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CATALYTIC ACTIVITY AND STABILITY OF HAMMERHEAD RIBOZYMES CONTAINING 2'-ACETAMIDO-2'-DEOXYRIBONUCLEOSIDES

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SUMMARY: Hammerhead ribozymes may be of great therapeutic importance, since they can, in theory, be developed to cleave any undesired target RNA. In order to design ribozymes which are stable against endogenous RNases, we incorporated 2'-acetamido-2'-deoxynucleosides in the catalytic core of a hammerhead ribozyme. The 2'-acetamido function has, like the hydroxyl group, both proton donor and proton acceptor capacities, which seem to be crucial for efficient catalytic activity. However, the presence of 2'-acetamido-2'-deoxypyrimidine residues in the catalytic core caused a considerable drop in cleavage rate. Replacement of the purine residues led to complete loss of the catalytic activity. Surprisingly, these 2'-modifications showed no beneficial influence on the stability of the ribozymes against RNases present in cell culture supernatant containing 10 % fetal calf serum.

The discovery of the ability of several classes of ribozymes to act as a biological catalyst has opened a new direction in the search for pharmaceutical agents. One type of ribozymes, the hammerhead ribozyme, requires the presence of a divalent metal ion, preferentially magnesium or manganese, and catalyses the site specific cleavage of the substrate RNA generating a 2',3'-cyclic phosphate [1]. These hammerhead ribozymes may be of great therapeutic importance, since they can theoretically be developed to cleave any undesired RNA containing a triplet amenable to cleavage [2,3]. However, one of the problems associated with exogenous use of ribozymes is the low stability of oligoribonucleotides against nucleases. This drawback necessitates the development of ribozymes with both maximum catalytic activity and high resistance to nucleases. The importance of some 2'-hydroxyl groups in the catalytic region of the hammerhead ribozyme for the catalytic efficiency has already been established by means of several 2'-substitutions, such as 2'-deoxy [4], 2'-NH_2, 2'-F [5-8], 2'-*O*-allyl or 2'-*O*-methyl [9]. These modifications gave an active ribozyme, though with less activity which is not desirable. Up to now the 2'-hydroxyl group has been replaced by an atom or a group which has obviously different physicochemical characteristics than a hydroxyl group. Here, we report the synthesis of 2'-acetamido-substituted

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ribozymes. An acetamido function has, like the hydroxyl group, both proton donor and proton acceptor capacities and is also a poor acid. Moreover, a better ability to complex divalent metal ions can be expected. In addition, its structure is sufficiently different from the hydroxyl group to expect ameliorated stability towards ribonucleases.

The 2'-acetamido-2'-deoxynucleosides were incorporated into hammerhead ribozymes targeted at the mRNA of interleukine 6 (IL-6). IL-6 is a multifunctional cytokine mainly involved in the regulation of inflammatory and immunological processes and it has been implicated in the pathogenesis of several cancers and other diseases [10,11]. In order to simplify the kinetic experiments a short synthetic RNA substrate, mimicking nucleotides 471 to 489 (19-mer) of human IL-6 mRNA [12], was used for the cleavage reactions. The influence of the chemical modifications on the degradation of the ribozymes by nucleases was investigated by incubating ribozymes in the supernatant of UAC cells.

EXPERIMENTAL PROCEDURES

Materials and methods - Tetrabutylammonium fluoride (1 M in tetrahydrofuran) was purchased from Acros Chimica. LCAA-CPG, purchased from Pierce, was functionalized with 1-*O*-dimethoxytrityl-1,3-propanediol analogously to the protocol reported by Tang et al. [13]. 2'-Acetamido-2'-deoxynucleoside building blocks were prepared as described previously [14]. The substrate oligoribonucleotide was ^{32}P-labeled at the 5'-end with T4 polynucleotide kinase (Gibco BRL) and [γ-^{32}P]ATP (3000 Ci/mmol, Dupont NEN) and purified on a NAP-10 column (Pharmacia). Cell medium DMEM and fetal calf serum were purchased from Gibco BRL. The fetal calf serum was heat-inactivated at 56 °C for 30 minutes.

Oligo(ribo)nucleotides were synthesized on an Applied Biosystems 392 DNA Synthesizer on a 1-2 µmol scale using phosphoramidites from Applied Biosystems (deoxy) and Milligen Biosearch (ribo). The oligo(ribo)nucleotides were worked-up as described previously [14]. Scanning laser densitometry was performed with a DeskTop Densitometer (pdi, NY, USA) equipped with The Discovery Series (Diversity One) software.

Cleavage kinetics - *Kinetic constants*, k_{cat} and K_m, for the unmodified ribozyme (Rib) were determined from Eadie-Hofstee and Lineweaver-Burk plots [15] from initial velocities under multiple turnover conditions. The ribozyme and the oligoribonucleotide substrate were incubated together at 75 °C for 3 min in 50 mM Tris-Cl, pH 7.5, followed by 5 min of incubation at 30 °C. Reactions were started by the addition of an equal volume of 40 mM $MgCl_2$ in 50 mM Tris-Cl, pH 7.5, which gave a final concentration of $MgCl_2$ of 20 mM. Cleavage reactions were performed at 30 °C in a total volume of 50 µL with concentrations of substrate between 50 and 300 nM and 30 nM of ribozyme. Aliquots were taken at appropriate time intervals between 2 and 50 min. Reactions were stopped by the addition of an equal volume of stop mix (50 mM EDTA, 0.1 % xylene cyanol and 0.1 % bromophenol blue in formamide) and analysed by denaturing 20 % PAGE (7.5 cm long) containing 8.3 M urea followed by autoradiography and scanning laser densitometry. The *comparative time course* of the cleavage reaction for modified and unmodified ribozymes was performed using 30 nM of ribozyme and 200 nM of substrate under the conditions described above. *Stoichiometric cleavage analyses* were performed under the same conditions with an excess of ribozyme (600 nM) in comparison to the substrate sequence (100 nM).

Stability of ribozymes - Supernatant of a 80 % confluent culture of UAC cells containing 10 % heat-inactivated fetal calf serum was used as a source of nucleases. As described by Heidenreich et al. [7], 5'-^{32}P-labeled ribozymes were dissolved in water, preheated at 90 °C for 1 min and chilled on ice. To 15 µL of the cell culture supernatant 3 µL of this ribozyme solution

(final concentration 300 nM) was added, followed by incubation at 37 °C. Aliquots were taken at appropriate time intervals and analysed by 20 % PAGE containing 8.3 M urea followed by autoradiography.

Melting temperatures - Melting curves were measured and evaluated the same way as described in reference 17. Oligomer concentrations were determined as described [16]. An extinction coefficient of 10000 for uridine was determined at 80 °C.

RESULTS

Nucleotide sequence 478 to 480 of the mRNA of IL-6 [12], containing the potential cleavage site GUC, was chosen as a target for ribozyme-mediated cleavage [17]. For kinetic analyses, a short RNA substrate consisting of 19 nucleotides, mimicking nucleotides 471 to 489 of the mRNA of IL-6, was chemically synthesized. The sequence is given in Fig. 1. By means of 1-*O*-dimethoxytrityl-1,3-propanediol functionalized LCAA-CPG, a propanediol moiety, rendering improved stability against 3'-exonucleolytic attack was attached at the 3'-end of the substrate. This 3'-group did not influence the hybridizing properties of the oligonucleotide.

The "unmodified" ribozyme (Rib, Fig. 1), 38 nucleotides long, was composed of ribonucleosides in the catalytic core and in helix and loop II, and deoxynucleosides in stems I and III [18,19]. Helix and loop II consisted of three base pairs attached to a thermodynamically stable tetraloop GCAA [20,21]. For all the ribozymes synthesized, 1-*O*-dimethoxytrityl-1,3-propanediol functionalized LCAA-CPG was used combined with 1-*O*-dimethoxytrityl-4-*O*-(2-cyanoethyl-N,N-diisopropylphosphoramidite)-1,4-cyclohexanediol as the first building block. This combination resulted in a 3'-protecting group (prop-hex, Fig. 1) which improved the half life of degradation by Snake Venom Phosphodiesterase from 7 min to 7 hrs (data not shown). The hexanediol building block was prepared as described by Ono et al. for a similar product [22].

Preliminary cleavage experiments at 25, 30 or 37 °C prompted us to use 30 °C as incubation temperature for all the kinetic experiments, since Rib gave the best cleavage activity at 30 °C. Moreover, at 37 °C a considerable amount of aspecific degradation of the substrate could

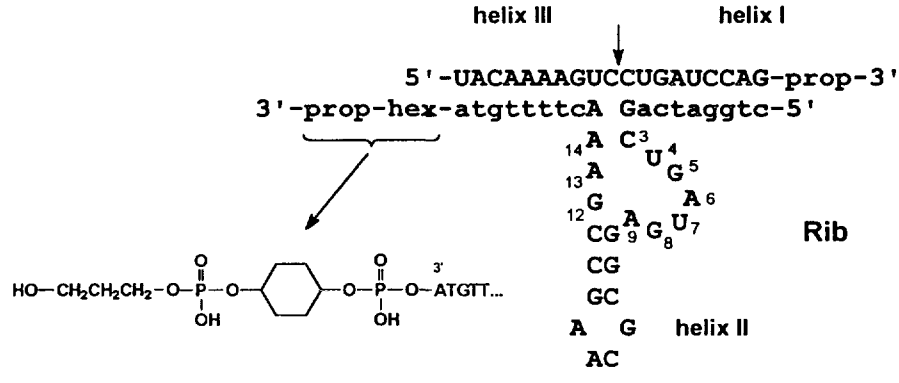


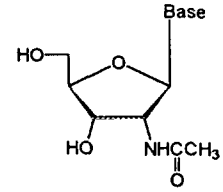
FIG. 1. **Structure of parent ribozyme-substrate complex.** Lowercase letters indicate DNA parts. Arrow indicates point of substrate cleavage.

be noticed (up to 17 % after 23 hrs). The kinetic parameters, k_{cat} and K_m, for the unmodified ribozyme Rib were determined from initial velocities under multiple turnover conditions: K_m = 44 nM, k_{cat} = 0.033 min^{-1}, k_{cat}/K_m = 0.765 x 10^{-3} $nM^{-1}min^{-1}$. These results indicate a rather low activity for Rib, notwithstanding the results of Hendry et al. [18] and Taylor et al. [19].

To investigate the influence of the 2'-acetamido-substitutions on the catalytic efficiency we systematically substituted the residues in the catalytic core by their 2'-acetamido analogues as indicated in table I. Substitution of residues in other, hybridizing parts of the ribozyme was not allowed since these 2'-acetamido substitutions caused a considerable drop in melting temperatures [14] and could thus deteriorate the formation of the appropriate secondary and tertiary structure of the ribozyme. Comparative cleavage reactions were performed using 30 nM of ribozyme and 200 nM of substrate. The time course of the cleavage (Fig. 2) shows that the cleavage of the substrate proceeded much slower after incorporation of the 2'-acetamido-2'-deoxynucleotides. The presence of only one 2'-acetamido-2'-deoxycytidine (Rib C) already caused a drop in cleavage rate from 36 % product formation for Rib to 20 % for Rib C after 3 hrs of incubation. The cleavage rate decreased further with increasing amount of 2'-modifications at pyrimidine residues (Rib U, Rib UC). The ribozymes containing 2'-acetamido purine residues (Rib A, Rib G, Rib AG) showed no catalytic cleavage at all, not even after 24 hrs of incubation (data not shown). Also no activity was observed for the ribozyme with a completely substituted catalytic core (Rib UCAG). The cleavage reaction was then further examined under stoichiometric conditions, with an excess of ribozyme (600 nM) in comparison to the substrate sequence (100 nM). It can be assumed that under these conditions the substrate should be entirely complexed and thus any effects due to product release avoided. Plots of the logarithm of

TABLE I

Melting temperature for complexes formed between the ribozymes and synthetic substrate RNA (19-mer)

substrate RNA hybridized to:	nucleosides substituted by 	Tm (°C)
Rib	-	40.1
Rib U	U4, U7	35.1
Rib C	C3	35.4
Rib UC	U4, U7, C3	35.1
Rib A	A6, A9, A13, A14	35.5
Rib G	G5, G8, G12	36.0
Rib AG	A6, A9, A13, A14, G5, G8, G12	33.9
Rib UCAG	U4, U7, C3, A6, A9, A13, A14, G5, G8, G12	33.8

Tm = melting temperature in °C determined under the following conditions: 0.1 M NaCl, 0.02 M potassium phosphate, pH 7.5, 0.1 mM EDTA, concentration = 1.5 µM for each oligonucleotide.

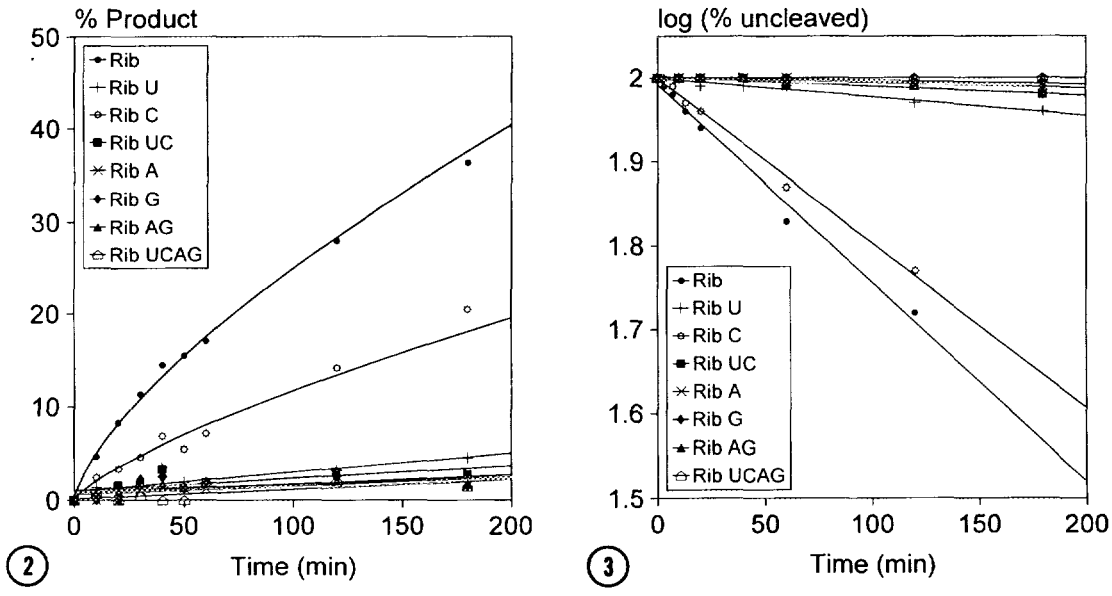


FIG. 2. **Percentage of product versus time for the cleavage reaction of the modified and unmodified hammerhead ribozymes.** The cleavage reaction was performed by treating 200 nM of substrate with 30 nM of ribozyme at 30 °C in the presence of 20 mM $MgCl_2$.

FIG. 3. **Plots of the logarithm of the concentration of the uncleaved substrate versus time for the cleavage reaction of the modified and unmodified hammerhead ribozymes.** The cleavage reaction was performed by treating 100 nM of substrate with 600 nM of ribozyme at 30 °C in the presence of 20 mM $MgCl_2$.

the concentration of the uncleaved substrate versus time (Fig. 3) confirm the previous observations.

In order to find an explanation for the reduced activity of the 2'-acetamido substituted ribozymes in terms of unability to hybridize to the substrate RNA, we determined the melting temperatures of the complexes formed between the ribozymes and the substrate RNA. The figures in table I indicate that all the substituted ribozymes have a melting temperature at least 4.1 °C lower than the unmodified Rib. Nevertheless all the melting temperatures are sufficiently higher than the incubation temperature of the cleavage reaction (30 °C) in order to allow hybridization and thus the formation of the ribozyme-substrate complex.

The influence of the 2'-acetamido substitutions on the stability of the ribozymes against nuclease digestion was investigated by incubating the ribozymes in the supernatant of a culture of UAC cells containing 10 % heat-inactivated fetal calf serum. For both the unmodified and modified ribozymes the same results were obtained as indicated by denaturing PAGE (data not shown). Surprisingly, no stability improvement could be observed for the modified ribozymes.

DISCUSSION

Hammerhead ribozymes may be of great therapeutic importance since they can theoretically be designed to catalyse site specific cleavage of any unwanted RNA sequence. However, for

exogenous delivery the problem of the low stability of ribozymes against nucleases has to be considered. In order to handle this drawback we developed ribozymes containing 2'-acetamido-2'-deoxy substitutions. The 2'-acetamido modification was chosen because of its physicochemical similarities with the natural 2'-hydroxyl group, the presence of which in some positions of the catalytic core of the ribozyme seems to be very important for the cleavage activity [4-9].

Kinetic evaluation (Fig. 2 and 3) of the systematic substitution of the natural nucleotides in the catalytic core revealed that only substitution of the pyrimidine residues (Rib U, Rib C and Rib UC) was allowed in order to retain catalytic activity. Ribozymes containing 2'-acetamido purine residues (Rib A, Rib G, Rib AG and Rib UCAG) showed no cleavage activity at all. This reduced activity could not be explained in terms of unability to hybridize to the substrate RNA, since melting temperatures determined for the complexes formed between the ribozymes and the substrate RNA (Table I) indicated that all ribozymes should hybridize sufficiently to the substrate at the incubation temperature of the cleavage reaction. It is likely that the 2'-acetamido group, because of steric hindrance, causes a distortion of the catalytically active structure of the ribozyme. The reduced activity might also be explained by the recently elucidated X-ray crystallographic structure of a hammerhead RNA-DNA ribozyme-inhibitor complex [23]. The crystal structure once more emphasizes the importance of certain 2'-hydroxyl groups, which also seem to be crucial for tertiary interactions (hydrogen bonds) stabilizing the catalytic core.

The contribution of this chemical modification to the stability of the ribozymes against nucleases was determined by incubating the ribozymes in the supernatant of UAC cells. Unexpectedly, all ribozymes showed the same level of degradation. These results are rather surprising, since earlier 2'-modifications such as 2'-NH_2 or 2'-F rendered improved stability to the ribozymes [5-8]. These data may also be explained by the destabilisation of the catalytic structure of the ribozyme after incorporation of the 2'-acetamido modified nucleotides, rendering the ribozyme more susceptible to nuclease degradation. These results confirm the critical role played by the 2'-hydroxyl groups for the formation of the catalytic core of ribozymes. Besides the hydrogen bonding and chelating capacities, also the steric effect of the 2'-substituent is important and it seems doubtful that all these functions may be taken over by another substituent than a hydroxyl group.

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