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Prolyl oligopeptidase stimulates the aggregation of α-synuclein

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ABSTRACT

Despite its thorough enzymological and biochemical characterization the exact function of prolyl oligopeptidase (PO, E.C. 3.4.21.26) remains unclear. The positive effect of PO inhibitors on learning and memory in animal models for amnesia, enzyme activity measurements in patient samples and (neuro)peptide degradation studies link the enzyme with neurodegenerative disorders. The brain protein α-synuclein currently attracts much attention because of its proposed role in the pathology of Parkinson's disease. A fundamental question concerns how the essentially disordered protein is transformed into the highly organized fibrils that are found in Lewy bodies, the hallmarks of Parkinson's disease. Using gel electrophoresis and MALDI TOF/TOF mass spectrometry we investigated the possibility of α-synuclein as a PO substrate. We found that in vitro incubation of the protein with PO did not result in truncation of full-length α-synuclein. Surprisingly, however, we found an acceleration of the aggregation process of α-synuclein using turbidity measurements that was reversed by specific inhibitors of PO enzymatic activity. If PO displays this activity also in vivo, PO inhibitors might have an effect on neurodegenerative disorders through a decrease in the aggregation of α-synuclein.

1. Introduction

α-Synuclein is a small, 140 amino acid long protein, which appears to be natively unfolded and represents about 1% of the cytosolic protein in the brain [27].

The protein has recently attracted much attention because of its involvement in neurodegenerative disorders. There is an important link between α-synuclein and Parkinson's disease (PD), based on several observations. First of all α-synuclein-derived fibrils were found to be the major filamentous component of Lewy bodies and Lewy neuritis [41]. These intracellular proteinaceous aggregates are hallmarks of neurodegenerative diseases such as PD [32]. Lewy bodies also occur in brains of patients with the classic clinical and

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pathological features of Alzheimer's disease (AD) [44]. A second observation is that point mutations (A53T, A30P or E46K) in the α-synuclein gene or α-synuclein gene duplication or triplication cause rare autosomal dominant forms of PD [5,20,35,40,50]. The three point mutations have been shown to accelerate the fibril and/or oligomer formation in vitro [6,25,33]. It has been suggested that the severity of the phenotype and the distribution of the neurodegeneration increase with the number of copies of the gene, since heterozygous triplication produces diffuse Lewy body disease, whereas heterozygous duplication results in a phenotype indistinguishable from idiopathic PD [5,16]. Finally the overexpression of wild-type and mutated human α-synuclein in rats, mice and *Drosophila* has been used to generate animal models of PD [7,18,23,24,27].

As the purified human α-synuclein is unstructured in aqueous solution [46], a fundamental question concerns how the essentially disordered protein is transformed into the highly organized fibrils with characteristic crossed β-sheet conformation [45]. The α-synuclein aggregation fulfills all criteria of a nucleation-dependent polymerization process: a lag phase, a seeding effect and a critical concentration of monomer at equilibrium. Once α-synuclein nuclei are present, fibril elongation is predicted to proceed via the deposition of soluble α-synuclein units onto the existing templates provided by these nuclei. The critical concentration for wild-type α-synuclein is estimated to be 28 μM [48]. The natively unfolded character of α-synuclein arises from its low intrinsic hydrophobicity and high net charge at neutral pH. A decrease in pH, an increase in temperature or in ionic strength transforms α-synuclein into a partially folded conformation and the presence of this intermediate strongly correlates with the enhanced formation of α-synuclein fibrils [45].

Prolyl oligopeptidase (PO, E.C. 3.4.21.26) hydrolyses peptide bonds at the carboxyl side mainly of L-proline. It has been purified and characterized from various mammalian and bacterial sources. In mammals PO was shown to have the highest concentration among brain peptidases. The exact function of the enzyme remains unclear, but several reports associate PO with various neurological disorders such as neuropsychiatric diseases [43], AD [17,28,36] and PD [14]. Furthermore, PO inhibitors have been shown to have a positive effect on learning, memory and memory-related behavior [30,31,39]. Known in vitro substrates of the enzyme, for example, thyroliberin, beta-endorphin, substance P and arginine–vasopressin are extracellular [34], while PO is most commonly reported to be cytosolic [8]. In order to elucidate the function of this enzyme, we looked for endogenous substrates in porcine brain homogenates [3]. We found a small peptide appearing upon incubation of one of the brain fractions with recombinant porcine PO. The peptide was sequenced by MALDI-TOF/TOF MS/MS and found to be a perfect match of porcine α-synuclein residues 133–138 using Mascot. This fragment, YQDYEP-EA, was cleaved after the proline. It is located at the end of the C-terminal tail of α-synuclein – which contains five prolines in a span of 30 amino acids – suggesting cleavage of a larger part of the polypeptide by this enzyme.

PO, being a prolyl-specific endo-peptidase, shares substrates with the immunophilins, enzymes that bind to immunosuppressant drugs and have a peptidylprolyl isomerase (PPIase) activity [12]. So both PO and the immunophilins can bind a proline residue in a polypeptide and either break the peptide bond after it or change its conformation. Within the immunophilins, three different families are recognized: the FK506 binding proteins (FKBP), the cyclophilins and the parvulins. FKBP12, a 12 kD member of the FKBP family, has been shown to accelerate the aggregation of α-synuclein [9] and different reports suggest the involvement of FKBPs and their inhibitors in PD [1,4,10,11,13,19].

So the common property of PO and the immunophilins to bind prolines in peptides, the potential links between neurodegenerative diseases and both PO and the immunophilins, together with the fact that a C-terminal fragment of α-synuclein was found to be a PO substrate, prompted us to investigate the influence of PO on the aggregation of α-synuclein and the possibility that α-synuclein is a substrate of PO.

2. Experimental

2.1. Enzymes

A clone of the wild-type porcine PO and the S544A variant were kindly provided by Prof. L. Polgár (Institute of Enzymology, Hungarian Academy of Sciences). The proteins were expressed in *E. coli* strain JM109. The enzyme was purified using published methods [42]. Enterokinase (E.C.3.4.21.9) and Bovine pancreas chymotrypsin (E.C.3.4.21.1) were purchased from Roche Diagnostics. Human recombinant Glutathione S-transferase-FKBP12 (GST-FKBP12) protein was kindly provided by Dr. Bultynck (Laboratory for Physiology, K.U. Leuven, Belgium). It has been shown that the properties of FKBP12 are not altered by the fusion of GST [49]. Therefore, GST-FKBP12 was used in our experiments. We will further refer to it as FKBP12.

2.2. α-Synuclein

α-Synuclein was expressed and purified as described before [9]. The incorporation of a his-tag at the N-terminus of the polypeptide does not substantially affect its aggregation rate [9].

2.3. Visualization of putative α-synuclein cleavage by means of SDS-PAGE

SDS/PAGE (15%) was carried out in the presence of 2-mercaptoethanol essentially according to the Laemmli method in 25 mM Tris/HCl, 190 mM glycine, pH 8.3, and 0.5% (w/v) SDS using a Mini Protean II apparatus (Bio-Rad) [22]. The gels were standardized with pre-stained broad-range molecular-mass-marker proteins from Bio-Rad. The gel was stained by a neutral silver-staining procedure modified after Heukeshoven and Dernick [15].

2.4. Visualization of putative α-synuclein cleavage by means of MALDI TOF/TOF

The following incubation mixtures were prepared: (1) 5 μL 500 μM α-synuclein, 15 μL 20 mM Tris–HCl pH 7.4 and 5 μL

ultra pure water; (2) 5 μL 500 μM α-synuclein, 15 μL 20 mM Tris–HCl pH 7.4 and 5 μL enterokinase (0.3 mg/mL); (3) 5 μL 500 μM α-synuclein, 15 μL 20 mM Tris–HCl pH 7.4, 5 μL ultra pure water, 1 μL PO (1000 U/L) and 1 μL 25 mM DTT; (4) 5 μL 500 μM α-synuclein, 15 μL 20 mM Tris–HCl pH 7.4, 5 μL enterokinase (0.3 mg/mL), 1 μL PO (1000 U/L) and 1 μL 25 mM DTT. The mixtures were incubated at room temperature for 6.5 h. A dilution series was made in 0.1% TFA.

One microliter of sample (diluted to 1, 5 and 10 pmol) was mixed with 1 μL of alpha-cyano-4-hydroxycinnamic acid (8 mg/mL in 50% acetonitrile and 0.1% TFA) and vortexed. One microliter of this mixture was applied on a 192-well stainless steel target plate and air-dried at room temperature. The target was then introduced into a MALDI TOF/TOF mass spectrometer, a 4700 Proteomics Analyzer (Applied Biosystems, Framingham, MA). The samples were measured in the linear mode. During analysis, the instrument was internally calibrated. The laser energy was adjusted until an optimal resolution and signal to noise ratio were obtained. The results of 2000 shots were averaged to obtain the final spectrum.

2.5. Turbidity measurements

140 μM of α-synuclein was incubated with different amounts of PO in 50 mM HEPES pH 7.4 with 1 mM DTT at room temperature for 30 min. If necessary, 1 μM of PO inhibitors, Z-Pro-Prolinal (ZPP) or UAMC-00021, were added to the sample. Then the sample (100 μL) was placed in the microtiterplate reader (Safire^2, Tecan Austria) at 37 °C and shaken (orbital, amplitude 5 mm, ~240 rpm). Shaking was continued for 1000 s followed by an absorbance reading at 350 nm. The change in turbidity was thus monitored every 1000 s during 160 h. The microtiterplate was covered with a transparent sealing film to prevent evaporation.

2.6. Peptidylprolyl cis/trans isomerase activity

For the determination of the *cis*/*trans* isomerase activity of purified PO, the method described by Küllertz et al. [21] was used. 80 μL of chymotrypsin solution (1 g/L chymotrypsin in 35 mM HEPES pH 7.8) was added to 7 μL of sample or 35 mM HEPES pH 7.8 (blanc). Reaction was started by adding 50 μL of 125 mg/L succ-Phe-Pro-Phe-4-nitroaniline (Bachem) in 35 mM HEPES pH 7.8. After mixing for 5 s, absorbance was measured every 10 s during 20 min. Measurements were performed at room temperature (±22 °C). Data were fitted to $y = (A_0(1 - e^{(-kt)})) + B_0$ in order to obtain the rate constants (k).

2.7. Prolyl-specific endopeptidase activity

PO catalyses the transformation of the substrate Z-Gly–Pro-*p*-nitroaniline (colorless) to Z-Gly–Pro and *p*-nitroaniline (yellow). The amount of product formed can be followed by the absorption at 405 nm (20 min at 37 °C, Spectramax, Molecular devices). One unit of PO activity is defined as the amount of enzyme responsible for the formation of 1 μmole *p*-nitroaniline per minute.

For the quantification of the inhibition of recombinant PO by UAMC-00021 in vitro, samples were incubated with different concentrations of inhibitor for 15 min at 37 °C prior to enzymatic activity assay. IC_{50} values were calculated based on activity measurements with an enzyme concentration of 2 U/L, a substrate concentration of 0.25 mM and at 8 different inhibitor concentrations (0, 0.25, 0.5, 1, 2, 2.5, 10, 25 and 50 nM). Z-Pro-Prolinal was purchased from Bachem. The PO inhibitor UAMC-00021 [4-phenyl-1-((S)-2-((S)-2-(5-phenylisoxazole-3-carbonyl) pyrrolidine-1-carbonyl)pyrrolidin-1-yl)butan-1-one] was synthesized as described for analogous compounds [2].

3. Results

3.1. PO does not cleave intact purified recombinant α-synuclein

Since α-synuclein, like PO, is a cytosolic protein and contains five proline residues in its C-terminal part (P108, P117, P120, P128 and P138), we decided to incubate the recombinant enzyme with the purified α-synuclein and look for possible degradation products.

Recombinant purified his-tagged α-synuclein (1 g/L) was incubated with different amounts of purified recombinant porcine PO in 35 mM HEPES pH 7.8 with 1 mM DTT at room temperature. No cleavage of this his-tagged protein was observed under these circumstances, not after 24 h incubation (Fig. 1) nor after longer time periods up to 160 h in the same conditions as the aggregation experiments (data not shown).

The N-terminal his-tag of the α-synuclein construct includes an enterokinase cleavage site. The incorporation of

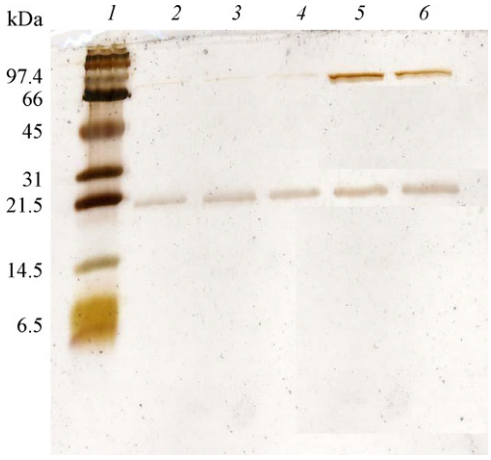

Fig. 1 – No cleavage of his-tagged recombinant α-synuclein by PO (SDS-PAGE). (Lane 1) Pre-stained broad-range markers (size indicated in kDa). (Lane 2) 42 μM α-synuclein and 40 U/L PO incubated for 1 h. (Lane 3) 42 μM α-synuclein and 40 U/L PO incubated for 3 h. (Lane 4) 42 μM α-synuclein and 40 U/L PO incubated for 24 h. (Lane 5) 42 μM α-synuclein and 700 U/L PO incubated for 2 h. (Lane 6) 42 μM α-synuclein and 700 U/L PO incubated for 24 h. All incubations proceeded at room temperature. After incubation 5 μL of sample buffer (2×) was added to 5 μL of the incubation mixture and loaded on the gel. The band at approximately 18 kDa is the his-tagged α-synuclein, the band at approximately 80 kDa is the recombinant porcine PO.

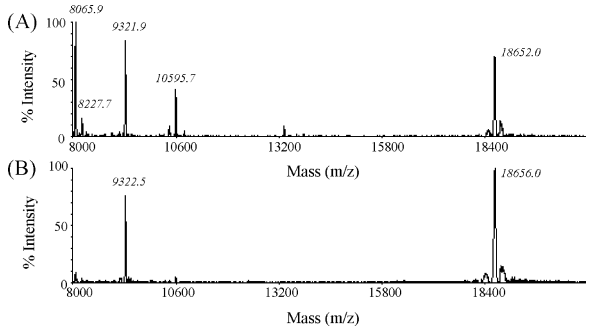

Fig. 2 – No cleavage of his-tagged recombinant α-synuclein by PO (MALDI TOF/TOF). (A) Control incubation of his-tagged α-synuclein. The peaks at 18,652 and 9321.9 represent single and doubly charged his-tagged α-synuclein, respectively. Other peaks result from an instability in the protein: 8065 (aa 1–76), 8227 (aa 1–77), 10,434 (aa 78–178) and 10,595 (aa 77–178). (B) His-tagged α-synuclein incubated with PO. The resulting peaks are the same as in the control incubation. No additional peaks pointing at PO-induced degradation were found.

a his-tag at the N-terminus of the polypeptide does not seem to affect its aggregation rate [9], but as size is an important criterion in the substrate selectivity of PO, the rather big N-terminal extension containing the his-tag (4 kDa) was removed from the protein by incubating it with enterokinase. The resulting protein, however, was also resistant to proteolysis by PO (data not shown).

The experiments were repeated using a MALDI-TOF-TOF mass spectrometer to detect peptide fragments. Again no PO-induced cleavage of the intact his-tagged α-synuclein by PO could be detected (Fig. 2).

3.2. *PO enhances the aggregation rate of recombinant α-synuclein in vitro*

Next we investigated whether PO could influence the aggregation of α-synuclein. Turbidity experiments with different α-synuclein protein preparations revealed that the aggregation rate of the protein was enhanced by addition of PO (Fig. 3a). Different concentrations of PO were used and a maximal effect was reached at around 20 nM of the enzyme. Thereafter, the effect was saturated and higher enzyme concentrations did not have a further positive effect on the aggregation of α-synuclein (Fig. 3a). Using a similar concentration of the S554A PO mutant, lacking prolyl oligopeptidase activity, did not result in an increased aggregation rate (Fig. 3b) but the turbidity vs. time curve differed in certain aspects from the control (broader, shorter lag phase, smaller final amplitude).

3.3. *The effect of PO on α-synuclein aggregation can be prevented by inhibitors of PO enzymatic activity*

Z-Pro-Prolinal (ZPP) is a classic prolyl endopeptidase inhibitor with high potency but relatively poor stability [47] and UAMC-00021 is a recently developed specific PO inhibitor, belonging

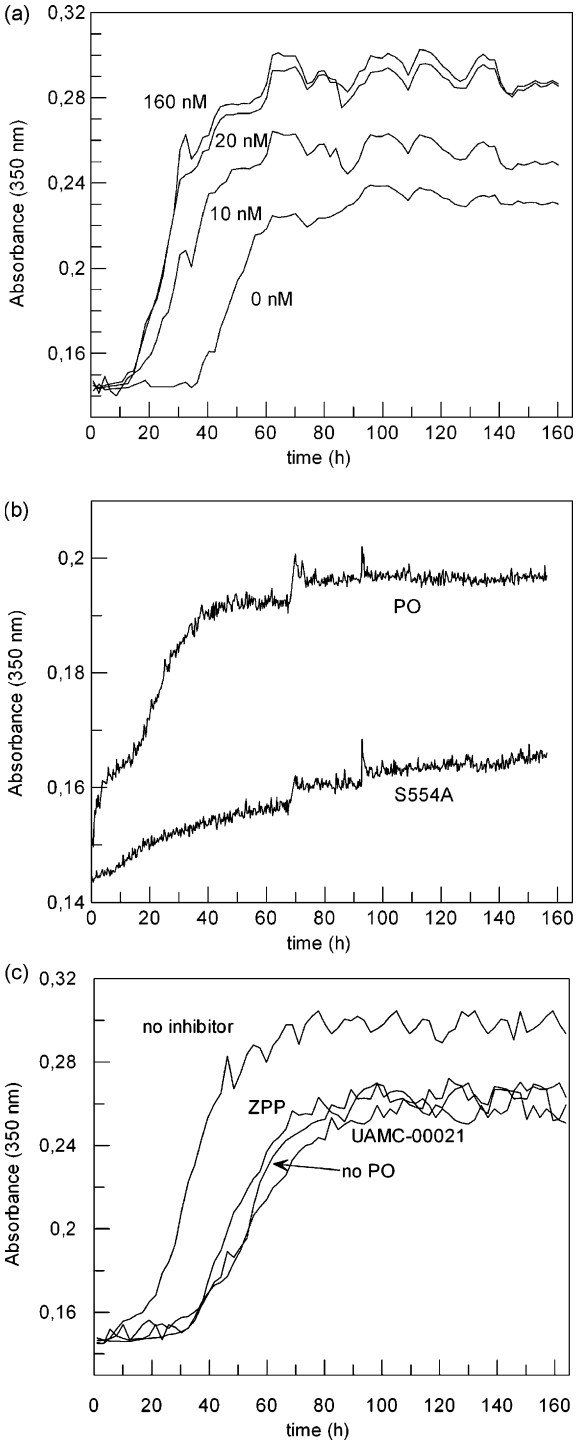

Fig. 3 – Concentration dependency of α-synuclein aggregation upon addition of PO. Turbidity measurement of the aggregation of 140 μM of α-synuclein in the presence of (a) 10, 20 and 160 nM PO and (b) 20 nM inactive mutant PO. Samples were incubated at 37 °C under continuous shaking; turbidity was measured at 350 nm, every 1000 s. (c) Acceleration of the aggregation is not observed in presence of PO inhibitors (1 μM Z-Pro-Prolinal (ZPP) or 1 μM UAMC-00021 in the presence of 20 nM PO).

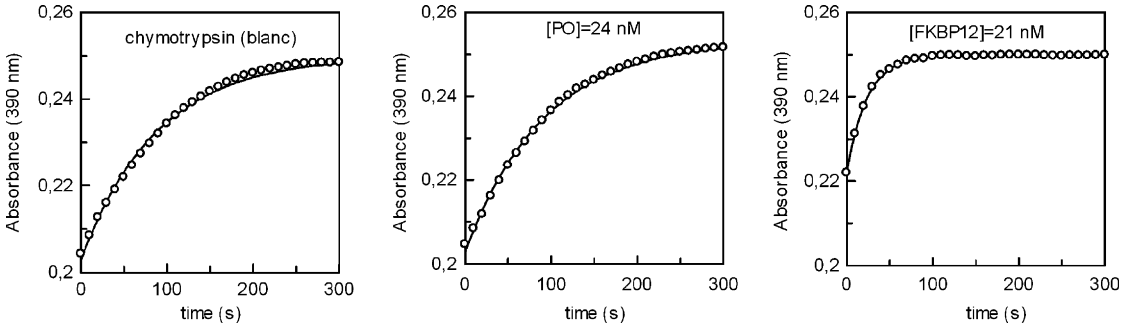

Fig. 4 – Investigation of the peptidylprolyl *cis/trans* isomerase activity of PO. The rate constants were measured following the microtiter plate assay as described by Küllertz et al. [21], but the assays were conducted at room temperature. Data were exported to Grafit and fitted to $y = (A_0(1 - e^{(-kt)})) + B_0$. The rate constant for the blanc reaction was 0.011 s^{-1}, 0.011 s^{-1} for the reaction with PO and 0.043 s^{-1} for the reaction with FKBP12.

to a series of ketoisoxazoles [2] which has not been described before. Using the PO assay described in Section 2, we determined that the IC_{50} of UAMC-00021 for PO is 1.6 ± 0.1 nM and classified UAMC-00021 as a slow tight binding inhibitor. Both in the presence of 1 μM ZPP or UAMC-00021, the accelerating effect of 20 nM PO on α-synuclein aggregation disappeared (Fig. 3c), suggesting that this effect is caused by the PO enzymatic activity.

3.4. *Peptidylprolyl cis/trans isomerase activity of PO*

We further investigated whether PO itself displayed peptidylprolyl *cis/trans* isomerase activity. The coupled assay exploits the high conformational selectivity of chymotrypsin towards chromogenic substrates of the type X-Pro-Phe-*p*NA. The hydrolysis of the C-terminal *p*-nitroanilide bond by chymotrypsin selectively occurs only in the trans X-Pro conformer. The assay is based on monitoring the time course of the chymotryptic cleavage of the 4-nitroanilide bond, which is kinetically coupled to the *cis/trans* isomerization of the peptidylprolyl bond of the substrate. As can be seen in Fig. 4, PO does not increase the rate constant of the release of *p*-nitroaniline under the circumstances used in our experiments while the same amount of FKBP12, a member of the peptidylprolyl *cis/trans* isomerase family and used as a control, did improve this rate constant.

4. Discussion

The intracellular localization of α-synuclein and the link between this protein and neurodegenerative disorders such as Parkinson's disease, makes it an interesting candidate as a PO substrate. However, in this study we show that purified recombinant α-synuclein is not cleaved by PO. This is not surprising considered that PO is known to cleave peptides of maximally 30 amino acids long. His-tagged α-synuclein (178 amino acids) and the enterokinase-truncated form (140 amino acids) do not meet this size restriction. The C-terminal part of α-synuclein that was detected as a PO cleavage product in the brain peptidomics experiment [3] may have been released from a proteolytic fragment of α-synuclein already present in the brain homogenate prior to the incubation with porcine PO. The truncation of α-synuclein has been reported before as a normal cellular process, enhanced in familial PD conditions [26]. So it is possible that the reported truncated variants and not the full-length α-synuclein protein serve as substrates for PO.

In vitro incubation of the full-length protein with PO did not result in truncation of α-synuclein, but, surprisingly, in an acceleration of the aggregation process. This effect was saturated upon addition of 20 nM PO. Incubation of α-synuclein with the inactive mutant S554A of PO did not cause such an acceleration, implicating the catalytic serine in the process. This was corroborated by the fact that two inhibitors of PO activity could prevent the accelerating effect of PO on α-synuclein aggregation. A plausible scenario to explain that the effect is saturated at a catalytic concentration of PO would be that a minor component of the α-synuclein preparation is proteolytically converted to a form that induces the formation of nuclei. At low PO concentration the proteolytic process is slow compared to the nucleation rate but at higher concentrations the substrate is rapidly and completely converted and the aggregation kinetics become independent of the enzyme concentration. Although outside the scope of this study, our result highlights the issue that really very little is known about the molecular events leading to aggregation and fibril formation in the in vitro (turbidity) model.

We also investigated other scenario's of how the enzyme may cause this intriguing phenomenon. Members of the FKBP family were reported to have a similar aggregation accelerating effect on α-synuclein [9]. It was hypothesized that, upon addition of FKBPs, through facilitation of the *cis* to *trans* isomerization of the prolines in the C-terminus, α-synuclein can rapidly adopt a partially folded conformation, which is a key step in the aggregation process. But in contrast to FKBP12, PO did not show peptidylprolyl *cis/trans* isomerase activity using the synthetic substrate. However, we cannot exclude that PO might display this activity towards natural proteins or protein fragments.

Since PO does not exert any proteolytic activity on full-length α-synuclein, another option is that its effect on the aggregation is due to stable or transient binding of PO to monomeric or aggregated α-synuclein. This binding could

shift the aggregation equilibrium to the right. The binding site on PO must then be located at or near its catalytic center, as both a mutation in this center and the presence of inhibitors binding to that center reverse the effect of PO on α-synuclein aggregation. It has been suggested that intrinsically unstructured proteins such as α-synuclein bind to grooves on protein surfaces and the ensemble of extended substrate binding sites on PO may represent such a binding site [29].

A report by Schulz et al. [38] suggests that PO might display chaperone functions, which have also been reported for the FKBPs. They found that PO is primarily localized in the perinuclear space and that it is associated with the microtubule cytoskeleton in human neuroblastoma and glioma cell lines. PO was found to bind to the C-terminus of tubulin in a yeast two-hybrid screening experiment. In the same report it was stated that tubulin itself and microtubuli-associated proteins bound to its C-terminus participate in protein folding due to their chaperone-like properties. We could not show chaperone activity of PO using classical tests with denatured lysozyme and rhodanese (data not shown). However, folding coupled to binding is a common element in interactions involving intrinsically unstructured proteins [37], as is the transient formation of differently structured forms. Since the folding of α-synuclein can lead to the initiation of its aggregation [45], the observed effect may originate in the nature of α-synuclein rather than PO. Current insights in the functions of intrinsically unstructured proteins correlate their ability to engage in a variety of protein–protein interactions with their roles as hubs, linkers or intermediates in signaling networks [37]. Therefore, our observation implicates PO, literally by association, in the deregulated pathways leading to pathological α-synuclein deposition.

Obviously, understanding the molecular basis and the function of the interaction between PO α-synuclein requires additional study. However, if we can validate PO as an accelerator of α-synuclein aggregation in the cell and UAMC-00021 as an inhibitor of this effect, as our in vitro results suggest, these findings might open new perspectives for the treatment of PD.

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REFERENCES

[1] Avramut M, Achim CL. Immunophilins in nervous system degeneration and regeneration. Curr Top Med Chem 2003;3:1376–82.
[2] Bal G, Van der Veken P, Antonov D, Lambeir AM, Grellier P, Croft SL, et al. Prolylisoxazoles: potent inhibitors of prolyloligopeptidase with antitrypanosomal activity. Bioorg Med Chem Lett 2003;13:2875–8.
[3] Brandt I, De Vriendt K, Devreese B, Van Beeumen J, Van Dongen W, Augustyns K, et al. Search for substrates for prolyl oligopeptidase in porcine brain. Peptides 2005;26:2536–46.
[4] Brecht S, Schwarze K, Waetzig V, Christner C, Heiland S, Fischer G, et al. Changes in peptidyl-prolyl *cis/trans* isomerase activity and FK506 binding protein expression following neuroprotection by FK506 in the ischemic rat brain. Neuroscience 2003;120:1037–48.
[5] Chartier-Harlin MC, Kachergus J, Roumier C, Mouroux V, Douay X, Lincoln S, et al. Alpha-synuclein locus duplication as a cause of familial Parkinson's disease. Lancet 2004;364:1167–9.
[6] Conway KA, Harper JD, Lansbury PT. Accelerated in vitro fibril formation by a mutant alpha-synuclein linked to early-onset Parkinson disease. Nat Med 1998;4:1318–20.
[7] Feany MB, Bender WW. A Drosophila model of Parkinson's disease. Nature 2000;404:394–8.
[8] Fruitier-Arnaudin I, Cohen M, Coitoux C, Piot JM. In vitro metabolism of LVV-Hemorphin-7 by renal cytosol and purified prolyl endopeptidase. Peptides 2003;24:1201–6.
[9] Gerard M, Debyser Z, Desender L, Kahle PJ, Baert J, Baekelandt V, et al. The aggregation of alpha-synuclein is stimulated by FK506 binding proteins as shown by fluorescence correlation spectroscopy. FASEB J 2006;20:524–6.
[10] Gold BG, Densmore V, Shou W, Matzuk MM, Gordon HS. Immunophilin FK506-binding protein 52 (not FK506-binding protein 12) mediates the neurotrophic action of FK506. J Pharmacol Exp Ther 1999;289:1202–10.
[11] Gold BG, Zeleny-Pooley M, Wang MS, Chaturvedi P, Armistead DM. A nonimmunosuppressant FKBP-12 ligand increases nerve regeneration. Exp Neurol 1997;147:269–78.
[12] Gothel SF, Marahiel MA. Peptidyl-prolyl *cis–trans* isomerases, a superfamily of ubiquitous folding catalysts. Cell Mol Life Sci 1999;55:423–36.
[13] Guo X, Dawson VL, Dawson TM. Neuroimmunophilin ligands exert neuroregeneration and neuroprotection in midbrain dopaminergic neurons. Eur J Neurosci 2001;13:1683–93.
[14] Hagihara M, Nagatsu T. Post-proline cleaving enzyme in human cerebrospinal fluid from control patients and parkinsonian patients. Biochem Med Metab Biol 1987;38:387–91.
[15] Heukeshoven J, Dernick R. Characterization of a solvent system for separation of water-insoluble poliovirus proteins by reversed-phase high-performance liquid chromatography. J Chromatogr 1985;326:91–101.
[16] Ibanez P, Bonnet AM, Debarges B, Lohmann E, Tison F, Pollak P, et al. Causal relation between alpha-synuclein gene duplication and familial Parkinson's disease. Lancet 2004;364:1169–71.
[17] Ichai C, Chevallier N, Delaere P, Dournaud P, Epelbaum J, Hauw JJ, et al. Influence of region-specific alterations of neuropeptidase content on the catabolic fates of neuropeptides in Alzheimer's disease. J Neurochem 1994;62:645–55.
[18] Kahle PJ, Neumann M, Ozmen L, Muller V, Jacobsen H, Schindzielorz A, et al. Subcellular localization of wild-type and Parkinson's disease-associated mutant alpha-synuclein in human and transgenic mouse brain. J Neurosci 2000;20:6365–73.
[19] Kato H, Oikawa T, Otsuka K, Takahashi A, Itoyama Y. Postischemic changes in the immunophilin FKBP12 in the rat brain. Brain Res Mol Brain Res 2000;84:58–66.
[20] Kruger R, Kuhn W, Muller T, Woitalla D, Graeber M, Kosel S, et al. Ala30Pro mutation in the gene encoding alpha-synuclein in Parkinson's disease. Nat Genet 1998;18:106–8.
[21] Küllertz G, Luthe S, Fischer G. Semiautomated microtiter plate assay for monitoring peptidylprolyl *cis/trans* isomerase activity in normal and pathological human sera. Clin Chem 1998;44:502–8.

[22] Laemmi UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970;227:680–5.
[23] Lakso M, Vartiainen S, Moilanen AM, Sirvio J, Thomas JH, Nass R, et al. Dopaminergic neuronal loss and motor deficits in Caenorhabditis elegans overexpressing human alpha-synuclein. J Neurochem 2003;86:165–72.
[24] Lauwers E, Debyser Z, Van Dorpe J, De Strooper B, Nuttin B, Baekelandt V. Neuropathology and neurodegeneration in rodent brain induced by lentiviral vector-mediated overexpression of alpha-synuclein. Brain Pathol 2003;13:364–72.
[25] Li J, Uversky VN, Fink AL. Effect of familial Parkinson's disease point mutations A30P and A53T on the structural properties, aggregation, and fibrillation of human alpha-synuclein. Biochemistry 2001;40:11604–13.
[26] Li W, West N, Colla E, Pletnikova O, Troncoso JC, Marsh L, et al. Aggregation promoting C-terminal truncation of alpha-synuclein is a normal cellular process and is enhanced by the familial Parkinson's disease-linked mutations. Proc Natl Acad Sci USA 2005;102:2162–7.
[27] Lo Bianco C, Ridet JL, Schneider BL, Deglon N, Aebischer P. Alpha-synucleinopathy and selective dopaminergic neuron loss in a rat lentiviral-based model of Parkinson's disease. Proc Natl Acad Sci USA 2002;99:10813–8.
[28] Mantle D, Falkous G, Ishiura S, Blanchard PJ, Perry EK. Comparison of proline endopeptidase activity in brain tissue from normal cases and cases with Alzheimer's disease, Lewy body dementia, Parkinson's disease and Huntington's disease. Clin Chim Acta 1996;249:129–39.
[29] Mészáros B, Tompa P, Simon I, Dosztányi Z. Molecular principles of the interactions of disordered proteins. J Mol Biol 2007;372:549–61.
[30] Miyazaki A, Toide K, Sasaki Y, Ichitani Y, Iwasaki T. Effect of a prolyl endopeptidase inhibitor, JTP-4819, on radial maze performance in hippocampal-lesioned rats. Pharmacol Biochem Behav 1998;59:361–8.
[31] Morain P, Robin JL, De Nanteuil G, Jochemsen R, Heidet V, Guez D. Pharmacodynamic and pharmacokinetic profile of S 17092, a new orally active prolyl endopeptidase inhibitor, in elderly healthy volunteers. A phase I study. Br J Clin Pharmacol 2000;50:350–9.
[32] Ohama E, Ikuta F. Parkinson's disease: distribution of Lewy bodies and monoamine neuron system. Acta Neuropathol (Berl) 1976;34:311–9.
[33] Pandey N, Schmidt RE, Galvin JE. The alpha-synuclein mutation E46K promotes aggregation in cultured cells. Exp Neurol 2006;197:515–20.
[34] Polgar L. The prolyl oligopeptidase family. Cell Mol Life Sci 2002;59:349–62.
[35] Polymeropoulos MH, Lavedan C, Leroy E, Ide SE, Dehejia A, Dutra A, et al. Mutation in the alpha-synuclein gene identified in families with Parkinson's disease. Science 1997;276:2045–7.
[36] Rossner S, Schulz I, Zeitschel U, Schliebs R, Bigl V, Demuth HU. Brain prolyl endopeptidase expression in aging, APP transgenic mice and Alzheimer's disease. Neurochem Res 2005;30:695–702.
[37] Russell RB, Gibson TJ. A careful disorderliness in the proteome: sites for interaction and targets for future therapies. FEBS Lett 2008;582:1271–5.
[38] Schulz I, Zeitschel U, Rudolph T, Ruiz-Carrillo D, Rahfeld JU, Gerhartz B, et al. Subcellular localization suggests novel functions for prolyl endopeptidase in protein secretion. J Neurochem 2005;94:970–9.
[39] Shinoda M, Miyazaki A, Toide K. Effect of a novel prolyl endopeptidase inhibitor, JTP-4819, on spatial memory and on cholinergic and peptidergic neurons in rats with ibotenate-induced lesions of the nucleus basalis magnocellularis. Behav Brain Res 1999;99:17–25.
[40] Singleton AB, Farrer M, Johnson J, Singleton A, Hague S, Kachergus J, et al. Alpha-synuclein locus triplication causes Parkinson's disease. Science 2003;302:841.
[41] Spillantini MG, Crowther RA, Jakes R, Hasegawa M, Goedert M. Alpha-synuclein in filamentous inclusions of Lewy bodies from Parkinson's disease and dementia with Lewy bodies. Proc Natl Acad Sci USA 1998;95:6469–73.
[42] Szeltner Z, Renner V, Polgar L. Substrate- and pH-dependent contribution of oxyanion binding site to the catalysis of prolyl oligopeptidase, a paradigm of the serine oligopeptidase family. Protein Sci 2000;9:353–60.
[43] Tarrago T, Sabido E, Kogan MJ, de Oliveira E, Giralt E. Primary structure, recombinant expression and homology modelling of human brain prolyl oligopeptidase, an important therapeutic target in the treatment of neuropsychiatric diseases. J Pept Sci 2005;11:283–7.
[44] Trojanowski JQ, Lee VM. Aggregation of neurofilament and alpha-synuclein proteins in Lewy bodies: implications for the pathogenesis of Parkinson disease and Lewy body dementia. Arch Neurol 1998;55:151–2.
[45] Uversky VN, Li J, Fink AL. Evidence for a partially folded intermediate in alpha-synuclein fibril formation. J Biol Chem 2001;276:10737–44.
[46] Weinreb PH, Zhen W, Poon AW, Conway KA, Lansbury Jr PT. NACP, a protein implicated in Alzheimer's disease and learning, is natively unfolded. Biochemistry 1996;35:13709–15.
[47] Wilk S, Orlowski M. Inhibition of rabbit brain prolyl endopeptidase by N-benzyloxycarbonyl-prolyl-prolinal, a transition state aldehyde inhibitor. J Neurochem 1983;41:69–75.
[48] Wood SJ, Wypych J, Steavenson S, Louis JC, Citron M, Biere AL. alpha-Synuclein fibrillogenesis is nucleation-dependent. Implications for the pathogenesis of Parkinson's disease. J Biol Chem 1999;274:19509–12.
[49] Xin HB, Timerman AP, Onoue H, Wiederrecht GJ, Fleischer S. Affinity purification of the ryanodine receptor/calcium release channel from fast twitch skeletal muscle based on its tight association with FKBP12. Biochem Biophys Res Commun 1995;214:263–70.
[50] Zarranz JJ, Alegre J, Gomez-Esteban JC, Lezcano E, Ros R, Ampuero I, et al. The new mutation, E46K, of alpha-synuclein causes Parkinson and Lewy body dementia. Ann Neurol 2004;55:164–73.