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Site-Specific Interaction between α-Synuclein and Membranes Probed by NMR-Observed Methionine Oxidation Rates

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Supporting Information

ABSTRACT: α-Synuclein (αS) is an intrinsically disordered protein that is water-soluble but also can bind negatively charged lipid membranes while adopting an α-helical conformation. Membrane affinity is increased by post-translational N-terminal acetylation, a common modification in all eukaryotic cells. In the presence of lipid vesicles containing a small fraction of peroxidized lipids, the N-terminal Met residues in αS (Met1 and Met5) rapidly oxidize while reducing the toxic lipid hydroperoxide to a nonreactive lipid hydroxide, whereas C-terminal Met residues remain unaffected. Met oxidation can be probed conveniently and quantitatively by NMR spectroscopy. The results show that oxidation of Met1 reduces the rate of oxidation of Met5 and vice versa as a result of decreased membrane affinity of the partially oxidized protein. The effect of Met oxidation on the αS−membrane affinity extends over large distances, as in the V49M mutant, oxidation of Met1 and Met5 strongly impacts the oxidation rate of Met49 and vice versa. When not bound to membrane, oxidized Met1 and Met5 of αS are excellent substrates for methionine sulfoxide reductase (Msr), thereby providing an efficient vehicle for water-soluble Msr enzymes to protect the membrane against oxidative damage.

α-Synuclein (αS) is a water-soluble 140-residue intrinsically disordered protein containing seven imperfect 11-residue repeats in its positively charged N-terminal domain followed by a highly acidic 40-residue C-terminal tail. Although a recent report proposed that in mammalian cells αS exists predominantly as a stably folded tetrameric α-helical structure,[1] subsequent studies excluded this possibility.[2] N-terminal acetylation of proteins is a common post-translational modification in eukaryotic cells that significantly enhances the affinity of αS for lipid membranes,[3] to which it can bind in an α-helical conformation.[4] The protein is associated with mitochondrial function,[5] but it is also found in red blood cells that lack mitochondria[6] and is present at particularly high levels in the presynaptic region of neuronal cells.[7] The normal function of αS in neurons remains ill-defined, but the protein has been implicated in membrane-related processes, including vesicular trafficking, as a chaperone in SNARE complex formation, and as a modulator of synaptic plasticity.[8] αS is also found in fibrillar form in Lewy body and Lewy neurite deposits in the brains of Parkinson's disease (PD) patients,[9] and both gene triplication[10] and αS mutations[11] have been correlated with inherited forms of the disease. Very recently, intrastriatal injection of nontransgenic mice with small amounts of synthetic αS fibrils was shown to result in pathology similar to that seen in PD.[12] These observations establish that αS plays a key role in PD, and it also has been implicated in a range of other neurological disorders, including Alzheimer's disease.[13] The neuronal tissue of PD patients exhibits elevated levels of lipid peroxides due to oxidative stress associated with high levels of dopamine or mitochondrial dysfunction.[14] The interaction of αS with membranes containing polyunsaturated alkyl chains results in oligomerization of the protein, a form that is believed to be highly toxic to the cells, potentially through an oxidative process involving lipid peroxide intermediates.[15] Lipid hydroperoxides (LOOH) occur naturally in cellular membranes and result from oxidation of unsaturated fatty acid chains. They have low stability and can decompose through reactive radical intermediates to aldehydic products,[16] thereby contributing to a chain reaction of oxidative damage to remaining unsaturated lipids.

Although αS was initially believed to be an apolipoprotein (ALP) on the basis of its amino acid sequence and its characteristic 11-residue repeats, its interaction with lipids is only transient, even though it involves the formation of an amphipathic helix motif similar to that of ALPs.[17,18] EPR and FRET studies of αS under saturating lipid concentrations confirmed the α-helical structure of the N-terminal domain deduced from prior circular dichroism (CD) experiments.[19] However, in view of the transient nature of αS−membrane binding, modification of the protein with spin labels or chromophores potentially can perturb the weak αS−lipid bilayer interaction. In contrast, Met oxidation can be followed conveniently and quantitatively by NMR spectroscopy and thus provides a valuable complement to those widely used methods for studying such interactions.

Oxidation of specific Met residues in ALPs upon interaction with LOOH occurs spontaneously and is coupled with reduction of LOOH to the inert lipid hydroxide.[20,21] Met oxidation can be monitored by mass spectrometry and has been used to probe the solvent accessibility of Met residues in proteins by addition of a hydrophilic oxidizing agent[22] and the lipid accessibility of Met in integral membrane proteins by using a peroxidation-sensitive polyoxyethylene detergent.[23] Fink[24] proposed that by removing toxic hydroperoxide species from the membrane through a mechanism involving oxidation of all four of its Met residues, αS

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can halt the oxidative chain reaction, thereby potentially playing an important functional role in preventing oxidative damage of brain lipids.

Here we show by NMR monitoring that Met oxidation of αS upon interaction with lipid vesicles containing LOOH is restricted to its two N-terminal Met residues M1 and M5, leaving M116 and M127 completely unaffected. The rates of oxidation as functions of lipid concentration report on the site-specific contacts between Met residues and the membrane and provide new insights into the membrane-binding mode of αS. Initially, we observed the effect of αS Met oxidation serendipitously when analyzing a time series of NMR spectra recorded in the presence of small unilamellar vesicles (SUVs) containing monounsaturated alkyl chains. These SUVs had been prepared by ultrasonication and stored at room temperature for an extended period of time prior to preparation of the NMR sample. Addition of SUVs containing a significant fraction of negatively charged lipids resulted in strong attenuation of the signal intensities for the N-terminal residues in the $^{1}H-^{15}N$ heteronuclear single-quantum correlation (HSQC) spectrum, even when the [lipid]:[αS] molar ratio (q) was very low (e.g., q = 1).[25] At higher q, the resonances for the first 100 residues completely disappeared.[26] Remarkably, when an old stock solution of SUVs was used to prepare the NMR sample, the resonances of the nine N-terminal residues in the $^{1}H-^{15}N$ HSQC spectrum were further attenuated over the course of the first few days, while new resonances appeared in their immediate vicinity. At the same time, the signals for residues 10−100, which were originally attenuated as a result of lipid binding, increased in intensity [Figure S1 in the Supporting Information (SI)]. $^{1}H-^{13}C$ 2D correlation spectra showed the appearance of several new resonances at (^{1}H, ^{13}C) frequencies of methionine sulfoxide (MetO) (Figure S2) in tandem with the attenuation of the methyl correlations of M1 and M5. The time-dependent increase in the signal intensities for N-terminal residues 10−100 upon oxidation of M1 and/or M5 pointed to weaker lipid binding by αS upon oxidation of its N-terminal Met residues but also interfered with direct quantitative measurement of the oxidation rates from the time course of the NMR spectrum. Thus, rather than directly quantifying the rates of Met oxidation in the NMR spectrometer, we carried out this reaction on the bench and periodically harvested aliquots from the αS/SUV stock solution. Lipids were rapidly removed from each aliquot by several cycles of methanol precipitation of αS, yielding an aqueous sample of lipid-free αS with very favorable spectral characteristics that permitted accurate quantification of the various αS oxidation states from the corresponding HSQC resonance intensities.

Figure 1 shows the $^{1}H-^{15}N$ HSQC resonances of residue L8 for four samples harvested at different times after excess lipid (q = 630) in the form of SUVs (5% w/v) was added to a freshly prepared sample of N-terminally acetylated αS. L8 falls in a particularly well resolved region of the HSQC spectrum, and four resonances are observed for it: the nonoxidized form of the protein (labeled "NN") gives the most-upfield L8 resonance, and those corresponding to protein with oxidized M1 and natural M5 ("ON"), natural M1 and oxidized M5 ("NO"), and both M1 and M5 oxidized ("OO") are increasingly shifted downfield.

Upon addition of fresh SUVs to a lipid-free wild-type (WT) αS sample containing a mixture of oxidation states of its Met residues, the binding of each form to the lipid could be monitored simultaneously by the attenuation of their signal intensities. As noted previously, exchange between the free and lipid-bound states of αS occurs in the slow-exchange limit, with

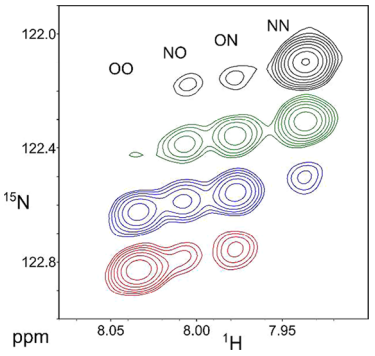

Figure 1. Overlays of the 800 MHz $^{1}H-^{15}N$ HSQC spectra of N-acetylated αS recorded after removal of lipid, showing the cross-peaks for residue L8 for samples harvested at different times [30 min (black), 2 h (green), 8 h (blue), and 16 h (red)] after incubation of the protein with a 5% w/v lipid mixture [100 μM αS, 150 mM NaCl, 20 mM sodium phosphate (pH 6), 15% DOPS, 25% DOPE, and 60% DOPC with 10 ± 0.5% peroxidization of the oleyl chains] at 20 °C. The black spectrum is shown in its original position, and the others are displaced in 0.2 ppm steps in the ^{15}N dimension for clarity. Oxidation state labels: NN, neither M1 nor M5 oxidized; ON, only M1 oxidized; NO, only M5 oxidized; OO, both M1 and M5 oxidized.

no significant impact on the line widths and positions.[25] The four forms of the protein were impacted to dramatically different extents by the presence of lipid (Figure 2). Complete obliteration

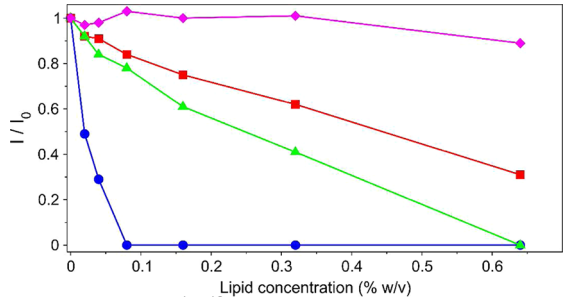

Figure 2. Ratios of $^{1}H-^{15}N$ HSQC peak heights in the presence (I) and absence (I_0) of lipid as functions of total lipid concentration. Results are shown for residue L8 in a partially oxidized sample of N-terminally acetylated WT αS containing a mixture of all four M1/M5 oxidation states: NN (blue), ON (red), NO (green), and OO (magenta). The αS sample initially was harvested 4 h after addition of 5% w/v partially oxidized SUVs (see the Figure 1 caption), and after lipid removal, nonoxidized SUVs were added to yield samples containing 100 μM αS in 20 mM sodium phosphate (pH 6). The SUV lipid composition was 30% DOPS, 50% DOPE, and 20% DOPC.

of the N-terminal intensities was observed for the NN protein upon addition of an 8-fold molar excess (800 μM total lipid, ∼0.08% w/v), whereas the OO species remained virtually unaffected up to 5 mM total lipid (∼0.5% w/v); the NO and ON species were attenuated at intermediate levels. The much smaller signal attenuations for NO and ON compared with NN are indicative of weaker binding of these partially oxidized states to the lipid, requiring the use of four different oxidation rate constants: $k_{NN\text{-}ON}$, $k_{NN\text{-}NO}$, $k_{ON\text{-}OO}$, and $k_{NO\text{-}OO}$. Fitting the harvest-time dependence of the resonance intensities of the four αS forms (Figure 3A) to the respective rate laws (eq S1 in the SI) gave rate constant values that confirmed the considerable decrease in the M1 (M5) oxidation rate after oxidation of M5 (M1) ($k_{NO\text{-}OO} < k_{NN\text{-}ON}$; $k_{ON\text{-}OO} < k_{NN\text{-}NO}$; Table 1).

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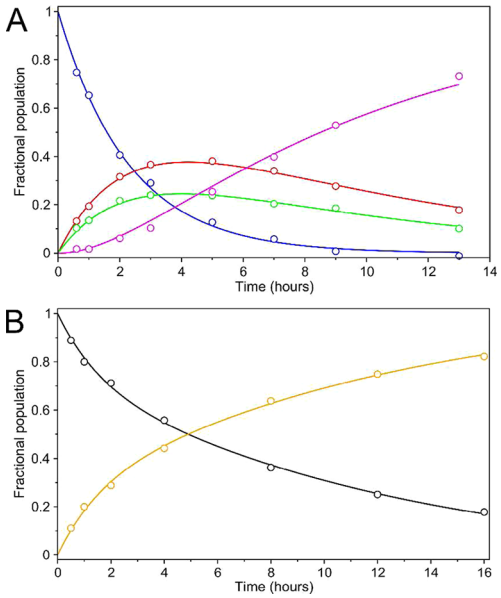

Figure 3. Fits of the populations of various oxidation states of αS as functions of incubation time in a 5% w/v SUV solution (see the Figure 1 caption) to the corresponding rate laws presented in the SI to obtain the rate constant values listed in Table 1. (A) Fits of the populations of the NN (blue), ON (red), NO (green), and OO (magenta) states of N-acetylated WT αS to eq S1. (B) Fits of the populations of the nonoxidized (black) and oxidized (orange) states of M49 in the N-acetylated V49M mutant of αS (obtained by monitoring the intensities of the HSQC cross-peaks of G51) to eq S3.

Table 1. Met Oxidation Rate Constants (in h^{-1}) for N-Terminally Acetylated WT and V49M αS[a]

	$k_{NN\text{-}ON}$	$k_{NN\text{-}NO}$	$k_{NO\text{-}OO}$	$k_{ON\text{-}OO}$	$k_{(N)N\text{-}(N)O}$[b]	$k_{(O)N\text{-}(O)O}$[b]
WT	0.259	0.174	0.124	0.110	–	–
V49M	0.209	0.159	0.100	0.076	0.217	0.089

[a]Incubated with 5% w/v SUVs (q = 630). Lipids were 15% DOPS, 25% DOPE, and 60% DOPC with 10.0 ± 0.5% (WT) and 8.8 ± 0.5% (V49M) oleyl peroxidation. For other conditions, see the Figure 1 caption. The errors in the reported k values (obtained from Monte Carlo analysis; see the SI) are ca. 0.006 h^{-1}. [b]For the V49M sample, the apparent M49 oxidation rate depends on four rate constants: $k_{NNN\text{-}NNO}$, $k_{NON\text{-}NOO}$, $k_{ONN\text{-}ONO}$, and $k_{OON\text{-}OOO}$. However, the last three could not be determined uniquely from the curves and instead were fitted as a single average value, $k_{(O)N\text{-}(O)O}$; $k_{(N)N\text{-}(N)O} = k_{NNN\text{-}NNO}$.

At first sight, the roughly 2-fold differences in the rate constants appear smaller than expected if the degree of attenuation of the NMR signals for the OO, NO, and ON species (Figure 2) is taken as a measure of their lipid affinity. However, it is important to note that the attenuation observed at low q (<50) reflects a different mode of interaction than the helix-on-membrane-surface binding mode active at high q. At low q, insufficient membrane surface is available to accommodate the latter binding mode. Instead, NMR data suggest the presence of lipid-binding modes involving a limited number of N-terminal residues under these conditions, and the rapid translational diffusion measured for the unattenuated C-terminal tail indicates minimal αS–lipid aggregation.[25]

The lipid-binding mode of αS at large q can be probed further by combining the Met oxidation probe with mutagenesis. As an example, we studied the kinetics of lipid oxidation in the V49M mutant. M49 is located near the center of the N-terminal lipid-binding domain, and its C^{α}–C^{β} bond is parallel to the membrane surface when αS adopts the predicted amphiphilic, slightly unwound 11/3 α-helical conformation.[18] Rates of M1, M5, and M49 oxidation were measured for three different high q values (150–630) using the same procedure as above. Since M49 is distant from M1 and M5, its oxidation does not impact the resonance positions of residues 1–10; conversely, neither M1 nor M5 oxidation perturbs the resonance positions of amides in the vicinity of M49. Therefore, the resonance intensity for nonoxidized M49 (Figure 3B) corresponds to the sum of the intensities for the M1/M5 NN, NO, ON, and OO oxidation states, while that for the M1/M5 NO oxidation state (for example) represents the sum of the NON and NOO signals. The time dependence of the observed intensities did not provide sufficient information to fit all 12 applicable rates independently. However, the initial buildup rates of the oxidized species at short harvesting times yielded the oxidation rates for the individual Met residues when the other two had not yet oxidized. After the slightly different levels of lipid oxidation used for WT αS and the V49M mutant were taken into account, the oxidation rates measured for M1 and M5 in the WT and mutant proteins were found to be very similar (Table 1), indicating that prior to oxidation of any of the Met residues, the V49M mutation does not significantly impact the interaction of M1 and M5 with the lipid. The initial M49 lipid oxidation rate was comparable to that of M1 but rapidly decreased at later times, after oxidation of M1 and M5 (Figure 3B). The fact that M49 oxidation is impacted by the oxidation states of M1 and M5 shows that the membrane interactions of residues M1, M5, and M49 are highly correlated.

The impact of M49 oxidation on the M1 (or M5) oxidation rate was more difficult to disentangle because the latter's oxidation rate is also affected by oxidation of M5 (or M1). However, with the effect of M5 oxidation on M1 already known from the WT protein measurements, the accelerated slowdown of M1/M5 oxidation relative to the WT indicates a significant impact of the M49 oxidation state on the interactions of M1 and M5 with the lipid (Table 1). Therefore, at the high q used in our harvesting experiments, binding modes where only a shorter N-terminal section of the protein engages the membrane (e.g., residues 1–20, which were distinctly attenuated at very low q[3]) do not play a significant role.

Comparison of the lipid oxidation rates at the three different q values showed a nearly 2-fold increase when q was doubled from 150 to 300 followed by a leveling off as q was further increased to 630 (Table S4 in the SI). These rates parallel the increase in the α-helical CD signature as measured by the ellipticity at 222 nm (Figure S5), which also approached its asymptotic value of −24 000 deg cm^2/dmol at this q (but using sample concentrations that were 20-fold more dilute). Interestingly, whereas at q = 150 the dependence of the M1 oxidation rate on the oxidation state of M5 was strong ($k_{NN\text{-}ON}/k_{NO\text{-}OO}$ = 2.6), this dependence decreased to a factor 1.7 for q = 630. Since the α-helicity and thus the αS–membrane binding were nearly complete at q = 630 even for samples that were 20-fold more dilute, this weaker dependence does not directly reflect the absolute affinity of the protein for the SUV. Instead, it must result from competition between the strongly binding nonoxidized protein and the slightly less strongly binding oxidized protein for the limited lipid surface available at q = 150.

NMR observation of Met oxidation through the interaction with a membrane or detergent micelle containing a small fraction of peroxidized lipids provides a convenient and quantitative method for site-specific probing of protein–lipid interactions.

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Our results for αS highlight the decrease in membrane affinity after Met residues become oxidized. In free solution, the oxidized M1 and M5 residues of N-terminally acetylated αS protein, as applies in mammalian cells, are excellent substrates for the methionine sulfoxide reductase (Msr) enzymes (Figure S4B), which convert the MetO residues back to their natural state. The decrease in αS−membrane affinity upon oxidation therefore makes the protein an effective shuttle between Msr and LOOH, allowing the water-soluble enzymes to play a role in the removal of highly toxic peroxide species from the membrane.

Our above in vitro findings do not prove that αS plays a pivotal role in minimizing oxidative damage in living organisms. Other enzymes, in particular GPx4, are known to be essential in protecting against lipid peroxidation.[27] Similarly, overexpression of apolipoprotein D (ApoD) increases hyperoxic stress resistance in *Drosophila*.[28] The antioxidant property of ApoD is likely related to a mechanism analogous to that described here for αS, where oxidation of one specific Met residue (M93) is believed to be key in removing toxic lipid peroxides.[21] The GPx4 and ApoD concentrations and their relative importance in protecting neuronal synapse membranes remain unknown, however. Msr enzyme levels in the substantia nigra are also unknown, but Msr has been found at high levels in the synapses of optical nerves,[29] suggesting that they are significantly present in other synapses too. αS is known to be present at high levels in neuronal synapses,[7] and its N-terminal Met residues (but not its C-terminal ones) are effective substrates for both MsrA and MsrB. β- and γ-synuclein have lipid-binding properties similar to those of αS and carry N-terminal Met residues in the same positions. Interestingly, αS, βS, and γS triple-knockout mice show late-onset neurological degeneration,[30] potentially consistent with accumulated oxidative damage.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Experimental procedures and additional data. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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