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In vivo EPR on spin labeled colicin A reveals an oligomeric assembly of the pore-forming domain in E. coli membranes

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Content

Materials and Methods

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Expression and purification of ColA wt and Cys mutants

Escherichia coli K-12 C600 mutants containing single cysteine mutations in the *caa* gene at positions 77 and 192 were prepared (the latter was kindly provided by Dr. D. Duche[1]) and the protein was purified as described[2] with the following changes of the published protocol: For the isolation of colicin A from the *E. coli* membrane fraction the cell pellet was resuspended in a small volume of culture medium and treated with lysozyme (0.5 mg/ml), DNase and 0.5 mM phenylmethylsulfonylfluoride (PMSF). Subsequently, cells were disrupted by sonication and solubilized with 2 % dodecyl-maltoside (DDM) overnight at 4°C. Afterwards, the crude extract was centrifuged for 20 min at 4500 rpm and 4°C to separate the cell debris. The supernatant was added to the rest of the culture medium and further processed as described before.[2]

Site-directed spin labeling

For labeling with MTSSL ((1-oxy-2,2,5,5-tetramethyl-pyrrolinyl-3-methyl) methanethiosulfonate) DTT was added to purified colicin A to a final concentration of 10 mM and incubated overnight at 4°C. DTT was removed by exchanging the buffer 5 times with 50 mM potassium phosphate buffer (pH 6.8) in centrifugal filter units (Millipore). Subsequently, a spin label stock solution (100 mM in acetonitrile) was added to the protein solutions to reach a final MTSSL concentration of 1 mM and incubated overnight at 4°C. Unbound spin label was removed again by buffer exchange (10 mM potassium phosphate buffer pH 6.8). Spin-labeled colicin A was stored at -80°C unless directly used for the EPR experiments. Spin labeling efficiencies have been determined by double integration of the first-derivative EPR spectra and comparison with references samples of known (spin) concentration. For both ColA cysteine mutants the labeling efficiency has been determined to be ~ 90-100%.

Viability test

500 µl of an *E. coli* K12 C600 culture that has been incubated overnight at 37°C was placed on a LB agar plate and spread with glass spreader. 1 µl of colicin A solutions with 5 different dilutions (from 10^0 to 10^5, starting from a 2 mg/ml ColA stock solution) were placed on the agar plates and they were incubated overnight at 37°C. After incubation the diameter of the bacteria free areola was determined.

Preparation of ColA-proteoliposomes

For the preparation of liposomes, native *E. coli* phospholipids (Polar Lipid Extract, Avanti Polar Lipids, Alabaster, USA) were used. Dried lipids were hydrated in 50 mM potassium buffer, pH 6.8 to a final concentration of 20 mg/ml and sonicated until a homogeneous solution was obtained. The resulting suspension 3 freeze-thaw cycles and stored at -80°C. Prior to use for reconstitution of colicin A the suspension was thawed slowly at room temperature and extruded (Avanti Mini-Extruder, Avanti Polar Lipids, Alabaster, USA) 11 times through 400 nm polycarbonate filters (Whatman, Dassel, Germany).

For protein reconstitution the liposomes were solubilized by stepwise addition of 0.1 % Triton X-100 (v/v, 50 mM NaPi, pH 6.8) until the turbidity of the solution (monitored at a wavelength of 540 nm) changes. The solubilized liposomes were mixed with spin labeled colicin A in a molecular ratio of 1:500 unless states otherwise and incubated by slowly seesawing for 30 min at RT. Afterwards the detergent was removed using washed (50 mM NaPi, pH 6.8) detergent removal beads (Calbiosorbs, Calbiochem). After removing the beads, the solution was centrifuged for 1.5 h at 80,000 rpm at 4°C (Sorvall Discovery, Thermo Fisher). The resulting pellet was resuspended in 50 µl buffer (50 mM NaPi, pH 6.8), frozen in liquid nitrogen, subjected to 3 freeze-thaw cycles and stored at – 80°C if not subjected directly to EPR experiments.

Sample preparation for EPR measurements on living E. coli cells incubated with spin labeled ColA

100 ml *E. coli* Origami cell culture (LB medium) was cultivated overnight at 37°C. This culture was used to inoculate 100 ml LB medium to obtain an optical density OD_{600} = 0.1. The cultures were further grown at 37°C. At an OD_{600} = 0.5 the cells were harvested by centrifugation at 4500 rpm, for 15 min at 4°C (Beckmann Coulter Allegra-X-15R, SX4750A). Cell pellets were resuspended in 2 ml 50 mM sodium phosphate buffer (pH 6.8), containing 0.2 % glucose and 0.3 mM KCl, and incubated on ice for 30 minutes. Afterwards the cells were harvested again by centrifugation and resuspended in 1 ml of the same buffer. 2 mg/ml of spin-labeled colicin A was added to 100-200 µl of the cell suspension. For cw EPR experiments at room temperature, potassium ferricyanide ($K_3[Fe(CN)_6]$) was added at a final concentration of 1 mM. After incubation for ~ 2 minutes the cells were pelleted at 13000 rpm, 4°C for 1 minute. The cell pellet was washed two times with 100-200 µl 50 mM sodium phosphate buffer (pH 6.8), 0.2 % glucose and 0.3 mM KCl to remove unbound colicin A. Finally, the cell pellet was resuspended in 30 µl of the same buffer containing 20 % deuterated glycerol and transferred into 3 mm (outer diameter) EPR quartz capillaries and immediately frozen in liquid nitrogen for the DEER measurements.

EPR spectroscopy

Room temperature continuous wave EPR spectra were obtained using a Magnettech (Berlin, Germany) Miniscope MS200 X-band benchtop spectrometer. All spectra were obtained at an incident microwave power of 10 mW and a field modulation of 0.15 mT with the scan time of 60 s. Glass capillaries with 1 mm diameter were used to load the samples.

Determination of rotational correlation times from room temperature cw EPR spectra

To obtain the rotational correlation times of the spin labeled side chains the stochastic Liouville equation is solved numerically using a series expansion. It includes the spin hamiltonian, the spin density and diffusion terms.[3] For the two spectral components the same A- and g-tensors have been used. Fitting of simulated spectra to the experimental data has been carried out with the program 'multicomponent' written by C. Altenbach.[4]

DEER spectroscopy

Pulse EPR experiments (DEER) were performed at X-band frequencies (9.3–9.4 GHz) with a Bruker Elexsys 580 spectrometer equipped with a Bruker Flexline split-ring resonator ER 4118X-MS3. 3 mm outer diameter EPR quartz capillaries were loaded with 25-50 µl of protein/liposome solution or bacteria in culture medium, both containing 20 % of deuterated glycerol. Temperature control was achieved using a continuous flow helium cryostat (ESR900; Oxford Instruments) regulated by an Oxford temperature controller ITC 503S. All measurements were performed using the four-pulse DEER sequence:[5]

$$\pi/2(\upsilon_{obs}) - \tau_1 - \pi\ (\upsilon_{obs}) - t' - \pi\ (\upsilon_{pump}) - (\tau_1 + \tau_2 - t') - \pi\ (\upsilon_{obs}) - \tau_2 - echo$$

For pulses at the observer frequency the ‹x› channels were used. A two-step phase cycling (+ ‹x›, - ‹x›) is performed on $\pi/2(\upsilon_{obs})$. Time t' is varied, whereas τ_1 and τ_2 are kept constant, and the dipolar evolution time is given by $t = t' - \tau_1$. Data were analysed only for $t > 0$. The resonator was over coupled to Q ~ 100; the pump frequency υ_{pump} was set to the center of the resonator dip and coincided with the maximum of the nitroxide EPR spectrum, whereas the observer frequency υ_{obs} was 67 MHz higher and coincided with the low field local maximum of the spectrum. All measurements were performed at a temperature of 50 K with observer pulse lengths of 16 ns for $\pi/2$ and 32 ns for π pulses and a pump pulse length of 12 ns. Deuterium modulation was averaged by adding traces at eight different τ_1 values, starting at $\tau_{1,0} = 400$ ns

and incrementing by $\Delta\tau_1 = 56$ ns. Analysis of the DEER traces was performed with the software Deer Analysis 2011.[6] Briefly, the phase corrected dipolar evolution data was background corrected assuming a homogeneous background, and distance distributions were obtained by assuming a single Gaussian distance distribution.

The number of coupled spins in a biomolecule can be determined from the modulation depth of the dipolar evolution function after background correction.[7] The modulation depth Δ is given by the equation $\Delta = 1 - (1-\lambda)^{N-1}$, where N describes the average number of interacting spins in the observed nanoobject and λ the fraction of spins excited by the pump pulse (inversion efficiency).[7]

References

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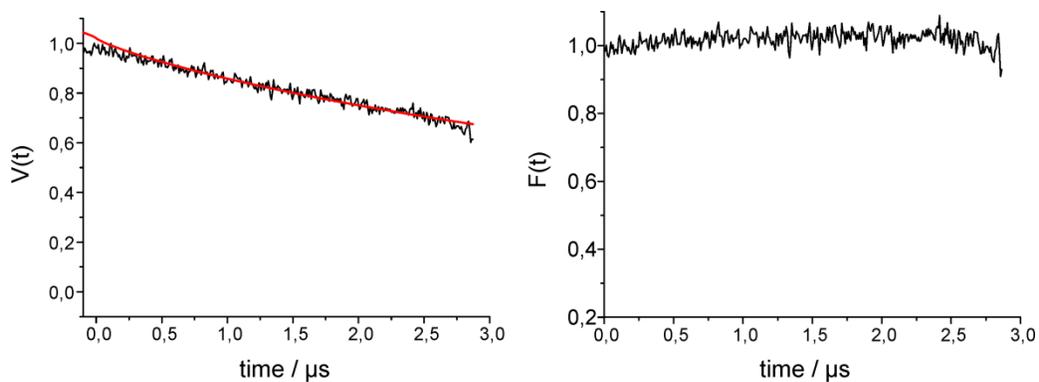
Fig. S1 DEER measurements on soluble ColA-A192R1. Left panel: Raw DEER data, V(t), with the corresponding background fit (red), assuming a homogeneous 3-dimensional spin distribution in the sample. Right panel: Resulting form factor F(t), showing the absence of dipolar modulations and thus supporting the notion that ColA in solution is predominantly monomeric.
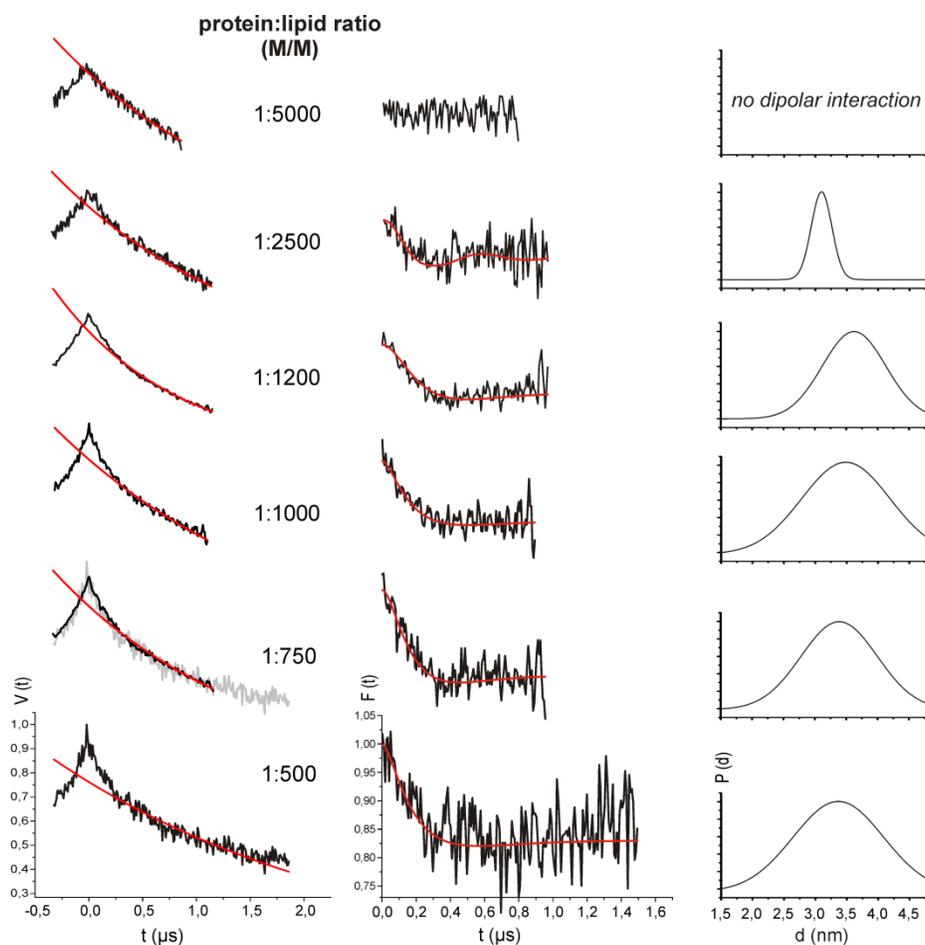
Fig. S2 DEER measurements on a dilution series with reconstitutions of ColA-A77R1 carried out with different protein:lipid ratios (1:500 – 1:5000 (M/M). Left panel: Raw DEER data, V(t), with the corresponding background fit (red). Middle panel: Resulting form factor F(t) with fits obtained by assuming a single gaussian distance distribution (red). Right panel: Resulting distance distributions.

The overlay of the raw DEER data obtained at the ratios 1:750 and 1:500 (grey trace) shows that the V(t)'s are similar within the experimental error (noise level).

Remarkably, the inter-spin distances in the range of about 2-5 nm for ColA-A77R1 on helix H4 suggests, together with the similar distance range observed for ColA-A192R1 on H10, that multiple helices might form the dimerization/oligomerization interface(s).

An analysis of the data in terms of determination of the dissociation constant for ColA oligomerization from the modulation depths of the DEER form factors has not been carried out due to significant uncertainties in the protein:lipid ratios of the final samples and in the modulation depths values.