The First Example of an RNA Urea Synthase: Selection through the Enzyme Active Site of Human Neutrophile Elastase

Dedicated to Professor Dr. K. P. C. Vollhardt on the occasion of his 57th birthday

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KEYWORDS:

diastereoselectivity · enzyme catalysis · peptides · RNA · stereoselection

RNA enzymes (ribozymes)[1, 2] have been found to be important in biochemistry, and RNA plays a crucial catalytic role in the ribosome.[3] In all of these biochemical processes substrate specificity can be essential for catalysis. In contrast to RNA catalysis, many examples of protein catalysis are known for which asymmetry or chirality is a key factor in the molecular recognition of substrates.[4] Most ribozymes known to date recognize their substrates through the templating of a nucleotide or oligonucleotide by easily determined Watson – Crick and other established nucleic acid hydrogen bonding patterns. It has recently been reported that RNA-catalyzed [4+2] cycloaddition reactions can show a moderate to high degree of substrate specificity[5] and stereoselectivity.[6] Herein, we describe for the first time RNA catalysts for urea bond formation and the degree of diastereoselectivity achieved in these reactions with peptide substrates. In addition, several recent reports have appeared on polymerase-catalyzed incorporation of modified bases[7] into DNA or RNA for enhanced catalytic activity. The two separate in vitro selections described herein were conducted with either modified or unmodified RNA, under identical conditions.

It was recently revealed that RNA can catalyze simple non-peptide amide bond formation.[8] Most importantly, these RNA amide synthases are the first examples of RNA catalysts in which nucleotide templating is not used in the recognition of the amine or carboxylate substrates. Other RNA catalysts that catalyze amide bond formation have also been discovered by similar in vitro selection techniques, which demonstrates that Watson – Crick templating of a substrate can be used to promote amide bond formation.[9] However, only nontemplated reactions have the potential to show the type of chiral molecular recognition and concomitant stereospecific catalysis exhibited by protein enzymes. Can the in vitro selection of RNA catalysts be dictated by stereoselectivity, as opposed to discovering[6] stereoselectivity once catalysts have been selected? The in vitro selection schemes previously published have not addressed this question because most rely on a streptavidin – biotin isolation procedure, which selects for all catalytic RNA independent of stereoselectivity. Herein, we describe the stereoselective selection of RNA catalysts by an isolation procedure (stereoselection) that utilizes the chirality of an enzyme (human neutrophile elastase,[10] HNE) active site.

To probe the stereoselection of RNA catalysis with peptide substrates a two-step scheme was devised in which streptavidin binding is replaced by capture in a protein protease (HNE) active site that can distinguish between closely related steroisomeric peptide – phosphonate suicide substrate inhibitors (Scheme 1 **A**, **B**). We envisaged that the peptide substrates (**A** or **B**) in this selection system might dictate the RNA stereoselectivity during RNA – peptide conjugation (Step 1, Scheme 1) if there was a bias for that peptide in enzyme suicide substrate capture (Step 2, Scheme 1). However, it was not known if the conjugated RNA – peptides would inhibit HNE or if the inhibition would be dominated by the RNA and not the suicide substrate, which would negate any possibility for stereoselection in Step 2 based on peptide structure. This problem caused significant concern since HNE is a basic protein (pI $\approx$ 10) and binds to a random pool of RNA sequences with a dissociation constant, K_d, of around 100 nM, and high affinity ($K_d = 6 - 12$ nM) has been reported for in vitro selected DNA aptamers.[11] The in vitro selection described herein tested whether the outcome would be dictated by the peptide – HNE or the RNA – HNE interaction. To maximize selection for the best peptide suicide substrate, highly denaturing polyacrylamide gel electrophoresis (PAGE) separation or reverse-transcription of RNA pools prior to separation with bead-coupled HNE was used to either unfold the RNA or make all sequences form similar double-stranded structures, presumably A-form DNA/RNA heteroduplexes less able to bind HNE.

Both RNA modified to include 5-imidazol-uridine[12] and unmodified RNA were employed in identical selection experiments to allow a direct comparison of RNA catalytic activity. The mixture of peptide substrates chosen, which on correct conjugation to the RNA would remain suicide inhibitors, were the small noncharged hydrophobic diastereomeric peptides **A** and **B**, activated at the N terminus by an *N*-hydroxysuccinimide (NHS)-carbamate moiety (Scheme 1).[13] The reaction to be catalyzed by the RNA (Step 1, Scheme 1) was substitution of the NHS-carbamate at the N terminus to give the urea of **A** or **B**. In Step 2 the active site serine hydroxy group of HNE could attack the phosphonate phosphorous of **A** or **B** and substitute one of the phenoxy groups at the C terminus of the tripeptide. The diastereomeric tripeptides **A** and **B** were used in a 1:1 ratio. It has previously been reported that highly reactive adenosine monophosphate-carboxy anhydrides can be used to select for carbonyl substitution.[14] Substitution of the significantly less reactive NHS-carbamate carbonyl group has not previously been reported for RNA catalysis.

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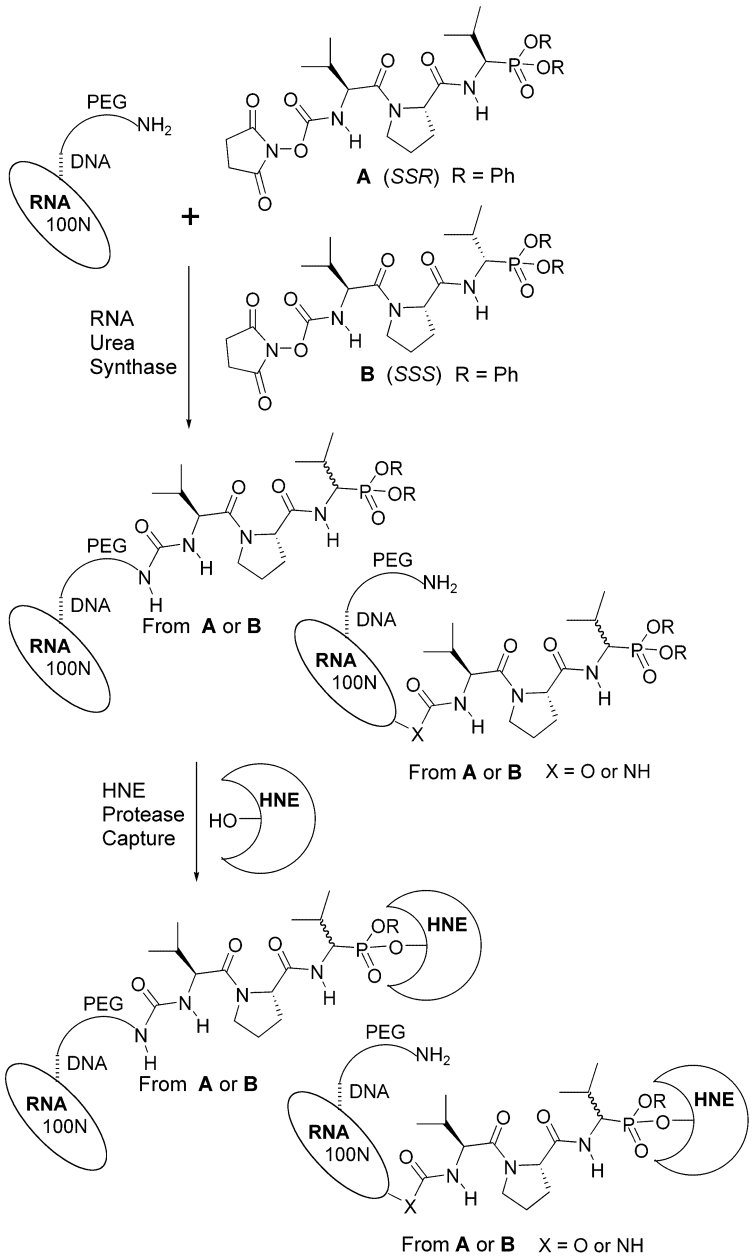
Supporting information for this article is available on the WWW under http://www.chembiochem.org or from the author.

 DOI: 10.1002/cbic.200300610 © 2003 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim



Scheme 1. Two-phase in vitro selection scheme for RNA. The RNA is attached to a tripeptide followed by tripeptide phosphonate capture with the serine protease human neutrophile elastase (HNE). PEG, polyethylene glycol.

The selection was started with an RNA sequence population of approximately 10^{14} random sequences.[15] Since peptide conjugation directly on the RNA could potentially interfere with the subsequent phosphonate suicide substrate reaction in the active site of HNE, a primary amine was tethered to the 5′ ends of the molecules in both the modified and unmodified RNA libraries through a 2000-MW polyethylene glycol linker – ssDNA (10 nucleotides),[16] which thus provided an additional nucleophile for NHS-carbamate substitution (Scheme 1). To further enrich the catalytic potential of the RNA, a mixture of divalent metal ions was included in the RNA peptide reaction buffer (125 mM NaCl, 125 mM KCl, 1.25 mM $MgCl_2$, 1.25 mM $CaCl_2$, 125 μM $MnCl_2$, 12.5 μM each of $ZnCl_2$, $CuSO_4$, $CoCl_2$, and $NiCl_2$, and 62.5 mM 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid, pH 7.5).

Nine cycles of in vitro selection[16] with the 5-imidazoluridine-modified RNA pool gave RNA – peptide conjugation, as indicated by PAGE with quantitation by phosphorimaging. No significant increase over background levels of conjugate was observed for selection with unmodified RNA even after 15 cycles. AMV reverse transcriptase sequencing of the imidazol-modified RNA following Cycles 11 – 14 indicated sequence convergence with little change after Cycle 13. The observed rate of conjugation in the unmodified RNA population was not above background (0.42% of Cycle 14 RNA reacted in 16 hrs; 250 nM RNA and 20 μM **A**/**B**). The significantly greater catalytic activity (48% of Cycle 14 RNA reacted after 4 min) of the modified RNA population is consistent with the idea that enhanced RNA functional diversity is important for catalysis.[17, 18]

PCR products from the Cycle-15 imidazol-modified RNA population were cloned and sequenced.[16, 19] Sequence alignment indicated four distinct RNA catalyst motifs among the 17 active clones isolated.[16] Only two isolates were inactive under the conditions investigated. The largest family (60% of all isolates) contained the conserved sequence GCAUGUCAUCGCUGCG. The next largest family (18% of isolates) also contained a fragment (GCAUGU) of this sequence at the 5′ end of its conserved region.

To measure the kinetics of RNA – peptide conjugation tritiated **A** (^{3}H-**A**) was prepared (78 Ci mmol^{-1}; American Radiolabeled Chemicals, Inc., St. Louis, MO) and the rates determined by PAGE separation of reaction products followed by quantitation (^{3}H-**A**, ^{32}P-RNA two-channel scintillation counting) of excised gel bands.[16] Similar observed rates (approximately 0.2 – 0.4 min^{-1} at 5 μM ^{3}H-**A**) were obtained for representatives of each sequence family. A more thorough kinetic analysis of two of the catalysts (**5** and **3**, see the Supporting Information for details) over a range of concentrations of ^{3}H-**A**, indicated that these catalysts follow Michaelis – Menton kinetics for a single turnover reaction. Values of the catalytic rate constant (k_{cat}), the Michaelis constant (K_m), and k_{cat}/K_m were determined for **3** (6.1 ± 0.3 min^{-1}, 90 ± 9 μM, and 6.6×10^4 M^{-1} min^{-1}) and **5** (3.2 ± 0.2 min^{-1}, 126 ± 12 μM, and 2.5×10^4 M^{-1} min^{-1}). The uncatalyzed reaction rate was estimated by using a random sequence of 5-imidazoluridine modified RNA (1 μM) and ^{3}H-**A** (250 μM). After 16 h, product formation above background levels (approximately 0.4% in this experiment) was not detected, which sets an upper limit of $k_{uncat} \leq 2 \times 10^{-2}$ M^{-1} min^{-1} if we assume the reaction is second order. This gives a rate enhancement ($[k_{cat}/K_m]/k_{uncat}$) of 10^6 for both **5** and **3**.

To determine the site of peptide conjugation to the RNA, ^{3}H-**A** was again used in the RNA catalyzed reaction to aid in HPLC analysis and double radioisotopic labeling experiments. Contrary to initial concerns about direct attachment of the inhibitor to the RNA, the RNA isolates **3** and **5** where found not to need the PEG-tethered primary amine as a nucleophile. Instead, the peptide conjugation reaction occurred at the 3′-terminal cytidine

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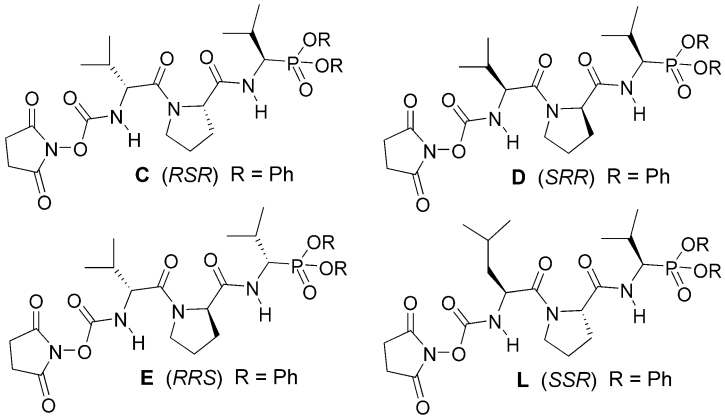
exocyclic amino group, as determined by chemical and enzymatic degradation followed by ESI-MS analysis and dual radioisotope counting experiments[16] (^{32}P-RNA and ^{3}H-peptide). These data support the formation of a urea linkage between the RNA terminal 3′-cytidine amino group and the N terminus of the peptide **A**, which means that these RNA catalysts are urea synthases.

The ultimate goal of these experiments was to determine the success of RNA-catalyzed stereoselection. In a set of kinetic competition experiments, the stereochemistry of the tripeptide suicide substrate was used as a probe to survey the asymmetry of the RNA active site. Scheme 2 shows the stereoisomer tripeptides (**C**, **D**, **E**, and **L**) synthesized stereospecifically in addition to **A** and **B**. Each diastereomer resulting from inversion of a single stereocenter in the tripeptide and the enantiomer **E** of the most potent inhibitor **A** was investigated.

Figure 1 shows the IC_{50} kinetic competition plots for representatives **3** and **5** of the two distinct sequence families. The data were obtained by using labeled ^{3}H-**A** and the competitive substrates **A**–**D** and **L**. The IC_{50} value for the unlabeled compound **A** was approximately the concentration of the labeled ^{3}H-**A** in the peptide conjugation (1.8 versus 2 μM, respectively). As expected,[20] **E** (the enantiomer of **A**) served as a negative control and did not competete in the peptide conjugation reaction. Inversion of any one stereocenter (**C** or **D**), except at the terminal valine phosphonate moiety (**B**), also results in essentially no competition with the labeled suicide substrate ^{3}H-**A**, a result consistent with highly specific asymmetric catalytic sites in the RNA urea synthases **3** and **5**. To test further the ability of these RNA catalysts to discriminate between different substrates, a single stereoisomer **L** was prepared, which had a subtle substituent change at the N-terminus of the tripeptide (leucine instead of valine) while maintaining the stereochemistry of **A** (*SSR*). This substitution tests the ability of these RNA catalysts to discriminate against the insertion of a CH_2 group between the α carbon atom and the isopropyl side chain of **A**. **L** was found to be a competitive substrate relative to ^{3}H-**A** with a 20–100-fold higher IC_{50} value, which demonstrates the power of these RNA catalysts to differentiate small changes in substrate structure. Strikingly, the IC_{50} value for the other diastereomer used in the selection, **B**, is at least 100 times greater for both **3** and **5** than that for **A**. Moreover, both **3** and **5** reacted preferentially with the more potent inhibitor of HNE. In fact, the modest diastereoselectivity between **A** and **B** observed in enzyme capture (Step 2, $K_i(\mathbf{A})/K_i(\mathbf{B}) = 3$) was amplified in the peptide–RNA conjugation kinetics (Step 1) of both **3** and **5** by more than 100 times.

In summary, Watson–Crick templating of the substrate was not required for these RNA urea synthase reactions with peptide substrates. Diastereoselective recognition of tripeptide substrates was achieved. It has now been shown that uncharged hydrophobic tripeptide substrates can be recognized stereoselectively in RNA catalysis. Even in the presence of a highly basic protein enzyme the outcome of the RNA catalysis selection was dictated by the stereochemistry of the tripeptide substrates not by protein–RNA interactions. Since the peptide conjugation occurred at the N terminus of the tripeptide substrate, and the stereochemistry of the C-terminal residue strongly influenced the rate of reaction, the extent of molecular recognition achieved by these RNA catalysts spans at least three peptide residues. The stereoselectivity observed for these RNA urea synthases suggests that it might eventually be possible to perform stereoselection on mixtures of peptides to identify the most potent enzyme inhibitors.



Scheme 2. *Tripeptide–phosphonate diastereomers used in the urea synthase competition kinetics experiments whose results are shown in Figure 1.*

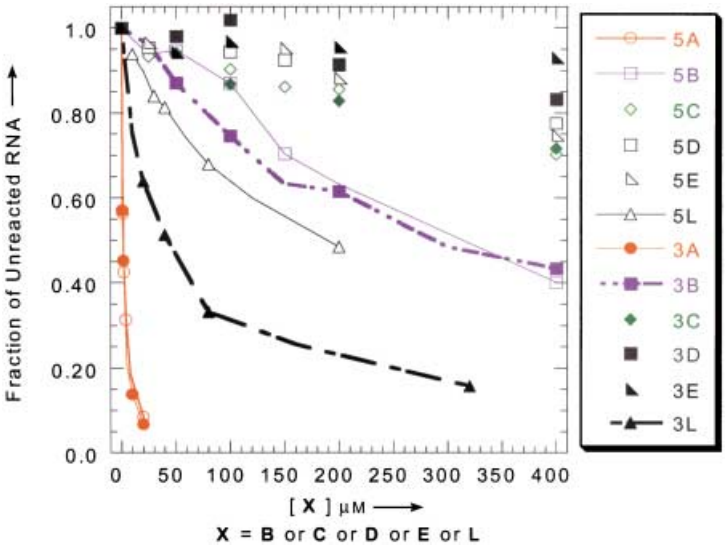


Figure 1. *IC_{50} plots for the RNA reaction with ^{3}H-**A** and competition by substrate **B**, **C**, **D**, **E**, or **L**.*

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Received: April 1, 2003 [Z 610]

Conserved Amino Acid Residues Correlating With Ketoreductase Stereospecificity in Modular Polyketide Synthases

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KEYWORDS:

biosynthesis · ketoreductases · polyketides · sequences · stereospecificity

The polyketide group of natural products includes antibiotics, immunosuppressants, anticancer drugs and other valuable bioactive compounds. The biosynthesis of these compounds is an area of considerable interest. Polyketide synthases (PKSs) resemble fatty acid synthases (FASs) and are composed of several different enzymes that assemble carbon chains.[1] An acyltransferase (AT) loads a dicarboxylic acid extender unit onto an acyl carrier protein (ACP), where it undergoes condensation with a starter acyl unit in a reaction catalysed by a ketosynthase (KS). The product is a β-ketoacyl intermediate that remains thioester-linked to the ACP. The β-ketone group may then be processed by ketoreductase (KR), dehydratase (DH) and enoyl reductase (ER) domains to yield an alcohol, enoyl or methylene group before the extended chain migrates to a KS for another cycle of extension. When the final polyketide chain length is reached, termination occurs, usually accompanied by cyclisation.[1]

FASs generally use acetyl starter and malonyl extender units. As a group, PKSs use a wide range of organic acids as starter units, whereas the extenders may be malonyl, methylmalonyl, ethylmalonyl or methoxymalonyl units. The diversity of polyketide structures results in part from combinatorial assembly of different starter and extender units, together with varying degrees of processing of β-ketone groups. Chiral centres are introduced by the use of branched extender units and by the reduction of β-ketone groups to alcohols. The introduction of chirality increases the potential diversity further.

The PKSs that assemble complex polyketides contain a module for every cycle of chain extension. Each module contains the requisite enzymatic domains, which are covalently linked in the order, KS-AT-reduction domain loop-ACP. Three main types of reduction domain loop exist. These contain KR, DH-KR or DH-ER-KR domains and determine the extent of β-ketone group processing in each cycle.[1]

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Supporting information for this article is available on the WWW under http://www.chembiochem.org or from the author.

 © 2003 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim DOI: 10.1002/cbic.200300581u