Published in final edited form as:

Angew Chem Int Ed Engl. 2009 ; 48(51): 9739–9742. doi:10.1002/anie.200903809.

Ca²⁺ Induces the Formation of Two Distinct Subpopulations of Single Group II Intron Molecules**

Dr. Miriam Steiner¹, Prof. Dr. David Rueda², and Prof. Dr. Roland K. O. Sigel¹

David Rueda: rueda@chem.wayne.edu; Roland K. O. Sigel: roland.sigel@aci.uzh.ch

¹ Institute of Inorganic Chemistry, University of Zürich, Winterthurerstrasse 190, CH-8057 Zürich, Switzerland, Fax: (+) 41 44 635 68 02, , Homepage: www.aci.uzh.ch/rna

² Department of Chemistry, Wayne State University, 5101 Cass Avenue, Detroit, MI 48202, USA, Fax: (+) 1 313 577 8822, Homepage: www.chem.wayne.edu/ruedagroup

Abstract

The folding pathway of the *Sc.ai5γ* derived group II intron ribozyme D135 is highly specific to the correct M²⁺ cofactor. Upon partial replacement of Mg²⁺ with Ca²⁺, the molecules split into two distinct static subpopulations that are not interchangeable. Type 2 molecules thereby form a compact but misfolded state.

Keywords

Multidomain RNA folding; single molecule Förster Resonance Energy Transfer; ribozyme; splicing; metal ions

The phosphate sugar backbone imposes a large negative charge on RNA and DNA that needs to be neutralized for three dimensional-structure assembly. In addition to proteins and polyamines, the most important cofactors that bind nucleic acids are metal ions. They help to overcome repulsion forces and mediate higher order structure formation, and they frequently participate directly in the chemical reaction of catalytic nucleic acids, i.e. ribozymes.[1] Catalytic RNAs show a distinct specificity for metal ions, both with respect to the kind as well as their concentration.[2–6] Mg²⁺ is the most abundant divalent metal ion in the cell and is often considered the natural cofactor for ribozymes.

Self-splicing group II introns rank amongst the largest ribozymes known and are found in organellar genes of lower eukaryotes, fungi, plants and bacteria.[7] They represent large

**This work was supported by the Swiss National Science Foundation and the University of Zurich (R.K.O.S.), as well as the NIH and the NSF (R01 GM085996 and MCB-0747285 to D.R.).

Correspondence to: David Rueda, rueda@chem.wayne.edu; Roland K. O. Sigel, roland.sigel@aci.uzh.ch.

molecular machines able to perform autocatalysis.[7] *In vitro*, the D135 ribozyme derived from the *S. cerevisiae* group II intron *Sc.ai5γ* displays an optimal activity at unphysiologically high metal ion concentrations (500 mM KCl, 50–100 mM MgCl₂).[8] This ribozyme consists of domains 1, 3, and 5, contains all the elements necessary for activity and represents the best investigated system for group II intron folding.[9,10] D135 folds in a Mg²⁺ dependent fashion.[10] Starting from the unfolded state U in the presence of monovalent metal ions only, two transient on-pathway intermediates I (extended intermediate) and F (folded intermediate) are observed before reaching the native state N by the addition of Mg²⁺. I and F are in fast dynamic equilibrium separated by low energy barriers.[10] Interestingly, increasing [Mg²⁺] activate the structural dynamics, directly linking dynamics to ribozyme activity.[10]

The hammerhead ribozyme retains activity with Ca²⁺ only,[3,11] and the *Tetrahymena* group I intron was shown to globally fold with Ca²⁺ only, but requires small [Mg²⁺] for catalysis.[12] Group II introns instead are inhibited already by very low [Ca²⁺];[4] Ca²⁺ thereby actively replaces Mg²⁺ ions in the folded state.[4] Calcium inhibition is particularly interesting because the *cox1* gene coding for *Sc.ai5γ* is located in mitochondria and these cellular compartments are not only involved in Ca²⁺ homeostasis, serving as Ca²⁺ storage pools, but both the *cox1* gene and altering Ca²⁺ levels are also involved in apoptosis.[13–15] Therefore, the ribozyme splicing activity and thus correct *cox1* expression is potentially regulated by local [Ca²⁺] in mitochondria.

Until now it was impossible to distinguish whether the role of Ca²⁺ is reflected in the disturbance of the overall ribozyme structure or the replacement of one or more Mg²⁺ ions directly involved in catalysis. Both of these effects would result in a similar, undistinguishable inhibition in a standard cleavage assay. To address this, we have characterized the folding of the group II intron D135 ribozyme in the presence of Ca²⁺ independently of catalysis using single-molecule Förster Resonance Energy Transfer (smFRET).

Calcium(II) ions affect the overall distribution of conformational states in the D135-L14 ribozyme

The fluorophore labeled D135-L14 ribozyme has been previously characterized[10] and shown to be catalytically competent in cleaving substrate RNA (Supporting Figure S1). We have now carried out smFRET experiments in the presence of 0, 2, 5, 7, or 10 mM Ca²⁺ in a background of Mg²⁺ to give a total [M²⁺] of 100 mM. Cumulative FRET distribution histograms from single-molecule time trajectories are shown in Figure 1a (30–50 molecules each). In the presence of Mg²⁺ only, the two intermediate folding states (I and F, FRET ≈ 0.25 and 0.4) are equally populated, while the native state (N, FRET ≈ 0.6) is clearly a minor population (Figure 1a, top).[10] Upon addition of Ca²⁺ a distinct population transfer takes place: The magnitude of the 0.4 FRET state decreases as the 0.6 FRET state increases dramatically (Figure 1a). Interestingly, the peak value of the highest FRET distribution also shifts from ~0.60 to ~0.54 as [Ca²⁺] increases to 10 mM.

The presence of Ca²⁺ leads to the appearance of two distinct subpopulations and a new misfolded species

To characterize the increase of the 0.6 FRET state in the presence of Ca²⁺, we analyzed the single time trajectories individually (Figure 2). In the presence of Mg²⁺ only, three reoccurring conformational states of D135-L14 are observed at FRET values ~0.25, 0.4 and 0.6, corresponding to I, F and N. These three states are in fast equilibrium and the 0.6 state is only transiently occupied (Figure 2, top). In the presence of Ca²⁺ two distinctively different

trace types appear. Type 1 traces are similar to those observed with Mg^{2+} only (Figure 2, middle). In contrast, type 2 traces show a completely new behavior: The molecules predominantly populate the high FRET state, and exhibit direct transitions from 0.25 to 0.6 FRET and fewer transitions to 0.4 FRET (Figure 2, bottom). The 0.4 state apparently describes a conformation along a minor folding pathway, although it is not clear whether the transitions from 0.6 to 0.25 are direct or if they include a very short intermission in the 0.4 FRET, which we miss because of our 33 ms time resolution. The intermediate 0.4 FRET state is reached from both the 0.25 and 0.6 states. Yet FRET 0.4 is not an on-pathway state as the molecules fall back to their original state rather than continuing to the respective third state. Interchanges from single molecules from type 1 to type 2 were also not observed within the ~1 min observation window. This reflects a certain memory effect of the single molecules similar to earlier observations.[16]

Cumulative histograms of the single molecule traces split into type 1 and type 2 traces strongly support the existence of two distinct subpopulations (Figure 1b). Type 1 histograms show large peak distributions at 0.25 and 0.4 FRET and a consistently minor distribution at ~0.6 FRET, which only increases slightly with $[\text{Ca}^{2+}]$. In contrast, type 2 traces primarily populate the low and high FRET states, but hardly the intermediate state. Careful inspection of the type 2 histograms also reveals a shift in the maximum of the high FRET distribution from 0.58 to 0.53, whereas the peaks at the two lower FRET states remain unchanged (Figure 1b). This can be explained by concluding that type 2 molecules no longer reach the native state N. Instead, they form a new conformational (misfolded) species M with a FRET state that is clearly distinguishable from the native state N at 0.6 FRET.

We further determined the relative amount of type 2 traces as a function of $[\text{Ca}^{2+}]$ (Figure 3). The fraction of type 2 molecules increases linearly up to 50% in 10 mM $[\text{Ca}^{2+}]$, which correlates nicely with the loss of function in the presence of Ca^{2+} ions.[4] This further supports the existence of two subpopulations and explains the observed progression in the overall FRET histograms that include both type 1 and type 2 traces (Figure 1a).

Folding rates in the presence of Ca^{2+} support the accumulation of the molecules in the high FRET state

Folding rates for type 1 molecules were determined in a range of $\text{Mg}^{2+}/\text{Ca}^{2+}$ ratios (25–50 molecules each) using dwell time analysis as described.[10] Both forward (k_{11} and k_{12}) and both backward rates (k_{-11} and k_{-12}) either increase linearly with increasing $[\text{Ca}^{2+}]$ or stay the same within the error limits (Figure 4a,b). The ratios of forward and backward rates agree with the observation that the high FRET state remains a minor species but its contribution increases in the presence of Ca^{2+} (Figure 1b).

For type 2 molecules, we assigned new folding rates describing the transition between 0.25 and 0.4 FRET as well as from the 0.25 or 0.4 states to the highest FRET state at 0.53 (k_{21}/k_{-21} , k_{22}/k_{-22} , and k_{23}/k_{-23} , respectively, Figure 4). Roughly one third of the type 2 molecules show transitions to and from 0.4 FRET, whereas all other traces show a direct I-M transition. Due to the rare occurrence of the transitions involving the F state (especially at low $[\text{Ca}^{2+}]$), the rates k_{21} , k_{-21} , k_{22} and k_{-22} were estimated as the inverse of the respective averaged dwell times. k_{21} and k_{-21} could be calculated at 10 mM Ca^{2+} ; 90 mM Mg^{2+} only but need to be interpreted with great care due to their large error. k_{22} and k_{-22} are independent of the $[\text{Ca}^{2+}]$. Instead, k_{23} increases with higher $[\text{Ca}^{2+}]$ and is the fastest rate under all conditions. The opposite transition (k_{-23}) becomes slower at high $[\text{Ca}^{2+}]$. Overall, the rapid accumulation of the molecules in the 0.53 FRET state is well explained by these rates.

Conclusion

This study is the first investigation at the single molecule level on the effect of an M^{2+} other than Mg^{2+} on RNA folding. The group II intron derived D135-L14 ribozyme is the largest protein-free RNA investigated by smFRET and represents a perfect model system for the investigation of folding of large RNAs. Its splicing activity is very sensitive to trace amounts of Ca^{2+} . [4] Although an (additional) inhibition of the catalytic step itself by Ca^{2+} cannot be ruled out, our results reveal an unprecedented behavior of RNA folding upon Ca^{2+} binding.

The addition of Ca^{2+} to the D135-L14 ribozymes induces the division of all single molecules into two distinct subpopulations:

- i. Type 1 molecules fold very similarly to those in the Mg^{2+} -only pathway, the individual folding rates increasing only moderately (if at all) by adding Ca^{2+} . We ascribe this type to a structure where Mg^{2+} still occupies the key sites of the three-dimensional architecture. Ca^{2+} might bind to some places in the RNA, but presumably only diffusely for charge compensation, thus having hardly any effect on the global structure. The faster ligand exchange rate and larger ionic radius of Ca^{2+} compared to Mg^{2+} might be reflected in the slight increase in folding rates, the higher dynamics of domain assembly, and the slightly less compact high FRET state.
- ii. In type 2 molecules, 0.4 FRET is highly destabilized and almost inexistent. It is an open question whether the molecules fold directly from the 0.25 state to the most compact and misfolded ~ 0.53 state, or if the 0.4 state is transiently reached but unobservable due to an occupancy shorter than the experimental time window of 0.33 ms. The high FRET state F is slightly less compact than the native one, and hence, within this second subpopulation, Ca^{2+} obviously occupies one or more key sites in the folded structure.

The differences in folding dynamics of the D135-L14 ribozyme with increasing $[Ca^{2+}]$ concur and can thus be explained with the different coordinating properties of Mg^{2+} and Ca^{2+} : Ca^{2+} is larger, has faster ligand exchange dynamics, and can enlarge its coordination number to 8, instead of only 6 with Mg^{2+} . In addition, Ca^{2+} has an intrinsic lower affinity towards nucleic acids than Mg^{2+} . As Mg^{2+} is in excess under all conditions, at least at one site Ca^{2+} must bind more tightly than Mg^{2+} .

One can speculate on the distinct role of Ca^{2+} for the individual steps of folding: The 0.25 FRET state I, which is attributed to a folded domain 1, [10] does not seem to be influenced by Ca^{2+} in its global fold. The split into two coexisting but distinct subpopulations upon Ca^{2+} addition becomes noticeable only after the subsequent docking of domains 3 and 5. It was previously shown [10] that high $[Mg^{2+}]$ increase the dynamics of group II introns, i.e. the 0.25 state is occupied on a regular basis. Presumably, one (or possibly several) Ca^{2+} ion bind to a specific junction within the I state, which controls the structural dynamics leading to an inevitable branching-off of the pathway towards the misfolded structure M. Maybe binding of the first Ca^{2+} leads to cooperative binding of subsequent ions, which would explain the coexistence of type 1 and type 2 molecules. Such a cooperative binding is corroborated by the linear increase in molecules of type 2 with increasing $[Ca^{2+}]$. Yet we do not observe traces that interchange from type 1 to type 2 behavior during our experimental time window. Although I shows the same FRET value for the two types of traces in our experiments, we cannot rule out that it differs already in the two subpopulations.

Our findings are well in line with previous observations that Ca^{2+} inhibition takes place in prefolded D135 molecules. [4] In earlier biochemical studies, the catalytic rate k_{cat} is reduced to 50% at 5 mM Ca^{2+} and splicing completely inhibited at 20 mM Ca^{2+} . A quantitative

comparison between splicing inhibition and the occurrence of two subpopulations with increasing $[Ca^{2+}]$ is not possible, because in the previous experiments D5 was added *in trans*, i.e. D5 is not covalently linked to the rest of the ribozyme. Such a two-piece setup obviously strongly impedes the last step of folding to the active structure.

Proving a functional role for folding heterogeneity has so far not been achieved. A recent bulk FRET study on the extended *Schistosoma* hammerhead ribozyme concluded that divalent metal ions other than Mg^{2+} have almost no effect on the global RNA fold, but strongly regulate catalysis.[6] However, the small differences in maximum FRET intensities found in our study would not be detected in bulk experiments aimed to study folding heterogeneity. This illustrates that only single molecule spectroscopy is able to reveal the subtle effect of M^{2+} binding during RNA folding.

Group II intron folding is of general interest because (i) no kinetic traps exist on the native folding pathway, (ii) the active state N is reached only transiently from a collapsed near-native state F, (iii) N is stabilized by substrate binding, and (iv) Mg^{2+} not only induces folding but also increases the inherent dynamics of the folded RNA.[10] Our presented results add two further aspects to this list: (v) It could be shown for the first time that the binding of specific metal ions has an effect on the global architecture of a large RNA. One can thereby distinguish between Mg^{2+} and Ca^{2+} bound RNA molecules on a single molecule level. (vi) By linking the biochemical data[4] with the here observed separation into two subpopulations, a functional role for folding heterogeneity could be demonstrated for the first time.

Experimental Section

RNA preparation and single molecule experiments

The D135-L14 RNA, derived from the *S. cerevisiae* intron *Sc.ai5γ*, was obtained by *in vitro* transcription under standard conditions with homemade T7 polymerase from *HindIII* digested plasmid pT7D135-L14 and the T-Biotin, Cy3- and Cy5 DNAs purified as described.[18–20] Single molecule experiments were performed as described.[10,21] Samples were incubated in mixtures containing 100 mM Mg^{2+} , 98 mM Mg^{2+} : 2 mM Ca^{2+} , 95 mM Mg^{2+} : 5 mM Ca^{2+} , 93 mM Mg^{2+} : 7 mM Ca^{2+} or 90 mM Mg^{2+} : 10 mM Ca^{2+} to test and compare the influence of increasing amounts of Ca^{2+} under conditions of equal ionic strength. For details see Supplementary Information.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

References

1. Sigel RKO, Pyle AM. Chem Rev. 2007; 107:97–113. [PubMed: 17212472]
2. Sigel RKO. Eur J Inorg Chem. 2005; 12:2281–2292.
3. Roychowdhury-Saha M, Burke DH. RNA. 2006; 12:1846–1852. [PubMed: 16912216]
4. Erat MC, Sigel RKO. J Biol Inorg Chem. 2008; 13:1025–1036. [PubMed: 18528718]
5. Freisinger E, Sigel RKO. Coord Chem Rev. 2007; 251:1834–1851.
6. Boots JL, Canny MD, Azimi E, Pardi A. RNA. 2008; 14:2212–2222. [PubMed: 18755844]
7. Michel F, Ferat JL. Annu Rev Biochem. 1995; 64:435–461. [PubMed: 7574489]
8. Su LHJ, Brenowitz M, Pyle AM. J Mol Biol. 2003; 334:639–652. [PubMed: 14636593]
9. Fedorova O, Zingler N. Biol Chem. 2007; 388:665–678. [PubMed: 17570818]
10. Steiner M, Karunatilaka KS, Sigel RKO, Rueda D. Proc Natl Acad Sci USA. 2008; 105:13853–13858. [PubMed: 18772388]

11. Chowrira BM, Berzal-Herranz A, Burke JM. *Biochemistry*. 1993; 32:1088–1095. [PubMed: 7678751]
12. Celander DW, Cech TR. *Science*. 1991; 251:401–407. [PubMed: 1989074]
13. Babcock DF, Hille B. *Curr Op Neurobiol*. 1998; 8:398–404. [PubMed: 9687353]
14. Carafoli E. *FEBS Lett*. 1979; 104:1–5. [PubMed: 383503]
15. Zamzami N, Hirsch T, Dallaporta B, Petit PX, Kroemer G. *J Bioenerg Biomembr*. 1997; 29:185–193. [PubMed: 9239543]
16. Zhuang XW, Kim H, Pereira MJB, Babcock HP, Walter NG, Chu S. *Science*. 2002; 296:1473–1476. [PubMed: 12029135]
17. Blanchard SC, Gonzalez RL, Kim HD, Chu S, Puglisi JD. *Nature Struct Mol Biol*. 2004; 11:1008–1014. [PubMed: 15448679]
18. Gallo S, Furler M, Sigel RKO. *CHIMIA*. 2005; 59:812–816.
19. Walter NG. *Methods*. 2001; 25:19–30. [PubMed: 11558994]
20. Rueda D, Walter NG. *Methods Mol Biol*. 2006; 335:289–310. [PubMed: 16785635]
21. Zhao R, Rueda D. *Methods*. 2009 online available. 10.1016/j.ymeth.2009.1004.1017

Figure 1.

Distribution histograms of smFRET time traces in the presence of Ca^{2+} . a) Upon addition of up to 10 mM Ca^{2+} (in a total divalent metal ion concentration of 100 mM completed with the addition of Mg^{2+}), the contribution of the high FRET state at ~ 0.6 dramatically increases. Concomitantly, the mean maximal FRET value shifts from 0.6 to 0.54 and the peak height of the 0.4 FRET state decreases. b) Histograms of single molecule trajectories separated into subpopulations type 1 (left) and type 2 (right) at the indicated $\text{Mg}^{2+}:\text{Ca}^{2+}$ mixtures. The two subpopulations are distinctly different.

Figure 2.

Exemplary single molecule traces at 100 mM Mg²⁺ (top) and of the two subpopulations at 90 mM Mg²⁺: 10 mM Ca²⁺ (middle and bottom). The FRET histograms (right) reveal two distinct types of traces in the presence of Ca²⁺.

Figure 3. Fraction of type 2 molecules as a function of $[\text{Ca}^{2+}]$. A linear relationship is revealed (slope = $4.5 \pm 0.4 \text{ mM}^{-1}$).

Figure 4.

Folding pathway of D135-L14 in the presence of Mg^{2+} and Ca^{2+} . a) In the presence of Mg^{2+} the ribozyme shows a linear folding pathway from the unfolded state U via the on-pathway intermediates I (unfolded intermediate) and F (folded intermediate) to the native state N.[10] b) In the presence of Ca^{2+} two different types of subpopulations appear. Type 1 traces depict a pathway similar to the Mg^{2+} case with slightly altered rates. Molecules of type 2 predominantly fold directly from I to a misfolded species M. Some type 2 molecules follow a minor pathway passing F on the way from I to M. The maxima of the FRET distributions are given in blue. All rates are in s^{-1} and correspond to 10 mM Ca^{2+} /90 mM Mg^{2+} . The individual error limits are estimated[17] to be about 50% of the given values. The first step of folding, i.e. the addition of monovalent ions to the RNA, is not shown. Note that we cannot distinguish if I is the dividing point or if it already differs for the two subpopulations (see also text).