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Novel Spermine–Amino Acid Conjugates and Basic Tripeptides Enhance Cleavage of the Hairpin Ribozyme at Low Magnesium Ion Concentration

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Abstract—Combinations of the polyamine spermine and magnesium ions synergize to dramatically enhance cleavage of the hairpin ribozyme. Certain synthetic basic tripeptides stimulate hairpin cleavage significantly at limiting magnesium ion concentration, notably the tripeptide of L-diaminobutyric acid (Dab). Of a range of novel synthetic spermine–amino acid conjugates, L-Dab-spermine (but not D-Dab nor other amino acid conjugates) was more effective than spermine itself.

Ions play essential roles in the folding and catalysis of small endonucleolytic ribozymes.[1] The hairpin ribozyme[2–4] is commonly studied in its minimum catalytic motif, consisting of two helical domains (A and B) each containing an internal loop flanked by two duplex regions (Fig. 1). The active conformation is achieved through antiparallel docking of the two domains and localized rearrangements involving the formation of new inter-domain hydrogen bonds.[5,6]

Folding of the natural four-way junction hairpin requires only μM magnesium ion concentration,[7] whereas mM concentrations are needed to fold the minimal form.[8] Efficient cleavage requires 10 mM magnesium ion concentration for both natural and minimal forms,[9,10] a concentration 20-fold higher than is present within cells. Metal ions do not participate directly in the hairpin cleavage mechanism[11,12] and instead appear to play structural roles, such as in docking and conformational change.

In place of metal ions, efficient hairpin ribozyme cleavage can be effected by mM concentrations of the tetra-amine spermine.[13] The shorter tri-amine spermidine is much less effective.[9,13] We showed previously that certain combinations of spermine and magnesium ions lead to up to 100-fold stimulation of hairpin ribozyme cleavage, particularly at limiting magnesium ion concentrations (0.5 mM).[13,14]

We looked for alternative scaffolds to place sequential amino groups of a similar pK_a range to spermine. Although a peptide backbone is less flexible than a polyamine in that rotation around the amide bond is more restricted, such molecules allow for ready chemical synthesis and the ability to locate specific functionalities on a flexible side chain. Since the natural amino acids lysine and arginine have long side chains and high pK_a values, we synthesized instead C-terminal amides of linear tripeptides of other basic amino acids containing primary amino groups spaced 3, 2 or 1 methylene groups from the α-carbon atom, namely ornithine (Orn), 2,4-diaminobutyric acid (Dab) and 2,3-diaminopropionic acid (Dap), respectively. Tripeptides composed of such amino acids have three side-chain amino groups with nominal pK_a values of 10.8, 9.8 and 9.5, respectively, and an N-terminus (pK_a 8–9), of similar range to that of spermine (8.6, 9.3, 10.5, 11.2). The peptides were synthesized by Fmoc methods of solid phase peptide synthesis[15,16] and characterized by MALDI-TOF mass spectrometry. His-His-His (pK_a 6.0) was also prepared as well as the mixed tripeptide Orn-Dap-Dab.

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A minimal three-stranded hairpin ribozyme was prepared from chemically synthesized oligoribonucleotides[17,18] (Fig. 1). This hairpin is known to fold and cleave well under standard (10 mM magnesium ions) conditions.[5,19,20] As expected, the cleavage rate (k_{obs}) of this construct under single turnover conditions and at limiting (0.5 mM) magnesium ion concentration at 37 °C at pH 7.4[21,22] was 0.0012 ± 0.0004 min^{-1}, some 100-fold lower than at standard 10 mM magnesium ion concentration,[9] and similar to our previous three-stranded hairpin.[14] The cleavage rate was measured in the presence of 0.5 mM magnesium ions plus 1, 5 or 10 mM of tripeptide ligand (Fig. 2). The k_{rel} (the k_{obs} measured in the presence of tripeptide relative to that obtained at 0.5 mM magnesium ions alone) was boosted 25-fold for 5 mM Dab-Dab-Dab and 18-fold for Dap-Dap-Dap. By contrast, Orn-Orn-Orn or His-His-His boosted cleavage only slightly.

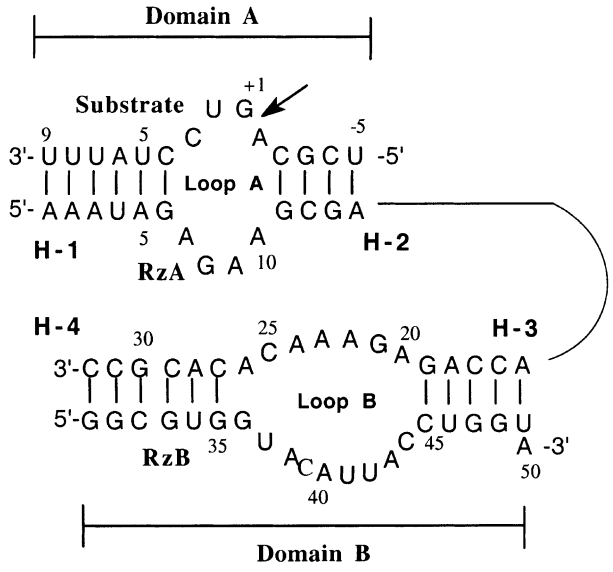

Figure 1. Secondary structure of the three-stranded hairpin ribozyme. The cleavage site is in domain A and is denoted by an arrow. The four helixes (H1–H4) are obtained by annealing of the substrate strand with the preformed complex of ribozyme strands A and B (RzA and RzB).

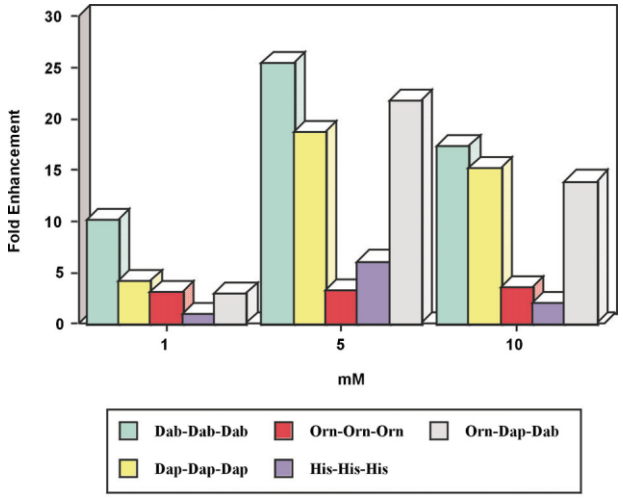

Figure 2. Relative cleavage efficiency (k_{rel}) values for hairpin ribozyme cleavage at three concentrations of tripeptide in the presence of 0.5 mM magnesium ions compared to magnesium ions alone.

The mixed Orn-Dap-Dab tripeptide at 5 mM stimulated cleavage 22-fold. Under the same conditions, 5 mM spermine boosted cleavage 54-fold (Table 1). These results show that tripeptides of the right size and pK_a range can cooperate with magnesium ions almost as well as spermine.

We asked if the cooperative cationic effect could be enhanced by a combination of the properties of spermine and a basic amino acid. Therefore, we synthesized amino acid–spermine conjugates by a solid-phase method (Fig. 3) through use of temporary primary amino protection with 2-acetyl-5,5-dimethyl-1,3-cyclohexanedione (Dde-OH).[23,24] Products were characterized as the expected conjugates by TLC, amino acid analysis and MALDI-TOF mass spectrometry.[25] Conjugates were tested at 2 mM and 5 mM concentrations for their abilities to enhance the cleavage of the hairpin ribozyme in the presence of 0.5 mM magnesium ion concentration under single turnover conditions (Table 1). Whereas spermine boosted cleavage 61- and 54-fold, respectively, almost all the amino acid–spermine conjugates showed a lesser ability to cooperate with magnesium ions than spermine.

By contrast, the spermine conjugate of L-Dab was a little more effective than spermine. L-Dap-spermine was the only other conjugate to show substantial activity on a par with spermine itself, but neither D-Dab nor D-Dap spermine conjugates were as effective as spermine. Dipeptide–spermine conjugates did not show enhancements in the cleavage rate.

To verify the activity of L-Dab-spermine, cleavage rates were measured over a concentration range in conjunction

Table 1. Values for k_{obs} and fold enhancement for amino acid–spermine conjugates at 2 mM (left) and 5 mM (right) in the presence of 0.5 mM magnesium ions

Ligand	k_{obs}[a]	Fold enhanced	k_{obs}	Fold enhanced
Spermine	0.073	61	0.065	54
L-Arg	0.016	15	0.034	32
L-His	0.037	35	0.013	12
L-Dab	0.085	71	0.080	67
L-Dap	0.061	51	0.045	38
L-Lys	0.016	15	0.013	13
Gly	0.017	16	0.030	28
β-Ala	0.018	17	0.030	28
4-Abu	0.0088	8	0.023	22
D-His	0.0085	8	0.026	25
D-Dab	0.023	22	0.027	26
D-Dap	0.020	19	0.022	21
D-Lys	0.0040	4	0.026	25
D-Orn	0.0086	8	0.034	32
L-Dab-Dab	0.021	19	nd[b]	
L-Dap-Dap	0.024	23	nd	
L-Dap-Dab	0.026	25	nd	
L-Dab-Dap	0.020	18	nd	

[a] k_{obs} values are the mean of 2–3 experiments. The error margins are ±20%.
[b] nd, not determined.

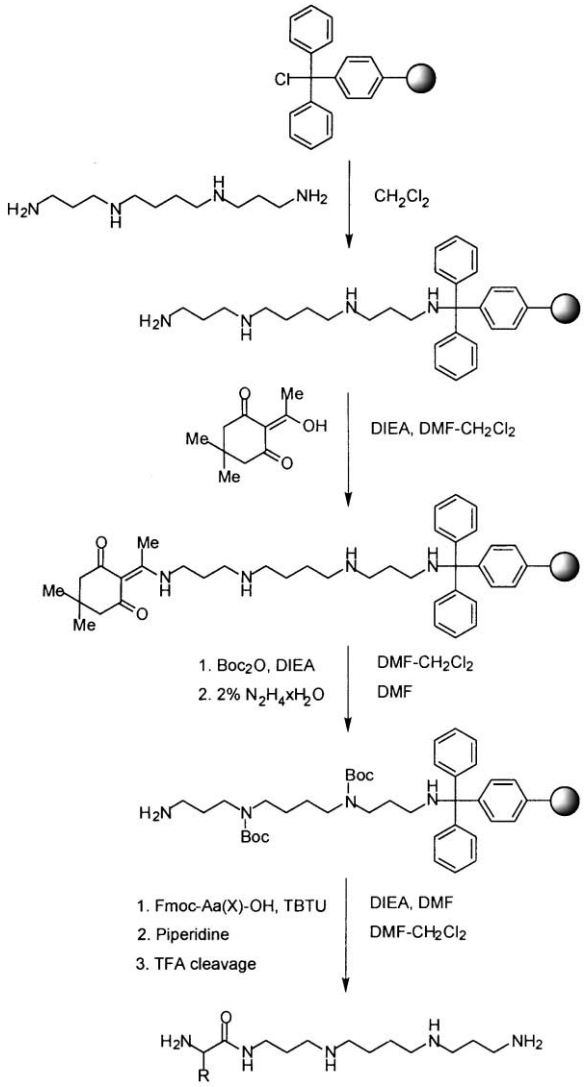

Figure 3. Scheme for solid-phase synthesis of amino acid–spermine conjugates. R = side chain of amino acids Gly, L-His, L-Arg, L-Lys, L-Orn, L-Dap, L-Dab, D-His, D-Lys, D-Orn, D-Dap and D-Dab. In the case of β-Ala and aminobutyric acid R = H and 1 or 2 extra methylene groups, respectively, are inserted between the amino and carboxylic groups. In the case of dipeptide–spermine conjugates, steps 1 and 2 are repeated in the final transformation prior to TFA cleavage.

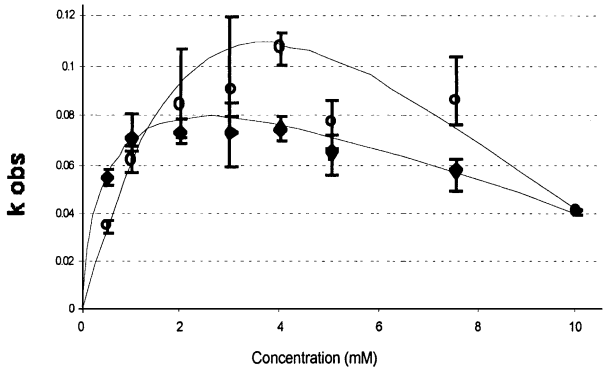

Figure 4. Graph showing the effect of L-Dab-spermine (open circles) compared to spermine (diamonds) on hairpin ribozyme cleavage at limiting magnesium ion concentration (0.5 mM). K_{obs} values were obtained over at least four determinations per ligand concentration.

with a fixed concentration (0.5 mM) of magnesium ions (Fig. 4). Each curve has a similar shape with rate maximum at 2–4 mM and gradually dropping at higher concentrations, presumably due to partial displacement of magnesium ions. A small improvement in rate is seen in the case of L-Dab-spermine to reach a k_{obs} value of about 0.1 min^{-1}. This shows that synthetic ligands can be found with better cooperative properties than spermine.

The mechanistic basis of ligand cooperativity is unknown. An established role for cationic ligands is in the docking of the two hairpin domains[8,10] and part of the cooperative effects observed may be due to improvements in docking rates. The shorter polyamine spermidine does not promote efficient docking of the minimal hairpin, but instead is able to boost the docking rate induced by magnesium ions.[8] However, no studies have appeared on whether the longer polyamine spermine can promote hairpin docking. The recent X-ray crystal structure of a four-way junction hairpin ribozyme has confirmed that docking is associated with rearrangements within the internal loop regions and the formation of new inter-domain contacts,[6] some of which were previously predicted.[5,26,27] Two ionic sites have been located in the major groove of domain B that may help to mediate domain rearrangements. Further ion placement may be required to attain an active configuration, perhaps by providing electrostatic stabilization for the anionic transition state. For example, a DNA-zyme has been selected that can carry out intramolecular RNase-like cleavage in the absence of metal ion by location of base-tethered imidazolyl and primary amino functional groups within the catalytic core.[28] Similar tethering of polycationic ligands to the hairpin ribozyme may reduce the free ligand requirement for cleavage.

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25. Amino acid analysis used a high-performance sodium column (Pharmacia) with sodium citrate buffers of pH 3.2, 4.25 and 6.45. In the case of Orn, Dab and Dap, the pH 6.45 buffer was replaced by borate/citrate buffer (pH 8.6). TLC on Alugram Sil G/UV254 silica gel plates (Macherey-Nagel) eluting with acetic acid/water/*n*-butanol (1:3:1) and development with 1% ninhydrin solution. The conjugates all showed a major product (>80%) with mobility marginally slower than spermine (R_f 0.5) and much slower than amino acids (R_f 0.7–0.8).
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