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Catalyzed and Spontaneous Reactions on Ribozyme Ribose

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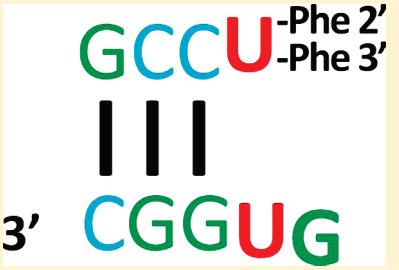
Supporting Information

ABSTRACT: The RNA world hypothesis requires that early translation be catalyzed by RNA enzymes. Here we show that a five-nucleotide RNA enzyme, reacting with a tetranucleotide substrate and elevated PheAMP, forms aminoacyl- and peptidyl-RNAs RNA—Phe through RNA—Phe_5. A second series of products is formed from RNA—Phe diesters, after *trans* migration of phenylalanine from the 2′- to the 3′- hydroxyl group of the substrate RNA, followed by reaminoacylation of the 2′-OH. While the ribozyme is required for initial attachment of phenylalanine to an RNA substrate, as well as reacylation (and thus for formation of all products), further extension into RNA—peptides appears to be an uncatalyzed, but RNA-stimulated reaction. The ribozyme readily turns over at high PheAMP and GCCU concentrations. Thus, GUGGC/GCCU comprises a true RNA enzyme. We define Michaelis—Menten parameters plus and minus divalent magnesium and characterize ca. 20 molecular species of aminoacyl-, peptidyl-, dipeptidyl-, and mixed peptidyl/aminoacyl-RNAs.



INTRODUCTION

Before the evolution of modern proteins, initial peptide synthesis reactions could not require protein enzymes. The discovery of RNA enzymes[1,2] led to the RNA world hypothesis, which supposes that RNA was the sole biological macromolecule in early evolution.[3] According to this hypothesis, early peptide synthesis was an RNA activity.[4]

A number of RNA enzymes have been discovered which catalyze reactions relevant to the process of protein synthesis, or translation. Each of the informational interactions and chemical group transfers that occur today in modern translation can, in fact, be catalyzed by RNA enzymes[4−7] isolated through in vitro selection,[8,9] or exemplified by rRNA.[10]

Several families of ribozymes have been isolated which catalyze RNA aminoacylation, the formation of an ester bond between an amino acid carboxyl and the terminal 2′- or 3′-hydroxyl group of RNA. A 95-mer produced the aminoacyl ester RNA—Phe when reacted with PheAMP,[6] and a 29-nucleotide descendant produced the RNA-peptide RNA—Phe_2.[11] A tiny ribozyme derived from the self-acylating ribozyme C3[7] was found not only to perform RNA acylation *in trans*, thus behaving as a true enzyme, but also to host the formation of RNA-peptides up to three amino acids long.[12] The initial attachment of Phe to GCCU tetranucleotide by ribozyme 5′-GUGGC-3′ was regiospecific to 2′-OH of the terminal uridine.

Upon further investigation, we find that more vigorous reaction conditions, such as increased PheAMP concentration, elevated pH, or high ribozyme concentration, reveal a second series of slower-migrating products, when monitored by gel electrophoresis. Here we characterize the new products and demonstrate that under these conditions the enzyme undergoes turnover. The second, slower-migrating products are diesters, resulting from the 2′ to 3′ aminoacyl migration commonly observed in charged tRNA.[13] We also investigate the mechanism of peptide bond formation, and show that the elongation of the enzyme-catalyzed products into peptides, while indirectly dependent on the enzyme, is primarily uncatalyzed, but rapid due to the inherently increased reactivity of free PheAMP with nucleophilic Phe-RNA products.

RESULTS

Previously, we showed that reaction of 10 μM 5′-GUGGC-3′, 20 μM 5′-GCCU-3′, and 1−2 mM PheAMP resulted in the formation of three acylated products (oligonucleotides are written 5′-left, 3′ right, by convention), as measured by gel electrophoresis of ^{32}P-labeled GCCU substrate RNA.[12] These were intrinsically limited reactions even at pH 7 and 4 °C, because the half-life of PheAMP is 21 min. Thus, long reactions stop because of hydrolysis of PheAMP. When PheAMP is increased to ∼10 mM, however, a second series of products becomes evident (products **4**−**8**, Figure 1). Interestingly, original and new products each seem to have their own modes; products **5**, **6**, and **7** appear to be higher in concentration than **4** after a 30-min acylation reaction (Figure 1). That is, the lower and higher products do not suggest progression of RNA-peptides from GCCU—Phe_4 to GCCU—Phe_8, unless we posit an unusual mechanism and/or peculiarly distributed rates.

To characterize the new products, we treated a completed aminoacylation reaction with leucine aminopeptidase (LAP), a protein enzyme which selectively hydrolyzes peptides. Both **1** (previously identified as an RNA—Phe ester) *and* **5** are the only products of extensive protease degradation, and, once formed, are resistant to protease (Figure 1). This suggests that **5** is an ester like **1**. While it migrates to the approximate position that one would expect for GCCU—Phe_5 in the gel, **5** cannot be pure

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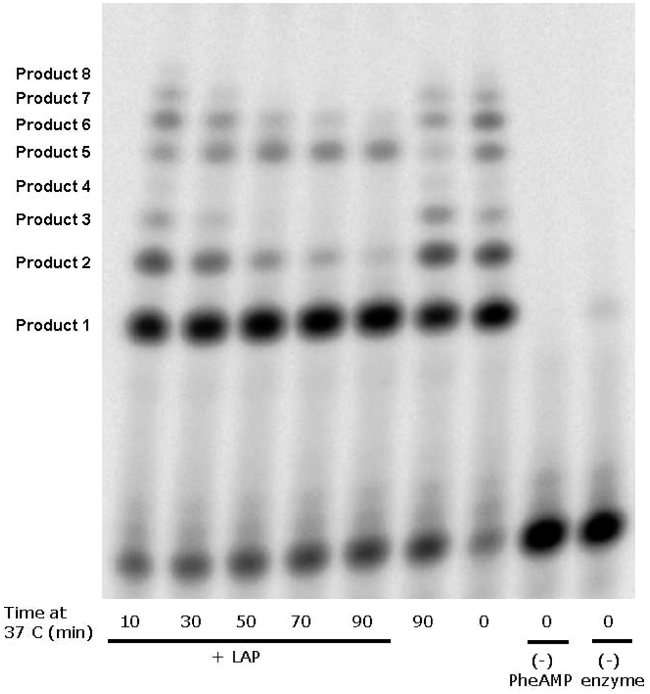

Figure 1. Product **5** is resistant to protease degradation. Aminoacylation reactions: 20 μM GCCU, 10 μM GUGGC, 100 mM KCl, 5 mM $MgCl_2$, 100 mM HEPES pH 7.0, 18.2 mM PheAMP; incubation at 4 °C for 30 min. LAP = leucine aminopeptidase, 8.9 ng/μL; reaction at pH 7.0, 37 °C. Product **1**−**8** origins are shown below in Figure 6, and specific structures are in the Supporting Information, Figure S1. The bottom band is [^{32}P]GCCU substrate.

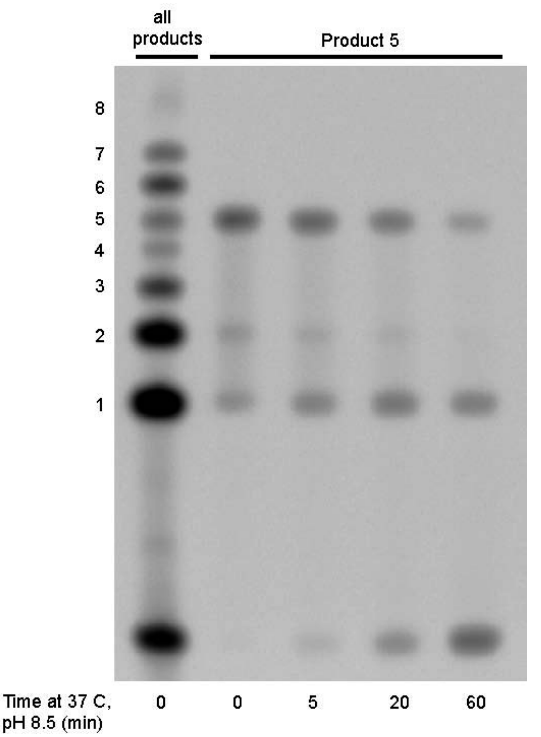

Figure 2. Product **5** yields product **1** by hydrolysis. Hydrolysis: 0.1 M Tris·HCl, pH 8.5, 37 °C. Product numbers indicated on left.

RNA−Phe_5 because of its significant resistance to LAP. All higher products appear to be smoothly converted to **5** by leucine aminopeptidase. Thus, they are peptides containing **5**, just as the lower products were converted by LAP to the initial ester GCCU−Phe (**1**), and were peptides of GCCU−Phe[12].

To further identify the new products, **5** was gel-purified, HPLC-purified, and then subjected to mild base hydrolysis (pH 8.5, 37 °C) (Figure 2). Under these conditions, **5** decays as **1** increases. A small **2** band is seen on the gel which does not increase during hydrolysis, but rather decays at the rate (0.034 min^{-1}) of purified **2** (Supporting Information, Table S1). We conclude that **2** is a result of contamination of faster-migrating products during purification (compare to Supporting Information, Figure S2B).

Given that **5** decays directly to **1**, it seems possible that **5** may be bis(2′,3′-*O*-phenylalanyl)-RNA (hereafter referred to as RNA-(Phe/Phe); **5**a, Figure S1). Diester could form by reacylation of the terminal 2′-OH of GCCU, following *trans*acylation of the initially esterified phenylalanine to the 3′-OH.[13]

If **5** is a Phe diester of ribose, then it must have two free phenylalanyl α-amino groups. In fact, when purified **5** is reacted with EZ-Link Sulfo-NHS-LC-biotin (reacts almost exclusively with primary amino groups), not one, but two biotinylated products are formed, as expected for singly and doubly biotinylated GCCU-(Phe/Phe) diester (Figure 3A, bands B and C). By way of contrast, both **1** (RNA−Phe) and **2** (RNA−Phe_2), which are present in the **5** reaction due to hydrolysis and the contamination of slower gel species with faster ones, yield only a single biotinylated product, at a mobility which differs from any normal ribozyme product (Figure 3, bands D and E). Therefore, **1** and **2** have one free amino group, and **5** has two, as predicted for Phe diester.

Finally, gel- and HPLC-purified **5** was subjected to MALDI mass spectrometry. Two peaks are evident: one at m/z = 1425.43, which corresponds to the quadruply protonated form of GCCU−Phe (again, likely from hydrolysis of **5** during handling), and one at m/z = 1572.48, which corresponds to the quadruply protonated form of GCCU-(Phe/Phe) diester, which has an exact theoretical mass of 1572.30 (Figure 4; **5a**, Figure S1). Notably, no peak appears at m/z = 2013 (Figure 4), where one would expect a peak for the peptidyl-RNA GCCU−Phe_5 (**5b**,**c** in Figure S1). Because **5** is HPLC-purified, GCCU−Phe_5 should not be present in the mass spectrum because a hydrophobic GCCU−Phe_5 will elute much more slowly than GCCU-(Phe/Phe).

The GCCU-(Phe/Phe) structure uniquely requires both the 2′ and 3′ ribose hydroxyls of GCCU. Therefore the structure can be tested thorough aminoacylation of the altered substrate GCC3′dU. This substrate, which lacks the 3′-terminal hydroxyl group, yields predominantly the lower set of four aminoacyl- and peptidyl-RNA products, even when reacted under high PheAMP concentrations (Figure S2A, lane 1).

However, a faint band corresponding to a fifth product also appears upon acylation of the 3′dU substrate (Figure S2A, lane 1). This product could represent a low level of spontaneous polymerization from GCC3′dU-Phe_4 to GCC3′dU-Phe_5 (discussed later; **5**b,c in Figure S1).

In addition, a faint band that migrates to approximately the same position appears to increase over time as the 3′-dU reaction is treated with leucine aminopeptidase (Figure S2A, lanes 2−4). This likely indicates a low level of ribose contamination in the

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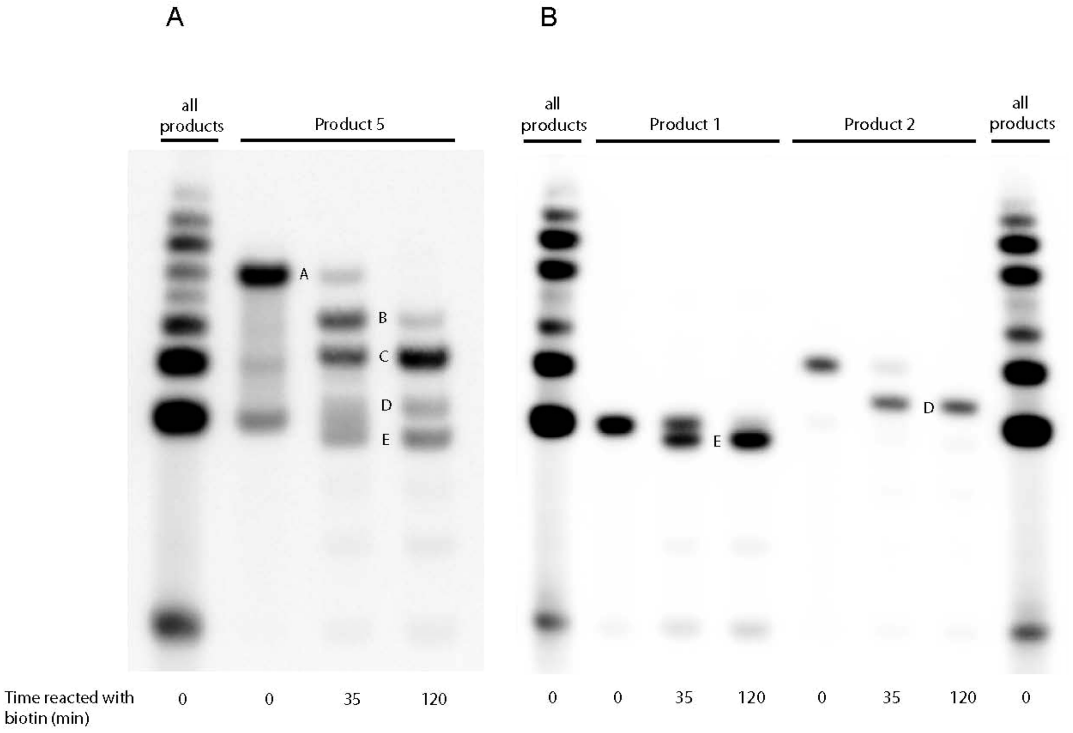

Figure 3. Biotinylation of **1**, **2**, and **5**. (A) Gel-purified **5** yields two unique products when reacted with Sulfo-NHS-LC-biotin. Band A = **5**, B = **5** + 1 biotin, C = **5** + 2 biotin, D = biotinylated **2**, E = biotinylated **1**. (B) Products **1** and **2** yield one biotinylated product each. Reactions were performed at 4 °C and contained 4.6 mM EZ-Link Sulfo-NHS-LC-biotin (Thermo Scientific), 0.1 M HEPES, pH 8.0, ~10000 cpm gel-purified RNA product.

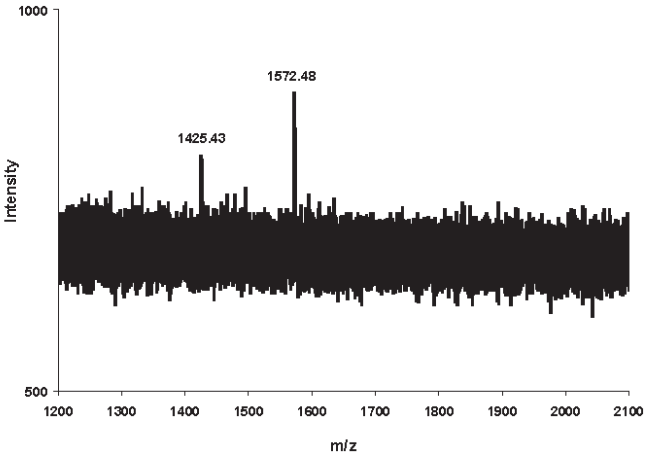

Figure 4. MALDI-TOF mass spectrum of gel-purified **5**. Previously measured mass for GCCU−Phe =1425.19, GCCU−Phe_2 = 1572.54 (the peptide, ref 12)—the peak at a similar mass is bisphenylalanylated GCCU-(Phe/Phe).

synthesis of GCC3′dU, resulting in trace amounts of higher products which are degraded and concentrated in **5** diester by LAP. Nevertheless, major quantitative products of LAP are uniformly the predicted ones for GCC3′dU.

Therefore, while the primary component of **5** in a standard reaction is GCCU-(Phe/Phe) diester, gel electrophoresis can also resolve a low level of GCCU−Phe_5. This can also be seen in Figure S2B, where all gel-purified and individually eluted products from a reaction with GCCU were refractionated in separate gel lanes. Product **5** is not as well-resolved as the other products, which supports the idea that it is a mixture of two species. Since there is a relatively low amount of GCCU−Phe_5 formed in most reactions, it is difficult to detect with the full manifold of products superposed over it (compare Figure 6).

Products **1**−**8** were purified by elution from gels and subjected to mild base to test our expectations for their hydrolyses. Product **2** (GCCU−Phe_2) hydrolyzes at a rate comparable to GCCU−Phe (Table S1; Figure 5, **1** and **2**). While peptidyl−RNA ester bonds are typically more stable than aminoacyl-RNA ester bonds,[14] GCCU−Phe_2 is uniquely labile due to spontaneous diketopiperazine formation; that is, cyclization to a six-membered ring and consequent loss of cyclic Phe−Phe.[15] Below we use "degradation" to mean hydrolysis or diketopiperazination for simplicity. Products **3** and **4** (Figure S1), in contrast, hydrolyze at slower rates (Table S1; Figure 5, products **3** and **4**), reflecting the increased stability normally expected of peptides linked to ribose via an esterified amino acid, but with a blocked amino group.

Mild base hydrolysis of **5** yields only **1**, followed by GCCU (Figure 2). Because of the interesting implications of synthesis of **2** from purified **5** (reaction would imply that diaminoacyl nucleoside ribose is a peptidyl transferase), we have carefully examined our kinetics for this reaction. However, peptide **2** formation from GCCU-(Phe/Phe) appears undetectable; orders of magnitude slower than the major reactions of **5** mild base hydrolysis (Table S1). This confirms previous negative experiments on peptide production by independent methods.[16]

Product **6** is related to **5** by peptide linkage (Figure 1, Figure S3) and so is a peptide extension of **5**, the GCCU-(Phe/Phe) ester. Thus, **6** is likely GCCU-(Phe/Phe_2), with peptide on either the 2′ or 3′ of terminal ribose (**6a** and **6b** in Figure S1). This is strongly supported by mild base hydrolysis, which yields **1** and **2**

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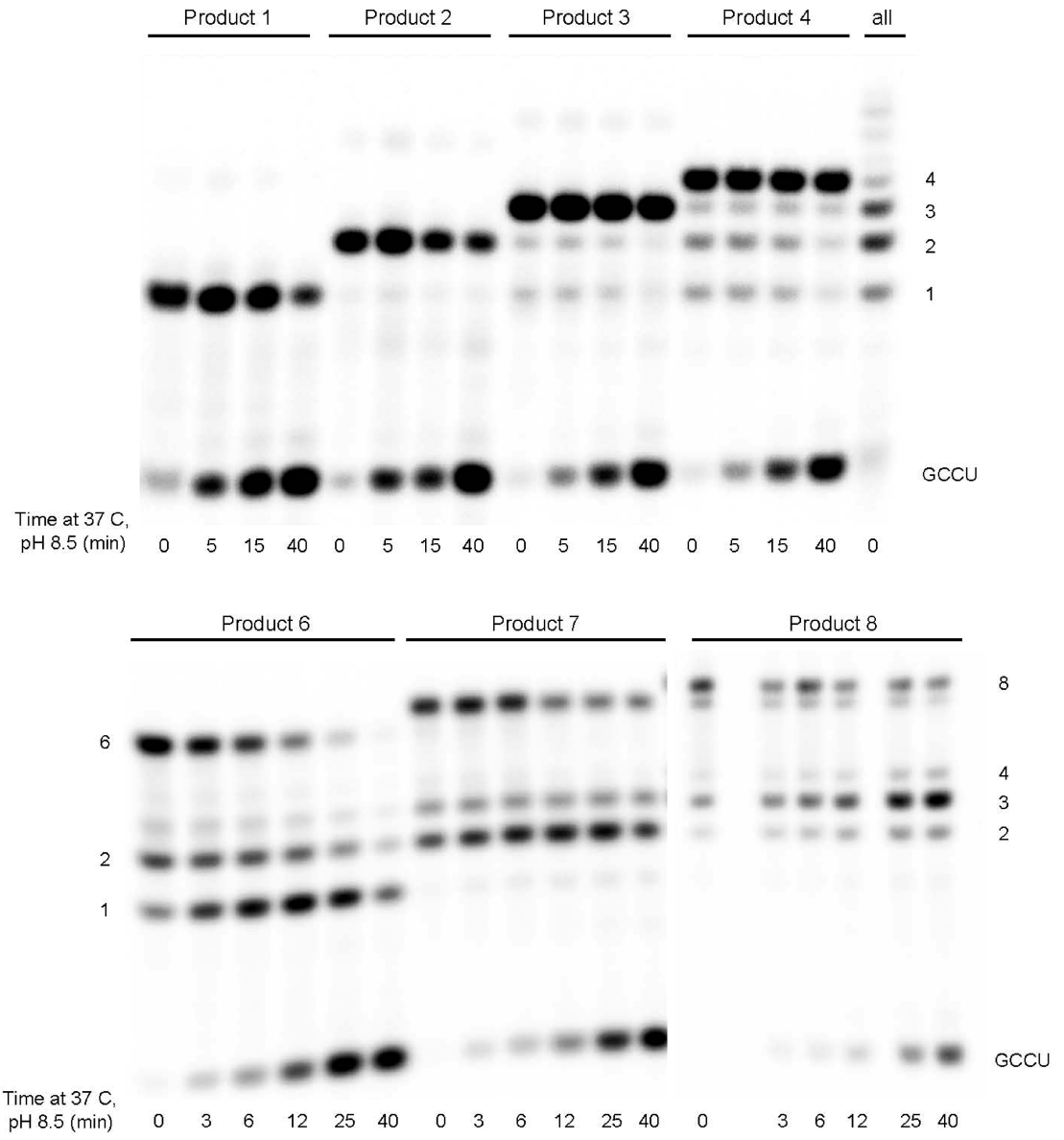

Figure 5. Degradation of gel-purified products. Relevant product numbers are indicated. Buffer: 0.1 M Tris·HCl, pH 8.5.

(Figure 5), via hydrolytic attack on the peptide and aminoacyl moieties, respectively. An unexpected twist appears (Table S1) in that the peptide of **6** reproducibly forms diketopiperazine 3-fold as fast (0.091 min^{-1}) as does an isolated dipeptide (**2**, 0.031 min^{-1}). It appears likely, therefore, that loss of the peptide to cyclization is catalyzed by the adjacent α-NH_2 of esterified phenylalanine. Accelerated degradation of bisaminoacylated oligonucleotide-(Ala/AcetylAla) esters has previously been reported by Duca, et al.[17] Such internal catalysis among these unusual aminoacyl/peptidyl-nucleotides could bear on their evolutionary role(s) during the appearance of translation.

Product **7** is also a peptide extension of **5** (Figure 1 and Supporting Information, Figure S3), and mild base hydrolysis yields **1**, **2**, and **3** (just as **6** yields **1** and **2**) (Figure 5). In fact, leucine aminopeptidase converts **7** to **6** followed by **5** (Figure S3). Thus, **7** from gel electrophoresis is likely a mixture of GCCU-(Phe_2/Phe_2) diester (**7c** in Figure S1) and GCCU-(Phe/Phe_3) diester (**7a** and **7b**, Figure S1; see also Figure 6). This identification is supported by the rates of mild base hydrolysis (Table S1). These were calculated by fitting the relative proportions of (Phe_2/Phe_2) and (Phe/Phe_3) products, along with the hydrolysis rate constants, to gel data. Loss of the Phe_3 peptide from **7** is slower than in the isolated peptide in **3**, but diketopiperazination is still faster than in **2** (which could reflect two sites of attack here), and loss of the ester (in GCCU-(Phe/Phe_3)) is of the order, but slower than, the rate in the unhindered ester of **1**.

Product **8** was also tested with mild base and leucine aminopeptidase. Mild base produces **2**, **3**, and **4** (Figure 5) and yields **7**, **6**, and ultimately **5** when treated with protease (Figure S3; **8a**−**8d**, Figure S1). While we do not trust detailed rates of degradation because the very hydrophobic **8** was difficult to handle reproducibly, these data imply that **8** is likely the expected mixture of GCCU−(Phe_2/Phe_3) and GCCU−(Phe/Phe_4), which would probably not be resolved by gel electrophoresis (compare Figure 6).

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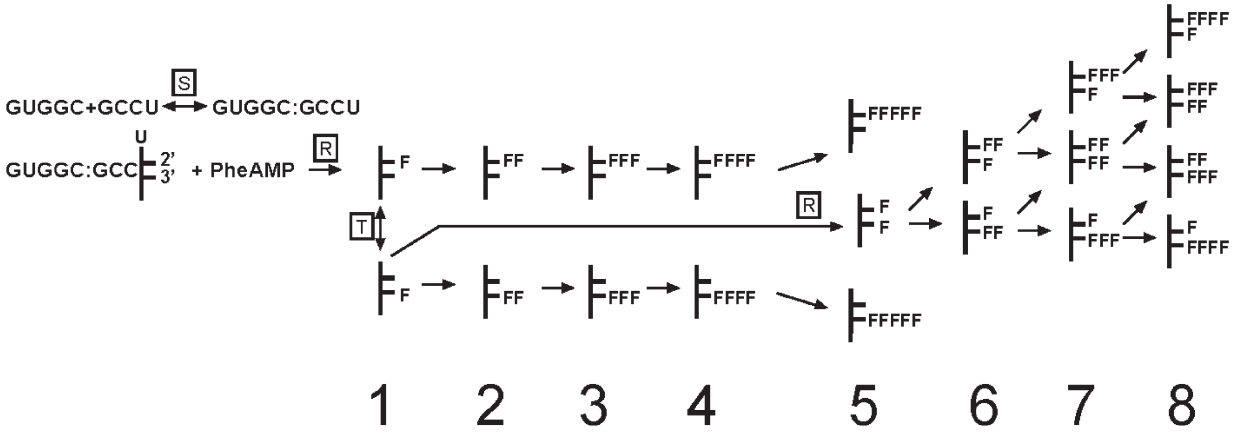

Figure 6. Schematic for PheAMP reactions. Bars on sticks portray 2′ (top) and 3′ (bottom) hydroxyl groups of GCCU terminal uridine; F is phenylalanine. S = strand association; T = transacylation; R = ribozyme-catalyzed aminoacylation. All other arrows indicate spontaneous peptide bond formation from PheAMP. Numerals below the structures indicate polyacrylamide gel fractions; 1 = product **1**, and so on.

Thus we believe we have observed all expected products from 2′ aminoacylation, 2′/3′ acyl migration, and RNA-peptide formation up to five total phenylalanines (Figure 6). Throughout, our methods do not distinguish 2′ and 3′ isomers. We assume both are formed to minimize assumptions, but data presented are in effect weighted averages of 2′ and 3′ behaviors.

To further investigate higher product formation, including the mechanism of peptide bond formation, products were purified and then also rereacted with PheAMP in the presence and absence of 10 μM GUGGC (Figure S4). The rate of higher product formation from GCCU−Phe is not accelerated in the presence of the enzyme, suggesting that peptide formation is lightly catalyzed or uncatalyzed (Figure S5). In fact, initial formation of GCCU−Phe_2 from GCCU−Phe is more rapid in the absence of the enzyme. This suggests that a more flexible or less hindered substrate facilitates peptide extension. GUGGC is required, however, for formation of **5**−**8** from purified **1** (Figure S4), because acylation (and reacylation) of the 2′-OH is highly dependent on the ribozyme.

When GCCU−Phe_2 and GCCU−Phe_3 are purified and reacted with PheAMP, diester products are not formed in appreciable amounts, either in presence or absence of GUGGC (Figure S4). The lack of diester products in the presence of GUGGC implies that ribozyme-catalyzed 2′-acylation of 3′-peptidyl products is slow or absent (cf. Figure 6). Thus acyl migration of peptidyl groups is slowed, as would be anticipated, and Figure 6 parsimoniously elects to omit this reaction. The low levels of **5** that can be seen are likely caused by migration and acylation of **1**, an unavoidable contaminant after gel purification (Figure S2B). Further, a noticeable amount of **5** is formed when **4** is reacted with PheAMP, which suggests that peptidyl extension from GCCU−Phe_4 to GCCU−Phe_5 is observed during reacylation (cf. Figure 6).

Spontaneous peptide bond formation in this system proceeds more rapidly than in a system containing acyl adenylate alone. Here, the rate of peptide formation from RNA−Phe to RNA−Phe_2 (in the presence of PheAMP), for example, is calculated to be ~20 M^{-1} min^{-1}, compared to ~0.3 M^{-1} min^{-1} for alanyl adenylate alone (extrapolated from data of ref 18). Polymerization of alanyl adenylate, however, was found to proceed by means of attack of free alanine on AlaAMP, a relatively slow reaction. In the case of RNA−Phe, the pK_a of the attacking amino group should be much lower than that of free amino acid (Phe methyl esters have an amino group p$K_a \approx 2$ pH units lower than Phe[19]). Therefore, the phenylalanyl amino moiety of RNA−Phe is likely to attack PheAMP under our conditions, leading to rapid polymerization to RNA−$Phe_{n>1}$. Peptide formation in this case, then, is RNA-accelerated, though not RNA-catalyzed.

To test whether the lack of secondary structure in GUGGC/GCCU is necessary for complex product formation, we repeated acylation experiments using the C3 ribozyme[7] (the parent of GUGGC/GCCU, more closely related to initially selected RNAs), as well as an *in trans* variant of C3 (transC3/GCCU, Figure S6). Previous experiments with C3 were conducted at pH 6.4 and with low PheAMP concentrations, and were analyzed using a relatively low-resolution streptavidin gel-shift assay that superposed products; it is thus not surprising that we did not observe more than one acylated product previously. However, using 12 mM PheAMP and acid gel electrophoresis, we observe at least four acylated products from C3, and eight from transC3/GCCU. Thus, the additional secondary structure of C3 does not appear to hinder RNA−peptide formation, even though the rate of peptide formation from purified GCCU−Phe is apparently enhanced in the absence of GUGGC (Figure S5). In addition, the full two-helix junction in transC3/GCCU apparently permits all higher products.

Analysis of the kinetics of GUGGC-catalyzed acylation reveals that the tiny ribozyme functions at rates comparable to other ribozymes independent of divalents (see Figure S7 and supplementary text). Reaction in the absence of magnesium (K_M^{GUGGC} = 2.1 × 10^{-5} M; K_M^{PheAMP} = 5.4 mM; k_{cat} = 0.068 min^{-1}) is quite similar to, but a little slower than, that with Mg^{2+} present (K_M^{GUGGC} = 7.4×10^{-6} M; K_M^{PheAMP} = 6.4 mM; k_{cat} = 0.12 min^{-1}), as we have noted before[12] (Table S2). Magnesium lowers the K_M for GUGGC/GCCU pairing by about a factor of 3. The other effect of the divalent is to elevate the apparent rate of aminoacyl transfer by perhaps 2-fold. In contrast, the several millimolar K_M for the PheAMP second substrate is not detectably altered by 5 mM $MgCl_2$. Because the K_M for PheAMP is high, in almost all usual reactions the enzyme complex is unsaturated by adenylate, with or without Mg^{2+}. We predict that there is no catalytically essential divalent in the active complex. Though small divalent effects on strand pairing and the action of the

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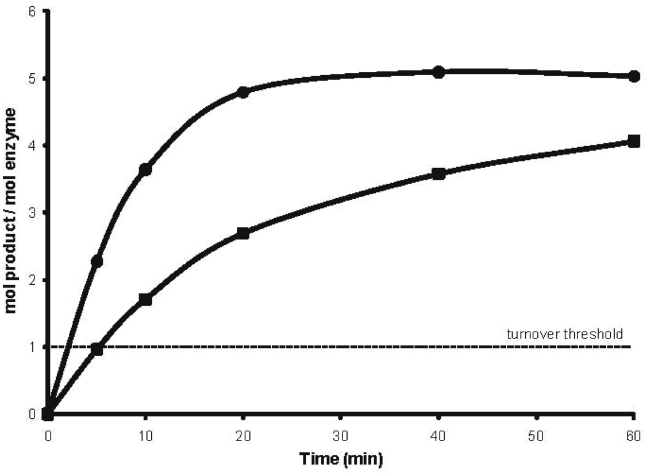

Figure 7. GUGGC/GCCU turns over. Reaction conditions: 10 μM GUGGC, 100 μM GCCU, 100 mM KCl, 5 mM $MgCl_2$, 0.1 M HEPES, 10.3 mM PheAMP, 4 °C. Squares = pH 7.0; circles = pH 7.5. The standard error of individual velocity assays is ±23%.

aminoacyl transfer center exist, these are probably mediated by nearby divalents nonspecifically bound to polar groups nearby.

The other striking thing about our kinetic results is the k_{cat}, which reflects aminoacylation of the majority of GCCU in a saturated complex in a few minutes at 4° and pH 7 (Figure S7, Table S2). Thus, despite the absence of tertiary support around the three nucleotides of the active center, the tiny ribozyme brings its substrates together precisely and transfers an aminoacyl group at rates typical in selected RNA catalysts.[7,11,20] This analysis can be appreciated more intuitively in the accompanying supplementary kinetic figures (Figure S7).

The same conditions that promote formation of the diester products also allow for turnover of the GUGGC enzyme, when the enzyme/substrate ratio is 1:10. Under high PheAMP concentration (10.3 mM) and increased pH (7.5), the reaction yields a 5-fold molar excess of products over the enzyme (Figure 7). At pH 7.0, and at the same time, the reaction yields a 4-fold molar excess. Therefore, each enzyme oligonucleotide is reacting with multiple substrate RNAs, remaining reactive itself after the first reaction has taken place. Thus GUGGC is unequivocally a true enzyme, the smallest known ribozyme capable of aminoacyl-RNA synthesis, and is probably rate-limited by 2′-oxyanion attack on PheAMP.

DISCUSSION

The processes of enzyme-catalyzed RNA aminoacylation, 2′/3′ acyl migration, and spontaneous peptide bond formation collaborate here to create an impressive variety of acyl- and peptidyl-RNA products. It is perhaps not surprising that the small RNA substrate hosts peptide extension and repeated acylation, given the dynamic nature of the RNA substrate−ribozyme interaction (mediated by only nine hydrogen bonds) and the unconstrained nature of the RNA substrate's terminal 2′- and 3′-hydroxyl groups.

Previous work involving a self-acylating ribozyme also yielded peptidyl-RNA under certain conditions, apparently only after the original 95-mer had been reduced to 29 nucleotides.[11] However, given that the C3 ribozyme was found to yield RNA−peptides once the assay was sensitive to these products (Figure S6), we may question whether other known enzymes could be catalyzing reactions that are not detectable at current levels of assay resolution. Nonetheless, enzyme−substrate complexes with little structure can allow for variety in type and number of products formed, as shown here.

The appearance of 2′/3′ diester products in our experiments raises the question of whether or not they are utilized by extant systems. Notably, it has been reported that phenylalanyl-tRNA synthetase from *Thermus thermophilus* can attach two phenylalanyl moieties to a single $tRNA^{Phe}$, resulting in a 2′/3′ tRNA diester.[21] Mechanistic studies revealed that these "tandemly activated" tRNAs can be utilized in both mammalian and bacterial *in vitro* assays. A tandemly activated tRNA donates one amino acid to a growing peptide chain, exits the translation complex, and can later return and donate its remaining amino acid.[16] Tandemly activated RNAs are also more stable than their monoacylated counterparts, representing an increased reservoir of activated amino acids queued for translation.

The five-nucleotide ribozyme GUGGC, then, is responsible for the formation of about 20 aminoacyl-, peptidyl- and mixed RNA products. Because of parallel tendencies, the variety of molecules formed in a world of simplified enzymes might greatly exceed our previous expectations.

More generally, modern protein enzymes are specific and rapid. They therefore direct the flow of carbon to a small set of biomolecules; this is essentially a kinetic function. Present data suggest that primitive enzymes/ribozymes may have a function better approximated as thermodynamic, making a broad chemical variety of products accessible from an activated precursor. Accordingly, early ribozymes instead increase the molecular variety visible to evolution/selection. In particular, it would be particularly plausible now to consider terminal RNA aminoacyl diesters, RNA-dipeptides, and mixed acylated RNAs as possible participants during the evolution of coded oligopeptide synthesis.

MATERIALS AND METHODS

Acylation and LAP Assays. RNA labeling, leucine aminopeptidase reactions, mass spectrometry, and gel electrophoresis were carried out as in ref 12. Aminoacylation assays were conducted as previously, but with modifications to conditions where indicated.

Product Purification. Products were purified after gel electrophoresis by exposing radioactive gel to Kodak X-OMAT Blue film, developing with a Kodak X-OMAT 2000A Processor, and excising the relevant bands. For products to be used for mass spectrometry, cold reactions were run in adjacent lanes to hot reactions, and then excised according to the position of the radiolabeled bands. Bands were crushed, mixed with water for 1 h at 4 °C, filtered, and lyophilized.

Gel-purified **5** was further purified using high performance liquid chromatography (HPLC) for hydrolysis and mass spectrometry experiments. HPLC used a Waters Atlantis T3 column (5 μm) and the following gradient: 95% water/5% acetonitrile for 10 min, ramp to 100% acetonitrile for 10 min, hold for 10 min, ramp to 95% water/5% acetonitrile for 10 min. Fractions were collected (1 mL/min) and radioactivity was quantitated using a scintillation counter. Fractions with high cpm (or cold fractions analyzed in parallel) were pooled and lyophilized.

Calculation of Hydrolysis Rate Constants. Hydrolysis rate constants were derived by fitting measured hydrolysis kinetics (Figure 5) to integrated degradation schemes using Twente-Sim version 2.3 (Controllab Products, Netherlands). Quoted constants are those

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yielding best least-squares fits to integrated systems of differential equations representing the mechanism.

■ ASSOCIATED CONTENT

Supporting Information. Details of kinetic analysis are described, showing how underlying K_M and k_{cat} values can be obtained from apparent measured values, along with product purification, product structure drawings, and data for protease degradation and purified product reacylation with PheAMP. This material is available free of charge via the Internet at http://pubs.acs.org.

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