J|A|C|S COMMUNICATIONS

Published on Web 01/15/2010

PELDOR Spectroscopy Reveals Preorganization of the Neomycin-Responsive Riboswitch Tertiary Structure

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Riboswitches are *cis*-acting RNA regulatory elements that control gene expression in the cell by sensing and quantifying the levels of specific small molecules.[1] These structured domains mostly reside in the 5′-untranslated regions of mRNAs. They bind their target molecules with high affinity and specificity and undergo conformational changes leading to transcription termination or translation inhibition. Using a novel, combined approach of *in vitro* selection and *in vivo* screening, an engineered RNA aptamer that is functional as a synthetic riboswitch in yeast has been identified.[2] This aptamer exhibits neomycin-dependent regulation of gene expression by interfering with translation initiation upon ligand binding. This 27 nt RNA element is the smallest functional riboswitch element known to date.

Neomycin B is a broad-spectrum aminoglycoside antibiotic with activity against Gram-positive and Gram-negative bacteria. It binds to the decoding site (A-site) on 16S rRNA and thereby inhibits protein biosynthesis.[3] This aminoglycoside also binds and inhibits the function of group I introns,[4] the Rev-responsive element of HIV-1,[5] the human hepatitis delta virus ribozyme,[6] and the hammerhead ribozyme.[7]

Electron paramagnetic resonance (EPR),[8] fluorescence resonance energy transfer,[9] and especially nuclear magnetic resonance[10] are highly utilized spectroscopic methods to elucidate structural information of complex biopolymers under biologically relevant conditions. Pulsed electron double resonance (PELDOR)[11] spectroscopy has established itself as a powerful tool to measure long-range distances (15−80 Å) in macromolecular systems such as polymers,[12] oligonucleotides,[13] and proteins,[14] using the dipolar spin−spin interaction between two paramagnetic centers. It allows us to investigate the global architecture and related structural changes of biomacromolecules in frozen samples.

The aim of the present study is to probe the conformational rearrangement of the neomycin-responsive riboswitch upon ligand binding using site-directed spin labeling of oligonucleotides with the nitroxide spin label 2,2,5,5-tetramethyl-pyrrolin-1-oxyl-3-acetylene (TPA)[15] and PELDOR spectroscopy. The rigidity of the TPA leads to relatively narrow spin−spin distance distribution. This small spin label provides a good correlation of an interspin distance with the structure of the nucleic acid polymer and thus can be used as a paramagnetic reporter of the orientation and dynamics of the nucleotide base.

Four double-labeled and five single-labeled aptamer mutants were synthesized via a Sonogashira cross-coupling reaction.[15] Optical melting experiments were performed to investigate the stability of the secondary structure of the unlabeled and labeled neomycin aptamers. The UV melting curves for each sample demonstrate a single cooperative and reversible melting transition. The modified RNA molecules have melting temperatures less than 3.5 °C lower than the unmodified RNA, indicating that the spin labels do not considerably disturb the secondary structure of the RNA (Table S2). Evidence of neomycin binding to the aptamer was provided by two independent experimental methods. First, measurement of UV melting curves upon addition of 1 equiv of neomycin (Figure S2) shows thermal stabilization of 20 ± 3 °C for the labeled sample, similar to the unmodified aptamer. Second, continuous-wave (cw) EPR spectra of the spin-labeled RNA alter upon the addition of neomycin clearly demonstrating that the local RNA dynamics at room temperature change as a result of introducing neomycin in the solution (Figures S8 and S9).

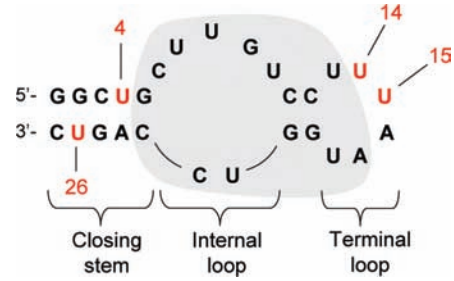

Figure 1. Secondary structure of the neomycin-responsive riboswitch with spin-labeled uridines (red). The putative binding pocket is highlighted in gray.

The nucleotide positions for the double-labeled samples U4−U14, U4−U15, U14−U26, and U15−U26 were chosen outside of the binding pocket (Figure 1) to prevent interference with binding. In addition, the interspin distances were expected to be effected if ligand binding induces alteration in the overall structure. U4 and U26 reside in the closing stem, while U14 and U15 are located in the terminal loop.[2] If there is a buckle of the terminal loop over the internal asymmetrical loop induced by association with the ligand, that should be noticeable in PELDOR as a change in the distance between the spin labels.

Dipolar evolution function with a good signal-to-noise ratio and modulation depth was observed for all double-labeled samples (Figure 2). Visible dipolar oscillations for the U14−U26 and U15−U26 labeled pairs derived well-defined distances providing evidence that the RNA aptamer was tertiary folded. In the case of the U4−U14 and U4−U15 samples, dipolar oscillation was overdamped leading to a broader distance distribution, presumably due to a larger conformational heterogeneity experienced by the labeled nucleobases. Nevertheless, good modulation depth dem-

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10.1021/ja9077914

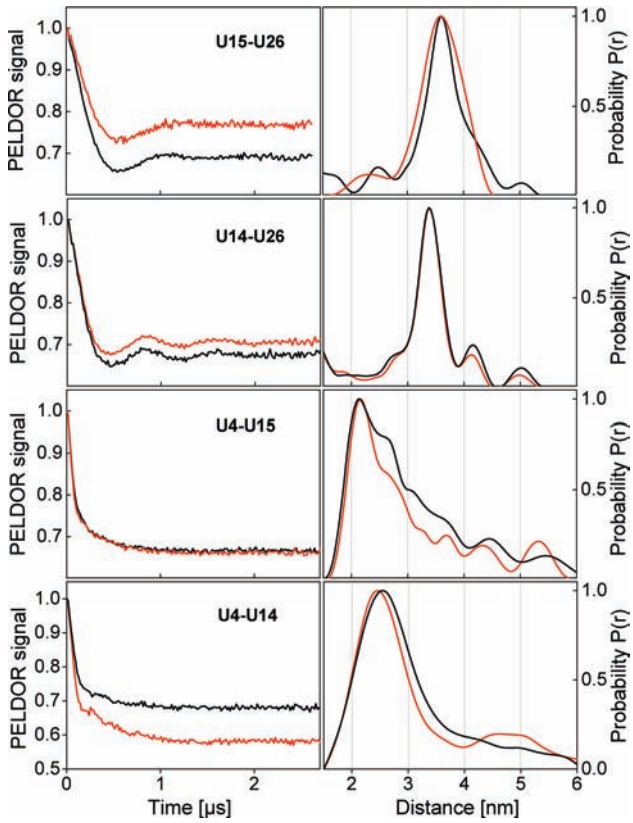

Figure 2. Left column: background corrected PELDOR time traces of double-labeled neomycin-responsive riboswitch samples with neomycin in ratio 1:1 (red) and without neomycin (black) using ethylene glycol as cryoprotectant. Right column: distance distribution derived by the Tikhonov regularization of the PELDOR data.

Table 1. Determined Spin−spin Distances and Regularization Parameters Used in Data Analysis by DeerAnalysis 2008[16]

Double-labeled aptamer mutant	Distance with/without ligand (nm)	Regularization parameter
U15−U26	3.6	100
U14−U26	3.4	10
U4−U15	2.1	100
U4−U14	2.5	10

onstrates that we probed homogeneous, efficiently double-labeled samples allowing a reliable interpretation of the obtained distances (Table 1).

The PELDOR time traces and thereof calculated distances are unchanged upon addition of neomycin in ratios 1:1 (Figure 2), 1:2, 1:3, and 1:10 (data not shown). The fact that there are no shifts in the measured distances implies the existence of a prearranged tertiary structure of the RNA aptamer without a significant global conformational change induced by ligand binding.

We tested the effect of neomycin analogues on the global aptamer structure. Although there was a significant difference in regulation of gene expression between the antibiotics neomycin and paramomycin,[3] a close analogue of neomycin in which a single amino group at ring I is substituted by a hydroxyl group, distances, and distance distributions determined by PELDOR spectroscopy remained unchanged upon addition of the ligand (Figure S7). The same was true for ribostamycin (Figure S6), an analogue of neomycin lacking ring IV, which is still able to regulate translation.[3] Eventual effects of the cryoprotectant on the global RNA organization were checked by using sucrose instead of ethylene glycol. The ligand-free and ligand-bound distances, deduced after Tikhonov regularization of the PELDOR time traces, were essentially identical (Figure S5). Therefore, we conclude that the overall stem-loop architecture, reflected in the measured U4−U14, U4−U15, U14−U26, and U15−U26 distances, is preserved in the presence or absence of the ligand.

All PELDOR measurements were performed at 50 K in frozen buffer solutions, and strictly speaking the conclusion holds at the glass transition temperature of the matrix. At this temperature, the balance between the enthalpic and entropic contributions to the free energy is shifted toward the former compared to biologically relevant temperatures. Differences in the local RNA flexibility upon docking of neomycin (contributing to the entropy), although not accessible to PELDOR, are clearly visible in the room temperature CW-EPR measurements on single-labeled aptamer mutants and should be considered for the detailed characterization of the binding process. Nevertheless, the reported low-temperature PELDOR data are sufficient to unambiguously demonstrate the existence of an enthalpically favorable set of RNA conformations ready to bind the ligand without major global rearrangement.

To the best of our knowledge, this is the first application of PELDOR spectroscopy on tertiary folded RNA reported in the literature. The measurements yield reliable intramolecular long-range distances, which reveal the intrinsic propensity of the global RNA architecture toward its ligand-bound form. This finding suggests that at room temperature neomycin probably binds to the identified ensemble of preexisting RNA conformations via conformational selection, rather than facilitating their formation through induced fit.

Acknowledgment. We thank Elke Durchardt and Jens Wöhnert for helpful discussions. This work was supported by the DFG (SFB 579) and BMRZ.

Supporting Information Available: Oligonucleotide synthesis and characterization; EPR parameters; PELDOR data with different ligands and different cryoprotectant; CW-EPR data. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Roth, A.; Breaker, R. R. *Annu. Rev. Biochem.* **2009**, *78*, 305–334.
(2) Weigand, J. E.; Sanchez, M.; Gunnesch, E. B.; Zeiher, S.; Schroeder, R.; Suess, B. *RNA* **2008**, *14*, 89–97.
(3) Moazed, D.; Noller, H. F. *Nature* **1987**, *327*, 389–394.
(4) von Ahsen, U.; Davies, J.; Schroeder, R. *Nature* **1991**, *353*, 368–370.
(5) Zapp, M. L.; Stern, S.; Green, M. R. *Cell* **1993**, *74*, 969–978.
(6) Rogers, J.; Chang, A. H.; von Ahsen, U.; Schroeder, R.; Davies, J. *J. Mol. Biol.* **1996**, *259*, 916–925.
(7) Stage, T. K.; Hertel, K. J.; Uhlenbeck, O. C. *RNA* **1995**, *1*, 95–101.
(8) Prisner, T. F.; Rohrer, M.; MacMillan, F. *Annu. Rev. Phys. Chem.* **2001**, *52*, 279–313.
(9) (a) Kobitski, A. Yu.; Nierth, A.; Helm, M.; Jäschke, A.; Nienhaus, G. U. *Nucleic Acids Res.* **2007**, 1–13. (b) Walter, N. G.; Hampel, K. J.; Burke, J. M. *EMBO J.* **1998**, *17*, 2378–2391.
(10) (a) Zhang, Q.; Stelzer, A. C.; Fisher, C. K.; Al-Hashimi, H. M. *Nature* **2007**, *450*, 1263–1268. (b) Noeske, J.; Buck, J.; Fürtig, B.; Nasiri, H. R.; Schwalbe, H.; Wöhnert, J. *Nucleic Acids Res.* **2007**, *35*, 572–583.
(11) (a) Milov, A. D.; Salikov, K. M.; Shirov, M. D. *Fiz. Tverd. Tela* **1981**, *23*, 975–982. (b) Schiemann, O.; Prisner, T. F. *Q. Rev. Biophys.* **2007**, *40*, 1–53. (c) Jeschke, G.; Polyhach, Y. *Phys. Chem. Chem. Phys.* **2007**, *9*, 1895–1910.
(12) Jeschke, G. *Macromol. Rapid Commun.* **2002**, *23*, 227–246.
(13) (a) Schiemann, O.; Piton, N.; Mu, Y.; Stock, G.; Engels, J. W.; Prisner, T. F. *J. Am. Chem. Soc.* **2004**, *126*, 5722–5729. (b) Cai, Q.; Kusnetzow, A. K.; Hideg, K.; Price, E. A.; Haworth, I. S.; Qin, P. Z. *Biophys. J.* **2007**, *93*, 2110–2117.
(14) Georgieva, E. R.; Ramlall, T. F.; Borbat, P. P.; Freed, J. H.; Eliezer, D. *J. Am. Chem. Soc.* **2008**, *130*, 12856–12857.
(15) Schiemann, O.; Piton, N.; Plackmeyer, J.; Bode, B. E.; Prisner, T. F.; Engels, J. W. *Nat. Protoc.* **2007**, *2*, 904–923.
(16) Jeschke, G.; Chechik, V.; Ionita, P.; Godt, A.; Zimmermann, H.; Banham, J.; Timmel, C. R.; Hilger, D.; Jung, H. *Appl. Magn. Reson.* **2006**, *30*, 473–498.

JA9077914