Enhancement of RNA self-cleavage by micellar catalysis

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Abstract **It has been reported recently that naturally occurring catalytic RNAs like hammerhead and hairpin ribozyme do not require metal ions for efficient catalysis. It seems that the folded tertiary structure of the RNA contributes more to the catalytic function than was initially recognized. We found that a highly specific self-cleavage reaction can occur within a small bulge loop of four nucleotides in a mini-substrate derived from *Arabidopsis thaliana* intron-containing pre-tRNA^{Tyr} in the absence of metal ions. NH_4^+ cations and non-ionic or zwitter-ionic detergents at or above their critical micelle concentration are sufficient to catalyze this reaction. The dependence on micelles for the reaction leads to the assumption that physical properties, i.e. the hydrophobic interior of a micelle, are essential for this self-cleavage reaction. We suggest that NH_4^+-ions play a crucial role for the entry of the negatively charged RNA into the hydrophobic interior of a detergent micelle. A change of the pattern of hydration or hydrogen bonds caused by the hydrophobic surrounding enhances the reaction by a factor of 100. These findings suggest that highly structured RNAs may shift pK_a values towards neutrality via the local environment and thereby enhance their ability to perform general acid-base catalysis without the participation of metal ions.**

Key words: Bulge loop; Metal ion; Micellar catalysis; Monovalent cation; Non-ionic detergent; RNA self-cleavage

1. Introduction

The discovery of RNA catalysis has created interest in using RNA enzymes or 'ribozymes' to target the degradation of specific RNA molecules in vivo. Ribozymes are extremely exciting molecules because of their ability to promote the hydrolysis and transphosphoesterification of the RNA phosphodiester backbone *in trans* or *in cis* that yield 3′-OH and 5′-phosphate or 2′,3′-cyclic phosphate and 5′-OH products [1]. Like proteins they retain the ability to act like a real catalyst, facilitating reactions without being changed themselves, accelerating reactions and reacting in a substrate-dependent manner [2]. Catalytic RNA activity, first discovered in the cellular RNA splicing and processing machinery as self-splicing group I introns [3] and precursor tRNA processing RNAse P [4], has been identified in a number of smaller RNAs. These naturally occurring catalytic RNAs are generally found in the genomes of RNA viruses and in virus-related RNAs [5]. Comparison of several self-cleavage RNA sequences and structures led to the identification of consensus motifs termed hammerhead and hairpin motif [6]. Recently two additional consensus motifs have been identified in the genomic RNA of hepatitis δ virus (HDV) [7] and in mitochondrial *Neurospora* RNA [8].

Almost all ribozymes require divalent metal ions (typically Mg^{2+}) for activation. However, it has been difficult to distinguish structural from catalytic roles for the metal ions and to identify the molecular mechanism of catalysis [9,10]. Especially the hairpin ribozyme seems to possess a distinct catalytic mechanism, where metal ions are not obligatory chemical participants in the reaction [11–13]. In this case the correctly folded structure is the fundamental requirement for catalysis and can be supported by the addition of high concentrations of monovalent cations in the absence of divalent metal ions such as Mg^{2+} [14].

Self-cleaving sequences, which do not resemble either of the motifs mentioned above, have been found in a precursor of an RNA from T4-infected *Escherichia coli* cells, i.e. p2Sp1 RNA [15], in human pre-tRNA^{Tyr} transcribed in vitro in HeLa cell nuclear extract [16] as well as in an in vitro transcribed pre-tRNA^{Tyr} from *Arabidopsis thaliana* [17]. Here we show that a mini-substrate derived from the *Arabidopsis* pre-tRNA^{Tyr} consisting of the anticodon stem including the intron and lacking the rest of the mature tRNA domain also undergoes highly specific self-cleavage at identical positions. Since the mature domain of the pre-tRNA is not involved in the self-cleavage reactions, we have studied the structure and sequence requirements of the reaction in a corresponding mini-substrate. The results indicate that the reaction can be distinguished from the self-cleavage activities of hammerhead and hairpin ribozymes. Monovalent cations, a non-ionic or zwitter-ionic detergent at or above its critical micelle concentration (CMC) and a single-stranded pyrimidine-A bond (U-A or C-A) are necessary and sufficient for this highly specific in vitro self-cleavage reaction.

2. Materials and methods

2.1. Enzymes and reagents

T7 RNA polymerase was prepared from an overproducing strain of *E. coli* kindly provided by Dr W. Studier [18]. Alkaline phosphatase and RNAse I were from Boehringer Mannheim, RNAse inhibitor from Fermentas and T4 polynucleotide kinase from Biozym. Oligodeoxynucleotides were synthesized on a 'DNA-Synthesizer-Gene Assembler Plus' from Pharmacia. [γ-^{32}P]ATP with a specific activity of 15 Tbq/mmol was purchased from Hartmann Analytic (Braunschweig, Germany). Triton X-100 ultrapure was from Pierce, other detergents were from Calbiochem. All other reagents were from commercial suppliers and autoclaved before use.

2.2. Preparation and purification of RNA mini-constructs

T7 RNA transcription was carried out with an oligodeoxynucleotide matrix following the protocol of Milligan and Uhlenbeck [19]. The matrix consisted of two oligodeoxynucleotides that were hybridized. For annealing, 500 pmol of the oligodeoxynucleotide containing the complete T7 promoter and 500 pmol of the oligodeoxynucleotide containing the sequence of the RNA mini-construct at its 5′-end and the T7 promoter at its 3′-end were incubated for 30 min at 5°C under their calculated T_m in a volume of 20 μl. After annealing, the DNA hybrid was transcribed to RNA with the help of T7 RNA polymerase.

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In a total volume of 30 μl, transcription of the annealed oligodeoxynucleotides was carried out with 60 ng T7 RNA polymerase/μl. Incubation was for 90 min at 37°C in 40 mM Tris-HCl, pH 8.1, 12 mM $MgCl_2$, 5 mM DTT, 1 mM spermidine and NTPs at 4 mM each. The reaction was terminated by the addition of 1 μl 0.5 M EDTA. The transcripts were purified by repeated phenol/$CHCl_3$ extractions and electrophoresis in a 20% polyacrylamide/8 M urea-TBE gel. After detection through fluorescence shadowing of the electrophoretically purified RNA it was eluted from the gel with TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5) and the concentration was measured photometrically.

2.3. Dephosphorylation of RNA mini-constructs

In a 15 μl assay 2 to 4 μg newly synthesized transcript were dephosphorylated by 0.1 U alkaline phosphatase/μl. Incubation was performed at 37°C for 2 h in the presence of 1 U/μl RNAse inhibitor. The reaction was stopped on ice, purified twice by phenol and phenol/$CHCl_3$/isoamylalcohol (25:24:1) extractions and precipitated with 3 vol of ethanol overnight at −20°C. After a second ethanol precipitation for 30 min at −80°C the RNA was vacuum dried and suitable for 5′-^{32}P-endlabelling.

2.4. Radioactive labelling of RNA mini-constructs

For 5′-^{32}P-endlabelling, the vacuum dried dephosphorylated RNA mini-constructs were incubated for 45 min at 37°C with 10 U T4 polynucleotide kinase/μl, 1 U RNAse inhibitor/μl and 1 MBq [γ-^{32}P]ATP. The reaction was stopped on ice and purified on a 20% polyacrylamide/8 M urea-TBE gel. The 5′-^{32}P-endlabelled RNA was detected by autoradiography and eluted in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5). After elution and purification the RNA was ready for in vitro cleavage assays.

2.5. Structure probing of RNA mini-constructs

Chemical and enzymatic structure probing was used to determine and confirm the postulated secondary structure of the RNA mini-constructs. Pb^{2+}-ions were used as a chemical probe [20]. Reaction was performed in a total volume of 20 μl buffer (10 mM Tris-HCl, pH 7.5, 20 mM NaCl, 10 mM $MgCl_2$), with 10 μg ultrapure tRNA from calf liver and 10 to 50×10^3 c.p.m. 5′-^{32}P-endlabelled RNA and incubated for 5 min at 30°C or 15 min at room temperature in the presence of 1 to 10 mM Pb^{2+}-ions. The reaction was stopped on ice and analyzed by autoradiography after a 20% polyacrylamide/8 M urea-TBE gel electrophoresis. Enzymatic structure probing with RNAse I from *E. coli* [21] was performed in 20 μl volumes. 10^4 to 5×10^4 c.p.m. 5′-^{32}P-endlabelled RNA were incubated in the presence of 10 mM Tris-HCl, pH 7.5, 20 mM EDTA, 10 μg ultrapure tRNA from calf liver and 0.1 U RNAse I/μl at 37°C for 10 min. The reaction was stopped on ice and analyzed by 20% polyacrylamide/8 M urea-TBE gel electrophoresis and autoradiography. Structure and cleavage site analysis was supported by partial cleavage of 5′-^{32}P-endlabelled RNA mini-construct through H^+-ions [22]. For this purpose 5×10^4 c.p.m. 5′-endlabelled mini-substrate transcript were incubated in 6 M urea, 0.1 M H_2SO_4, 0.03% Xylencyanol (w/v) and 10 μg ultrapure tRNA from calf liver for 3 min at 95°C in a total volume of 8 μl. The reaction was stopped on dry ice and loaded on a 20% polyacrylamide/8 M urea-TBE gel.

2.6. In vitro cleavage of synthetic RNA mini-constructs

5 to 10×10^3 c.p.m. of the purified 5′-^{32}P-endlabelled RNA mini-constructs were incubated in the self-cleavage buffer with 100 mM NH_4OAc, pH 7.0, 10 mM $MgCl_2$, 0.5 mM spermine and 0.4% Triton X-100 established by van Tol et al. [16]. Standard incubation was for 1 h at 37°C. The samples were analyzed by electrophoresis on 20% polyacrylamide/8 M urea-TBE gels. Autoradiograms were evaluated by densitometry using an LKB UltroScan XL laser densitometer for quantification of the self-cleavage products.

2.7. Fluorimetric determination of critical micelle concentration

Fluorimetric determination of critical micelle concentration was performed using a method developed by Chattopadhyay and London [23]. Fluorescence measurements were performed with a spectrofluorometer using 1 cm path-length quartz cuvettes. The excitation wavelength was 358 nm and the emission wavelength was 430 nm. Fluorescence was averaged over 5 s for each sample reading. For determination of the critical micelle concentration (CMC) 1 μl of 10 mM 1,6-diphenyl-1,3,5-hexatriene (DPH) in tetrahydrofuran (THF) was added to various amounts of Triton X-100 in ddH_2O or self-cleavage buffer in a total volume of 1.5 ml of aqueous solution. Tubes were vortexed and incubated in the dark for 30 min at room temperature. Background samples lacking DPH were prepared, and their fluorescence intensity was subtracted. Duplicate sets of samples were prepared in each case. To reverse photoisomerization of DPH, samples were kept in the dark in the fluorimeter for 30 s before the excitation shutter was opened and fluorescence measured.

3. Results and discussion

3.1. Self-cleavage reaction of the mini-construct AtY3II/M

Previously we have shown that a human intron-containing $tRNA^{Tyr}$ precursor transcribed in vitro in HeLa cell nuclear extract [16] and an *A. thaliana* pre-$tRNA^{Tyr}$ transcript (AtY3II*-T7) generated in vitro by T7 RNA polymerase [17] possess self-cleavage activity. Both pre-tRNAs undergo self-cleavage at the authentic 3′-splice site and near the authentic 5′-splice site. The reactions take place under nearly physiological conditions (100 mM NH_4OAc, pH 7.0, 10 mM $MgCl_2$, 0.5 mM spermine, 0.4% Triton X-100). To investigate whether the mature domain of the pre-tRNA is important for these highly specific self-cleavage reactions, the mini-substrate AtY3II/M (Fig. 1b) was constructed and transcribed in vitro by T7 RNA polymerase. It is derived from *A. thaliana* pre-$tRNA^{Tyr}$ (Fig. 1a) and consists simply of its anticodon stem and loop including the intron and an extension at the 5′- and 3′-ends by three G:C pairs for a clamp effect and an improved transcription rate [19]. Incubation of the mini-substrate AtY3II/M under identical conditions as the full-length pre-$tRNA^{Tyr}$ in the self-cleavage buffer [17] resulted in hydrolysis at corresponding sites, i.e. self-cleavage occurred at the 3′-splice site in the bulge loop between the nucleotides U_{26}/A_{27} and one nucleotide downstream of the authentic 5′-splice site in the single-stranded anticodon loop region between U_{15} and A_{16} (Fig. 2a). An additional self-cleavage site due to secondary cleavages could be detected between U_{12}/A_{13} after 30 min of incubation. To ensure that the cleavage is not caused by any contamination with ribonuclease the solutions were pretreated with SDS and/or proteinase K. These treatments and an addition of up to 20 μg ultrapure tRNA from calf liver had no inhibitory effect on the self-cleavage reaction (not shown). From the specific self-cleavage reactions in the mini-substrate we conclude that the reaction is not dependent on the sequence and structure of the mature domain of the tRNA, i.e. that the extended anticodon stem with the intron is sufficient enough for this reaction.

3.2. Influence of structure and sequence on the self-cleavage reaction

In all three reported cases (human pre-$tRNA^{Tyr}$, plant pre-$tRNA^{Tyr}$ AtY3II*-T7 and mini-substrate AtY3II/M) self-cleavage exclusively took place in single-stranded regions between pyrimidine-A (U-A and C-A) bonds. We addressed the question whether double-stranded regions can undergo self-cleavage as well and whether the two splice sites can be cleaved independently of each other. For that purpose, we synthesized the mini-substrates AtY3II/M-DS3 and AtY3II/M-DS5 with either the 3′- or 5′-splice site being located in double-stranded regions. In case of AtY3II/M-DS3 the nucleotides from the 3′-bulge loop are base-paired with four added bases in the opposite strand (Fig. 1c), whereas in the construct AtY3II/M-DS5 the 5′-splice site is located in a dou-

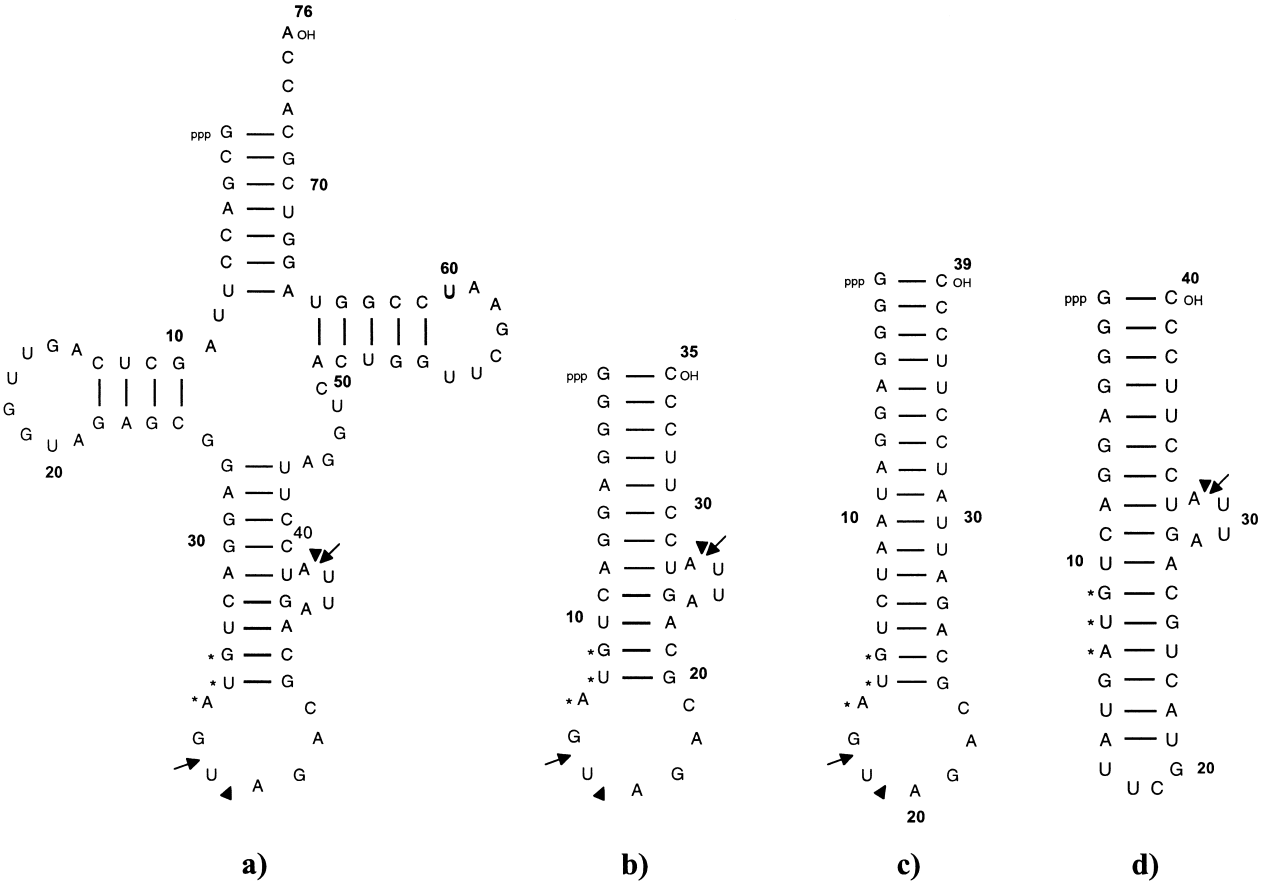

Fig. 1. Secondary structures of intron-containing pre-tRNA^{Tyr} and mini-substrates derived from it. a: Pre-tRNA^{Tyr} (AtY3II*-T7) from *A. thaliana*; b: mini-substrate AtY3II/M; c: mini-substrate AtY3II/M-DS3; and d: mini-substrate AtY3II/M-DS5 synthesized by in vitro transcription with T7 RNA polymerase. Dots identify the anticodon. Arrows indicate the authentic enzymatic 5′- and 3′-splice sites. The sites of self-cleavage are indicated by arrowheads.

ble-stranded region and the anticodon loop is exchanged by a very stable UUCG tetraloop [24] (Fig. 1d). In both cases the former 3′- and 5′-self-cleavage sites were still present, although in double-stranded regions. The secondary structure of all three mini-substrates was chemically and enzymatically probed to confirm the postulated structure. Pb^{2+}-ions were used as a chemical probe and the single-strand specific RNAse I from *E. coli* as an enzymatic probe (not shown).

To compare directly the three substrates with each other, they were all transcribed and incubated at the same time under identical conditions. After 1 h of incubation the mini-substrate AtY3II/M was cleaved to the expected shorter fragments (Fig. 2a). Self-cleavage of AtY3II/M-DS3 exclusively took place at the single-stranded 5′-splice site. No reaction was detectable at the double-stranded 3′-splice site and after 2 h of incubation 60% of the precursor were cleaved between the nucleotides U_{19}/A_{20} (Fig. 2b). Double bands in Fig. 2 are due to an inhomogenous transcript after T7 RNA polymerase transcription that could not be separated by gel electrophoresis [25]. In the mini-substrate AtY3II/M-DS5 self-cleavage exclusively occurred at the single-stranded 3′-splice site. No reaction was detectable at the double-stranded 5′-splice site. After 2 h of incubation under standard reaction conditions nearly 80% of the substrate AtY3II/M-DS5 were converted to shorter fragments by cleavage between U_{31}/A_{32} (Fig. 2c). From these results it is clear that the reaction is dependent on single-stranded regions and that the splice sites can undergo self-cleavage independently.

Previously we have shown that this self-cleavage creates 5′-OH and 2′,3′-cyclophosphate ends [16,17]. These product ends are the same as those generated by the small ribozymes hammerhead and hairpin [5], indicating that the reaction follows an S_N2 mechanism in which the attacking group, the leaving group as well as the electrophilic phosphor atom are nearly 'in-line' to each other [26]. To achieve this, a high flexibility of the RNA backbone as present in single-stranded regions is necessary. In double-stranded regions, a pseudorotation at the phosphor atom with a higher activation energy does not occur and hence does not allow self-cleavage [27].

With the mini-construct AtY3II/M-DS5 (Fig. 2c) being cleaved nearly quantitatively after a short incubation time, we subsequently focussed our interest on the 3′-bulge loop. All further investigations were accomplished particularly with the mini-substrate AtY3II/M-DS5 and its derivatives. At first we examined different nucleotide combinations at the self-cleavage site U_{31}/A_{32}, to answer the question whether only U-A bonds are capable of self-cleavage. The results indicate that cleavage is restricted to U-A and C-A bonds. No reaction was detected between a purine-purine bond, pyrimidine-pyrimidine bond and a purine-pyrimidine bond (not shown).

3.3. Influence of Mg^{2+}-ions, spermine and NH_4^+ on the self-cleavage reaction

After having elucidated the structure and sequence requirements of the self-cleavage reaction we further studied in details the optimal reaction conditions in the mini-construct AtY3II/M-DS5. The reaction has a broad pH optimum ranging from slightly acid (pH 6.5) to alkaline (pH 8.2) and a broad temperature range from 10°C to 50°C. To keep reaction conditions as physiological as possible the buffer was adjusted to pH 7.0. The optimal temperature was around 37°C. As expected, unspecific cleavage increased at higher temperatures whereas the reaction rate decreased at lower temperature. To investigate the contribution of the reaction buffer components NH_4OAc, $MgCl_2$, spermine and Triton X-100, we stepwise omitted each particular component from the standard reaction mixture. Fig. 3 (lanes e and f) indicates that neither magnesium ions nor spermine are essential for the cleavage reaction. Compared with reactions in standard self-cleavage buffer (Fig. 3, lanes b and c) a similar specificity and efficiency of the reaction was detected upon exclusion of one of these cations. Even an incubation in the absence of Mg^{2+}-ions and in the presence of up to 15 mM EDTA did not inhibit the reaction at all (not shown). Omission of both components from the reaction buffer resulted in unspecific and increased secondary cleavage (Fig. 3, lanes g and h). From these results

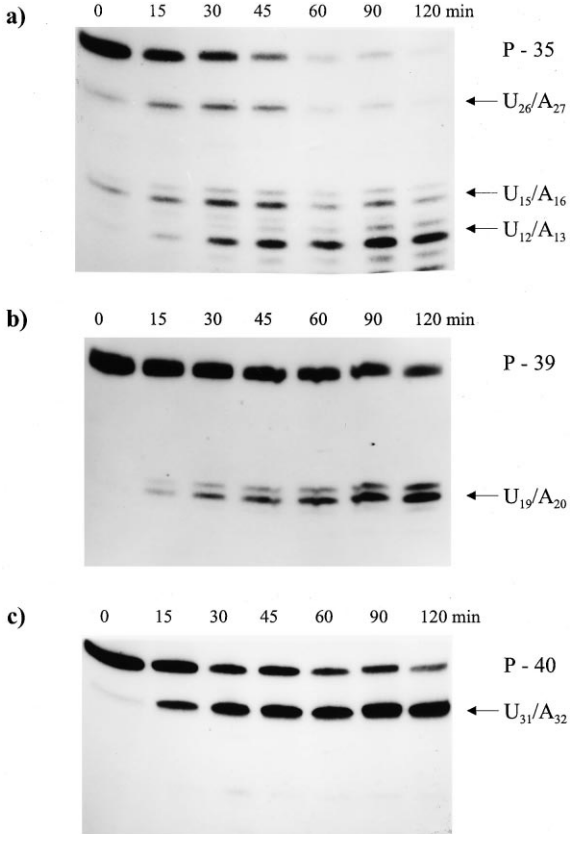

Fig. 2. Self-cleavage of mini-substrates in the absence of mature domain. a: AtY3II/M (P-35); b: AtY3II/M-DS3 (P-39); and c: AtY3II/M-DS5 (P-40). The 5′-^{32}P-endlabelled RNA was incubated in the self-cleavage buffer with 100 mM NH_4OAc, pH 7.0, 10 mM $MgCl_2$, 0.5 mM spermine and 0.4% Triton X-100 at 37°C. Incubation times (min) are indicated on top. Arrows indicate the fragments after self-cleavage and their origin. Cleavage products were analyzed on a 20% polyacrylamide/8 M urea-TBE gel.

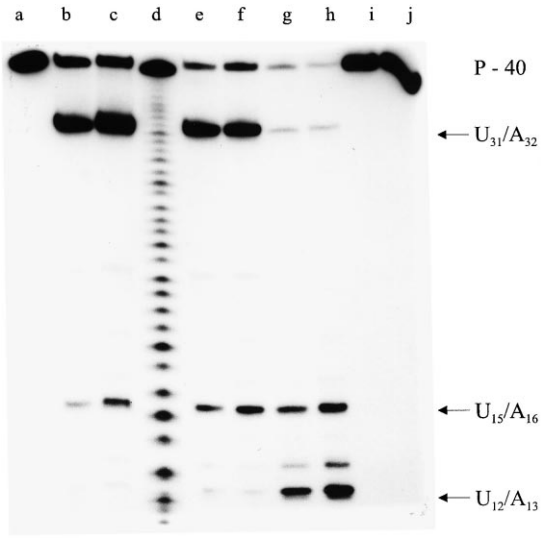

Fig. 3. Effect of different buffer components on the self-cleavage reaction. 5′-^{32}P-endlabelled AtY3II/M-DS5 (P-40) was transcribed in vitro with T7 RNA polymerase and used as mini-substrate in the following reactions: control lane, 3 h incubation in ddH_2O (lane a), 2 h and 3 h incubation in self-cleavage buffer (100 mM NH_4OAc, pH 7.0, 10 mM $MgCl_2$, 0.5 mM spermine and 0.4% Triton X-100) (lanes b and c), acid ladder of 5′-^{32}P-endlabelled AtY3II/M-DS5 (lane d), 3 h incubation in self-cleavage buffer without $MgCl_2$ (lane e), 3 h incubation in self-cleavage buffer without spermine (lane f), 2 h and 3 h incubation in self-cleavage buffer without $MgCl_2$ and spermine (lanes g and h), 2 h and 3 h incubation in self-cleavage buffer without Triton X-100 (lanes i and j). All incubations were performed at 37°C. Arrows indicate the fragments after primary and secondary self-cleavage reactions between the nucleotides U_{31}/A_{32}, U_{15}/A_{16} and U_{12}/A_{13}, respectively. Cleavage products were analyzed on a 20% polyacrylamide/8 M urea-TBE gel.

we conclude that Mg^{2+} and/or spermine are not directly involved in the reaction itself but improve the specificity and preciseness of the cleavage reaction by stabilizing the structure of the RNA substrate.

Divalent metal ions were supposed to be responsible for the stability and catalytic activity of RNA [10]. However, the role of metal ions in catalysis of ribozyme action still remains elusive [28]. A differentiation between a catalytic and a structural role is often difficult, since the charged phosphodiester backbone of RNA is coated with metal ions [29]. All of the known, naturally occurring ribozymes catalyze phosphoryl transfer reactions, particularly suited for using metal ions. However, the hairpin ribozyme seems to use a catalytic mechanism without metal ions [11–13,28]. Very recently it has been reported that even hammerhead and the VS ribozyme of *Neurospora* mitochondria are capable of catalyzing RNA cleavage reactions efficiently in the absence of divalent cations and in the presence of high amounts of ammonium ions [14]. These observations are similar to the self-cleavage reactions studied here, where Mg^{2+}-ions do not exert a catalytic role (Fig. 3, lane e). They can be exchanged for spermine without any differences in specificity and efficiency. Hence it appears that both positively charged components are important for the stability of the RNA mini-substrates, but not essential for the reaction itself.

A significant reduction of the cleavage was observed when NH_4OAc was excluded from the incubation buffer. A decrease of the cleavage rate was also observed when NH_4^+-ions were

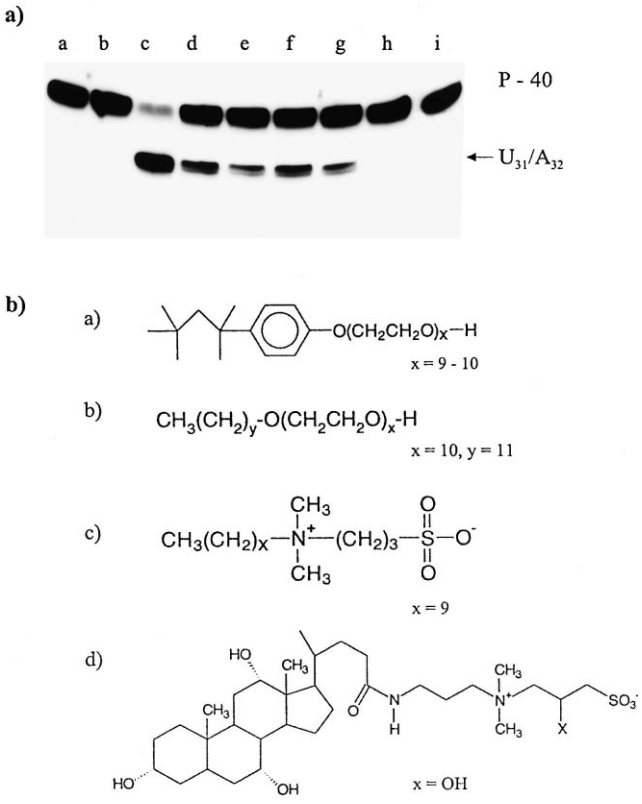

Fig. 4. a: Influence of different detergents on the self-cleavage reaction of in vitro transcribed 5′-^{32}P-endlabelled mini-substrate AtY3II/M-DS5 (P-40). Standard reaction conditions (1 h at 37°C) were chosen. Substrate was incubated in ddH_2O (lane a), in self-cleavage buffer without Triton X-100 (lane b), in self-cleavage buffer in the presence of 0.5% Triton X-100 (lane c), in self-cleavage buffer with different types of detergents at a final concentration of 0.5% (v/v) (lanes d–i): addition of non-ionic detergents Genapol X-100 and Tween-80 (lanes d and e), addition of zwitter-ionic detergents Zwittergent 3–10 and CHAPS (lanes f and g), addition of ionic detergents Deoxycholacid and SDS (lanes h and i). The arrow indicates the fragment after self-cleavage reaction between the nucleotides U_{31}/A_{32}. Cleavage products were analyzed on a 20% polyacrylamide/8 M urea-TBE gel. b: Structure of different detergents. a) non-ionic detergent Triton X-100; b) non-ionic detergent Genapol X-100; c) zwitter-ionic detergent Zwittergent 3–10; and d) zwitter-ionic detergent CHAPS.

replaced by K^+- and Na^{2+}-ions (not shown), indicating that NH_4^+-ions are more specifically involved in the reaction. A similar dependence of RNA cleavage on NH_4^+-cations was observed with a T4 RNA precursor from infected *E. coli* cells [15,30,31].

3.4. Influence of detergents on the self-cleavage reaction

The most pronounced effect on the self-cleavage reaction was achieved by the non-ionic detergent Triton X-100. This compound not only enhances the self-cleavage reaction of different substrates by a factor of 100 but also stimulates the activity of the yeast [32] and wheat germ [33] pre-tRNA splicing endonuclease. In the presence of the detergent the half-life of the substrate was around 30 min (Fig. 2a and c). Exclusion of the detergent from the assay resulted in the complete loss of the cleavage reaction under standard reaction times of 2 to 4 h (Fig. 3, lanes i and j). In these cases the half-lifes of the mini-substrates were about 48 h and uncleaved substrate was still detectable after 96 and 120 h (not shown). Incubation of the mini-substrates in 0.4% Triton X-100 alone did not cause any cleavage either (not shown), indicating that the detergent itself is not sufficient for the reaction.

Detergents are small molecular compounds that contain both a polar and a hydrophobic group. Due to their amphipathic structure they exhibit unique properties in water. Their polar groups form hydrogen bonds with water molecules, whereas the hydrocarbon chains aggregate due to hydrophobic interaction. Based on the nature of their hydrophilic head group, they can be broadly classified as non-ionic, zwitter-ionic and ionic detergents (Fig. 4b). Interestingly, the same efficiency and specificity of the cleavage reaction was observed when the non-ionic detergent Triton X-100 was replaced by other non-ionic detergents such as Genapol X-100 or Tween-80 (Fig. 4a, lanes d and e) or even by zwitter-ionic detergents like CHAPS or Zwittergent 3–10 (Fig. 4a, lanes f and g). However, addition of an ionic detergent such as Deoxycholacid or SDS instead of Triton X-100 resulted in a complete inhibition of the reaction (Fig. 4a, lanes h and i). From these results we conclude that functional groups of the detergents are not involved in the reaction. The complete absence of cleavage in case of the ionic detergents SDS and Deoxycholacid (Fig. 4a, lanes h and i) may be based on an electrostatic repulsion between the highly negatively charged RNA backbone and the negatively charged head groups of the anionic detergents.

To investigate the contribution of the detergent, different physical parameters describing detergent behavior were considered. Two of the most important parameters are the aggre-

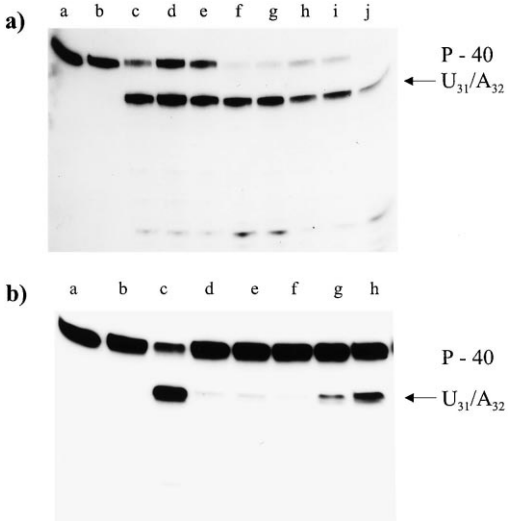

Fig. 5. Influence of the concentration of non-ionic detergents on self-cleavage of 5′-^{32}P-endlabelled AtY3II/M-DS5 (P-40). a: Incubation in Triton X-100 for 4 h: control lane, incubation in ddH_2O (lane a), in self-cleavage buffer without Triton X-100 (lane b), in the presence of 0.001%, 0.005%, 0.01%, 0.02%, 0.03%, 0.04%, 0.05% and of 0.4% Triton X-100, respectively (lanes c–j). b: Incubation in Genapol X-100 for 1 h: control lane, incubation in ddH_2O (lane a), in self-cleavage buffer without detergent (lane b), in standard self-cleavage buffer (0.4% Triton X-100) (lane c), in the presence of 0.001 mM, 0.005 mM, 0.01 mM, 0.05 mM and of 0.1 mM Genapol X-100, respectively (lanes d–h). Arrows indicate the fragments after self-cleavage reaction between the nucleotides U_{31}/A_{32}. Cleavage products were analyzed on a 20% polyacrylamide/8 M urea-TBE gel.

gation number (N) and the critical micelle concentration (CMC). It is known that above this particular concentration detergent molecules self-associate to form thermodynamically stable aggregates, called micelles. The CMC is usually determined by measuring some physical property as a function of detergent concentration. The aggregation number (N) is the average number of monomers per micelle by which an indirect evidence of the micelle dimension is given. To ensure that the CMC and the aggregation number N of the analyzed detergents in the self-cleavage buffer are equivalent to published values [34], a fluorimetric determination of the CMC was performed. This method, based upon the enhancement of 1,6-diphenyl-1,3,5-hexatriene (DPH) fluorescence intensity upon incorporation into the hydrophobic interior of micelles is one of the most sensitive and convenient assays for CMC measurement [34]. We showed in different experiments that the CMC value of Triton X-100 in the self-cleavage buffer was identical with the value measured in ddH_2O [34]. Hence, an influence of the reaction buffer on CMC was not detected (not shown).

Subsequently, we examined the influence of different Triton X types on the self-cleavage reaction. These Triton X types (Triton X-45, Triton X-100, Triton X-114 and Triton X-405) have distinct aggregation numbers due to different lengths of their hydrophobic tails and therefore form micelles of different sizes. Studies with different Triton X types indicated that the aggregation number and hence the size of the micelle does not play a crucial role in the self-cleavage reaction. No obvious correlation was observed between N and the efficiency of the self-cleavage reaction with non-ionic as well as with zwitter-ionic detergents (not shown).

Since neither the size nor the functional groups of the different detergents seem to be involved in the reaction we focussed our interest on the CMC. Interestingly, a direct relation of the CMC to self-cleavage activity was detected (Fig. 5). Incubation of the mini-substrate AtY3II/M-DS5 below the CMC of different detergents did not stimulate the reaction (Fig. 5a; Fig. 5b, lanes d–f). An increase of the detergent concentration to the CMC (Triton X-100 between 0.001–0.01%, Genapol X-100 between 0.06–0.15 mM) caused cleavage to a minor extent (Fig. 5a, lanes c–e; Fig. 5b, lanes g and h). An efficient and quantitative self-cleavage was only achieved when the detergent concentration was raised above the CMC (Fig. 5a, lanes f–j). These results suggest that the physical property of a micelle, i.e. its hydrophobic interior is absolutely important for this reaction and that the micelle itself reacts as a catalyst for this highly specific self-cleavage reaction, comparable with the rapid cleavage of a model phosphodiester bond within Brij 35 micelles by complexed ceric ions [35]. This and latest results on cleavage activity of the hairpin ribozyme in the absence of metal ions [36] encourage us to postulate an enhanced cleavage reaction within micelles, whereby the hydrophobic interior is a crucial factor for the reaction catalyzed by the functional groups within the RNA. This assumption explains why different micelles with different head groups and distinct aggregation numbers are able to support the reaction. It would further explain the necessity of NH_4^+-ions: RNA, being extremely hydrophilic with its negatively charged phosphodiester backbone, would be neutralized by NH_4^+-ions and thus become able to enter the hydrophobic interior of a micelle. In this context it is interesting to note that especially NH_4^+-ions are used for liposome-mediated transfection of cells to enclose DNA for delivery [37]. Moreover, NH_4^+-ions are able to support the reaction of hammerhead, hairpin and VS ribozymes [14], by aiding the correct folding.

The hydrophobic interior of a micelle appears to provide the optimal environment for the catalytic conformation necessary for the reaction, i.e. a change of the pattern of hydration and hydrogen bonds within the RNA molecule which makes sequence-specific hydrolysis possible. This conformational change may shift pK_a values of functional groups of the RNA towards neutral, thus allowing them to react via the S_N2 mechanism. Such a pK_a shift of functional groups in RNA has been reported by Legault and Pardi [38] due to a specific secondary and tertiary structure of the RNA. In case of the hairpin ribozyme latest results indicate that all essential elements of catalytic function are provided by the structure of the folded hairpin ribozyme-substrate complex, with cations functioning especially in stabilizing the active structure by electrostatic screening of the charged phosphodiester backbone [36].

The mini-construct AtY3II/M-DS5 represents the bulge loop that contains the 3′-splice site of *Arabidopsis* intron-containing pre-tRNA^{Tyr} (Fig. 1a, d). Our previous studies comparing autolytic and enzymatic intron excision suggest that notably the 3′-cleavage site seems to have preserved an intrinsic autolytic cleavage activity in many eukaryotic tRNA precursors due to its highly conserved secondary structure [17]. The well-documented stimulation of pre-tRNA splicing endonuclease by Triton X-100 [32,33] may indicate an active involvement of the hydrophobic nuclear membrane in the splicing reaction.

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