarkosyl-insoluble material and ThS fluorescence. This indicates that the presence of a Cys residue at position 136 does not significantly influence filament assembly of α-syn in vitro. It agrees with a recent study, which concluded that inclusion formation of α-syn was increased for mutations Y39C and Y125C, but not for mutations Y133C and Y136C [17].

In conclusion, the present findings show that a substantial proportion of bacterially expressed human α-syn carries a Tyr to Cys change at position 136. Dimerization of α-syn was significantly increased by the Cys miscorporation. This unexpected difficulty, which may well give rise to artefactual findings, can be avoided by expressing recombinant α-syn from a construct, where codon 136 has been mutated from TAC to TAT.

Acknowledgment: We thank M. Mikami for assistance with the analysis of SDS–PAGE.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2006.02.032.

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