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At Low Concentrations, 3,4-Dihydroxyphenylacetic acid (DOPAC) Binds Non-covalently to α -Synuclein and Prevents Its Fibrillation

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Abstract

Several studies have shown that catecholamines can inhibit the fibrillation of α -synuclein, a small presynaptic protein whose aggregation is believed to be a critical step in the etiology of Parkinson's disease and several other neurodegenerative disorders. However, the mechanism of this inhibition is uncertain. We show here that substoichiometric concentrations of DOPAC (3, 4-dihydroxyphenylacetic acid), a normal product of the metabolism of dopamine, can inhibit the fibrillation of α -synuclein (α -Syn), due to non-covalent binding of DOPAC to α -Syn monomer. Intriguingly, the presence of α -synuclein accelerates the spontaneous oxidation of DOPAC, and the oxidized form of DOPAC (the quinone) is responsible for the fibrillation inhibition. In addition, the presence of DOPAC leads to the oxidation of the methionine residues of α -Syn, probably due to the H_2O_2 production as a by-product of DOPAC oxidation. The lack of fibrillation results from the formation of stable oligomers, which are very similar to those observed transiently at early of the α -Syn fibrillation. A possible explanation for this phenomenon is that DOPAC stabilizes the normally transient oligomers and prevents them from subsequent fibril formation. The analysis of the α -synuclein Y39W variant suggests that DOPAC binds non-covalently to the same N-terminal region of α -Syn as lipid vesicles, probably in the vicinity of residue 39. In contrast to the compounds with 1,2-dihydroxyphenyl groups (DOPAC, catechol), their 1,4-dihydroxyphenyl isomers (hydroquinone, homogentisic acid) are able to modify α -Syn covalently, probably due to the less steric hindrance in the Michael addition.

Keywords

α -synuclein; DOPAC; DOPAC-stabilized oligomer; amyloid fibril; oxidative modification; methionine oxidation; dopamine

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Footnote: Prof. Anthony L. Fink has passed away on March 2, 2008

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The abnormal movements associated with Parkinson's disease (PD) result from the death of the specific dopaminergic neurons in the *substantia nigra*. The oxidative instability of dopamine and its several metabolites create potentially greater predisposition for oxidative stress in the dopaminergic neurons, which is assumed to be associated with the particular vulnerability of these neurons. Several investigations have shown that catecholamines can inhibit the fibrillation of α -synuclein, a protein whose aggregation is believed to be a critical step in the etiology of PD and several related neurodegenerative diseases.

The diagnostic hallmark of PD is the presence of intracellular fibrillar inclusions, Lewy bodies and Lewy neuritis, in the surviving dopaminergic neurons¹⁻³. α -Synuclein (α -Syn), a 140-residue presynaptic protein of unknown function, is the primary component of these fibrillar inclusions³. *In vitro*, far-UV CD, Fourier transform IR, fluorescence and NMR spectroscopies, as well as SAXS and several hydrodynamic technique show that α -Syn is intrinsically disordered⁴⁻⁶. Intrinsic disorder refers to the lack of a fixed structure in proteins and many biologically active proteins were shown to remain unstructured, or incompletely structured, under physiological conditions⁷⁻³⁴. Intrinsic disorder has been reported both at a region as well as a whole protein level. There are several crucial differences between amino acid sequences of intrinsically disordered proteins and regions and structured globular proteins and domains^{9;24;35}. Intrinsically disordered proteins are highly abundant in nature and the overall amount of disorder in proteins increases from bacteria to archaea to eukaryota³⁶⁻³⁹. These proteins carry out numerous biological functions, many of which obviously rely on high flexibility and lack of stable structure. These functions are diverse and complement those of ordered proteins and protein regions²⁷⁻²⁹. Many intrinsically disordered proteins are associated with human diseases such as cancer⁴⁰, cardiovascular disease⁴¹, amyloidoses⁴², neurodegenerative diseases, diabetes and others³⁴. Based on these intriguing links among intrinsic disorder, cell signaling and human diseases, suggesting that protein conformational diseases may result not only from protein misfolding, but also from misidentification and missignaling, the “disorder in disorders” or D² concept was recently introduced³⁴.

α -Syn assembles into filaments and fibrils after incubation under physiological conditions *in vitro*. Morphologies of these filaments and fibrils are similar to those extracted from the diseased brain⁴³⁻⁴⁶. A variety of factors, such as low pH, high temperature, naturally occurring polyamines, and environmental PD risk factors (including heavy metals, pesticides and herbicides), accelerate fibrillation of α -Syn *in vitro*^{5;47-51}. Substantial evidence indicates that α -Syn aggregation is a critical step in the etiology of Parkinson's disease^{51;52}, as well as in a number of other neurodegenerative diseases, collectively known as synucleinopathies⁵¹. However, the question on whether the mature fibrils, protofilaments, protofibrils, specific oligomers or some folding intermediates are the neurotoxic species responsible for the cell death in these diseases is still a subject of great controversy⁵³⁻⁵⁸. Oligomers of α -Syn with globular, annular or chain-like conformations have been observed^{59;60}. Some of these oligomers were suggested to form pores that can permeabilize membranes⁶⁰⁻⁶⁴. Since it is primarily dopaminergic neurons that appear to be affected in PD, there has been considerable speculation regarding the role of dopamine and its metabolites in the disease.

Although the normal function of α -Syn is still unknown this protein is believed to be involved in regulation of the dopamine neurotransmission via effects on vesicular dopamine storage and trafficking⁶⁵⁻⁶⁹. Interestingly, dopamine and related compounds that have vicinal dihydroxy groups were shown to be the effective inhibitors of the α -Syn fibrillation⁷⁰⁻⁷⁴. It was proposed that α -Syn is able to form α -Syn-dopamine-quinone (DAQ) adducts with oxidized dopamine, and that these adducts block the α -Syn fibrillation and stabilize the potentially most toxic α -Syn oligomers or protofibrils⁷⁰. However, the mechanism of fibrillation inhibition by α -Syn-

DAQ adduct remains uncertain because the yield of adducts is very low as observed by the lack of characteristic signals from the dopamine adducts detectable by mass spectrometry ⁷⁰.

A better understanding of the mechanism of inhibition of α -Syn fibrillation by catechols is of particular interest for a number of reasons, including an attempt to understand whether the resulting oligomers are toxic ⁷⁵. It has been shown that the neurotoxicity of α -Syn is dopamine-dependent ⁷⁶ and that α -Syn also facilitates the toxicity of oxidized catechol metabolites ⁷⁷. SDS-PAGE analysis of α -Syn incubated with dopamine and other catechol compounds revealed a ladder of SDS-stable oligomers that suggested covalently cross-linked species. However, these effects were at concentrations of catechol compounds (0.18~2 mM) far greater than those occurring *in vivo* ⁷⁸. Recently it has been shown that intracellular catechols such as DA and DOPAC have the ability to modulate α -Syn aggregation in cultured human cells ⁷⁹. In particular, an increase in cytosolic catechol levels was associated with a decrease in α -Syn-containing inclusions, possibly through the formation of catechol-induced oligomeric intermediates.

Here, we take advantage of the good water solubility of DOPAC (3, 4-dihydroxyphenylacetic acid), a normal product of the dopamine metabolism, to show that: (a) lower concentrations of DOPAC than those used in previous studies are sufficient to inhibit fibrillation of α -Syn; (b) DOPAC binds to α -Syn non-covalently at low concentration but the covalent modifications of α -Syn occur at higher concentrations; (c) DOPAC can oxidize methionine groups of α -Syn; (d) in the absence of DOPAC, the formation of transient oligomers preceded the α -Syn fibrillation. Although in the presence of low DOPAC concentrations, the α -Syn oligomers were also formed, these oligomers did not assemble into fibrils.

Results

Unless otherwise noted, the conditions for α -synuclein incubation were 20 mM phosphate, pH 7.4, 100 mM NaCl, 37°C, with 70 μ M α -Syn and agitation. Under these conditions, α -Syn formed fibrils with a lag phase of approx. 20 h, and fibrillation was complete by 35-40 h; the fibrillation kinetics were very sensitive to small changes in the rate of agitation.

Oxidation of DOPAC

Solutions of DOPAC at physiological pH and 37°C were spontaneously oxidized by dissolved oxygen to form DOPAC-quinone and other oxidized compounds. We will refer to this mixture as DOPAC-Q. This oxidation was accompanied by a color change and induction of fluorescence (excitation λ_{max} 360 nm; emission λ_{max} around 460 nm, Figure 1, ⁷⁰). Sodium azide is frequently added to α -synuclein solutions to be incubated for long time periods in order to prevent the microbial contamination of the sample. Monitoring the fluorescence changes from oxidation of DOPAC during incubation showed that NaN_3 increased the intensity of fluorescence from DOPAC-Q, indicating that NaN_3 might accelerate the DOPAC oxidation. This could be explained as follows: since azide is a free radical scavenger, it could react with free radical products of DOPAC oxidation and thus could enhance the oxidation by mass action effects. Interestingly, the presence of 70 μ M α -Syn greatly enhanced the fluorescence signal of DOPAC compared to 5 mM NaN_3 (Figure 1). Although the fluorescence could come from formation of di-tyrosine cross-linked α -synuclein or an α -synuclein-DOPAC complex, incubation of DOPAC with a Tyr-minus mutant (the four tyrosines substituted with phenylalanines) of α -Syn showed no significant decrease in fluorescence compared to the wild-type α -Syn. This clearly indicated that the Tyr residues of α -Syn are not involved in the fluorescent species. Furthermore, incubation of α -Syn alone did not show this fluorescence signal. Therefore, these observations implied that α -Syn itself accelerated the oxidation of DOPAC or alternately that binding of oxidized DOPAC to α -Syn increased the fluorescence intensity of DOPAC-Q.

DOPAC inhibits fibrillation of α -synuclein at micromolar concentrations

The effects of different concentrations of DOPAC on α -Syn fibrillation were analyzed at 37°C, pH 7.4 using the ThT fluorescence assay. DOPAC showed significant inhibitory effects on α -Syn fibrillation and the extent of inhibition was DOPAC concentration-dependent (Figure 2A). DOPAC, even at concentration of only one tenth of that of α -Syn, increased the elongation time and decreased the ThT fluorescence signal, which was roughly proportional to the amount of fibrils formed. The data indicated a stoichiometric inhibitory effect. At concentrations exceeding 1:1 molar ratio over α -Syn, DOPAC completely inhibited α -Syn fibrillation evidenced by ThT signal, EM image (which showed mostly oligomers) and nearly all α -Syn were soluble and could not be pelleted by centrifugation (Figure 2B) (α -Syn fibrils are insoluble and can be pelleted). Small amount of short fibrils and protofibrils in a form of small, thin, curvy fibrils, were also observed by EM image (see Figure 2Bb'). However, the amount of such fibrils and protofibrils was very small and they were not abundantly present in analyzed EM fields. These fibrils might originate from a subset of α -Syn molecules aggregating prior their interaction with DOPAC. Furthermore, no such fibrils were detected by AFM image (see below, Figure 3B), suggesting either these fibrils are not stable and dissociate due to the dilution during the AFM samples preparation or the chance to observe these aggregated species by AFM is small. The inhibitory effects of DOPAC on α -Syn fibrillation were greatly decreased in the presence of sodium thiosulfate ($\text{Na}_2\text{S}_2\text{O}_3$), a reducing reagent. Freshly prepared DOPAC (in the presence of thiosulfate) showed little inhibition whereas pre-oxidized DOPAC still showed significant inhibition of α -Syn fibrillation even when $\text{Na}_2\text{S}_2\text{O}_3$ was added to the solutions at the beginning of incubation (Figure 2C). Since $\text{Na}_2\text{S}_2\text{O}_3$ does not reduce oxidized DOPAC, these observations indicate that it is the oxidized form of DOPAC that is responsible for the fibrillation inhibition. The possibility of α -Syn methionine oxidation by non-oxidized DOPAC (at low concentrations) was excluded by the failure to detect a change in the mass of α -Syn by MS.

DOPAC stabilizes transient oligomers of α -Syn

The fact that DOPAC (100 μM) completely inhibited fibrillation of α -Syn (70 μM) was supported by both the low ThT signals and EM images (Figure 2B). To better characterize the time-course of changes in the heights of these DOPAC-stabilized oligomers, AFM was used to study α -Syn incubated alone or with DOPAC. For α -Syn alone, AFM showed that oligomers (~5 nm high) built up during the first ~ 19 hours followed by fibrils (~7 nm high) at ~48 hours and the final mature fibrils (~ 10 nm high) (Figure 3A). In the presence of DOPAC, increasingly larger oligomers formed (~5 nm high at 20 hrs; ~7 nm high at 44 hr and ~10 nm at 72 hrs), and no fibrils were observed (Figure 3B). The heights of these DOPAC-induced α -Syn oligomers were comparable to heights of the oligomers or fibrils formed during the incubation of α -Syn alone at similar incubation time points. It appears that these oligomers were stabilized by DOPAC in such a way that they cannot enter the fibrillation pathway.

AFM data showed that the oligomers were built up during the first 20 hours of incubation with agitation (i.e., during the lag time) for both α -Syn incubated alone and in the presence of DOPAC. These oligomers were soluble and unlike fibrils were not pelletable by the centrifugation. Importantly, these oligomers dissociated easily during the gel-filtration experiments, as the SEC HPLC profiles contained predominantly the α -Syn monomeric peak, although small amount of oligomeric peak was also detected when incubating α -Syn with DOPAC (Figure 4). This suggests that oligomers were not stable and dissociated to monomers on SEC HPLC due to the sample dilution. The decrease of the monomeric peak intensity during incubation of α -Syn alone indicated the formation of insoluble fibrils, while the increase and the shift to right of the monomeric α -Syn peak after incubation with DOPAC suggested that DOPAC was bound to α -Syn and that these DOPAC-bound α -Syn species were slightly more compact than the non-incubated protein.

Taken together these data indicated that oligomers formed either during the incubation of α -Syn alone or in the presence of DOPAC were structurally similar. In fact, they shared similarity in morphology (heights) and in the stability on SEC HPLC. However, in contrast to the oligomers accumulated during the incubation of α -Syn alone, the oligomers “stabilized” by DOPAC did not transform into the fibrils.

At low concentrations, DOPAC binds to α -Syn non-covalently

Without agitation, α -Syn does not form fibrils being incubated for several weeks. To test whether DOPAC binds to monomeric α -Syn, SEC FPLC was used to analyze the mixture of α -Syn (70 μ M) with DOPAC (100 μ M) during incubation without agitation (Figure 5). The area of the monomeric α -Syn peak increased and area of the DOPAC peak decreased with time. This suggested that DOPAC was bound to α -Syn slowly in a course of incubation. Finally, after the prolonged incubation, the DOPAC peak disappeared and there was a large increase in the α -Syn monomer peak. This indicated that most of the DOPAC was bound tightly to α -Syn, although a small amount of polymerized DOPAC was also observed at later stages. Reactive groups of α -Syn, such as Lys, Tyr and the N-terminal alpha-amino groups have been suggested to react with DOPAC-Q⁷⁰. However, no mass changes of α -Syn were observed from ESI-MS even when a 3 or 4-fold excess of DOPAC was used (see below, Figure 8). The possibility that a Schiff base, which is sensitive to acid hydrolysis, formed between α -Syn and DOPAC-Q is unlikely because the treatment of the incubated α -Syn and DOPAC solution with even 100-fold excess of NaBH₄, which reduces Schiff bases, did not yield any new MS peaks corresponding to α -Syn-DOPAC-Q complex. Also, there was no obvious increase in the α -Syn peak area on reverse phase HPLC (data not shown), while SEC showed a big increase in α -Syn monomer peak area (Figure 5). These observations suggested that DOPAC at low concentration was bound to α -Syn non-covalently. This was also supported by the failure to detect crosslinked α -Syn on SDS-PAGE or by MS. Furthermore, no α -Syn-DOPAC-Q bands were observed after NBT red-ox staining of the SDS-PAGE gels at low concentrations of DOPAC (see below Figure 7). The DOPAC-bound monomeric α -Syn was separated by SEC FPLC, concentrated and incubated under normal fibrillation conditions. No fibrils were detected by ThT fluorescence assay and EM. This meant that the non-covalent binding of DOPAC to α -Syn monomer was sufficient for the complete inhibition of the α -Syn fibrillation.

What is the binding site for DOPAC on α -Syn?

It is interesting to know where DOPAC (at low concentrations) binds non-covalently to α -Syn since the physiological concentration of dopamine-like chemicals is quite low ($\leq$ nano-molar range). Taking the advantage that the WT α -Syn has no Trp residue, three mutants of α -syn with Tyr to Trp substitutions at positions 39, 125, 136 were created and incubated with equivalent molar ratios of DOPAC. The idea here was that if DOPAC-Q (excitation $\lambda_{\text{max}} = 360$ nm) binds to α -Syn at a position close to Trp (excitation $\lambda_{\text{max}} = 290$ nm, emission $\lambda_{\text{max}} \sim 350$ nm), it might quench the fluorescence of Trp by fluorescence resonance energy transfer (FRET). Y39W α -Syn showed a much faster decrease in Trp fluorescence than the other two mutants (Figure 6A). Unfortunately, no fluorescence of DOPAC from FRET was detected when solutions were excited at 290 nm. This could be due to the low intensity of DOPAC-Q fluorescence (only 2~5 % of Trp fluorescence intensity). It is also possible that DOPAC-Q quenches Trp fluorescence through direct interactions rather than through FRET. Monitoring the time-dependence of DOPAC-Q fluorescence showed that WT α -Syn and its Trp mutants had similar effects on DOPAC oxidation (data not shown). This means that fast Trp fluorescence decrease of Y39W in the presence of DOPAC was not due to the fast oxidation of DOPAC.

The Trp fluorescence of the Y39W, Y125W, and Y136W mutants showed that the environment of Trp in Y39W was more hydrophobic (emission $\lambda_{\text{max}} = 350$ nm) than the Trp environments

in Y125W and Y136W mutants (emission $\lambda_{\text{max}} = 357$ nm). We propose that DOPAC-Q binds to the hydrophobic region of α -Syn. There was no obvious Trp fluorescence peak shift after incubating the Y125W or Y136W mutants with DOPAC, and there was a small (2~3 nm) red shift for Y39W. This implied that the residues at positions 39, 125, 136 were still, or even more, solvent exposed in the transient α -Syn oligomers (Figure 6B). In contrast, the Trp emission λ_{max} was blue-shifted ~7 nm (from 350 to 342 nm) after fibrillation of the Y39W mutant while no peak shifts were observed for Y125W and Y136W α -Syn fibrils (Figure 6C). These observations suggest that the residue 39 is buried inside the fibrils but is still solvent exposed in the DOPAC-stabilized oligomers.

Covalent modifications of α -Syn by DOPAC occur at high DOPAC concentrations

At higher concentrations of DOPAC (1 mM), dimers, trimers and higher order oligomers of α -Syn were observed on SDS-PAGE. NBT red-ox stain (which only detects quinone modified protein) showed that all monomeric and oligomeric α -Syn were associated with DOPAC quinones and most α -Syn was still in its monomeric form (Figure 7). The smear above the monomeric α -Syn band may indicate that the polymerized DOPAC-Q bind to α -Syn monomer. This smear was most noticeable when higher concentrations of DOPAC (5 mM) were used. Interestingly, for dopamine, SDS gel electrophoresis analysis after NBT staining showed only dopamine-Q-associated oligomeric α -Syn and the extended α -Syn monomer bands, but no dopamine-Q associated α -Syn monomer band. Therefore, it appears that dopamine-Q preferentially modifies oligomeric α -Syn and only polymerized dopamine quinones can modify monomeric α -Syn. This could be due to faster polymerization of dopamine than DOPAC. The cross-links between α -Syn and DOPAC or dopamine quinones might occur after oligomerization of α -Syn, which could result in the side-chains involved in cross-linking being brought together.

Regio-isomers of DOPAC and homogentisic acid or catechol and hydroquinone react with α -Syn differently

It is interesting that there were no covalent modifications of α -Syn induced by low concentrations of DOPAC or dopamine. It has been reported that hydroquinone (HQ) but not catechol modified α -Syn by Michael addition⁷⁵. This observation suggested that compounds with the 1,2-dihydroxyphenyl groups did not covalently modify α -Syn, whereas their para-isomers; i.e. 1,4-dihydroxyphenyl derivatives, can modify α -Syn covalently. We chose catechol and DOPAC (Figure 8) as representatives of the former, and HQ and homogentisic acid (HGA) as representatives of the latter. All four compounds showed substantial inhibition of α -Syn fibrillation by ThT assay (data not shown).

These four compounds were incubated with α -Syn at 3:1 molar ratio. Based on ESI MS analysis, catechol and DOPAC did not form covalent α -Syn adducts, although a small amount of methionine oxidized α -Syn was formed after the prolonged incubation in the presence of NaN_3 . However, covalent modifications of α -Syn, through Michael additions of the quinones, were observed with HQ and HGA by ESI-MS already after one day of incubation, and longer times of incubation gave more covalent α -Syn adducts (Figure 8). Methionine oxidation of α -Syn was also seen by MS after the incubation for two days. This implies that, if covalent modification of α -Syn occurs by catechol-related quinones, it occurs before methionine oxidation of α -Syn. The Michael addition of HGA (mass 168) or HQ (mass 110) quinones to α -Syn, resulted in mass changes of 162 or 104 daltons respectively on α -Syn, which correspond to the adducts with mass (chemical + α -Syn – 6) daltons. The mass loss of 6 daltons comes from the three times of oxidation of the phenols to the quinones after Michael addition of two amino groups from α -Syn to one quinone molecule⁷⁵. Interestingly, no mass loss of 4 daltons, corresponding to one amino group adding to one quinone, was detected by ESI-MS. This

indicated that the addition of two amino groups of α -Syn to the quinones was a rather cooperative reaction.

For HGA and HQ, amino groups from α -Syn can be added to the quinones from opposite sides of the phenyl ring. But for DOPAC and catechol, amino groups have to attack quinones from the same side on two adjacent carbon atoms, which suggests an unfavorable steric effect. This could be the reason why no Michael addition occurred for DOPAC and catechol. For HGA and HQ, which did modified α -Syn through Michael addition, at a 1:1 molar ratio, only small amounts of α -Syn were modified (only mono-adduct) after many days of incubation and no modification occurred for the first 2 days (data not shown). However, at a 3:1 ratio, substantial amounts of mono-adduct, and some di-adduct were observed on ESI-MS after only one day of incubation. Even after incubation for many days, tri-adducts were never observed. This indicated that some α -Syn sites were involved in the non-covalent binding of HGA and HQ. These α -Syn sites were likely incompatible with the Michael addition taking place.

DOPAC can oxidize the methionine groups of α -Syn

Dopaminergic neurons are likely to be subjected to higher levels of ROS than most cells due to the oxidative instability of dopamine. Previous studies have shown that the four methionine groups of α -Syn are vulnerable to oxidation by H_2O_2 leading to formation of soluble oligomers and inhibition of fibrillation⁸⁰⁻⁸³. When 70 μM α -Syn was incubated with 100 μM or higher concentration of DOPAC for 3 days at 37°C, ESI MS showed that α -Syn was not oxidized, although DOPAC underwent oxidation. However, under the same conditions but in the presence of 5 mM NaN_3 the methionine groups of α -Syn were oxidized by DOPAC (data not shown). The excess of methionine oxidation depended on DOPAC concentration: the more DOPAC added, the greater the level of α -Syn oxidation observed. In contrast, the addition of higher concentrations of NaN_3 (e. g. 100 mM), caused no more methionine oxidation of α -Syn than oxidation induced by 5 mM NaN_3 . Co-incubation of α -Syn (70 μM) with 5 mM NaN_3 did not lead to the methionine oxidation. The possible catalytic effects of trace amounts of iron from the buffer were excluded by the addition of the chelator DETAPAC (200 μM) during incubation. Furthermore, the methionine oxidation of α -Syn by DOPAC was inhibited by the reducing reagent $\text{Na}_2\text{S}_2\text{O}_3$. This implied that DOPAC was responsible for the methionine oxidation, and NaN_3 acted as an activator, probably by interacting with certain ROS from the oxidation of DOPAC and leading to oxidation pathways that generate more peroxide.

Since methionine oxidation is also known to inhibit α -Syn fibrillation⁸⁰, it was not clear what was the main cause of the α -Syn fibrillation inhibition when DOPAC was present, DOPAC binding or methionine oxidation. ESI-MS was used to monitor methionine oxidation of α -Syn (70 μM) by DOPAC (100 μM) under the fibrillation condition. The noticeable methionine oxidation of α -Syn by DOPAC was only detected after at least 30 hours of incubation (a time point where some fibrils were already formed for intact α -Syn). More specifically, ESI-MS revealed that 1 or 2 Met residues per α -Syn were oxidized under these conditions whereas there are four oxidizable methionine residues in α -Syn. Figure 5 shows that more than 60% DOPAC is bound to α -Syn during the same time period. Furthermore, partial methionine oxidation of α -Syn (i.e., oxidation of only two Met residues) did not completely inhibited α -Syn fibrillation⁸³. This suggested the binding of DOPAC to α -Syn preceded methionine oxidation of α -S and that this binding dominated in the inhibition of α -Syn fibrillation.

Discussion

Our data showed that even substoichiometric concentrations of DOPAC can inhibit the fibrillation of α -Syn, and that the non-covalent binding of DOPAC to α -Syn monomer was sufficient to inhibit the fibrillation process. Determining the molecular mechanism of DOPAC-induced inhibition of α -synuclein fibrillation was complicated by the oxidative instability of

the compound. The results of the experiments using sodium thiosulfate which prevented the oxidation of DOPAC but did not reduce the oxidized forms indicated that the oxidized form of DOPAC (the quinone) was mostly responsible for the inhibition. The presence of α -synuclein accelerated the spontaneous oxidation of DOPAC, possibly due to the presence of trace metal ions associated with α -synuclein⁸⁴, or due to interactions with potentially catalytic side chains of the protein in the DOPAC- α -synuclein complex. We also found that the presence of DOPAC led to the oxidation of the methionine residues in α -synuclein. This probably reflects the production of H₂O₂ as a by-product of DOPAC oxidation, since peroxide is well known to oxidize the methionines of α -synuclein to the sulfoxide.

At concentrations of DOPAC equivalent to, or less than that of α -synuclein, DOPAC bound to the protein in a non-covalent manner. Various observations demonstrated that the non-covalent interaction of DOPAC with α -Syn induced the formation of α -Syn oligomers, which did not form fibrils.

Non-covalently bound DOPAC appeared to bind to the same N-terminal region of α -synuclein as lipid vesicles, probably at a location close to residue 39, based on the analysis of the Y39W variant of α -synuclein. It is interesting to note that AFM imaging indicated that the oligomers induced by non-covalently bound DOPAC were very similar to those observed transiently during the incubation of α -Syn alone and their heights were similar to those of fibrils. One possibility was that the oxidized DOPAC stabilized the normally transient oligomers and prevented them from entering the fibril formation pathway, perhaps by altering or blocking the oligomer interface involved in fibrillation. Regardless of the exact mechanism, it was clear that the oxidized DOPAC stabilized species, oligomers or protofibrils, prior to fibril formation.

In contrast, at higher DOPAC concentrations ($\geq 3:1$ stoichiometry) α -Syn was covalently cross-linked by DOPAC quinones, and Michael addition products were formed. Interestingly, formation of these species involved dimerization, indicating that two Michael additions occurred. This suggested that appropriately located Lys side-chains of two adjacent α -synuclein molecules in an oligomer were optimally oriented for such a reaction. The possible products of the α -Syn interaction with DOPAC are summarized in Figure 9.

It is notable that there were no covalent modifications of α -Syn by low concentrations of DOPAC or dopamine quinones, but it has been reported that the structurally related compound, hydroquinone, modified α -Syn by Michael addition⁷⁵. We confirmed that HQ did indeed form covalent adducts via Michael addition when present at low concentrations, and showed that HGA, which also has a 1,4-dihydroxyphenyl group, also formed such adducts, whereas catechol, with its 1,2-dihydroxyphenyl structure, did not. These observations indicated that the outcome was determined by whether the polyphenol had 1,2- or 1,4-dihydroxy (or quinone) groups. Here, compounds with 1,2-dihydroxyphenyl groups did not covalently modify α -Syn, whereas their 1,4-dihydroxyphenyl isomers made covalent modifications of α -Syn. We attributed this difference to a steric hindrance effect for Michael addition to the quinones from the 1,2-dihydroxyphenyl compounds. Since both the 1,2- and 1,4-dihydroxyphenyl compounds examined were strong inhibitors of α -synuclein fibrillation it appeared that the covalent modification was not directly related to the prevention of fibril formation.

With HQ and HGA, the MS data indicated that the Michael addition occurred between the two nucleophilic groups of α -Syn (presumably lysine side-chains) and only one HGA or HQ molecule. This observation, along with the stoichiometry of the reaction, suggested that the dihydroxyphenyl compounds bound at a specific site at the interface of two α -synuclein molecules in an oligomer.

DOPAC, HGA, catechol and HQ were able to oxidize the methionine groups of α -Syn to the methionine sulfoxide in the presence of sodium azide. This suggested that more hydrogen

peroxide was produced during oxidation of these chemicals in the presence of azide, presumably due to its radical scavenging activity leading to a shift in the distribution of oxidation products.

In summary, at physiologically relevant concentrations, DOPAC binds non-covalently to α -Syn and results in the formation of specific α -Syn oligomers that do not form fibrils. Spontaneous oxidation of DOPAC leads to the formation of the quinone, which at high concentrations, can result in covalent modification of α -synuclein via a Michael addition.

Materials and Methods

Materials

Recombinant human α -Syn and its mutants Y39W, Y125W, Y136W were expressed in *Escherichia coli* and purified as described previously⁸⁵. Lyophilized protein was freshly prepared before use by dissolving protein in 2 mM phosphate buffer at pH 7.4 and centrifuged at 95000 rpm with a Beckmann Airfuge. 3, 4-dihydroxyphenylacetic acid (DOPAC), dopamine, catechol, hydroquinone, homogentisic acid (HGA) and diethylenetriaminepentaacetic acid (DETAPAC) were obtained from Aldrich Chem. Co. All other chemicals were analytical grade and purchased from Fisher chemicals or EM science.

Methionine oxidation of α -Syn by DOPAC

α -Syn (70 μ M) was incubated with 100 μ M or higher concentration of DOPAC at 37°C in 20 mM phosphate buffer pH 7.4 with or without 5 mM or 100 mM NaN_3 for 1-3 days. To prevent methionine oxidation of α -Syn, 5 mM $\text{Na}_2\text{S}_2\text{O}_3$ was added to the above solutions. 200 μ M DETAPAC was used to remove trace amounts of metal ion in buffer solutions. The methionine oxidation of α -Syn was detected by ESI-MS.

Covalent modification of α -Syn by Catechol, DOPAC, Hydroquinone and Homogentisic acid

70 μ M α -Syn was incubated with these compounds at the indicated molar ratios at 37°C, pH 7.4 for 1-3 days. Modifications were detected by ESI-MS and SDS-PAGE.

Oxidation of DOPAC by air

100 μ M DOPAC was incubated in 20 mM phosphate buffer (pH 7.4) with or without 5 mM NaN_3 at 37°C for 1-3 days. In some experiments, 100 mM NaN_3 was used, as indicated in the text. "Complete" and "incomplete" pre-oxidized DOPAC were obtained by incubating 10 mM DOPAC for periods of several months or by incubating 200 μ M DOPAC for 2 weeks with 5 mM NaN_3 at 37°C. The oxidation of DOPAC was confirmed by color change from colorless to brown or dark red depending on the extent of oxidation and increase in fluorescence (excitation: 360 nm; emission: 400-520 nm)⁷⁰. Oxidized DOPAC was a mixture of the quinone and many other compounds and the total mixture was named DOPAC-Q herein.

Fibril formation and ThT assay

Fibril formation was monitored by ThT (thioflavine T) fluorescence using a Fluoroskan Ascent CF plate reader (Labsystems, Inc.). Protein solutions with 70 μ M α -Syn and 20 μ M ThT were incubated in 100 mM NaCl and 20 mM phosphate buffer (pH 7.4) in a 96-well plate, each well containing a 1/8 inch diameter Teflon spherical bead. The plate was incubated at 37°C with shaking at 120 rpm, 20 mm diameter or 600 rpm, 2 mm diameter. The fluorescence was measured at 30 min intervals with excitation at 450 nm and emission at 485 nm. Data from six wells were averaged and plotted as a function of time. Lag times were calculated based on the equation described previously⁸⁶.

Size-exclusion HPLC and FPLC measurements

Aliquots of solutions were removed during incubation and centrifuged for 20 min at 14000 rpm to remove any insoluble materials before running SEC chromatography. For HPLC, 15 μ L of incubated solutions were injected on a TSK-GEL G2000SW_{XL} size-exclusion column (7.8 mm ID $\times$ 30 cm) at a flow rate of 0.5 mL/min and for FPLC, 300 μ L of incubated solutions were injected on a SUPERDEXTM 200 size-exclusion column at a flow rate of 2.0 mL/min in 10 mM phosphate buffer (pH 7.0) and 100 mM Na₂SO₄. HPLC was performed using a Waters 2695 separations module with a Waters 996 Photodiode Array (PDA) detector, and data were collected and analyzed by Millennium software. Absorbance of the eluant was monitored over the wavelength range from 220 to 450 nm with bandwidth of 1.2 nm. For FPLC (AKTA instrument), spectra were monitored at 280 nm.

SDS-PAGE gel electrophoresis measurements

Samples for sodium dodecyl sulfate PAGE (SDS-PAGE) gel electrophoresis were boiled in a water bath for 5 to 10 min in 4% SDS prior to electrophoretic separation. A PhastSystem with 8-25% gradient gels (Amersham Biosciences) was used and experiments were run with SDS buffer strips containing 0.20 M Tricine, 0.20 M Tris and 0.5% SDS, pH 7.5 for 30 min at 250 V. Gels were stained with Coomassie blue. For NBT redox cycling stain, SDS-PAGE was run on a 15% polyacrylamide gel, proteins was transferred to nitrocellulose membrane and stained with 0.24 mM NBT (NitroBlue Tetrazolium chloride) in 2 M potassium glycinate at pH 10 in the dark for 1 hour. The same membrane was stained with Ponceau S for all proteins after NBT staining⁸⁷.

Mass spectrometry

Samples for MS analysis were desalted with C8 RP column and eluted with 4:1 acetonitrile:H₂O (pH adjusted to 2.0 by TFA) solution. The desalted solution was directly loaded onto the mass spectrometer. Mass spectra were obtained using a MicroMass Quattro II electrospray mass spectrometer operating in positive ion mode. Injection was carried out using a syringe pump (Harvard Apparatus, Holliston, MA) at a flow-rate of 20 μ L/min. The source temperature was set to 80 °C and the capillary voltage was 3.25 kV. Protein molecular weight was determined from mass-to-charge ratios by MassLynx software.

Electron Microscopy Measurements

Samples for electron microscopy (EM) were deposited on carbon-coated pioloform 300 mesh copper grids and negatively stained with 1% (w/v) aqueous uranyl acetate. EM images were obtained on a JEOL JEM-100B transmission electron microscope operated at 80 kV.

Atomic force microscopy (AFM) measurements

AFM images were collected with a PicoScan LE system (Molecular Imaging, Phoenix, AZ) equipped with Acoustic AC mode (Tapping mode) for *ex-situ* experiment. Triangular cantilevers with 160 kHz resonance frequency and 2 N/m spring constant, a V-shaped cantilever NSC16/AIBS (MikroMasch), were used in Tapping mode imaging. The imaging was carried out at a scan rate of 1 line/s with 512 data points per line, at a drive current of 10 ± 4 Å. Aliquots of 10 μ L of sample containing 100 mM NaCl were placed on a freshly cleaved mica substrate. After incubation at least 60 min, the substrate was rinsed with water several times to remove salt and loosely bound protein and dried with high-purity Nitrogen.

Heights ranging from 0.1 to 100 nm were estimated by section analysis, and lateral sizes were calibrated with standard calibration grid. At least four regions of the mica surface were examined to verify that similar structures existed through the sample. No filter treatment was

used to modify the images. SPIP 4.0 (Image Metrology) was used to analyze the height, area and volume distribution of the α -Syn aggregations.

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Figure 1.

Oxidation of DOPAC leads to formation of fluorescent species. A: Fluorescence (excited at 360 nm) of freshly prepared DOPAC (short dash); after 4 days of incubation (long dash); and after 1 year of incubation (solid line). DOPAC concentration was 100 μ M, excitation was 360 nm. B: Kinetics of DOPAC fluorescence (excitation at 360 nm, emission at 456 nm) on incubation with 70 μ M α -synuclein (●); with 5 mM NaN₃ (▼); and DOPAC alone (○).

Figure 2.

The presence of DOPAC inhibits alpha-synuclein fibrillation. **A:** ThT fluorescence assays of fibrillation of 70 μ M α -Syn in the presence of 0 ($\circ$), 7 ($\blacktriangle$), 14 (∇), 28 ($\bullet$), 35 ($\square$), 42 ($\blacktriangledown$), 49 ($\triangle$), and 100 ($\blacksquare$) μ M DOPAC in 20 mM pH 7.4 phosphate buffer and 100 mM NaCl at 37 $^{\circ}$ C. **B:** Incubation of α -synuclein with DOPAC leads to formation of soluble oligomers. EM images and SDS gel of incubated α -Syn (70 μ M) without (**a**) or with DOPAC (100 μ M) (**b** and **b'**) in 20 mM phosphate buffer (pH 7.4) and 100 mM NaCl at 37 $^{\circ}$ C, the scale bar is 200 nm. Panel **b'** represents a rear field where some protofibrils are seeing. The side panel shows the SDS gel of incubated and centrifuged solutions, S and M represent supernatant and non-

centrifuged mixture respectively. **C:** Thiosulfate prevents DOPAC oxidation. ThT assay of fibrillation of 70 μM $\alpha\text{-Syn}$ and 5 mM $\text{Na}_2\text{S}_2\text{O}_3$ in the presence of 0 ($\square$), 100 μM fresh ($\blacksquare$) or pre-oxidized ($\diamond$) DOPAC in 20 mM phosphate buffer (pH 7.4), 100 mM NaCl at 37 °C.

Figure 3A

Figure 3B

Figure 3.

Formation of oligomers during the incubation of α -synuclein monitored by AFM. **A:** Time course of α -Syn (70 μ M) incubated without DOPAC in 20 mM pH 7.4 phosphate buffer and 100 mM NaCl at 37°C, scale bar is 300 nm. The lower panels show the height distribution.

B: Time course of incubated α -Syn solution (70 μ M) with DOPAC (100 μ M) in 20 mM pH 7.4 phosphate buffer and 100 mM NaCl at 37°C, scale bar is 300 nm. The lower panels show the heights distribution. Note: sometimes, due to the variance in tip's condition, particles on AFM images were stretched and looked “wider” than their real sizes. However, the heights of these particles are reliable.

Figure 4.

Time course monitoring of incubating α -Syn (70 μ M) alone (A) or with DOPAC (100 μ M) (B) at pH 7.4, 37°C under fibrillation condition by size exclusion HPLC. Absorbance spectra were monitored at 280 nm. In these experiments, 15 μ L of incubated solutions were injected on a TSK-GEL G2000SW_{XL} size-exclusion column at a flow rate of 0.5 mL/min.

Figure 5. Monitoring the interaction of α -synuclein with DOPAC by SEC FPLC. UV absorbance (280 nm) traces of incubated solutions (no agitation) of α -Syn (70 μ M) with DOPAC (100 μ M) at pH 7.4, 37°C by SEC FPLC. In these experiments, 300 μ L of incubated solutions were injected on a SUPERDEX™ 200 size-exclusion column at a flow rate of 2.0 mL/min.

Figure 6.

Changes in Trp fluorescence of Trp-containing mutants of α -synuclein (Y39W, Y125W, and Y136W) on incubation with DOPAC. Note, the wild type α -synuclein has no tryptophan residues. **A:** the kinetics of Trp fluorescence emission change for Y39W ($\circ$), Y125W (Δ) and Y136W ($\square$) α -Syn (70 μ M) in the presence of one molar equivalent of DOPAC (pH 7.4, 37°C). **B:** Trp Fluorescence of α -Syn (70 μ M) mutants before and after incubation with one molar equivalent of DOPAC: Y39W before (black solid) and after incubation (red solid); Y125W before (black long dash) and after (red long dash); Y136W before (black short dash) and after (red short dash). **C:** Tryptophan fluorescence of α -synuclein mutants: Y39W in fibril (blue solid) and native monomer (black solid); Y125W in fibril (dash dot) and in native form (dash); Y136W in fibril (dotted) and in native form (dash dot dot). Fluorescence intensities were normalized.

Figure 7.

Covalent modification of α -synuclein by the quinones of DOPAC and dopamine. SDS PAGE of incubated α -Syn (70 μ M; for lane 6, 140 μ M was used) with different concentrations of DOPAC (lane 1-6) or dopamine (lane 7-8): Lane 1 (0 μ M); 2 (10 μ M); 3 (50 μ M); 4 (100 μ M); 5 (1000 μ M); 6 (5000 μ M); 7 (100 μ M); 8 (1000 μ M). Panel **A** shows the results with the NBT stain, which only detects quinone-modified proteins. Panel **B** shows all proteins present on the same gel using Ponceau S stain after NBT staining.

Figure 8.

Formation of covalent adducts during incubation of α -synuclein with hydroquinone or homogentisic acid. Mass spectra of: a) α -Syn alone; b) α -Syn incubated with 3 molar equivalent of DOPAC or dopamine for 1 day; c) α -Syn incubated with 3 molar equivalent of HGA for 1 day and d) α -Syn incubated with 3 molar equivalent of HQ for 1 day.

Figure 9.

Potential reactions between DOPAC and α -Syn: i) at low concentration, DOPAC quinone monomer binds α -Syn non-covalently ($M\alpha$ -S + DOPAC); ii) at high concentration, monomeric DOPAC quinone can react with α -Syn monomer to form covalent products ($M\alpha$ -S-DOPAC); iii) at high concentration, oxidized DOPAC can polymerize first. Polymerized DOPAC can react covalently either with one α -Syn molecule forming poly-DOPAC modified α -Syn monomer ($M\alpha$ -S-Poly-DOPAC) or with several α -Syn molecules to get polymerized α -Syn (Poly- α -S-DOPAC).