RNA Folding

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Ca^{2+} Induces the Formation of Two Distinct Subpopulations of Group II Intron Molecules**

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The phosphate–sugar backbone of RNA and DNA imposes a large negative charge 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 the formation of higher order structures. Metal ions also frequently participate directly in the chemical reactions of catalytic nucleic acids, that is, ribozymes.[1] Catalytic RNAs show a distinct specificity for metal ions, both with respect to the kind as well as their concentration.[2–6] Mg^{2+} 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 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 $MgCl_2$).[8] This ribozyme consists of domains 1, 3, and 5, contains all the elements necessary for activity, and represents the best investigated system for the folding of group II introns.[9,10] D135 folds in a Mg^{2+}-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 the native state N is reached upon addition of Mg^{2+}. I and F are in fast dynamic equilibrium separated by low energy barriers.[10] Interestingly, increasing Mg^{2+} concentration activates the structural dynamics, directly linking dynamics to ribozyme activity.[10]

The hammerhead ribozyme retains activity with Ca^{2+} ions only,[3,11] and the *Tetrahymena* group I intron was shown to globally fold with Ca^{2+} only, but requires low concentrations of Mg^{2+} for catalysis.[12] In contrast, group II introns are inhibited already by very low levels of Ca^{2+};[4] Ca^{2+} ions thereby actively replace Mg^{2+} ions in the folded state.[4] Calcium inhibition is particularly interesting since the *cox1* gene coding for *Sc*.ai5γ is located in mitochondria. These cellular compartments are not only involved in Ca^{2+} homeostasis, serving as Ca^{2+} storage pools, but both the *cox1* gene and altering Ca^{2+} levels are also involved in apoptosis.[13–15] Therefore, the ribozyme splicing activity and thus correct *cox1* expression is potentially regulated by local Ca^{2+} levels in mitochondria.

Until now it was impossible to distinguish whether the role of Ca^{2+} is reflected in the disturbance of the overall ribozyme structure or the replacement of one or more Mg^{2+} 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 Ca^{2+} independently of catalysis using single-molecule Förster resonance energy transfer (smFRET).

The fluorophore-labeled D135-L14 ribozyme has been previously characterized[10] and shown to be catalytically competent in cleaving substrate RNA (Figure S1 in the Supporting Information). We have now carried out smFRET experiments in the presence of 0, 2, 5, 7, or 10 mM Ca^{2+} along with Mg^{2+} to give a total concentration of M^{2+} ions 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 Mg^{2+} 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.[10] Upon addition of Ca^{2+} 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 the Ca^{2+} concentration increases to 10 mM.

To characterize the increase of the 0.6 FRET state in the presence of Ca^{2+}, we analyzed the single-molecule time trajectories individually (Figure 2). In the presence of Mg^{2+} only, three reoccurring conformational states of D135-L14 are observed at FRET values of approximately 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 only has a short lifetime (Figure 2, top). In the presence of Ca^{2+} 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

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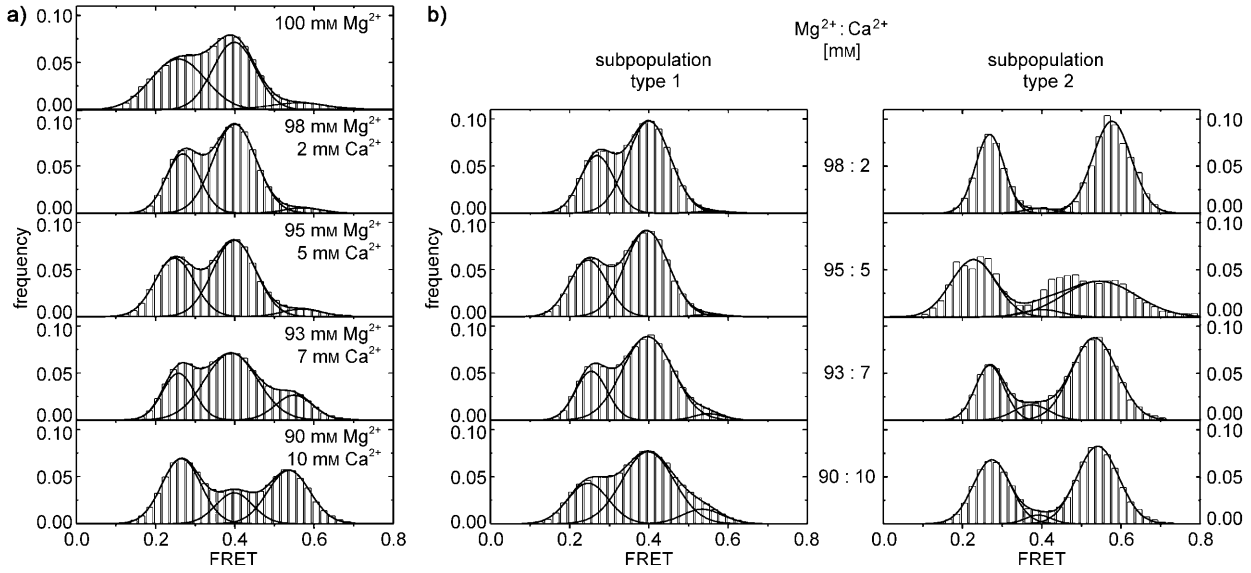

Figure 1. Distribution histograms of smFRET time traces in the presence of Ca^{2+}. a) Upon addition of up to 10 mM Ca^{2+} (total concentration of divalent metal ions of 100 mM completed with the addition of Mg^{2+}), the contribution of the high FRET state at ≈0.6 dramatically increases. Concomitantly, 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 Mg^{2+}/Ca^{2+} ratios.

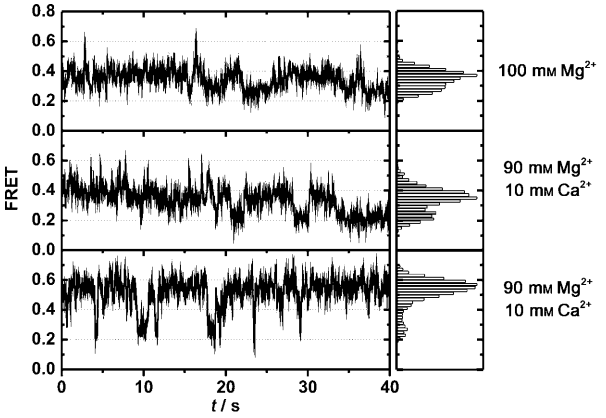

Figure 2. Exemplary single-molecule traces at 100 mM Mg^{2+} (top) and traces of the two subpopulations at Mg^{2+}/Ca^{2+} = 90 mM:10 mM (type 1 middle and type 2 bottom). The FRET histograms (right) reveal two distinct types of traces in the presence of Ca^{2+}.

direct transitions from the 0.25 to the 0.6 state and fewer transitions to the 0.4 state (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 the 0.6 to the 0.25 state are direct or if they include a very short intermission in the 0.4 state, which we do not observe because of our time resolution of 33 ms. The intermediate 0.4 FRET state is reached from both the 0.25 and 0.6 states. Yet the 0.4 state is not an on-pathway state as the molecules fall back to their original state rather than continuing to the respective third state. Interchanges of 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 that in 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 values and a consistently minor distribution at ≈0.6, which only increases slightly with increasing Ca^{2+} concentration. 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 distribution of the high FRET states from 0.58 to 0.53, whereas the peaks at the two lower FRET states remain unchanged (Figure 1b). We by conclude 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 a FRET value of 0.6.

We further determined the relative amount of type 2 traces as a function of Ca^{2+} concentration (Figure 3). The fraction of type 2 molecules increases linearly up to 50% in 10 mM Ca^{2+}, which correlates nicely with the loss of function

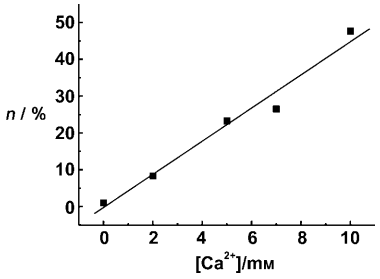

Figure 3. Fraction of type 2 molecules (n) as a function of Ca^{2+} concentration. A linear relationship is revealed (slope = (4.5±0.4) mM^{-1}).

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 for type 1 molecules were determined in a range of Mg^{2+}/Ca^{2+} ratios (25–50 molecules each) using dwell-time analysis as described.[10] The rates of the forward (k_{11} and k_{12}) as well the back reaction (k_{-11} and k_{-12}) either increase linearly with increasing Ca^{2+} concentration or stay the same within the error limits (Figure 4). The ratios of the rate constants for the forward and back reactions 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 the 0.25 and the 0.4 state 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 the 0.4 state , 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 Ca^{2+} levels), 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 $Mg^{2+}/Ca^{2+} = 90$ mM:10 mM only but should be interpreted with great care owing to their large error. k_{22} and k_{-22} are independent of the Ca^{2+} concentration. In contrast, k_{23} increases with higher Ca^{2+} concentration and is the fastest rate under all conditions. The opposite transition, k_{-23}, becomes slower at high Ca^{2+} concentrations. Overall, the rapid accumulation of the molecules in the 0.53 FRET state is well explained by these rates.

This study is the first investigation at the single-molecule level on the effect of a divalent metal ion 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 the 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:

1) Type 1 molecules fold very similarly to those in the Mg^{2+}-only pathway; the individual folding rates increase only moderately (if at all) upon addition of Ca^{2+}. We ascribe this type to a structure where Mg^{2+} still occupies the key sites of sites 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.

2) In type 2 molecules, the 0.4 FRET state is highly destabilized and almost nonexistent. 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. If the occupancy of the 0.4 state is shorter than our experimental time window of 33 ms, it cannot be observed. The high FRET state F is slightly less compact than the native one, and hence, within this second subpopulation, Ca^{2+} likely occupies one or more key sites in the folded structure.

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

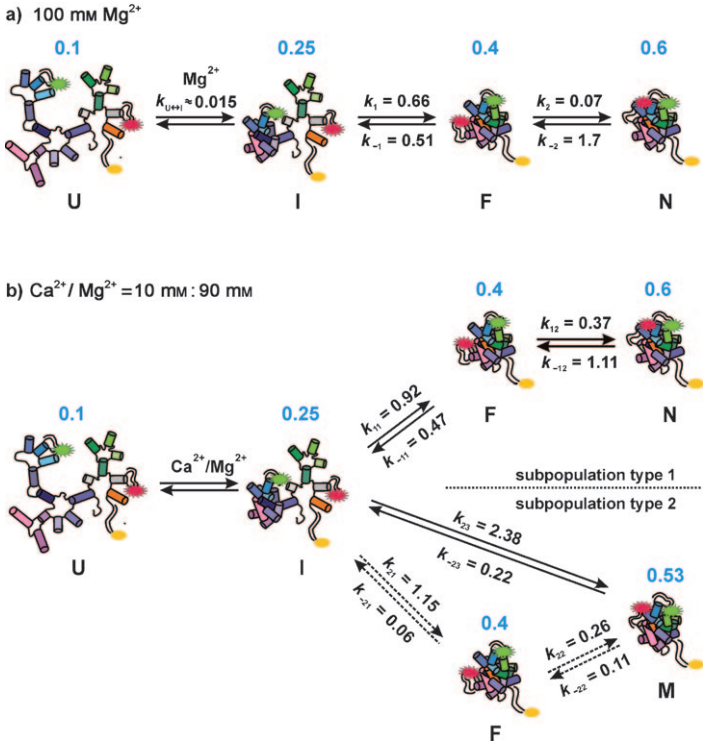

Figure 4. Folding pathway of the D135-L14 ribozyme in the presence of Mg^{2+} and Ca^{2+}. a) In the presence of Mg^{2+} the ribozyme shows a linear folding pathway from the unfolded state U via the on-pathway intermediates I (unfolded intermediate) and F (folded intermediate) to the native state N.[10] 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 intermediate I to a misfolded species M. Some type 2 molecules follow a minor pathway via 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 were determined at concentrations of $Mg^{2+}/Ca^{2+} =$ 90 mM:10 mM. The individual error limits are estimated[17] to be about 50% of the given values. The first step of folding, that is, the addition of monovalent ions to the RNA, is not shown. Note that we cannot distinguish whether I is the dividing point or if it already differs for the two subpopulations (see text).

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+} levels increase the dynamics of group II introns, that is, the 0.25 state is occupied on a regular basis. Presumably, one or possibly several Ca^{2+} ions 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+} ion 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+} concentration. Yet we do not observe traces that interchange from type 1 to type 2 folding behavior during our experimental time window. Although state 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 is completely inhibited at 20 mM Ca^{2+}. A quantitative comparison between splicing inhibition and the occurrence of two subpopulations with increasing Ca^{2+} concentration is not possible, because in the previous experiments D5 was added in trans, that is, D5 is not covalently linked to the rest of the ribozyme. Such a two-piece setup obviously strongly impedes the last step of fold

A functional role for folding heterogeneity has not been proved so far. 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 the maximum FRET intensities found in our study would not be detected in bulk experiments aimed at studying folding heterogeneity. This illustrates that only single-molecule spectroscopy can reveal the subtle effect of M^{2+} binding during RNA folding.

The folding of group II introns is of general interest because 1) no kinetic traps exist in the native folding pathway, 2) the active state N is reached only transiently from a collapsed near-native state F, 3) N is stabilized by substrate binding, and 4) Mg^{2+} not only induces folding but also increases the inherent dynamics of the folded RNA.[10] Our here presented results add two further unprecedented aspects to this list: 5) 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. 6) Linking the biochemical data[4] with our observed separation into two subpopulations, a functional role for folding heterogeneity could be demonstrated.

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 *Hind*III-digested plasmid pT7D135-L14 and the T-Biotin, Cy3 and Cy5 DNAs purified as described.[18–20] Single-molecule experiments were performed as described elsewhere.[10,21] Samples were incubated in Mg^{2+}/Ca^{2+} mixtures containing 100:0, 98:2, 95:5, 93:7, and 90 mM:10 mM to test and compare the influence of increasing amounts of Ca^{2+} under conditions of equal ionic strength. For details see the Supporting Information.

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