Structural Basis for Dityrosine-Mediated Inhibition of α-Synuclein Fibrilization

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α-Synuclein (α-Syn) is a 140-residue intrinsically disordered protein whose exact physiological role remains unknown.1 However, it is strongly associated with Parkinson’s disease (PD) and forms inclusions known as Lewy bodies in the brains of PD patients.2 Metal-ion-catalyzed oxidation (MCO) is believed to play a significant role in the origin and progression of PD.2,3 However, common MCO modifications, including carbonylation of the side chains of Lys, Pro, Arg, and Thr residues, occur only to a low extent with α-Syn.4 MCO of α-Syn predominantly leads to the oxidation of Met to Met sulfoxides5,6 and the formation of dityrosine (diTyr) linkages.5 These modifications favor assembly into soluble aggregates rather than fibrils.7−9 DiTyr cross-linkages, both intra- and intermolecular, are associated with oxidative stress8,10−12 and have been identified post mortem in brains of PD patients.13 Intermolecular diTyr formation connecting Tyr39-Tyr39 results in covalent α-Syn dimers that have been shown to have various effects on α-Syn aggregation.10,12,13 At early time points of α-Syn MCO, the formation of intramolecular diTyr cross-linked α-Syn monomers was favored over the formation of diTyr-linked dimers.7 This raises the question of how early oxidative modifications influence the fibrillation mechanism.

To answer this question, we employed an MCO protocol5 combining Cu2+ and H2O2 to investigate how early α-Syn modifications, mainly Met oxidations and diTyr cross-links, affect structure and amyloidogenic properties.

α-Syn contains four Tyr residues, one in the N-terminal region (Tyr39) and three in close proximity in the C-terminal tail (Tyr125, Tyr133, and Tyr136) (Figure 1A). Monitoring Tyr and diTyr fluorescence in parallel, it is seen that diTyr is formed rapidly in α-Syn upon MCO with a half-life of 0.86 min, in reasonable accord with a half-life of 1.41 min for Tyr fluorescence decay (Figure 1B). We used mass spectrometry (MS) to characterize modifications within the intact protein. MCO of α-Syn showed up to three +16 Da increases for 15 min of oxidation (Figure S1A) and more for longer incubation times. LC-MS/MS supported the presence of Met sulfoxides and sulfones (data not shown). The deconvoluted mass spectrum further showed a −2 Da loss for the oxidized wild-type (wt) protein (Figure 1C and Figure S1B), corresponding to an intramolecular Tyr cross-link formation (loss of 2 H). Tyr→Phe mutations in either the N-terminal (Tyr39Phe) or in the C-terminal tail (Tyr125/133/136Phe) of α-Syn led to similar modifications, i.e. Met oxidations, as well as loss of 2 H, suggesting MCO-induced cross-links. However, since Tyr125/133/136Phe α-Syn contains only one Tyr residue, other cross-links can be formed, e.g., between Tyr39 and one of the 15 Lys residues14 or His50. A shift in charge state distribution toward lower charge states for wt and the Tyr125/133/136Phe variant (Figure S1C) indicates a shift toward compact conformations.15 The rate of Met oxidation was not a function of mild oxidation on monomeric α-Syn and its aggregation. Using a combination of biophysical methods, small-angle X-ray scattering, and native ion mobility mass spectrometry, we find that oxidation leads to formation of intramolecular dityrosine cross-linkages and a compaction of the α-Syn monomer by a factor of √2. Oxidation-induced compaction is shown to inhibit ordered self-assembly and amyloid formation by steric hindrance, suggesting an important role of mild oxidation in preventing amyloid formation.
were detected, with an increase in intensity at 15 min for both the wt and the Tyr39Phe samples. SDS-PAGE analysis showed formation of faster-migrating species (Figure 1D, bottom, and Figure S4). Higher molecular weight bands detected for wt α-Syn under oxidative conditions. Data fitted to a single-exponential decay with linear drift. Fit shown with a solid line. (C) Deconvoluted MS spectra of wt, Tyr39Phe, and Tyr125/133/136Phe α-Syn either unmodified (black) or 15 min oxidized (red). Arrows showing decrease and increase of molecular mass. (D) Top: DiTyr detection on an immunoblot of wt, Tyr39Phe, and Tyr125/133/136Phe α-Syn, oxidized for 0, 3, 15, and 60 min. Monomeric diTyr is indicated by an arrowhead. Positive control: oxidized α-casein. Bottom: Coomassie-stained SDS-PAGE showing different migration patterns of unmodified and oxidized α-Syn.

Figure 1. DiTyr formation of α-Syn. (A) Schematic of α-Syn (PDB: 1XQ8, SDS micelle bound). Possible oxidation sites indicated. Insert: diTyr formation. (B) Time course of Tyr and diTyr fluorescence for wt α-Syn under oxidative conditions. Data fitted to a single-exponential decay with linear drift. Fit shown with a solid line. (C) Deconvoluted MS spectra of wt, Tyr39Phe, and Tyr125/133/136Phe α-Syn either unmodified (black) or 15 min oxidized (red). Arrows showing decrease and increase of molecular mass. (D) Top: DiTyr detection on an immunoblot of wt, Tyr39Phe, and Tyr125/133/136Phe α-Syn, oxidized for 0, 3, 15, and 60 min. Monomeric diTyr is indicated by an arrowhead. Positive control: oxidized α-casein. Bottom: Coomassie-stained SDS-PAGE showing different migration patterns of unmodified and oxidized α-Syn.

were detected, with an increase in intensity at 15 min for both the wt and the Tyr39Phe samples. SDS-PAGE analysis showed formation of faster-migrating species (Figure 1D, bottom, and Figure S4). Higher molecular weight bands detected for wt suggest cross-linked oligomers. As expected, no diTyr was detected in the triple mutant (Figure 1D top). The loss of 2 Da supports formation of an intramolecular cross-link (Figures S2 and S3). The immunoblot highlights the importance of diTyr cross-links in the oxidation of α-Syn but does not rule out alternative cross-links formed in parallel.

We then asked whether these intramolecular diTyr links affect the conformational preferences of α-Syn. Small-angle X-ray scattering (SAXS) data for unoxidized and 60 min oxidized α-Syn display Guinier behavior, i.e., a relatively constant level at low values of the modulus of the scattering vector, $q$, followed by a power-law behavior (linear decline in the log–log plot) at intermediate $q$, characteristic of polymer-like structures (Figure 2). Radii of gyration ($R_g$) from indirect Fourier transformation (IFT, see the Supporting Information (SI)) are given in Table 1. Native α-Syn showed an $R_g$ of 3.96 nm, in good agreement with an extended α-Syn conformation (Figure 2, Table 1, SS). $R_g$ was reduced to 2.69 nm (a factor 1.47) upon MCO. An ideal ring (joined at the two termini) will have an $R_g$ that is $\sqrt{2}$ ($\sim$1.4) smaller than that of an ideal chain (see SI), suggesting that MCO induced a conversion from disordered to compact monomer.

The scattering curves were subsequently fitted by the models derived in the SI (fits in Figure 2, summarized in Table 1). The native monomer is in good agreement with the linear chain model, giving a concentration value identical to the one determined by absorbance measurements and a Kuhn length, only slightly larger than the expected value of 1.51 nm. The oxidized monomer is best described by a loop-containing model, which represents a structure with a link from Tyr39 to one of the three C-terminal Tyr residues.

Based on the SAXS data, we hypothesize that MCO could promote α-Syn compaction through intramolecular diTyr formation. To investigate this, we used native ion mobility mass spectrometry (IM-MS) (Figure 3A). Briefly, from the time it takes ions to traverse a gas-filled drift cell, we can calculate their collision cross sections (CCSs), giving information on their conformational preferences. Analysis of unmodified α-Syn revealed a CCS distribution centered around 2400 Å² for all major charge states (Figure 3B). Five minutes of MCO did not notably increase dimers (cf. Figure 1) but shifted the CCSs of the monomers toward a compact state with CCS ≈ 1900 Å² for lower charge states (Figure 3B), consistent with our extended-to-ring-transformation hypothesis. A direct correlation between molecular weight and CCS is observed (Figure S6). To investigate the conformational stability of different α-Syn populations, we employed collision-induced unfolding (CIU). Here, the protein ions are subjected to increasing collisional activation in the ion trap of the mass spectrometer. The resulting change in CCS informs about the conformational stability of the ion. Interestingly, oxidized α-Syn showed no significant increase in CCS as the collisional activation was increased from 5 to 50 V, at which protein fragmentation (not unfolding) occurred (Figure 3C). The high resistance of the compact states to unfolding indicates covalent stabilization rather than altered non-covalent interactions in the oxidized monomer.
To corroborate that the compaction stems from intramolecular cross-links, we performed IM-MS of the Tyr39Phe single mutant and the Tyr125/133/136Phe triple mutant. To exclude effects from altered solution conformations in response to the Tyr-to-Phe mutations, we performed IM-MS under denaturing conditions using 50% acetonitrile with 0.1% formic acid (Figure S7). Under these conditions, all variants exhibited the same extended conformation as native wt monomer (Figure 5, black line). Following MCO, the denatured wt monomer underwent the same extended-to-compact shift as seen under native conditions. However, the oxidized Tyr39Phe mutant showed two populations with similar intensities, one more compact and the other extended. We speculate that, in this variant, the central His50 could be linked to a C-terminal Tyr, resulting in a smaller compact population. His50’s role as a Cu

Table 1. Results from SAXS Analysis

|                | \(c\) [mg/mL] | \(R_g\) (IFT) [nm] | \(c\) (model) [mg/mL] | \(R_g\) (model) [nm] | \(b\) (model) [nm] | \(\chi^2\) (model) |
|----------------|---------------|---------------------|-----------------------|----------------------|-------------------|-----------------|
| monomer        | 4.0           | 3.96 ± 0.02         | 4.00 ± 0.03           | 3.80                 | 1.71 ± 0.02       | 1.0             |
| oxidized monomer| 2.2           | 2.69 ± 0.02         | 2.55 ± 0.02           | 2.95                 | 1.96 ± 0.03       | 1.1             |

\(c\), concentration measured by absorbance; \(R_g\) (IFT), radius of gyration from IFT; \(c\) (model), concentration determined from the model fits (linear chain model for native monomer and ring model for oxidized monomer); \(R_g\) (model), radius of gyration of the two models determined numerically from the low-q range of the model curves; \(b\), Kuhn length; \(\chi^2\), reduced weighted chi-square.

Figure 3. IM-MS analysis of oxidized \(\alpha\)-Syn. (A) Spectrum of 5 min oxidized wt \(\alpha\)-Syn and ion mobiligrams showing +11 to +8 charge states. (B) Overlay of CCSs of wt untreated \(\alpha\)-Syn (red) and oxidized wt \(\alpha\)-Syn (black). (C) CIU shown from 5 to 45 V for the same charge states as given in the CCS plots (B).
In conclusion, we show that MCO associated with PD can induce long-range intramolecular diTyr cross-links which induce a compact, yet disordered, α-Syn monomer species. Steric hindrance from the diTyr linkage prevents aggregation of the monomer through β-sheet formation. Interference with the extended conformation of α-Syn opens up new interpretations of α-Syn function and pathology, as well as strategies to prevent α-Syn aggregation and ultimately treat PD.

ASSOCIATED CONTENT

* Supporting Information
The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/jacs.2c03607.

Additional discussion, experimental data, and materials and methods (PDF)

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Figure 4. MCO inhibits amyloid formation. (A) ThT fluorescence was measured for α-Syn oxidized for 0, 0.1, 1, 5, 30, and 60 min prior to aggregation. (B) After the ThT signal had plateaued, the secondary structure was analyzed by FTIR, where the second derivative is shown for all time points. (C) CD spectra of untreated and 60 min oxidized monomer, and after reaching plateau in the ThT assay. (D) TEM images of untreated and oxidized α-Syn samples from the ThT assay. Scale bar: 200 nm.
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Notes
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ABBREVIATIONS
α-Syn, α-synuclein; ATD, arrival time distribution; CIU, collision-induced unfolding; CCS, collision cross section; CD, circular dichromism; FTIR, Fourier transformed infrared spectroscopy; IM-MS, ion mobility mass spectrometry; MS, mass spectrometry; MCO, metal-catalyzed oxidation; PD, Parkinson’s disease; Rg, radius of gyration; SAXS, small-angle X-ray scattering; THT, thioflavin T
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