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Unveiling the role of the pesticides paraquat and rotenone on α-synuclein fibrillation *in vitro*

Maurício Guilherme Valente Maturana [a,1], Anderson Sá Pinheiro [b,1], Theo Luiz Ferraz de Souza [c], Cristian Follmer [a,*]

[a] Department of Physical Chemistry, Institute of Chemistry, Federal University of Rio de Janeiro, Rio de Janeiro 21941-909, Brazil
[b] Department of Biochemistry, Institute of Chemistry, Federal University of Rio de Janeiro, Rio de Janeiro 21941-909, Brazil
[c] Faculty of Pharmacy, Federal University of Rio de Janeiro, Rio de Janeiro 21941-909, Brazil

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ABSTRACT

Epidemiological data have suggested that exposure to environmental toxins might be associated with the etiology of Parkinson's disease (PD). In this context, certain agrochemicals are able to induce Parkinsonism in different animal models *via* the inhibition of mitochondrial complex I, which leads to an increase in both oxidative stress and the death of nigrostriatal neurons. Additionally, *in vitro* experiments have indicated that pesticides are capable of accelerating the fibrillation of the presynaptic protein α-synuclein (aS) by binding directly to the protein. However, the molecular details of these interactions are poorly understood. In the present work we demonstrate that paraquat and rotenone, two agrochemicals that lead to a Parkinsonian phenotype *in vivo*, bind to aS *via* solvent effects rather than through specific interactions. In fact, these compounds produced no significant effects on aS fibrillation under physiological concentrations of NaCl. NMR data suggest that paraquat interacts with the C-terminal domain of the disordered aS monomer. This interaction was markedly reduced in the presence of NaCl, presumably due to the disruption of electrostatic interactions between the protein and paraquat. Interestingly, the effects produced by short-term incubation of paraquat with aS on the protein conformation resembled those produced by incubating the protein with NaCl alone. Taken together, our data indicate that the effects of these agrochemicals on PD cannot be explained via direct interactions with aS, reinforcing the idea that the role of these compounds in PD is limited to the inhibition of mitochondrial complex I and/or the up-regulation of aS.

1. Introduction

Parkinson's disease (PD) is the second most common age-related disorder after Alzheimer's disease and effects 1–2% of the population over 65 years of age (Langston, 2006). The clinical symptoms (resting tremor, bradykinesia, rigidity and postural dysfunction) are due to a loss of dopaminergic neurons in the substantia nigra pars compacta and a resultant dopamine (DA) deficiency in the striatum (Langston et al., 1983; Langston, 2006; Sulzer, 2007). Although the etiology of PD remains unknown, both the formation of potentially toxic intracellular α-synuclein (aS) deposits (called Lewy bodies and Lewy neurites) (Spillantini et al., 1998) and exposure to environmental toxins (Petrovitch et al., 2002; Kamel et al., 2007) are believed to correlative of neuropathogenesis.

Epidemiological studies have indicated an increased incidence of PD within populations that have been exposed to agrochemicals, including herbicides and insecticides (Priyadarshi et al., 2000). These compounds are also capable of inducing Parkinsonism in different animal models *via* the inhibition of mitochondrial complex I, which results in increased reactive oxygen species (ROS) production as well as selective degeneration of nigrostriatal neurons (Hartley et al., 1994). Furthermore, it has been reported that certain agrochemicals might affect aS aggregation directly or indirectly. For instance, exposing rodents to MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine), paraquat or rotenone leads to the up-regulation of aS (Manning-Bog et al., 2002; Chorfa et al., 2013). Moreover, a direct binding of aS to agrochemicals has been reported to increase the rate of aS fibrillation *in vitro*, thereby contributing to PD pathogenesis (Uversky et al., 2001a,b; Silva et al., 2013a,b). Among these compounds are herbicides (paraquat,

* Corresponding author. Tel.: +55 21 2562 7752; fax: +55 21 2562 7265.
E-mail address: follmer@iq.ufrj.br (C. Follmer).
[1] These authors contributed equally to this work.

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diuron, trifularin), fungicides (dithiocarbamate), and botanical (rotenone) or synthetic (kepone, DDT) pesticides.

There exists compelling evidence indicating that oligomeric intermediates are the toxic entity generated during aS fibrillation (Follmer, 2014). In this context, compounds that stabilize aS oligomers rather than fibrils have been associated with the formation of toxic aS aggregates (Winner et al., 2011). If aS fibrillation represents a non-toxic pathway by shifting the equilibrium toward the consumption of potentially toxic oligomers, the fact that agrochemicals accelerate the aS fibrillation process argues against the hypothesis that direct interaction of these compounds with aS contributes to aS-mediated PD pathogenesis. To clarify this inconsistency, the molecular details of the interactions between aS and the agrochemicals paraquat and rotenone were investigated.

2. Experimental

Fibrillation of aS. The expression and purification of aS was performed as previously described by our group (Coelho-Cerqueira et al., 2013). The molar concentration of the aS monomer was determined by measuring absorbance at 276 nm using a molar extinction coefficient of 5600 M^{-1} cm^{-1}. aS fibrillation was carried out by incubating 35 μM purified aS monomer in 10 mM sodium phosphate, pH 7.5, in the presence of varying concentrations of NaCl (0–200 mM). The aggregation assay was performed at 37 °C under agitation (350 rpm) using a Thermomixer Comfort equipment (Eppendorf, Hamburg, Germany) in a 96-well plate (Corning NBS 96-well white plate) which contained a single 3 mm glass (borosilicate) bead per well. Fibril formation was monitored by measuring Thioflavin-T (ThT) fluorescence using a single-time point dilution protocol. ThT fluorescence was evaluated in a Cary Eclypse Fluorimeter (Agilent Technologies, Santa Clara, USA) by excitation at 446 nm and collection of fluorescence emission at 485 nm. To investigate the effect of rotenone on aS fibrillation, 35 μM aS monomer was incubated in the presence of 100 μM rotenone, dissolved in 100% DMSO. An equivalent amount of pure DMSO (5% as final concentration) was used as a control. Concentrations of rotenone higher than 100 μM were not achievable in aqueous solution with 5% DMSO, a condition in which DMSO did not interfere significantly in the fibrillation process; however, paraquat is largely soluble in water and was evaluated in concentrations ranging from 0.1 to 1 mM. Both paraquat and rotenone were purchased from Sigma–Aldrich Co (St. Louis, USA).

Nucleation-dependent fibrillation of aS. For the fibrillation in seeding conditions, 35 μM aS monomer in 10 mM sodium phosphate, pH 7.5, in the presence or absence of 100 mM NaCl, was incubated with aS seeds (5%, w/w) at 37 °C without agitation. aS seeds were prepared by allowing 100 μM of aS monomer to incubate for 8–10 days at 37 °C under agitation (350 rpm) until mature fibrils were created. Fragmented fibrils were then produced *via* ultrasound sonication (60 min; 40 kHz). To induce fibrillation induced *via* sodium dodecyl sulfate (SDS), 50 μM aS monomer was incubated at 37 °C, without agitation, in the presence of 600 μM of SDS (12 molecules of SDS per aS monomer).

NMR. All NMR experiments were performed at 15 °C on a Bruker Avance III 800 MHz spectrometer (Bruker Biospin GmbH, Reinstetten, Germany) using an inverse-detection triple resonance *z*-gradient probe. The sample contained 200 μM purified ^{15}N-aS in 10 mM sodium phosphate, pH 7.5, [10% D_2O (v/v)], in the presence or absence of 100 mM NaCl. Agrochemicals were evaluated at concentrations of either 100 μM rotenone or 1 mM paraquat. aS plus 5% DMSO was used as a control for rotenone. Insoluble aggregates were removed *via* centrifugation. Spectra were acquired both before and after incubation of the samples at 37 °C with agitation (350 rmp) for 24 h. Phase-sensitive two-dimensional ^{1}H–^{15}N Heteronuclear Single Quantum Coherence (HSQC) spectra were recorded using Echo-anti Echo gradient selection. TopSpin 3.1 was used for data acquisition. All spectra were processed with NMRPipe (Delaglio et al., 1995) and analyzed with NMRViewJ (Johnson and Blevins, 1994). Amide resonance assignments were made according to previously reported chemical shift assignments for the unfolded aS monomer (Rao et al., 2009) and chemical shift perturbations (CSP) were calculated as follows:

$$\mathrm{CSP} = \sqrt{\delta H^2 + \left(\frac{\delta N}{10}\right)^2}$$

where δH and δN refer to the chemical shift of ^{1}H and ^{15}N, respectively.

Isothermal titration calorimetry (*ITC*). ITC measurements were performed using a VP-ITC calorimeter from MicroCal, Llc (Northampton, USA). The titration of 50 μM aS with rotenone (100 μM) or paraquat (2 mM) involved 10 injections (10 × 25 μL or 10 × 10 μL, respectively) at 5-min intervals with constant stirring at 307 rpm. The temperature was set at 37 °C. The reference cell was filled with Milli-Q water. For the assays using rotenone, exactly the same concentration of DMSO (5%) was added to both aS solution (or buffer alone) and rotenone to minimize the thermal effects of dilution of DMSO in water. Any heat produced by the study compounds following dilution into buffer was subtracted from the raw data obtained with aS.

TEM. 10 μL aliquots of aS samples were deposited on a carbon-stabilized, parlodion-coated copper grid (300 Mesh). Samples were dried at room temperature. Images of Energy Filtered Transmission Electron Microscopy (EFTEM) were acquired at the Institute of Chemistry, University of Campinas (Unicamp), São Paulo, using a Carl Zeiss Libra 120 transmission electron microscope operated at 80 kV.

3. Results

Rotenone and paraquat fail to accelerate aS fibrillation under physiological concentrations of NaCl. aS fibrillation can be described as a nucleated polymerization process in which the unfolded monomer undergoes self-assembly to form oligomeric intermediates (nuclei) at the onset of aggregation, followed by monomer accretion and fibril growth. Thus, aS fibrillation kinetics display sigmoidal behavior with an initial lag-time phase (nucleation) followed by an exponential-growth phase (elongation) that evolves until a plateau is reached (formation of mature amyloid-fibrils). Fig. 1A and E indicates that both agrochemicals (100 μM rotenone or 500 μM paraquat) accelerate aS fibrillation in the presence of sodium phosphate, pH 7.5, which is in accordance with previous reports (Uversky et al., 2001a,b; Silva et al., 2013a,b). By analyzing the kinetic parameters extracted from the normalized curves displayed in Fig. 1C and G we verified that the duration of the lag-time and the $t_{½}$ (time required to reach 50% of maximum fibrillation) significantly decreased in the presence of the pesticides, while the elongation rates increased (Table 1). Our assays utilized a molar ratio of aS monomer:pesticide of ~1:3 (for rotenone) and ~1:15 (for paraquat). Rotenone did not produce significant effects on aS fibrillation at concentrations below 100 μM and higher concentrations were not evaluated due to the very low solubility of this compound in aqueous medium. However, paraquat is highly soluble in water and a concentration-dependent effect on aS fibrillation was observed in the absence of NaCl (Supplementary Fig. S1 A).

Next, we investigated the manner in which NaCl concentration effected the interaction of aS with paraquat or rotenone. The intention of this assay was not to reproduce the physiological