α-Synuclein Shows High Affinity Interaction with Voltage-dependent Anion Channel, Suggesting Mechanisms of Mitochondrial Regulation and Toxicity in Parkinson Disease*

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Background: The intrinsically disordered protein α-synuclein, a hallmark of Parkinson disease, is involved in mitochondrial dysfunction in neurodegeneration and directly interacts with mitochondria.

Results: α-Synuclein regulates VDAC permeability; α-synuclein toxicity in yeast depends on VDAC.

Conclusion: α-Synuclein both blocks VDAC and translocates via this channel across the mitochondrial outer membrane.

Significance: (Patho)physiological roles of monomeric α-synuclein may originate from its interaction with VDAC.

Participation of the small, intrinsically disordered protein α-synuclein (α-syn) in Parkinson disease (PD) pathogenesis has been well documented. Although recent research demonstrates the involvement of α-syn in mitochondrial dysfunction in neurodegeneration and suggests direct interaction of α-syn with mitochondria, the molecular mechanism(s) of α-syn toxicity and its effect on neuronal mitochondria remain vague. Here we report that at nanomolar concentrations, α-syn reversibly blocks the voltage-dependent anion channel (VDAC), the major channel of the mitochondrial outer membrane that controls most of the metabolite fluxes in and out of the mitochondria. Detailed analysis of the blockage kinetics of VDAC reconstituted into planar lipid membranes suggests that α-syn is able to translocate through the channel and thus target complexes of the mitochondrial respiratory chain in the inner mitochondrial membrane. Supporting our in vitro experiments, a yeast model of PD shows that α-syn toxicity in yeast depends on VDAC. The functional interactions between VDAC and α-syn, revealed by the present study, point toward the long sought after physiological and pathophysiological roles for monomeric α-syn in PD and in other α-synucleinopathies.

Emerging evidence establishes the critical role of mitochondria in the pathogenesis of neurodegenerative diseases including Parkinson disease (PD) and Alzheimer disease (1, 2). Dysfunction of mitochondrial enzyme complexes, production of reactive oxygen species, mitochondrial outer membrane (MOM) permeabilization, enhanced apoptosis, and structural alterations of mitochondria have been associated with these pathologies (1–3). Neurons are especially sensitive to mitochondrial dysfunction because of their high demand for energy and their characteristic subcellular distribution of mitochondria. α-Synuclein (α-syn) is a small, intrinsically disordered neuronal protein that is involved in the etiology of PD (4) and various α-synucleinopathies (5). This protein is a major structural component of intracellular protein inclusions, or Lewy bodies, that constitute a pathological hallmark of PD (4). Recent research demonstrates the involvement of α-syn in mitochondrial dysfunction in neurodegeneration and in induction of neuroapoptosis (3, 6–9) and suggests direct interaction of α-syn with mitochondria (6, 7, 9, 10). However, the exact mechanism of α-syn toxicity and its effect on neuronal mitochondria in particular remain elusive. It was shown that α-syn gene mutations cause early onset of PD (11–13). The observation that Lewy bodies are enriched with fibrillar α-syn has led to a hypothesis of the neurotoxicity of fibrillar components (14). Therefore, most of the studies so far have been focused on the role of α-syn aggregates in PD, where α-syn monomers are regarded simply as supplies for the aggregates.

There are three distinctive amino acid regions of α-syn: the membrane-binding amphipathic N-terminal domain (residues 1–60), the mostly hydrophobic central part, also called the non-amyloid β component domain (residues 61–95), and the highly acidic C-terminal tail containing 15 negative charges (residues 96–140) (14). In physiological salt solutions, α-syn

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4 The abbreviations used are: PD, Parkinson disease; MOM, mitochondrial outer membrane; α-syn, α-synuclein; VDAC, voltage-dependent anion channel; FL, full-length; ROS, reactive oxygen species; PT, permeability transition; ANT, adenine-nucleotide translocator.
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exists in an intrinsically disordered form (15). When α-syn binds to the negatively charged lipid membranes, the N terminus adopts a helical form, and the acidic C terminus remains unstructured and does not interact with the membrane (16). Interestingly, it was also found that α-syn preferentially interacts with mitochondrial membranes compared with other native cell membranes, such as endoplasmic reticulum or plasma membranes (17), and the specificity of α-syn binding to the mitochondrial membranes does not depend on the functional state of mitochondria. However, another group (6) reported that import of human α-syn to mitochondria under in vivo and in vitro conditions depends on the mitochondrial membrane potential and mitochondrial ATP level. It was shown that α-syn is predominantly associated with the inner mitochondrial membrane in human dopaminergic neurons (6) and HEK cells (18) and that accumulation of α-syn in mitochondria impairs complex I of the mitochondrial electron transport chain inducing oxidative stress. Other groups reported accumulation of α-syn on the MOM of mouse brain (10) or HEK cells (19) but the absence of the inhibition effect of α-syn on complex I (8). Therefore, questions regarding α-syn localization in mitochondria, the mechanism underlying selective α-syn binding to the mitochondrial membranes, and the role of mitochondrial bioenergetics in the α-syn interaction with mitochondria remain open. Notably, most studies agree on the inhibitory effect of α-syn on the mitochondrial oxidative phosphorylation capacity and on the promotion of oxidative stress.

Surprisingly, there have been no serious attempts to identify the pathway(s) for the translocation of water-soluble α-syn across the MOM from the cytosol to the mitochondrial inner membrane, although the latest reports suggest that α-syn can bind to voltage-dependent anion channel (VDAC), the main channel in the MOM. Lu et al. (20) showed that α-syn overexpressed in the substantia nigra of rats co-immunoprecipitated with VDAC. Human A53T-mutant α-syn associated with dysmorphic neuronal mitochondria also co-immunoprecipitated with VDAC in the brainstem, striatum, and cortex of early and late symptomatic human α-syn transgenic mice (9). These reports raise the possibility that VDAC, a large β-barrel channel suitable for transport of metabolites and polypeptides, could be a pathway for α-syn translocation into the mitochondria.

VDAC controls a significant portion of the outer membrane function (21–24). Because VDAC has been shown to be involved in a wide variety of mitochondria-associated pathologies, including neurodegenerative disorders, such as PD, Alzheimer disease, and amyotrophic lateral sclerosis, VDAC is emerging as a promising pharmacological target (25). This multifunctional channel is regarded as a conjunction point for a variety of cell signals mediated by various cytosolic proteins (26–28). Any restriction to the metabolite exchange through VDAC affects the mitochondrial functions.

Here, we study the functional interaction of α-syn with VDAC reconstituted into lipid bilayers and find that nanomolar concentrations of recombinant monomeric α-syn reversibly block VDAC in a highly voltage-dependent manner. Furthermore, a detailed kinetic analysis of the blockage events suggests that α-syn is able to translocate through VDAC. Experiments with a yeast strain deficient in VDAC1 (por1Δ) demonstrate that α-syn toxicity in yeast depends on VDAC, revealing an α-syn interaction with VDAC in living cells. Considering that VDAC is a major conduit for respiratory substrates across the MOM, our results suggest that the functional interaction of monomeric α-syn with VDAC could be essential for both physiological adaptation of mitochondrial respiration and dysfunction in PD and other α-synucleinopathies.

Experimental Procedures

Protein Purification—VDAC was isolated from frozen mitochondrial fractions of rat liver that were a generous gift of Dr. Marco Colombini (University of Maryland, College Park, MD) and purified following the standard methods (29). WT α-syn full-length (FL) was expressed, purified, and characterized as described previously (30). Purified protein was buffer-exchanged (20 mM Tris and 0.1 M NaCl, pH 8), using Amicon Ultra-15 centrifugal filter units (molecular mass cut-off 3 kDa; Millipore) and stored at −80 °C. Protein concentrations were determined using an extinction coefficient of 5120 M−1 cm−1 at 280 nm using a Cary 300 biospectrophotometer (Varian). Plasmid for α-syn carrying residues 1–115 (α-syn N115) was constructed using the QuikChange site-directed mutagenesis kit (Stratagene) through insertion of a stop codon. Mutation was verified by DNA sequencing. Mutant α-syn N115 was expressed, purified, and characterized as described previously (31). α-Syn mutants A53T and A30P and the C terminus peptide corresponding to residues 96–140 of α-syn (C45) were purchased from rPeptide (Bogart, GA).

Channel Reconstitution—Planar bilayer membranes were formed from diphytanoyl-phosphatidylcholine (Avanti Polar Lipids, Alabaster, AL), and channel currents were analyzed as described previously (31, 32). VDAC insertion was achieved by adding purified VDAC in a 2.5% Triton X-100 solution to the aqueous phase of 1 M (1 mol/liter) KCl buffered with 5 mM Hapes at pH 7.4 in the cis compartment while stirring. Potential was defined as positive when it is greater at the side of VDAC addition (cis). α-Syn was added to the membrane-bathing solutions after VDAC channel reconstitution; statistical analysis of the blockage events was started 15 min after the α-syn addition to ensure a steady state.

Analysis of Open and Blocked Times—Blockage events were identified using a threshold detection algorithm implemented with custom MATLAB (Mathworks) code. The absolute values of the current traces were median-filtered (order 3) and compared with a threshold equal to 80% of the open pore current. The times at which each threshold crossing occurred were recorded and designated as “positive” or “negative” based on the slope of the current at the threshold crossing. Histograms of τ on were compiled on a logarithmic scale from the intervals between each positive threshold crossing and the subsequent negative threshold crossing. Histograms of τ on were compiled on a logarithmic scale from the intervals between each positive threshold crossing and the subsequent negative threshold crossing. Each distribution bin was weighted by Poisson statistics and fit to an exponential function (33) using a Levenburg-Marquardt algorithm. Unless otherwise stated, error bars are calcu-
lating from bootstrap distributions obtained by random resampling of the experimentally observed time distributions.

**Yeast Strains, Plasmids, and Media**—Saccharomyces cerevisiae BY4742 (MATa his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0) and por1Δ (ATCC) strains were used in this study. Plasmid pESC-SYN (34) was a kind gift from Dr. F. Madeo (University of Graz, Graz, Austria), and plasmid pYX212-hVDAC1 (35) was a kind gift from Dr. Vito DePinto (University of Catania, Italy). Transformants were selected and grown on synthetic complete (SC) media lacking uracil and histidine. SC media were prepared as described elsewhere (36). 2% glucose (non-inducing media) or 2% galactose (inducing media) were used as carbon sources. Yeast were washed and resuspended in water at OD1 at a density of 10^6 cells/ml and then subjected to serial 10-fold dilution. Yeast were spotted on plates in duplicate and incubated in a non-controlled atmosphere at 30 °C for 3 days. Plates were imaged, and growth was quantitated from the scanned image using Adobe Photoshop version CS5.1 by measuring the sum of pixel intensity of the yeast spot less an equal area of medium without yeast.

**Western Blotting**—Strains were pregrown in SC medium supplemented with 2% raffinose (non-inducing condition) at 30 °C and inoculated for overnight growth in SC medium with 2% galactose at 37 °C. Cell lysates were prepared as described (37). Briefly, cells were harvested by centrifugation, washed in double-distilled H2O. Cells were resuspended in 1× LDS sample buffer (Invitrogen) with 2.5% β-mercaptoethanol with the protease inhibitor mixture (Roche Applied Science) and boiled for 5 min. Samples were run on 4–12% SDS-PAGE (Invitrogen) and blotted onto a PVDF membrane. α-Syn-FLAG was detected using a mouse monoclonal anti-FLAG antibody (Sigma) and anti-mouse IRDye 800RD secondary antibodies (LI-COR Biosciences). Human VDAC1 was detected using a rabbit polyclonal anti-VDAC1 antibody (Millipore), anti-rabbit HRP-conjugated secondary antibodies (Jackson Immunoresearch), and enhanced chemiluminescence substrate (Pierce).

**Results**

**α-Syn Reversibly Blocks VDAC Reconstituted in Planar Lipid Bilayers**—Ion channel reconstitution is so far the best available method for direct functional studies of organelle channels, such as mitochondrial VDAC. When reconstituted into planar lipid membranes, VDAC forms large, 4-nanosiemens (in 1 M KCl) anion-selective channels permeable for non-charged polymers up to a few kDa (38–40) and for ATP (29, 42). Depending on experimental conditions, such as lipid composition and salt concentration, the channel can stay open for a few seconds even at an applied voltage as high as 60 mV. The addition of nanomolar concentrations of α-syn to the membrane-bathing solution changes VDAC behavior dramatically. A representative experiment is shown in Fig. 1A, where the addition of 50 nM α-syn to a single VDAC causes time-resolved reversible blockages of the channel conductance (right traces). α-Syn induces two distinct blocked states, depending on its concentration and the applied voltage. The first blocked state (B1) is characterized by a conductance of ~40% that of the open state (Fig. 1A, a). A second, deeper blocked state (B2) with a conductance of ~17% that of the open state is observed at potentials |V| ≥ 30 mV (Fig. 1A, b and c); the incidence of this state increases with the applied voltage (event amplitude histograms in Fig. 1A, a–c). α-Syn blocks VDAC from both sides of the channel, but only when a negative potential is applied from the side of the α-syn addition, suggesting that the negatively charged C-terminal region of α-syn is responsible for the observed VDAC blockage.

The distribution of times between blockage events when the channel is open, τoff, is well described by a single exponential function at all applied potentials (Fig. 1C, a). The on-rate of the blockage, ⟨τon⟩−1, is highly voltage-dependent (Fig. 1B, top). The blockage is adequately described as a first-order binding reaction with the on-rate first increasing proportionally to the α-syn concentration, followed by saturation at ~50 nM α-syn (Fig. 2A). The characteristic time the channel spends in the blocked state, τoff = ⟨τon⟩−1, calculated at 25 mV, where only B1 is observed (see Fig. 1A, a), is virtually independent of the α-syn concentration (Fig. 2A, inset). The voltage dependence of the equilibrium constant of α-syn binding to VDAC, K(α-syn) defined as τoff/(⟨τon⟩C), where C is the bulk concentration of α-syn, spans 6 orders of magnitude and, at voltages between −40 and −15 mV, can be fit with an effective “gating charge” of 11.4 ± 1.4 (solid line in Fig. 2B).

In order to analyze binding kinetics at α-syn concentrations well below saturation, a series of experiments were performed at 1 nM α-syn. Representative traces in Fig. 3A demonstrate that at 1 nM, the number of blockage events is dramatically reduced compared with 50 nM (traces a–c in Figs. 1A and 3A obtained at the same voltages). The on-rates are also highly voltage-dependent (Fig. 3B, top) and can be approximated by exponential dependences.

Interestingly, at 1 nM α-syn, B2 is only observed at the high applied voltage of −60 mV (Fig. 3A, d). The distributions of blockage times τoff are adequately described by single exponentials for all voltages at 1 nM α-syn (Fig. 3C) or when the probability of B2 is relatively small compared with that of B1 at 50 nM α-syn (Fig. 1C, b). At 50 nM α-syn for |V| ≥ 45 mV, when the probability of B2 becomes comparable with the probability of B1 (event amplitude histogram in Fig. 1A, c), a single exponential no longer satisfactorily describes the histogram of τoff (Fig. 1C, c). A two-exponential fit of the blockage time histogram (dashed line in Fig. 1C, c) fits long-time events satisfactorily but not the short-time blockages. The exponential increase of τoff with voltage at |V| ≤ 43 mV suggests that the mechanism of α-syn blockage of VDAC, similarly to that of tubulin (27), is a reversible block (Fig. 4). At higher potentials, however, the voltage dependence of τoff changes dramatically, and τoff begins to decrease with voltage. The decrease is better seen when τoff is plotted on a linear scale (bottom panels in Figs. 1B and 3B), whereas the exponential increase of τoff at lower applied voltage is more evident on a logarithmic scale (middle panels in Figs. 1B and 3B). The decrease of the residence time at high voltages is seen in Fig. 3C as a shift of the blockage time distribution at ~60 mV toward shorter times compared with that at ~45 mV. The traditional interpretation of this decrease is a voltage-driven translocation (31, 43–47), so that the biphasic behavior of the residence time voltage dependence suggests translocation of α-syn through the VDAC pore (Fig. 4).
To rule out the possibility that at higher α-syn concentrations, the residence time in state 1 (B1) and “blocked state 2” (B2), with B2 seen at |V| ≥ 30 mV. The event amplitude histograms show that the relative probability of B2 increases with voltage. The dashed lines indicate open (O) and blocked (B1 and B2) conductance states and zero current. The membrane-bathing solution contained 1 M KCl buffered with 5 mM HEPES, pH 7.4. Current records were additionally filtered using a 5-kHz 8-pole digital Bessel filter. B, voltage dependences of the on-rate of α-syn blockage, τ\text{on}, in the presence of 50 nM α-syn (top panel) and of the residence time, τ\text{off} = (τ\text{on}), where τ\text{on} is the time of the blockage event. The residence time is presented in both logarithmic (middle panel) and linear (bottom panel) scales. τ\text{off}(B1) was calculated as the average time at the first blocked conductance level; τ\text{off}(B1 + B2) gives the average time in the blocked states without discrimination between the two states. Error bar, S.E. C, corresponding log-binned distributions (33) of the open time, τ\text{on}, at −25 mV (a) and of the time of the blockage events, τ\text{off}, calculated for both closed states (B1 + B2) at −35 mV (b) and 45 mV (c) from statistical analysis of the current records at 50 nM α-syn such as those shown in A. Solid lines, logarithmic single exponential fittings with characteristic times (τ\text{on}) equal to 3 ± 0.1 ms (a) and (τ\text{off}) equal to 6.5 ± 0.1 ms (b) and to 4.7 ± 0.1 ms (c). A two-exponential fit of the blockage time histogram (dashed line) at 45 mV (c) with characteristic times of 3.5 and 22.1 ms fits the long-time events satisfactorily but not the short-time blockages.

In the two experiments presented in Figs. 1 and 3, α-syn was added to both compartments. Therefore, when the potential is positive, it drives the anionic C terminus of α-syn in the trans compartment to the pore, and, vice versa, when the potential is negative, it acts on the α-syn terminus in the cis compartment.

To test the suggested importance of the negatively charged C-terminal tail of α-syn for VDAC blockage, we performed experiments with a mutant α-syn N115 (amino acids 1–115) in which the last 23 amino acids representing about half of the C-terminal region were truncated. The ability of this mutant to block VDAC was strongly reduced. Fig. 5A illustrates that 100
nm α-syn N115 starts to induce rare blockage events only at the elevated voltage of 40 mV (top trace). This is in striking contrast with α-syn FL, which, already at 50 nm, induces a massive VDAC blockage at a lower voltage of 20 mV (Fig. 5A, bottom trace). At the same time, a C-terminal peptide, C45, consisting of the last 45 amino acids of α-syn (amino acids 96–140) did not have a measurable effect on VDAC up to 500 nm (Fig. 5B, top trace) even at 45 mV of applied voltage. When 50 nm α-syn FL was added to the cis compartment following the C45 addition, it induced typical VDAC blockages (Fig. 5B, bottom trace), confirming that the channel was fully functioning and that C45 could not block the channel. These experiments demonstrate that the C terminus of α-syn is essential but not sufficient for VDAC blockage. The N terminus of α-syn, which binds to the lipid membranes in a form of an α-helical conformation (16) is also required for this interaction.

A53T and A30P α-syn mutations were identified in rare familial cases of PD (11, 48) and are located in the N-terminal region of α-syn. Therefore, it seemed reasonable to test both mutants for their interaction with VDAC. It turned out that both A53T and A30P mutants induced VDAC blockage similar to the WT. Kinetic analysis of the on-rates and the residence times of VDAC blockage induced by a 50 nm concentration of both mutants in comparison with α-syn WT is shown in Fig. 6. The lack of an effect of physiologically important mutations in the N-terminal domain on both the on- and off-rates of α-syn interaction with VDAC supports our model (Fig. 4) in which the negatively charged C terminus plays an essential role in VDAC blockage.

It is well accepted that the major function of VDAC is to transport and regulate ATP and ADP fluxes across the MOM. ATP permeates through the VDAC open state but not its voltage-induced “closed” state (29). Blockage by α-syn decreases the open channel conductance to a similar extent as the voltage-induced VDAC closure, by ∼60% (B1) or ∼83% (B2). This suggests that α-syn creates a steric obstruction for ATP and ADP translocation through the VDAC pore. The permeability ratios for Cl− and K+ ions, P_{Cl}/P_{K}, measured in a 1.0 M cis versus 0.2 M trans KCN gradient were 1.0 ± 0.2 and 1.3 ± 0.3 (mean values ± S.E. of three experiments) for B1 and B2, respectively. Thus, B1 is essentially non-selective, and the selectivity of B2 is...


only modestly reduced from the open state selectivity characterized by a permeability ratio $P_{Cl}/P_K = 1.5 \pm 0.1$.

It was reported that at concentrations of 140–700 nM, $\alpha$-syn could form pores in the planar membranes, but only in the presence of the negatively charged or nonlamellar lipids (49). In our control experiments without VDAC, a pore formation activity of $\alpha$-syn has never been observed in neutral diphytanoyl-phosphatidylcholine membranes used in our experiments and at $\alpha$-syn concentrations up to 300 nM.

**Yeast Model of $\alpha$-Syn Cytotoxicity—**Experiments with reconstituted VDAC demonstrate that $\alpha$-syn efficiently blocks the VDAC pore, obstructing ADP/ATP exchange between mitochondria and the cytosol. The biphasic character of the residence time voltage dependences also suggests that $\alpha$-syn translocates through the channel, which could lead to $\alpha$-syn targeting of the protein complexes of the electron transport chain in the mitochondrial inner membrane. Therefore, it is natural to ask whether these in vitro results are relevant to the $\alpha$-syn toxicity in living cells. To answer this question, we used a yeast model of PD in which human $\alpha$-syn is expressed in *S. cerevisiae*, which does not contain an endogenous homolog of $\alpha$-syn. We introduced plasmids carrying $\alpha$-syn (controlled by an inducible promoter), human VDAC1 (controlled by a strong constitutive promoter), or empty vectors into the wild type BY4742 strain (Fig. 7, A and B) or the congenic por1Δ strain, which lacks the endogenous major VDAC (Fig. 7, C and D). Plasmid-transformed strains were plated in serial 10-fold dilutions on medium supplemented with galactose (inducing conditions, $\alpha$-syn expressed) or glucose (repressing conditions, $\alpha$-syn not expressed) and grown at 37 °C or 30 °C for 3 days. Wild type yeast with or without hVDAC1 alone grew well on galactose medium (Fig. 7A, lanes 1 and 4). In contrast, wild type yeast expressing $\alpha$-syn alone grew slowly on galactose (lane 1 versus lane 2), and yeast expressing both hVDAC and $\alpha$-syn exhibited a strong inhibition of growth on galactose (lane 3). All plasmid-transformed, wild type yeast grew equally well on glucose, indicating that growth differences between strains were due to plasmid-encoded gene expression and not due to unequal plating. Western blot analysis confirmed the expression of $\alpha$-syn and hVDAC1 (at low levels) in yeast (Fig. 7B).


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In contrast to the wild type strain, expression of α-syn alone in the 
por1Δ strain, which lacks the major endogenous VDAC, did not cause slowed growth on galactose medium (Fig. 7C, lanes 1 and 2). Expression of high levels of hVDAC in the por1Δ strain led to moderate inhibition of growth, both on glucose and galactose (lanes 3 and 4), but co-expression of both α-syn and hVDAC in the por1Δ strain led to a very strong inhibition of growth on galactose (lane 3). Again, Western blotting confirmed expression of both exogenous genes in the por1Δ strain (Fig. 7D). Together, these data indicated that co-expression of α-syn and VDAC (either yeast or human) produced cytotoxicity and that the toxic effects of α-syn were absent in yeast lacking VDAC. This genetic interaction between α-syn and VDAC is consistent with a physical interaction between the two proteins in cells.

**Discussion**

To summarize, we propose a model of α-syn interaction with VDAC, in which the negatively charged C terminus of α-syn enters the net positive VDAC (Fig. 4). At small applied voltages, the C terminus moves back and forth, with the α-helical bundle of the N terminus bound to the membrane, thus preventing translocation and increasing the residence time of the C terminus inside the pore (Fig. 4). This mechanism is similar to that suggested for the effect of tubulin’s negatively charged C-terminal tails on VDAC (50). However, contrary to the findings with tubulin, at $|V| > 43$ mV, the driving force of the applied potential acting on the negatively charged C terminus is large enough to detach the helical N-terminal part of α-syn from the membrane surface and allow the whole molecule to translocate through the channel. Based on the finding that the probability of the second blocked state, B2, increases with α-syn concentration faster than that of the first blocked state, B1, (compare traces at 50 and 1 nM in Figs. 1A and 3A), we suggest that B2 is not a signature of translocation through the pore (51) but rather is a result of a second α-syn molecule interacting with the pore when the first molecule is already there. The internal dimension of the VDAC pore of ~2.7 nm in its narrowest part (52) is sufficient to accommodate two disordered polypeptides.

A propensity of α-syn for aggregation in solution and at membrane surfaces is well known (16, 53), but aggregation is unlikely under our experimental conditions. α-Syn aggregation in solution occurs at orders of magnitude higher concentrations (hundreds of μM) (54) than those that have been used in our experiments (tens of nM). It was shown that the presence of the negatively charged lipid is a requirement for α-syn aggregation at the membrane surface (16, 55) and that there is no noticeable aggregation at concentrations below tens of μM (56). These results suggest that a likelihood of measurable aggregation of α-syn at the surface of neutral membrane (diphytanoylphosphatidylcholine) and at a 1–100 nM concentration range of α-syn is negligibly low. The same is true for the C terminus-truncated α-syn N115. It was shown in a number of publications (e.g. Refs. 54, 57, and 58) that C-terminal deletion increases aggregation in comparison with α-syn WT. However, the aggregation could be achieved only at concentrations of 100–700 μM truncated α-syn, orders of magnitude higher than the concentrations used in our experiments and only under conditions of constant shaking for 1–3 days at 37 °C (57). Serpell et al. (54) reported that the minimal concentration for the assembly of the C terminus-truncated human α-syn is 50 μM, which is still well above concentrations that have been used in our work. Thus, in our experiments, α-syn exists in a predominantly monomeric form.

The kinetic analysis of α-syn binding to VDAC suggests at least two physiological consequences for mitochondrial function (Fig. 8). First, α-syn sterically blocks the VDAC pore and thus obstructs fluxes of ATP and ADP across the channel. This leads to a depletion of the electron transport chain complexes of cytosolic ADP, inducing dissipation of the mitochondrial transmembrane potential (59), and eventually to a decrease of oxidative phosphorylation. Likewise, the reversible blockage of VDAC by α-syn may constitute a regulation mechanism of normal mitochondrial respiration in neurons through the modulation of VDAC permeability, pointing to a non-pathological function of monomeric α-syn. This could be a step forward in understanding the involvement of endogenous α-syn in maintaining normal mitochondrial bioenergetics, which is currently an open question (6, 60). Second, our data suggest that VDAC is able to serve as a pathway for α-syn into the intermembrane space, which may result in its direct interaction with the elec-
We speculate that other proteins in the mitochondrial intermembrane space might also interact with α-syn. In this case, α-syn translocation into the intermembrane mitochondrial space could have a potentially significant impact on mitochondrial dysfunctions. In neurons or cancer cells with overexpressed α-syn, for example, the mitochondria exhibited a loss of oxidative phosphorylation capacity and enhanced generation of ROS (14, 64). This mechanism (Fig. 8) also explains the co-localization of α-syn with the MOM (10, 19) and with the inner membrane (6) because our data suggest that α-syn may associate with either mitochondrial membrane. Depending on physiological conditions in the cell, such as the total level of mono- and/or VDAC-facilitated α-syn translocation. Studies in yeast have demonstrated association of α-syn with the MOM (65). Toxicity of α-syn in the yeast model of PD depends on mitochondrial function and ROS production by mitochondria (66). Mitochondrial dysfunction is the first symptom of α-syn toxic-
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In addition, interaction of α-syn with VDAC is highly voltage-dependent. Therefore, at 10–20 mV, IC_{50} of α-syn-VDAC binding is in the range of 3–100 μM (Fig. 2B), which is already physiologically relevant. This immediately raises a question about the potential across the MOM in vivo, which is usually believed to be close to zero due to the presence of VDAC pores in the MOM. The actual potential across the MOM, including its possible variations with the mitochondrial state, is still a subject of ongoing debate (21). The estimates for the voltage span from 10 mV (75) to as high as 46 mV (76). Depending on α-syn concentration, which might reach ~20 μM in synaptic terminals (77), a 10-mV potential could be enough to promote a significant α-syn binding to VDAC (Fig. 2B). By contrast, translocation of α-syn through VDAC requires relatively high voltages of more than 40 mV, which may seem to be unrealistically high for the potential across the MOM. Interestingly, a recently published model (41) suggests that a VDAC complex with hexokinase is a generator of the potential across the MOM with the Gibbs free energy of kinase reactions being used as a driving force. In the framework of this model, the estimated MOM potential is as high as 50 mV, negative at the cytoplasmic side of the MOM. Whatever the MOM potential, it is important to note that α-syn translocation through VDAC, which requires relatively large voltages under the grossly simplified conditions of our reconstitution experiments, may occur much more readily in the crowded, compartmentalized cell environment and in the presence of different chaperons.

Conclusions

Using the channel reconstitution technique, we have demonstrated the existence of a functional interaction between α-syn and VDAC that suggests a previously unknown mechanism of MOM permeability regulation. Our data indicate that α-syn is able to both block VDAC in a concentration- and voltage-dependent manner and translocate via this channel across the MOM. We have also explored a yeast model of PD and shown that α-syn toxicity in yeast depends on VDAC, thus supporting our in vitro results. Based on these findings, we believe that our study reveals the evasive physiological and pathophysiological roles for monomeric α-syn and reconciles previous observations of various α-syn effects on mitochondrial bioenergetics.

Author Contributions—T. K. R., P. A. G., and S. M. B. initiated the project. T. K. R., P. A. G., O. P., and S. M. B. designed all experiments, performed data analysis, and wrote the manuscript. T. K. R., P. A. G., and O. P. performed experiments. D. P. H. performed data analysis. T. L. Y. and J. C. L. purified and characterized proteins. D. P. H., J. C. L., and C. C. P. contributed to manuscript writing. All authors reviewed the results and approved the final version of the manuscript.

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**α-Synuclein Regulates VDAC Permeability**

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