FEBS Letters 452 (1999) 26–30 FEBS 22005

Minireview

Folding and catalysis by the hairpin ribozyme

David M.J. Lilley*

CRC Nucleic Acid Structure Research Group, Department of Biochemistry, The University of Dundee, Dundee DD1 4HN, UK

Received 13 April 1999

Abstract The hairpin ribozyme undergoes a site-specific transesterification cleavage of the phosphodiester backbone. The natural form of the ribozyme is a four-way helical junction, where two arms contain unpaired loops. This folds by pairwise coaxial stacking of helical arms, and a rotation into an antiparallel conformation in which there is close association between the loops. This probably generates the local conformation required to facilitate the trajectory into an in-line S_N2 transition state. Folding is induced by the cooperative binding of at least two divalent metal ions, which are probably distributed between the junction and the loop-loop interface. The junction forms the structural scaffold on which the geometry of the ribozyme is built, and structural perturbation of the junction leads to impaired catalytic activity.

Key words: RNA structure; Transesterification reaction; RNA-mediated catalysis; Metal ion

1. RNA-mediated catalysis

RNA can behave like an enzyme and accelerate chemical reactions [1]. Perhaps the simplest examples of ribozymes are the small RNA self-cleaving nucleases found in plant viroids [2,3], the transcript of newt satellite DNA [4], tobacco ring spot virus satellite RNA [5,6], *Neurospora* VS RNA [7] and the hepatitis delta virus [8]. These ribozymes are self-cleaving motifs that function in the processing of RNA produced by rolling-circle replication. They undergo site-specific cleavage of the backbone by a transesterification reaction, the only exogenous requirement for which is the presence of certain metal ions. The products of the phosphodiester bond cleavage are 5′-hydroxyl and 2′,3′-cyclic phosphate termini [9,10], and the reactions proceed with inversion of configuration at the phosphorus [11]. This indicates that the reaction occurs by attack of the 2′ hydroxyl on the 3′ phosphorus in an S_N2 mechanism. The structure of A-form RNA does not provide the alignment of 2′ oxygen-3′ phosphorus-5′ oxygen required for the transition state of an S_N2 mechanism, and therefore conformational distortion is likely to play an important role in the catalytic mechanism. Ribozymes might therefore be expected to fold into a conformation that achieves a pre-activation of the phosphodiester bond. Given the polyelectrolyte nature of RNA, metal ions are likely to be important in this. Metal ions held in the folded RNA structure generated are also likely to participate directly in the chemistry of bond scission, although this aspect is currently somewhat controversial [12–15].

2. The hairpin ribozyme

The negative strand of the satellite RNA of the tobacco ring spot virus is cleaved by the hairpin ribozyme [5,6,16], and a similar self-cleaving sequence is used in other plant viruses [17]. The hairpin ribozyme undergoes site-specific self-cleavage in the presence of magnesium ions; it also catalyses the ligation reaction [16], generating a circularised RNA species [18] and leading to an equilibrium between cleavage and ligation [19]. As with the other nucleolytic ribozymes such as the hammerhead, the reaction mechanism is clearly S_N2, and an important component of the rate enhancement is likely to come from structural distortion in order to facilitate achieving the correct alignment of O_2', P_3', and O_5' in the transition state. For this reason we have explored the folding of the hairpin ribozyme, and its dependence on the presence of metal ions.

The secondary structure of the satellite RNA around the region of ribozyme cleavage is shown in Fig. 1. The scissile phosphodiester bond is contained in a formally unpaired loop (the A loop), which is located on one arm of a four-way helical junction. The minimal active form of the ribozyme consists of two loop-containing duplexes, that containing the cleavage site and the adjacent helix containing another loop (the B loop). Most of the functional groups that are essential for catalytic activity lie in the two loops and it seemed probable that the active form of the ribozyme becomes generated when these loops interact. This was generally supported by a number of experiments in which the loop-containing duplexes were connected in a variety of non-natural ways with retention of activity [20], and by experiments studying the cleavage activity of RNA modified by the introduction of 2′-2′ disulphide crosslinks [21].

We felt that the four-way helical junction would act as a structural scaffold on which the ribozyme is built, and that its conformation would be a significant factor in the activity of the ribozyme. We showed that a construct comprising all four arms is active as a ribozyme, cleaving at the same site as the hinged form and with very similar kinetics [22].

3. The conformation of the four-way junction

We have demonstrated previously that four-way helical junctions in RNA are always found in a conformation formed by pairwise helical stacking of arms [23,24]. These can exist in one of two possible conformers that differ in stacking partner choice. For the majority of junctions, the axes of the two pairs of stacked helices are approximately perpendicular in the ab-

*Fax: (44) (1382) 201063.
E-mail: dmjlilley@bad.dundee.ac.uk

PII: S0014-5793(99)00544-X

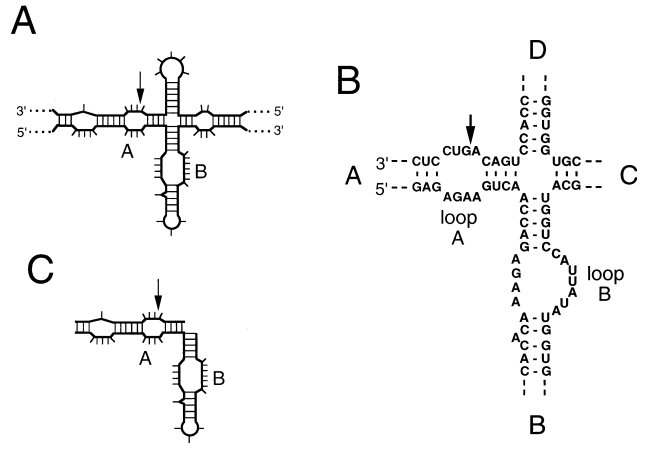

Fig. 1. Secondary structure of the hairpin ribozyme. A: Schematic to show the secondary structure of the tobacco ring spot virus satellite (−) RNA around the site of self-cleavage indicated by the arrow. The RNA adopts a four-way helical junction, consecutive arms of which contain the A and B loops. B: The base sequence around the four-way junction and the loop-carrying arms. We refer to the arms as A and B (containing the A and B loops respectively), continuing to C and D in a circular manner. The arrow shows the point of ribozyme cleavage in the A loop. C: The minimised form of the hairpin ribozyme. This form consists of the A and B arms held together by the continuity of one strand. The resulting nick forms a hinge in the structure.

sence of divalent cations, but rotate in the direction of an antiparallel structure on addition of sufficient magnesium or calcium ions. If we place the two loops of the hairpin ribozyme onto such a junction, then in order to bring them together to create an active ribozyme it is necessary that they are located on different stacked helical pairs, and that the structure of the junction is antiparallel.

Initial studies of the isolated junction of the hairpin ribozyme (i.e. in the absence of the loops) by comparative gel electrophoresis showed that it is folded by the pairwise stacking of the loop-carrying arms onto the remaining two arms, placing them on different axes [22]. This means that they can potentially interact given an antiparallel rotation, and this is just what happens with addition of magnesium or calcium ions in the millimolar range of concentration. Thus the junctions have the required conformational propensity to permit loop-loop interaction. These conclusions were supported and extended by FRET studies [22,25] (Fig. 2). Fitting the fluorescence data indicated the rotation of the junction into a symmetrical antiparallel conformation was induced by the binding of a single magnesium ion. The same conformational transition was induced by calcium or manganese ions, but monovalent sodium ions were totally ineffective.

4. The conformation of the hairpin ribozyme in its natural four-way junction form

We have studied the global conformation of the hairpin ribozyme in its natural junction form using FRET [22,26] (Fig. 3). In order to prevent ribozyme cleavage occurring during the analysis, we use a deoxyribose substitution at the cleavage site, thus removing the attacking nucleophile. Like the isolated junction, the arms of the hairpin ribozyme are coaxially stacked. The choice of stacking partners remains the same as that of the junction. This pattern of stacking (A on D and B on C) places the loops on opposite helical stacked pairs, making them potentially able to interact given an antiparallel rotation. In the absence of added metal ions, the arms adopt a slightly parallel disposition, placing the two loops far apart, but with the addition of low concentrations (e.g. 10 μM) of divalent ions the axes rotate to become close to perpendicular. Under these conditions the loops must still be held

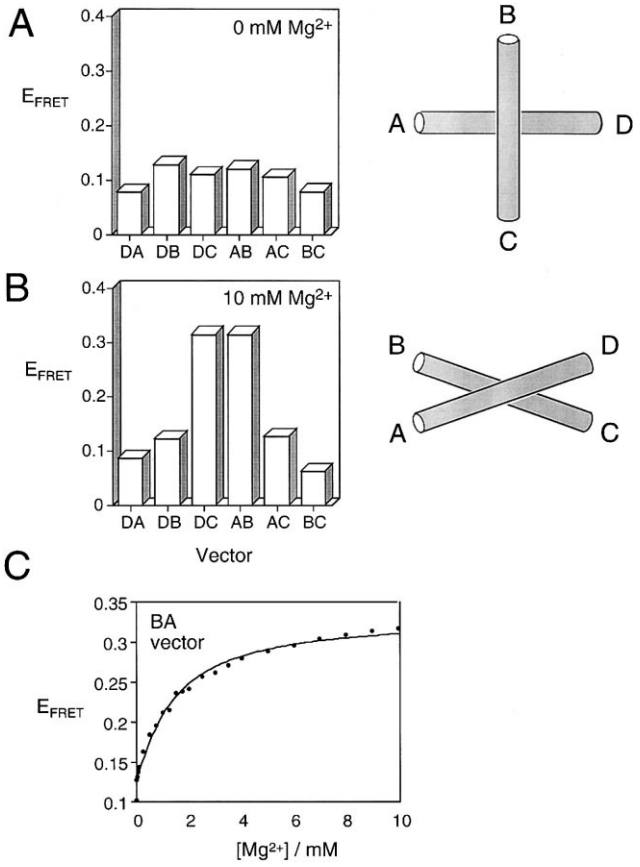

Fig. 2. Global folding of the four-way junction of the hairpin ribozyme, studied by FRET. The six different end-to-end vectors have been prepared by 5′ labelling with donor and acceptor fluorophores, and FRET efficiency (E_{FRET}) measured by steady-state fluorimetry. The values of E_{FRET} are plotted for the six end-to-end vectors as a function of ionic conditions. The vectors are named according to the labelled arms, in the order donor-acceptor. A: E_{FRET} values in the absence of added divalent ions. The pattern of efficiencies is consistent with the schematic model of the global structure shown on the right, in which there is coaxial stacking of A on D and B on C arms, with the two axes mutually perpendicular. The resulting structure has four end-to-end distances (A-B, D-B, D-C and A-C) that are slightly shorter than the remaining two (D-A and B-C), thus explaining the smaller E_{FRET} values for the DA and BC vectors. B: E_{FRET} values in the presence of 10 mM magnesium ions. Under these conditions the efficiencies can be described by DC ≈ AB > DB = AC > DA = BC. These values are consistent with an antiparallel stacked structure based on coaxial stacking of A on D and B on C stacking as indicated schematically on the right. In this structure the lengths of the end-to-end vectors are described by D-A = B-C > A-C = D-B > A-B = D-C. The handedness of this structure has not been determined, and this is drawn arbitrarily. C: Folding of the junction over the range 0–10 mM magnesium ions followed by the change of E_{FRET} for the short vector BA. The experimental data (●) were fitted by regression to a simple binding model where the binding of n ions to the RNA induces a global structural change from the square structure to the antiparallel structure. These data show that the transition to the antiparallel structure is induced by the binding of a single magnesium ion.

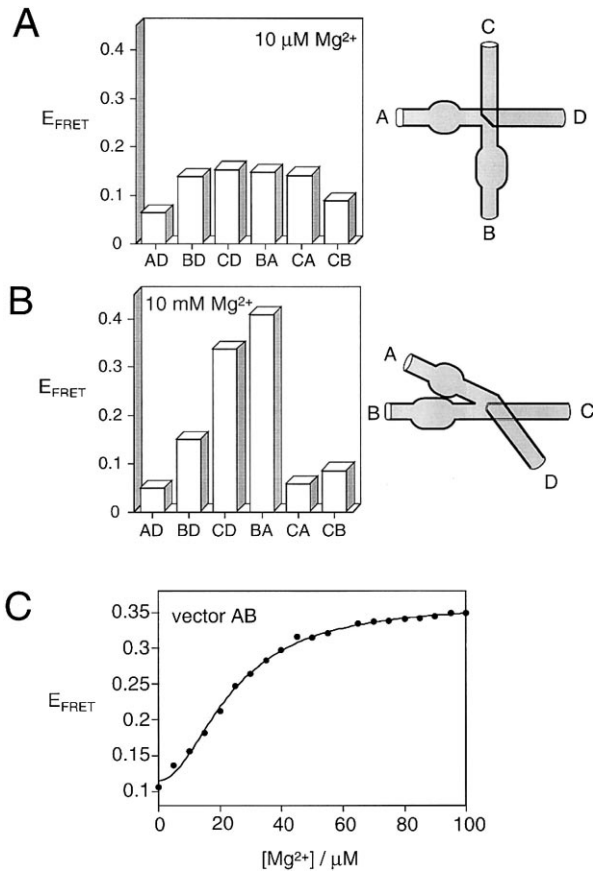

Fig. 3. Global folding of the complete hairpin ribozyme, studied by FRET. The six different end-to-end vectors have been prepared by 5′ labelling with donor and acceptor fluorophores, and FRET efficiency (E_{FRET}) measured by steady-state fluorimetry. The values of E_{FRET} are plotted for the six end-to-end vectors as a function of ionic conditions. A: E_{FRET} values in the presence of 10 μM magnesium ions. Under these conditions all six values are relatively low, indicating an extended structure. The slightly lower values for AD and CB are consistent with a 90° crossed structure based on A on D and B on C stacking (indicated right). B: E_{FRET} values in the presence of 10 mM magnesium ions. Under these conditions the structure has clearly changed, with BA exhibiting highest efficiency. This is consistent with the formation of an antiparallel structure in which the end-to-end vector for helices A and B is the shortest such distance. However, the structure is clearly less symmetrical than that of the simple junction (Fig. 2), and the relative values of E_{FRET} are consistent with the structure indicated (right) where there is close interaction between the A and B loops that distorts the overall global structure. C: Folding of the hairpin ribozyme over the range 0–100 μM magnesium ions followed by the change of E_{FRET} for the short vector AB. The shape of this curve is quite different from that exhibited by the simple junction (compare with Fig. 2C), indicating a cooperative binding of magnesium ions. Fitting the experimental data (●) to a simple binding model suggests that the transition is induced by the binding of two ions.

apart. However, with the addition of divalent ions such as magnesium to a concentration ≥100 μM, there is a further rotation of the axes into a pronounced antiparallel geometry. In contrast to the loop-free junction, the full ribozyme is significantly distorted, with the closest end-to-end distance being that between the two loop-carrying arms (A and B). This indicates a strong interaction between the loops that pulls these arms together, apparently distorting the entire global structure of the ribozyme.

The rotation of the hairpin ribozyme is induced by magnesium, calcium or strontium ions, all of which lead to cleavage activity [26]. Manganese ions induce a global structure that is indistinguishable by FRET analysis, yet the ribozyme is inactive under these conditions. Monovalent ions such as sodium are completely incapable of folding the hairpin ribozyme, but hexammine cobalt(III) ions and spermidine induce a global folding that is indistinguishable from that in the presence of the divalent ions. Corresponding results have been recently obtained for the hinged form of the ribozyme [27]. In contrast to the isolated junction, the magnesium ion-induced folding indicates a more complex binding model. The simplest model producing an acceptable fit to the fluorescence data consists of the cooperative binding of two ions, with an association constant in the region of 10^9 M^{-2} [22,26]. This apparent affinity corresponds to the micromolar range of magnesium ion concentration for the transition, in contrast to the isolated junction which requires millimolar concentrations to induce folding [25].

5. Junction conformation and the activity of the hairpin ribozyme

The four-way junction has the properties of a scaffold on which the structure of the hairpin ribozyme is built, and the conformational characteristics of the junction are likely to determine the facility with which the two loops can interact. This has been confirmed experimentally in the following experiments.

The kinetics of cleavage for the junction form of the hairpin ribozyme were very similar to those of the minimal hinged form [22]. We changed the sequence around the four-way junction with that of a different junction (junction 3). Although the resulting sequence is quite different, the conformational preferences of this junction should be unaltered from the natural hairpin sequence [23,24], i.e. the stacking of arms A on D and B on C and thus the overall structure should be preserved. Loop-loop interaction should therefore still be possible in this conformer, and the result was found to be a hybrid ribozyme with a very similar rate of cleavage to the natural sequence [28]. In another experiment we left the natural sequence of the junction untouched, but rotated the positions of the arms about the central junction in a cyclical manner. This should lead to a tendency to adopt the alternative stacking conformer, and in this form the two loops are now located on helices that are coaxially stacked. Loop-loop interaction would require disruption of this structure in some manner, and the result was a significant reduction in the rate of cleavage [22]. Lastly, we replaced the sequence of the junction by that of the U1 snRNA. This was done in such a way that it should preserve the correct stacking conformer. However, the U1 junction has a marked tendency to remain in the 90° crossed structure, thus hindering rotation into the antiparallel structure [23,24]. Even though the stacking conformer is correct for loop-loop interaction, contact would be expected to be impaired due to the reluctance of this junction to undergo ion-induced rotation. We found that this change also led to a significant reduction in cleavage rate [28].

The junction of the hairpin ribozyme can be replaced with one of different sequence, but so long as this has the same conformational properties, ribozyme cleavage is largely unaffected. However, if the conformation is altered, either by

changing the choice of stacking partner (thus placing the loops onto the same pair of stacked helices) or by interfering with the rotation that brings the loops together, the activity of the ribozyme is reduced. Clearly the conformation of the junction has a major influence on the function of the ribozyme. However, it should be noted that the resulting activity of the conformationally impaired ribozymes remains significant. This is probably because the loop-loop interaction is sufficiently strong that it can partially overcome the conformational preferences of the junction, perhaps pulling the equilibrium towards the A on D conformer in the case of the rotated junction, and forcing the rotation in the case of the U1 junction. The relatively asymmetrical global structure of the ribozyme (see Fig. 3) compared to the simple junction [25] also suggests a strong interaction between the loops.

6. Further ion-induced conformational changes in the activation of the hairpin ribozyme

The transition of the global structure of the hairpin ribozyme into the antiparallel structure (with loop-loop interaction) occurs largely in the magnesium ion concentration range 0–100 μM, and the FRET analysis suggests cooperative binding, possibly of two ions (Fig. 3C) [22,26]. However, this is quite different from the concentration range required for activation of ribozyme activity, which becomes maximal in the millimolar range [28]. The kinetic data suggest that the cleavage rate is sensitive to the non-cooperative binding of a single ion. We conclude that the FRET and the cleavage experiments are therefore detecting the consequences of different binding events. Since no change in FRET efficiencies is detected during titration of magnesium ion over the millimolar range of concentration, any structural effects must be local such that the global shape of the ribozyme is essentially unaffected. It is possible that the additional metal ion is required to participate directly in the chemistry of the transesterification cleavage of the phosphodiester backbone. However, demonstration of cleavage activity in the presence of the substitutionally inert hexammine cobalt(III) complex [12–14] or high concentration of monovalent ions [15] appears to exclude a direct role for the metal ion as either a Lewis acid or in general base catalysis. This seems to leave only a structural role for the additional ion. Since the effect of its binding is not detectable by FRET, it seems probable that it produces a local change in conformation that is a prerequisite for cleavage. Since the ribozyme is inactive in manganese ions, despite apparently folding the RNA normally [26], it seems likely that the transition metal ion behaves similarly to magnesium in terms of the overall folding, but it is unable to participate in this local manner.

7. The distribution of structural metal ions in the folding of the hairpin ribozyme

The simplest interpretation of the ion-induced, FRET-detected folding of the hairpin ribozyme is that the cooperative binding of two magnesium ions is required to bring about the folding into the form with close interaction between the loops. When we remove the loops to leave an isolated junction, the folding becomes induced by the non-cooperative binding of a single magnesium ion [25]. We have also obtained point mutations in the loop sequence with very similar folding properties (Z. Zhao and D.M.J. Lilley, unpublished data). If we remove the junction, to leave a form equivalent to the minimal hinged ribozyme, this junction-free form of the RNA also folds by the non-cooperative binding of a one magnesium ion (F. Walter, A.I.H. Murchie and D.M.J. Lilley, unpublished data). Putting these observations together suggests that in the full ribozyme two ions bind cooperatively to bring about the folding, and these are distributed between the junction and the loop-loop interface. Uranyl ion-induced photocleavage has revealed ion binding at the point of strand exchange of the four-way DNA junction [29], and selective phosphate neutralisation by substitution with methyl phosphonates [30] suggests that charge screening at the point of strand exchange is essential. Hexammine cobalt(III) binding has recently been observed at the corresponding position in the crystal structure of a hybrid RNA-DNA junction [31]. While most multivalent metal ions fold the complete hairpin ribozyme into the global structure observed in the presence of magnesium ions, a novel structure is induced by cadmium ions. In this case the results can be interpreted in terms of close loop-loop interaction in the absence of a correctly folded junction [26], suggesting that this ion can bind at the loop interface but not the junction, and has thus separated the two functions.

8. Generation of catalytic activity

The folding of the hairpin ribozyme is quite different from that of the hammerhead ribozyme. In the latter case we observe two sequential single-ion-induced transitions that can be associated with the formation of different features within the overall structure [32,33]. Nevertheless, both the hammerhead and hairpin ribozymes exhibit ion-induced conformational changes associated with folding into their active forms, and RNA structure is the key to catalytic activity. Conformation is probably the single most important factor in the generation of site-specific transesterification rate enhancement, facilitating the molecular trajectory into the transition state in which there is alignment of [O_2'-P_3'-O_5']. By contrast, the direct role of metal ions in the chemistry appears to be dispensable [12–15], although current data do not preclude a role under normal circumstances. Thus RNA conformation is almost certainly the major player in the action of the small nucleolytic ribozymes, and in the case of the hairpin ribozyme the four-way junction provides the scaffold that facilitates the formation of the tertiary structure required to achieve the catalytically competent conformation.

Acknowledgements: The original research summarised in this review of Drs Alastair Murchie, Frank Walter, James Thomson and Z. Zhao is acknowledged. This work has been supported by the Cancer Research Campaign and the Biotechnology and Biological Sciences Research Council.

References

[1] Eckstein, F. and Lilley, D.M.J. (1996) RNA Catalysis, Springer-Verlag, Berlin.
[2] Forster, A.C. and Symons, R.H. (1987) Cell 49, 211–220.
[3] Haseloff, J.P. and Gerlach, W.L. (1988) Nature 334, 585–591.
[4] Epstein, L.M. and Gall, J.G. (1987) Cell 48, 535–543.
[5] Feldstein, P.A., Buzayan, J.M. and Bruening, G. (1989) Gene 82, 53–61.
[6] Hampel, A. and Tritz, R. (1989) Biochemistry 28, 4929–4933.

[7] Beattie, T.L., Olive, J.E. and Collins, R.A. (1995) Proc. Natl. Acad. Sci. USA 92, 4686–4690.
[8] Sharmeen, L., Kuo, M.Y., Dinter-Gottlieb, G. and Taylor, J. (1988) J. Virol. 62, 2674–2679.
[9] Hutchins, C.J., Rathjen, P.D., Forster, A.C. and Symons, R.H. (1986) Nucleic Acids Res. 14, 3627–3640.
[10] Uhlenbeck, U.C. (1987) Nature 328, 596–600.
[11] Koizumi, M. and Ohtsuka, E. (1991) Biochemistry 30, 5145–5150.
[12] Hampel, A. and Cowan, J.A. (1997) Chem. Biol. 4, 513–517.
[13] Nesbitt, S., Hegg, L.A. and Fedor, M.J. (1997) Chem. Biol. 4, 619–630.
[14] Young, K.J., Gill, F. and Grasby, J.A. (1997) Nucleic Acids Res. 25, 3760–3766.
[15] Murray, J.B., Seyhan, A.A., Walter, N.G., Burke, J.M. and Scott, W.G. (1998) Chem. Biol. 5, 587–595.
[16] Buzayan, J.M., Gerlach, W.L. and Bruening, G. (1986) Nature 323, 349–353.
[17] DeYoung, M.B., Siwkowski, A.M., Lian, Y. and Hampel, A. (1995) Biochemistry 34, 15785–15791.
[18] van Tol, H., Buzayan, J.M. and Bruening, G. (1991) J. Virol. 280, 23–30.
[19] Hegg, L.A. and Fedor, M.J. (1995) Biochemistry 34, 15813–15828.
[20] Komatsu, Y., Shirai, M., Yamashita, S. and Ohtsuka, E. (1997) Bioorg. Med. Chem. 5, 1063–1069.
[21] Earnshaw, D.J., Masquida, B., Müller, S., Sigurdsson, S.T., Eckstein, F., Westhof, E. and Gait, M.J. (1997) J. Mol. Biol. 274, 197–212.
[22] Murchie, A.I.H., Thomson, J.B., Walter, F. and Lilley, D.M.J. (1998) Mol. Cell 1, 873–881.
[23] Duckett, D.R., Murchie, A.I.H. and Lilley, D.M.J. (1995) Cell 83, 1027–1036.
[24] Walter, F., Murchie, A.I.H., Duckett, D.R. and Lilley, D.M.J. (1998) RNA 4, 719–728.
[25] Walter, F., Murchie, A.I.H. and Lilley, D.M.J. (1998) Biochemistry 37, 17629–17636.
[26] Walter, F., Murchie, A.I.H., Thomson, J.B. and Lilley, D.M.J. (1998) Biochemistry 37, 14195–14203.
[27] Walter, N.G., Hampel, K.J., Brown, K.M. and Burke, J.M. (1998) EMBO J. 17, 2378–2391.
[28] Thomson, J.B. and Lilley, D.M.J. (1999) RNA 5, 180–187.
[29] Møllegaard, N.E., Murchie, A.I.H., Lilley, D.M.J. and Nielsen, P.E. (1994) EMBO J. 13, 1508–1513.
[30] Duckett, D.R., Murchie, A.I.H. and Lilley, D.M.J. (1990) EMBO J. 9, 583–590.
[31] Nowakowski, J., Shim, P.J., Prasad, G.S., Stout, C.D. and Joyce, G.F. (1999) Nature Struct. Biol. 6, 151–156.
[32] Bassi, G., Møllegaard, N.E., Murchie, A.I.H., von Kitzing, E. and Lilley, D.M.J. (1995) Nature Struct. Biol. 2, 45–55.
[33] Bassi, G.S., Murchie, A.I.H., Walter, F., Clegg, R.M. and Lilley, D.M.J. (1997) EMBO J. 16, 7481–7489.