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Alpha-Synuclein Binds to the Inner Membrane of Mitochondria in an α-Helical Conformation

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The human alpha-Synuclein (αS) protein is of significant interest because of its association with Parkinson's disease and related neurodegenerative disorders. The intrinsically disordered protein (140 amino acids) is characterized by the absence of a well-defined structure in solution. It displays remarkable conformational flexibility upon macromolecular interactions, and can associate with mitochondrial membranes. Site-directed spin-labeling in combination with electron paramagnetic resonance spectroscopy enabled us to study the local binding properties of αS on artificial membranes (mimicking the inner and outer mitochondrial membranes), and to evaluate the importance of cardiolipin in this interaction. With pulsed, two-frequency, double-electron electron paramagnetic resonance (DEER) approaches, we examined, to the best of our knowledge for the first time, the conformation of αS bound to isolated mitochondria.

The protein alpha-Synuclein (αS), a major component of Lewy bodies, is of particular interest because of its association with Parkinson's disease (PD) and related neurodegenerative disorders.[1] The 140-amino-acid intrinsically disordered protein[2] is characterized by the absence of a well-defined structure in solution, and displays remarkable conformational flexibility upon macromolecular interaction.[3,4] Although the exact function(s) of αS remains unknown, recent reports have highlighted its roles in mitochondrial dysfunction,[5] oxidative stress,[6] mitochondrial lipid abnormalities,[7] and the mitochondrial fusion–fission cycle.[8] Artificial-membrane binding studies with various techniques, including EPR (electron paramagnetic resonance) spectroscopy, have shown that the N-terminal part of αS (~100 residues) preferentially binds to negatively-charged lipids and acquires an alpha-helical structure, whereas the negatively charged C terminus remains unbound and unstructured.[9–11] EPR measurements have been used to unravel the conformation of membrane-bound αS.[12–15] The protein can associate with mitochondrial membranes in different ways,[16,17] but its modes of binding[18] have been only sparsely characterized. Site-directed spin-labeling[19] in combination with EPR spectroscopy[20] enabled us to study the local binding properties of αS with artificial membranes mimicking the inner (IMM) and outer (OMM) mitochondrial membranes, and to assess the importance of cardiolipin (CL) in this interaction.[7] With the aid of double-electron electron paramagnetic resonance (DEER; a pulsed, two-frequency EPR method),[21] we examined—to the best of our knowledge for the first time—the conformation of αS bound to isolated mitochondria.

We isolated mitochondria from human HEK293 cells.[22] Biochemical analysis and immunogold electron microscopy studies showed high purity of the mitochondrial fraction and intact mitochondria upon binding of αS (Supporting Information).

Binding of wild-type αS (αSwt) was detected in gel electrophoresis experiments (Figure 1). A band at 17 kDa[23] (mitochondria-bound αSwt) increased in intensity with increasing

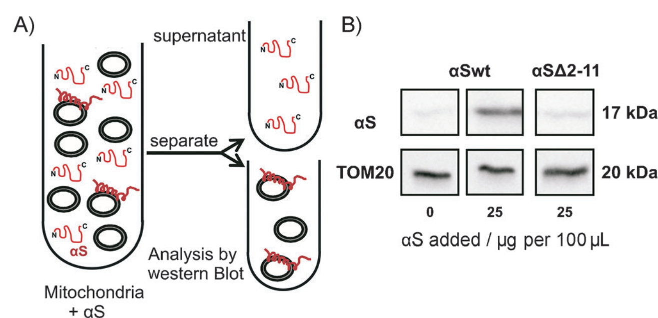

Figure 1. A) Isolated mitochondria were incubated for 5 min with recombinant wild-type αS (αSwt) or with αS lacking N-terminal amino acids 2–11 (αSΔ2–11). Unbound αS was removed by centrifugation. B) The pellet was subjected to western blot analysis. To ensure equal amounts of mitochondria in all samples, the OMM protein TOM20 was stained as a loading control.

αSwt concentrations. The absence of significant binding for αSΔ2–11 (αS lacking amino acids 2–11) shows that the N terminus of the protein is also crucial for binding to mitochondria.[18,23]

We prepared nine αS derivatives, spin-labeled at single cysteines introduced at positions 9, 18, 27, 35, 41, 56, 69, 90, and 140 (αS9, αS18, etc.; Figure S1 in the Supporting Information). EPR spectroscopy was used to quantify the local interaction of αS with large unilamellar phospholipid vesicles (LUVs) mimicking either the IMM or the OMM. We employed LUVs with a mixture of POPC/POPE/Ch (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phos-

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phocholine/1-hexadecanoyl-2-(9*Z*-octadecenoyl)-*sn*-glycero-3-phosphoethanolamine/cholesterol; molar ratio 4.0:2.0:0.9), which corresponds to the composition of the OMM; LUVs corresponding to the IMM were composed of POPC/POPE/cardiolipin(CL)/Ch (2.0:1.3:1.0:0.6, м/м/м/м).[24] The spectra of αS9 in the presence of OMM LUVs (representative spectra in Figure 2A, blue lines) consisted of three narrow lines with no significant difference in shape from the αS spectra obtained in solution. In the presence of IMM LUVs, the spectra of almost all mutants showed drastic changes (Figure 2B, red lines, experimental spectra and corresponding fits in the Supporting Information). The observed changes in the spectral shape are indicative of lower mobility of the spin labels as a result of local membrane binding.[23] Only the spectra of αS140 remained unchanged upon addition of LUVs, as expected for the unbound C-terminal tail of the protein.[9] These observations show that the N-terminal region of αS binds significantly to IMM LUVs but not to those mimicking the OMM, thus indicating the importance of CL in the binding. It is noteworthy that αS has been shown to translocate across the plasma membrane[25,26] (mediated by its N-terminal motif),[27] and it is thus reasonable to expect that it is capable of translocating across the OMM to give access to the IMM.

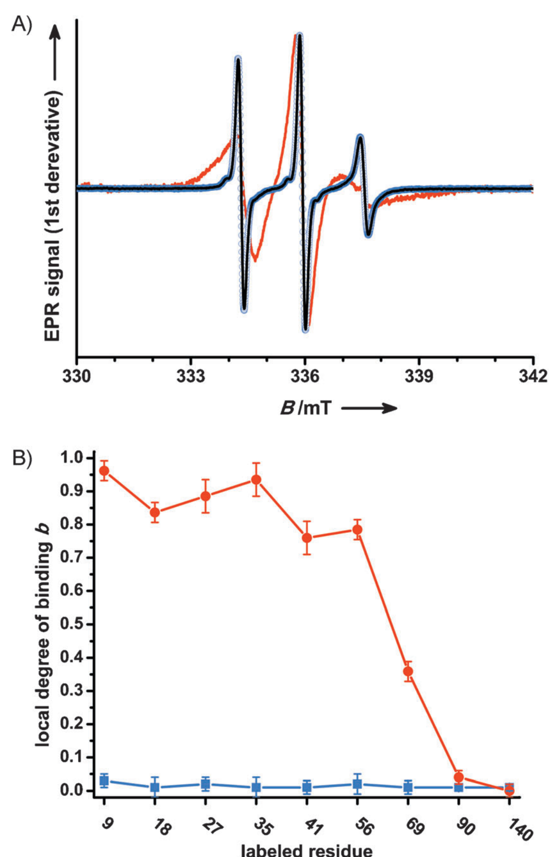

Figure 2. A) Spectra of αS9 in solution (black line) and in the presence of IMM LUVs (red) and OMM LUVs (blue); B) Local degree of binding in the proximity of investigated αS residues in the presence of LUVs mimicking IMM (red) and OMM (blue).

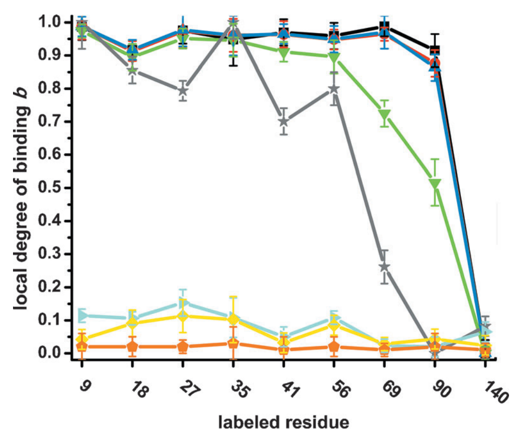

Figure 3. Local degree of binding in the proximity of different residues of αS in the presence of LUVs with different charge surface density (ρ) made from diverse CL/POPC compositions ($\rho=0$ orange, $\rho=0.05$ yellow, $\rho=0.1$ cyan, $\rho=0.2$ grey, $\rho=0.3$ green, $\rho=0.5$ blue, $\rho=0.7$ red, and $\rho=1$ black).

As CL is mitochondria-specific,[28] we obtained spectra to investigate the effect of CL concentration on the binding of αS to POPC/CL LUVs (Figure 3). The CL content results in a surface charge: $\rho=[CL]/([CL]+[POPC])$. The spectra can be analyzed by superposition of two spectral components featuring different rotational mobilities: a slow component (S_B) and a fast component (S_A; $S=(1-b)S_A+bS_B$, spectra and corresponding simulations in the Supporting Information). For the spectral simulations, raw experimental spectra of the respective singly labeled αS mutants in solution were used as S_A. For S_B, the simulation parameters of the singly labeled mutants on CL LUVs ($\rho=1$) were used, where quantitative binding of the protein was detected. The remaining parameter to be fitted was *b*, which reflects the local degree of binding proximal to the labeled position (for simulation parameters see the Supporting Information). Figure 3 shows the changes in *b*, that is, the degree of local binding as a function of the labeled position. At low CL content (up to $\rho=0.1$) there was almost no binding for all of the investigated αS residues. At $\rho=0.2$ (corresponding approximately to the CL content of the IMM), a drastic increase in the degree of local binding of the N-terminal part of the protein was observed, whereas the C-terminal tail of the protein did not bind.

As EPR spectroscopy in combination with site-directed spin-labeling is virtually background free, it can be exploited to study αS binding not only to artificial membranes but even in a complex environment, for example to human mitochondria. In order to unravel the conformation of mitochondrial membrane-bound αS, we exploited the doubly-labeled αS mutant αS9/27. These labeling positions were chosen because they are in the N-terminal part of αS (high local binding affinity according to Figures 2 and 3). NMR data suggest that these residues point outwards to the aqueous environment.[11] We used singly labeled αS9 and αS27 to monitor the local binding affinity to mitochondria (Supporting Information). Under the conditions used, we observe that more than 60% of the molecules

bound to mitochondria (at least for the 27 N-terminal residues). However, the results suggest that the fraction of αS bound varied significantly between different samples, thus illustrating the exceedingly difficult nature of these kinds of experiments. Therefore, we decided to determine the fraction of N-terminally bound αS in the very same sample to be used for distance measurements, that is, αS9/27. Figure 4 shows data

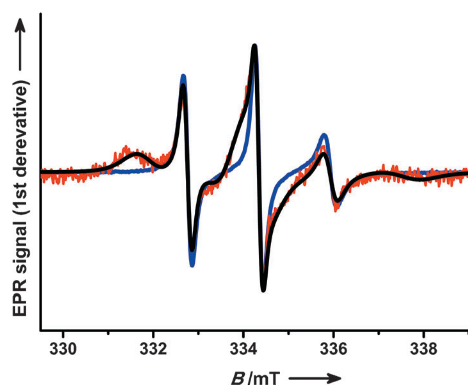

Figure 4. Spectra of αS9/27 in the absence (blue) or presence (red) of mitochondria. The low concentration (~8 μM) of labeled αS in the mitochondria sample results in a more noisy spectrum; this could be accurately analyzed by spectral simulation (black fit).

from the corresponding EPR experiment measuring binding of αS9/27 to mitochondria. The spectrum of αS9/27 in the presence of mitochondria (red curve) clearly differs from that without mitochondria (blue curve). The spectral simulation of the former (black fit, see the Supporting Information) indicates that under these conditions (75 ± 5) % of the αS9/27 molecules bound to the mitochondria.

In order to monitor the conformation of αS bound to mitochondria, EPR distance measurements were performed (Figure 5 A). As a reference, we measured αS9/27 in solution (blue) as well as in the presence of artificial membranes made from POPG lipids, for which quantitative binding has been reported (green).[23] Figure 5 B shows the corresponding distance distributions (blue) for αS9/27 in its disordered state as well as in its alpha-helical state bound to artificial POPG membranes. The latter is in full agreement with the theoretically predicted distance distribution (Figure 5 B, black) based on the NMR structure of micelle-bound α-helical αS (Figure 5 C). The model assumes a completely rigid macromolecular conformation and accounts for flexibility of the spin-label linker by a rotamer library approach.[29] The width of the distance distribution for αS9/27 measured in solution was only slightly broader than for the membrane-bound state. This means that αS in solution on the corresponding length scale is structurally more defined than would be expected for a random coil.[30]

The distance distribution obtained for αS9/27 in the presence of mitochondria was obtained by using a model-free Tikhonov regularization procedure (resulting distance distribution in Figure 5 B, red circles). In spite of the rather low signal-to-noise ratio (because of the low concentration of αS9/27 in the mitochondrial sample), this distance distribution can be described by a superposition (red line) of the distance distribution of intrinsically disordered αS9/27 and the distance distribution of alpha-helical membrane-bound αS9/27. The fraction of the latter (69 %) corresponds well with the estimate of the bound fraction determined independently by the mobility measurement (Figure 4).

We thus observed directly, for the first time to the best of our knowledge, an α-helical binding mode in the complex environment of the intact mitochondrial membrane, a setting that is difficult to access spectroscopically. This helical binding mode is in accordance with that reported for αS interacting with artificial membranes. αS does not interact with membranes mimicking the OMM but binds to membranes mimicking the IMM (Figure 2), most likely attributable to the CL content of the inner membrane (Figure 3). Finally, we show that, as expected, αS binding is triggered by the N-terminal region of the protein (Figures 2 and 3).

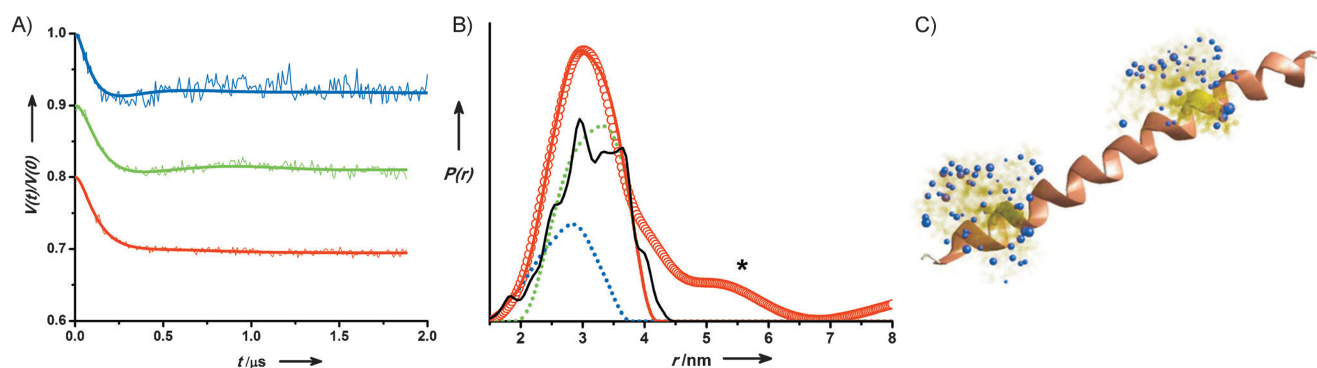

Figure 5. A) DEER data for αS9/27 with background correction in solution (blue), in the presence of POPG LUVs (green), and in the presence of mitochondria (red), with corresponding fits (solid lines). B) Corresponding distance distributions of αS9/27 in its intrinsically disordered state (blue dotted), in its alpha-helical state (experimental: green dotted, theoretical: black) and in the presence of mitochondria (red circles). The latter can be described by a superposition (red line) of the experimental distance distributions obtained for αS9/27 in solution and in the presence of POPG LUVs. Asterisks mark artefacts due to Tikhonov regularization (Supporting Information). C) Visualization of the rotamer approach used for theoretical prediction of the distance distribution for αS9/27 in its membrane-bound state (model PDB ID:1XQ8). Purple spheres show the positions of the electron spins and are scaled according to the populations of the respective rotamers.

Experimental Section

Mitochondria were isolated from HEK293 cells by using a commercially available kit (Qproteome Mitochondria Isolation Kit, Qiagen). The LUVs were made by extrusion through 100 nm polycarbonate membrane. αS cysteine mutants were labeled with 3-maleimido-PROXYL spin label. DEER experiments were performed in Q-band at 45 K with an Elexsys E580 spectrometer (Bruker); data were analyzed by using DEERAnalysis2011[31] and model-free Tikhonov regularization.[32] For details of sample preparation, measurements and analysis, see the Supporting Information.

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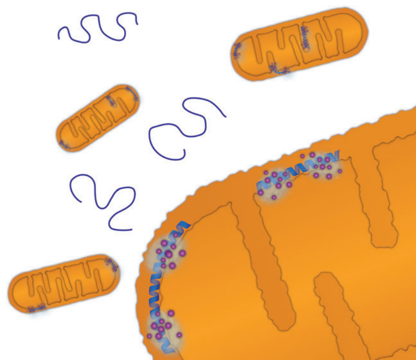
The complex environment of mitochondria requires background-free methods to determine the conformation of membrane-bound alpha-synuclein (αS). EPR spectroscopy in combination with site-directed spin labeling revealed that the α-helical binding mode often observed for αS interacting with artificial membranes also holds for the inner membrane of mitochondria.



Alpha-Synuclein Binds to the Inner Membrane of Mitochondria in an α-Helical Conformation