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REPORT

A tiny RNA that catalyzes both aminoacyl-RNA and peptidyl-RNA synthesis

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ABSTRACT

A 29-nt RNA catalyst successively forms the aminoacyl ester phe-RNA, and then peptidyl-RNA (phe-phe-RNA), given phenylalanine adenylate (phe-AMP) as substrate. Catalysis of two related reactions at similar rates supports the argument that RNA catalysts would evolve as groups with similar mechanisms. In particular, successive aminoacyl- and peptidyl-RNA synthesis by one RNA suggests that uncoded but RNA-catalyzed peptide synthesis would evolve before the synthesis of coded peptides.

Keywords: acylation; evolution; ribozyme; RNA world; translation

INTRODUCTION

The self-acylating ribozymes (Illangasekare et al., 1995) are aminoacyl-RNA forming catalysts, selected in vitro by incubating randomized RNAs with biologically activated amino acids, amino acid adenylates (aa-AMP). RNAs that acylate themselves were isolated by selecting for RNAs that exhibit an amino acid's α-amino group. Self-aminoacylated RNA products are terminal 2′(3′) aminoacyl esters, and therefore chemically similar to aminoacyl-transfer RNAs (Illangasekare et al., 1995). Self-aminoacylating RNAs are frequently calcium ribozymes (Illangasekare et al., 1997), and can be relatively small. One such calcium-requiring RNA enzyme can be repetitively acylated at the same rate, and has been reduced from 95 to 43 nt (Illangasekare et al., 1997). Particular self-aminoacylating RNAs can exhibit very rapid aminoacyl transfer, and high specificity for the phenylalanine side chain comparable to or greater than modern protein aaRS (Illangasekare & Yarus, 1999). The properties of these RNAs therefore offer support for the existence of an ancestral translation system using RNA catalysts.

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RESULTS

Small catalysts

We now report simpler catalysts than previously observed. Figure 1A shows an initially selected 95-mer, isolate #29 (Illangasekare et al., 1995). Figure 1B is a related 29-mer ribozyme derived by deletion of most of the right-hand hairpin of isolate #29 (and capping with a stabilizing tetraloop), as well as replacement of the left-hand hairpin by a short, apparently unstructured 3′ sequence. Figure 1C is a related 32-mer (with an added –UUC– in the 3′ extension) whose properties can be usefully compared with the 29-mer (below).

The 29-mer (Fig. 1B) retains only 13 nt corresponding to randomized positions in the initial selection. Despite deletion of about 70% of the parental 95-mer, these RNAs continue to form aminoacyl-RNA. Rates vary with the molecule; the 29-mer forms aminoacyl-RNA approximately as rapidly as does the parental 95-mer, demonstrating the simplicity of the structural requirement for catalysis of this reaction. The proficiency of such simple RNA molecules in the aminoacylation reaction adds weight to the hypothesis that translation appeared in an RNA world, catalyzed by ribozymes.

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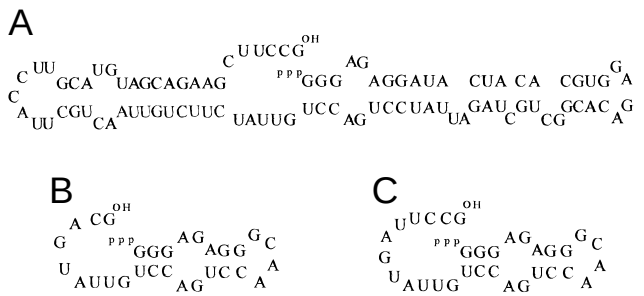

FIGURE 1. Self-aminoacylating RNAs. **A**: The parental self-acylating 95-mer RNA, isolate #29 (Illangasekare et al. 1995). **B**: 29-nucleotide derivative of selection isolate RNA #29, **C**: 32-nt derivative of isolate RNA #29, to illustrate the differences between smaller catalysts.

A second product

However, a further reaction is evident for the 29-mer and 32-mer RNAs: after initial aa-RNA formation, these RNAs appear to extend the 3′ aminoacyl residue into a peptide. This process is illustrated in Figure 2. As shown in Figure 2A, the phosphorimage of a polyacrylamide-gel fractionation shows the formation of a primary product, and with a delay, the formation of a second, slower-migrating product. Although the second reaction is detectable in the initial isolate, it is increased in rate in molecules with an unstructured 3′ acceptor domain. We shall show below that the first product is the phe-

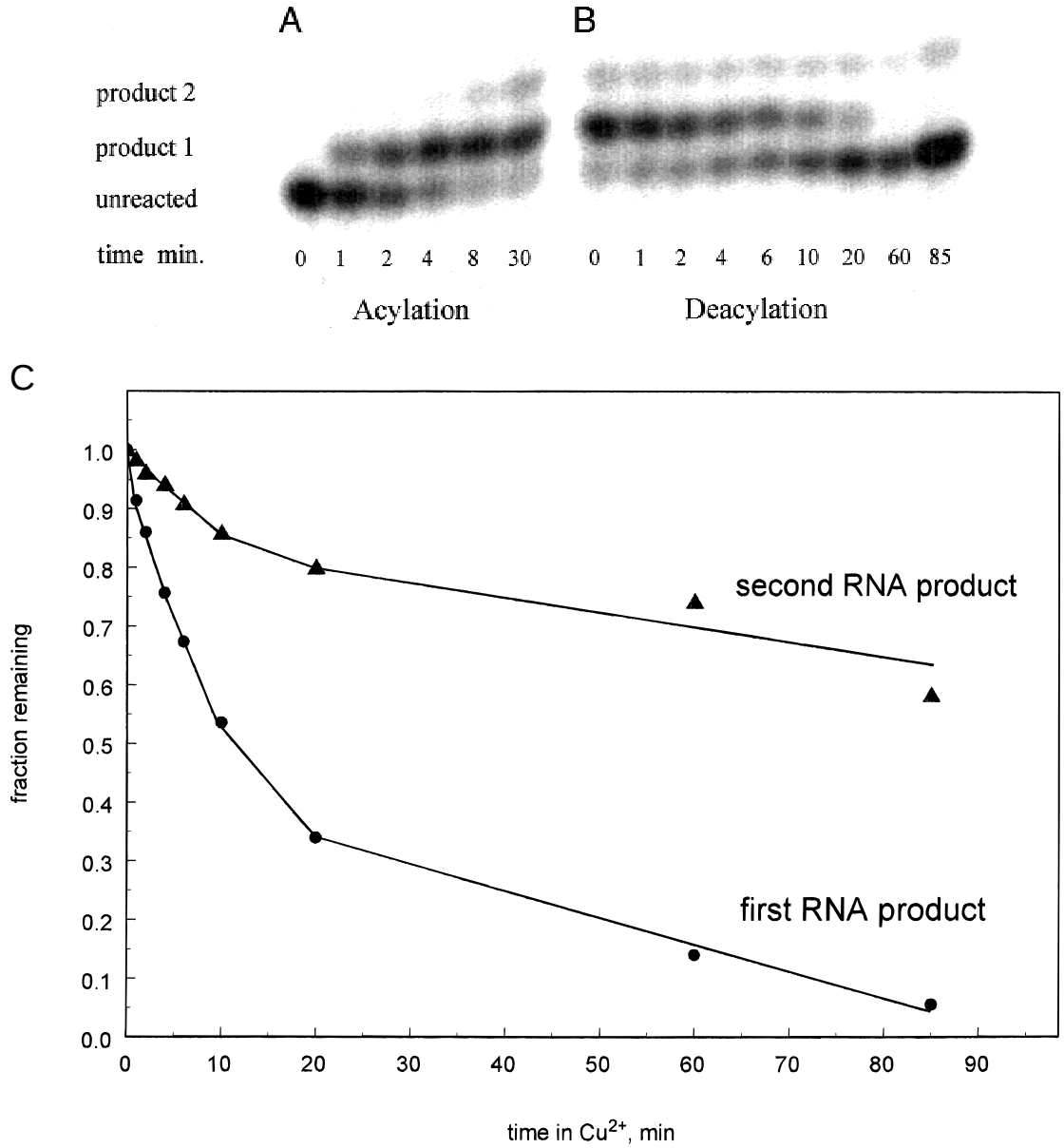

FIGURE 2. Acylation of 29-mer and deacylation of the product with Cu^{2+}. Products of acylation (3.5 mM phe-AMP) have been fractionated by gel electrophoresis at pH 5 (7 M urea, 8% acrylamide), and quantitated by phosphorimaging. **A**: Acylation reaction. **B**: Deacylation of the same products in the presence of Cu^{2+}. **C**: Normalized kinetics of deacylation by Cu^{2+}. Circles: first product of acylation; triangles: second product of acylation.

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RNA, as expected, and the second product is peptidyl-RNA, phe-phe-29-mer.

Characterization of the second product

Stability to hydrolysis is consistent with peptide formation. A simple test of the structure of the two products, hydrolysis by Cu^{2+}, is shown in Figure 2B. Copper-stimulated hydrolysis is more rapid with a free α-amino group (Schofield & Zamencnik, 1968). Thus Figure 2B shows that the initial product of 29-mer catalysis hydrolyzes rapidly, consistent with the existence of a free amino group, as would be anticipated for phe-RNA. In contrast, the normalized kinetics (Fig. 2C) show that the second product is more resistant to Cu^{2+}, and therefore the α-amino of phe-RNA is likely blocked, as it would be after peptide formation, in phe-phe-RNA.

To characterize the products more specifically, we synthesized molecular markers using the method of Gottikh et al. (1970). These nucleotide markers are identical to expected P1 nuclease products from aminoacyl- and peptidyl-RNAs, phe-GMP and phe-phe-GMP. Phe-GMP was the same material we had previously used as an acylation standard (Illangasekare et al., 1995), and phe-phe-GMP was made using the same method, but acylating 5′ GMP with tBOC-phe-phe. The acylated, deprotected nucleotides were resistant to periodate, proving the protection of the 2′ (3′) hydroxyls by esterification, and had a phosphatase-accessible 5′ phosphate, and an unaltered guanosine spectrum. Furthermore, electron spray mass spectroscopic characterization of phe-phe-GMP suggested a mass of 658.3 for the positive ion and 656.3 for the negative ion. Phe-phe-GMP's mass is calculated to be 657.2. Thus the mass of this peptidyl-nucleotide standard is exactly as expected. The positive ion of phe-GMP was found at 511.2 and the negative ion at 509.1, compared to a calculated mass of 510.1. The mass of the aminoacyl-nucleotide is therefore also exactly as predicted.

29-mer RNA transcript was uniformly labeled with [α-^{32}P]GTP to produce a labeled product. After treatment with P1 nuclease (which releases 5′ nucleotides), the expected products are: 1 mol/mol chain of GTP from the 5′ terminus of the transcript; 9–10 mol/mol chain of GMP from internal and 3′ positions; and, finally, 1–0 mol/mol chain of the acylated 3′ terminal GMP should be observed, dependent on the extent of the acylation and subsequent reactions.

These [^{32}P] nucleotides have been fractionated in four chromatography systems along with the synthetic phe-GMP and phe-phe-GMP markers: three thin-layer chromatography (TLC) systems and high-resolution high-performance liquid chromatography (HPLC). These results are shown in the four panels of Figure 3, which illustrates the cofractionation of the major initial product with the phe-GMP marker, and the less prevalent second product with phe-phe-GMP. This identity of the RNA product and molecular markers is particularly evident from Figure 3D. In high resolution HPLC, the absorbance of the authentic phe-GMP and phe-phe-GMP markers precisely tracks the radioactivity of the terminal-acylated products from 29-mer RNA. RNA 29 therefore initially forms phe-RNA, the 2′(3′) phenylalanine ester, as did the molecules from which it is derived (Illangasekare et al., 1995). It then apparently proceeds, relatively efficiently, to utilize phe-RNA for synthesis of phe-phe-RNA, the 3′ peptidyl ester of the RNA.

Successive reactions with adenylate

A second adenylate substrate molecule can be used to form a phe-phe peptide bond. Because the process is first order in RNA and aminoacyl-RNA (Fig. 2 and M. Illangasekare & M. Yarus, unpubl.), both reactions likely involve a single catalytic RNA molecule:

$$\text{RNA} + \text{phe-AMP} \rightarrow \text{phe-RNA} + \text{AMP} \quad (1)$$

$$\text{Phe-RNA} + \text{phe-AMP} \rightarrow \text{phe-phe-RNA} + \text{AMP}. \quad (2)$$

These two reactions are probably quite similar. The first involves nucleophilic attack at carbonyl carbon in adenylate with the ribose hydroxyl as the nucleophile (Illangasekare & Yarus, 1997). The second reaction involves nucleophilic attack at carbonyl carbon in adenylate with the α-amino group of the amino acid as nucleophile. Thus though the products (an ester bond and a peptide bond) differ, the two reactions, and particularly their transition states, can easily be imagined in the same RNA active site. We conjecture that the flexibility of the 3′ tail (Fig. 1) is advantageous to present the amino group of phe-RNA for the second reaction.

A mechanism postulating direct reaction of the phe-RNA with a second adenylate is the simplest explanation of peptide synthesis. Neither copper hydrolysis (Fig. 2B) nor extensive characterization of reacting molecules (Fig. 3) reveals any other product than phe-GMP and phe-phe-GMP. Thus there is no evidence for another intermediate, and the evidence is particularly extensive against another intermediate involving the 3′ nucleotide.

An alternative reaction path can be excluded

We can also eliminate an alternative, partially uncatalyzed, pathway. It might seem possible that two molecules of phe-AMP react in solution to yield phe-phe-AMP. Newly formed phe-phe-AMP (phe-phe-adenylate) could then directly acylate RNA to give phe-phe-RNA. We have examined this possibility by synthesis of the adenylate phe-phe-AMP (Illangasekare et al., 1995) for use as a molecular marker and as a substrate for the 29-mer self-acylator.

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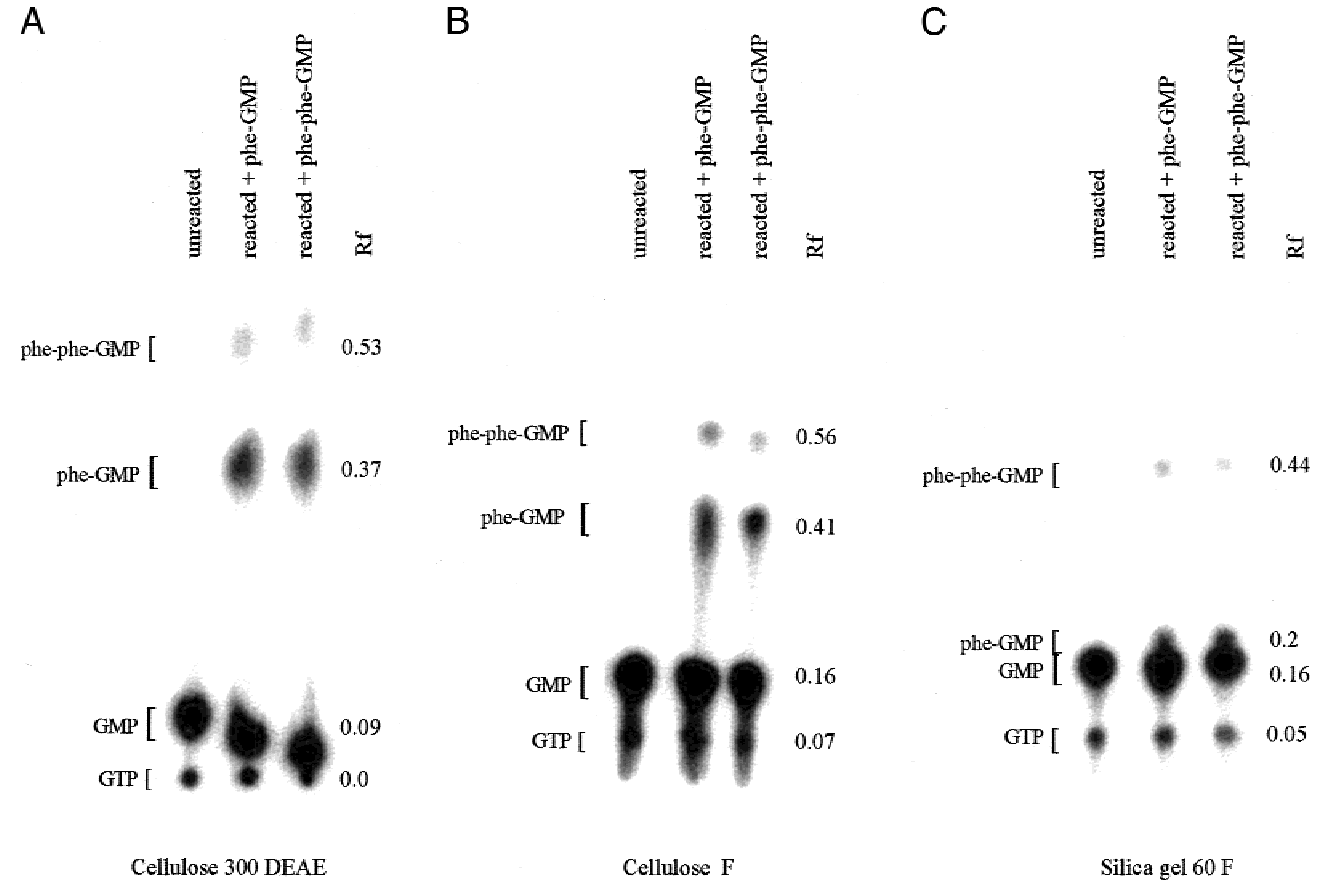

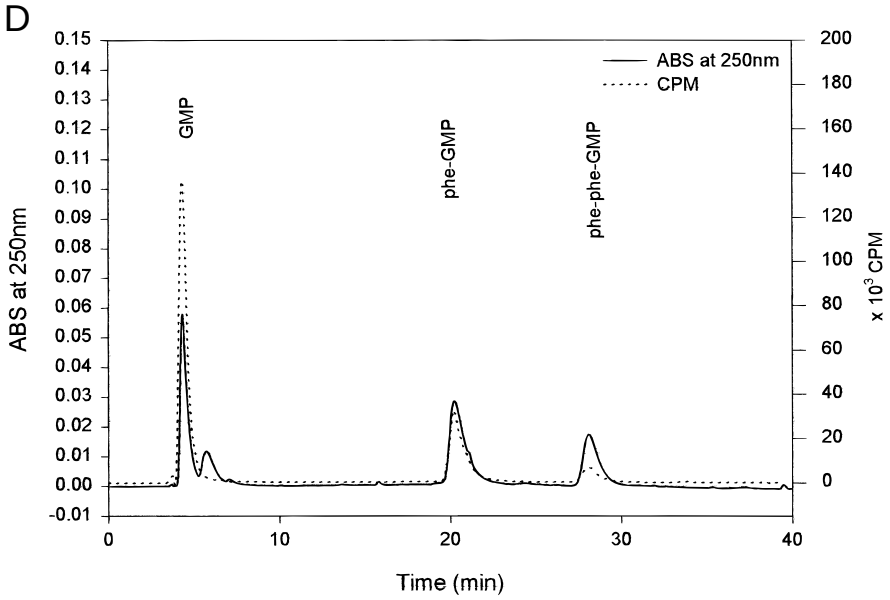

FIGURE 3. Fractionation of the ^{32}P aminoacylation product with standards. Markers phe-GMP and phe-phe-GMP were cofractionated with the acylation product on four chromatographic systems. **A**: DEAE cellulose TLC phosphorimage; **B**: cellulose F TLC; **C**: silica gel TLC; all eluted with butanol:acetic acid:water. Brackets show the approximate position of marker absorbance. Rfs shown are the mobility of the spots relative to the solvent front. Marker distribution was always identical with ^{32}P, to the limits of the technique. **D**: HPLC in gradient of acetonitrile-0.1 M NH_4OAc on a C4 glass column. Solid line: absorbance at 250 nm; dashed line: CPM.

First, the formation of phe-phe-AMP from two phe-AMP cannot be detected, and this must occur at a very low net rate. Synthetic phe-phe-AMP is relatively stable under acylation reaction conditions ($t_{1/2}$ = 140 min) and should persist if formed. Presumably this implies that its disappearance is slow not only by hydrolysis, but also by cyclic peptide (diketopiperazine) formation (Weber & Orgel, 1979). Nevertheless, using our synthetic marker, we could not observe net synthesis of phe-phe-AMP when phe-AMP was incubated under our reaction conditions and analyzed by HPLC. This implies, given our conditions, a second-order rate constant for

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phe-AMP reaction to give phe-phe-AMP of less than 2×10^{-1} M^{-1} min^{-1}. This agrees with literature on other adenylates; for example, ala-ala-AMP formation from ala-AMP was also undetectable (Lewinsohn et al., 1967).

Second, even if it were formed, phe-phe-AMP is a very poor substrate for the 29-mer RNA. We could not observe direct peptidylation of 29-mer RNA with phe-phe-AMP when the two were incubated together. Taken conservatively, this implies a second-order rate constant less than 2×10^{-3} M^{-1} min^{-1} for reaction of phe-phe-AMP and RNA. Therefore we do not detect either of the two reactions required for the existence of this alternative pathway. However, by calculating using the limits of detection, the reactions via phe-phe-AMP are more than 10^5-fold too slow to account for the observed phe-phe-RNA synthesis (Fig. 2). Peptide formation therefore occurs via catalysis on the RNA, not via reaction of adenylates in solution.

Rates for aminoacyl- and peptidyl-RNA formation

To interpret the rates of these RNA-catalyzed reactions, models were fitted to the observed kinetics (e.g., equations 1–2, Fig. 2). Fitting takes account not only of reactions that form the two successive RNA products, but also of the rapid hydrolytic decay of phe-AMP under these acylation conditions ($t_{1/2} = 5.8$ min). The results are that a precursor–product relationship between phe-RNA and phe-phe-RNA is supported. A reaction mechanism assuming successive phe-AMP Michaelis–Menten complexes with the RNA (to form phe-RNA), then with the phe-RNA (to form phe-phe-RNA) fits the data. The fits yield plausible rate constants, for example, rates are similar to acylation rates measured independently and more simply. If it is assumed that both reactions have the same K_m for phe-AMP, then the relative k_{cat}/K_m for these successive reactions are well constrained by the kinetics in the figure. For the aminoacylation of the 29-mer RNA by phe-AMP, k_{cat}/K_m = 154 M^{-1} min^{-1}, compared to 170 M^{-1} min^{-1} for the 95-nt parental molecule measured under the same conditions. Peptide production is slower, having a k_{cat} that is 13-fold less ($k_{cat}/K_m \approx 12$ M^{-1} min^{-1}) than for initial 29-mer phe-RNA formation.

If desired, assumption of equal K_m's can be avoided when comparing rates of aa-RNA and peptidyl-RNA formation. If the reactions are taken as successive second-order reactions with phe-AMP (thereby ignoring varying saturation with phe-AMP), the data can still be fit closely to kinetic models. Here peptide formation is again 13-fold slower than phe-RNA formation, in agreement with the ratio obtained with the more complex Michaelis–Menten mechanism. The calculated kinetics corresponding to these fitted numbers for successive second-order steps is shown as solid lines in Figure 4.

Much slower peptidyl-RNA than aminoacyl-RNA formation is not a necessary property of the RNA-catalyzed reaction. The relative rate of peptide synthesis is somewhat affected by divalent concentrations, but particularly by the structure of the 3′ extension in similar molecules (M. Yarus & M. Illangasekare, unpubl.). For example, for 32-mer RNA (Fig. 1C), phe-RNA formation is about one-third the rate of the 29-mer (k_{cat}/K_m = 47 M^{-1} min^{-1}). However, the rate of phe-phe-RNA (peptide) formation is ≈60% the rate of phe-RNA formation ($k_{cat}/K_m \approx 29$ M^{-1} min^{-1}). In all such reactions, prolongation of the reactions leads to discrete, even more slowly migrating, copper-stable products than phe-phe-RNA. The likely inference is that longer peptides can be produced.

Comparison with similar reactions

These phe-RNAs form peptide from adenylate at second-order rates of 10–30 M^{-1} min^{-1}. Similar reactions also occur on protein aminoacyl-tRNA synthetases. For example, AspRS, HisRS, LeuRS, and TyrRS purified from *Bacillus stearothermophilus* all catalyze reaction between their bound adenylates and free amino acid derivatives to give dipeptides (Nakajima et al., 1986). The second-order rate for tyr-leu-NH_2 formation by TyrRS protein using this route was 2.8×10^{-3} M^{-1} min^{-1}, calculated by correcting published data to pH 7.25 and 0 °C, as in our experiments. Peptide formation from reaction of adenylate with free amino acids or peptides does not require catalysis, and occurs in solution (Lewinsohn et al., 1967). The reaction of the peptide ala-ala with ala-AMP has a second-order rate of 0.3 M^{-1} min^{-1}, again corrected to our assay conditions. The present self-acylating RNAs therefore accelerate peptide formation about 35- to 100-fold over a comparable solution reaction, and more than 50- to 150-fold over reactions between phe-AMP molecules (discussed above). Comparison to reaction on the surface of a protein aminoacyl-RNA synthetase, where the adenylate is evidently protected from nucleophiles in solution, suggests an acceleration of about 3,500 to 10,000-fold on the RNA versus the protein enzyme.

DISCUSSION

A single 29-mer RNA catalyzes two of the essential chemistries of genetic translation, aminoacylation of RNA and peptide synthesis. Although such aminoacyl-RNA formation mirrors the biological reaction, peptide synthesis utilizes the adenylate, which is not the normal activated form of amino acid for ribosomal peptide formation. Nonetheless, this double catalysis adds to the preexisting body of evidence that RNA catalysts are capable of originating the process of translation (reviewed in Yarus, 1999).

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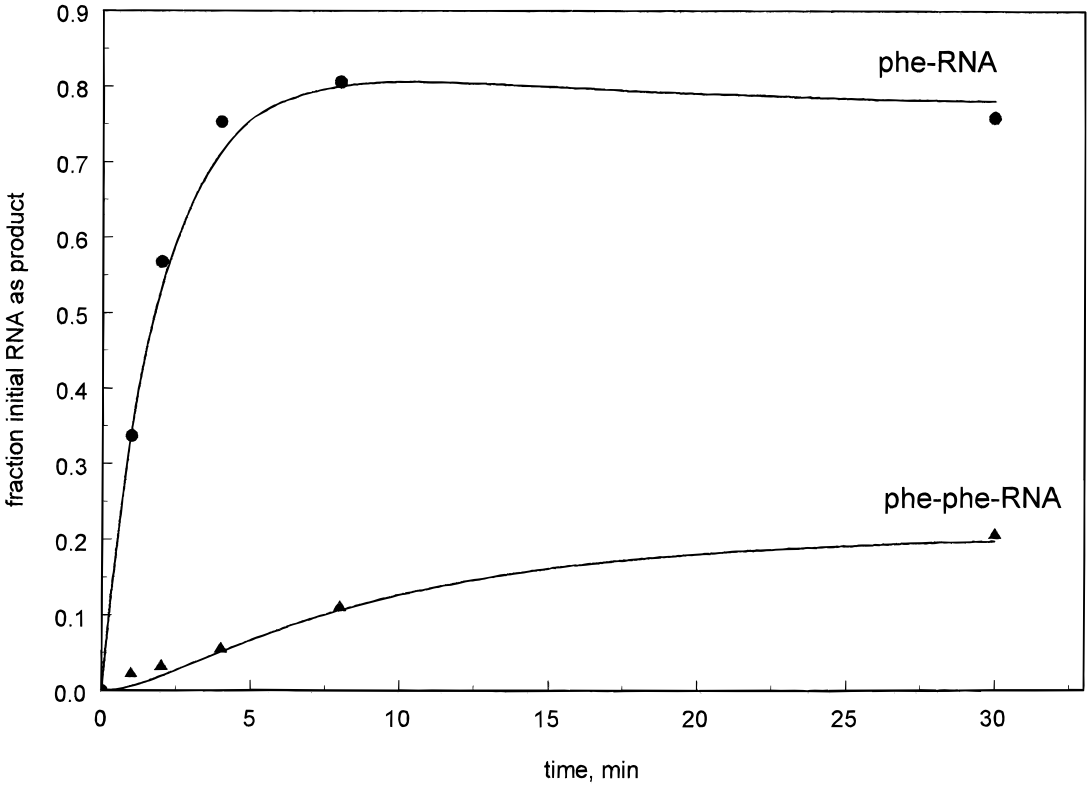

FIGURE 4. Kinetics for the formation of phe-RNA and phe-phe-RNA. The points are phosphorimage data for 29-mer RNA from Figure 2, normalized to 85% total acylation. The lines are the calculated fits, which include hydrolysis of the phe-AMP, formation of phe-RNA and its subsequent conversion to phe-phe-RNA, using rate constants given in the text. Circles: first acylation product; triangles: second acylation product.⟨FC⟩

Two further implications of these data seem notable. First, it has been suggested (Yarus, 1999) that RNA catalysis can be predicted to some extent. Reactions likely to exist (1) link substrates whose binding by RNA has been established (2) via transition states similar to those already known to be stabilized within RNA. The 29-mer RNA supports this proposition, as it shows that similar reactions involving the carbonyl of aminoacyl adenylates can indeed take place successively in the same active site. Previous work established (Lohse & Szostak, 1996) that an RNA can form both esters and amides if the amino group for the second reaction is chemically substituted for the attacking RNA hydroxyl in the active site for the ester-transfer reaction. However, the self-aminoacylating RNAs above comprise the first example of two successive, related reactions by the same RNA catalyst. To host these two reactions, 29-mer RNA overcomes inevitable steric difficulties. For example, the α-amino group of phe-RNA whose attack forms peptide is potentially displaced $\approx$4 Å and differs geometrically from the terminal hydroxyl group of an RNA whose attack forms aa-RNA. However, no such difference is sufficient to prohibit the second reaction.

Second, the properties of the 29-mer suggest an evolutionary route to translation. Highly evolved catalysts can be very specific, and accordingly less likely to perform related reactions. However, ancient newly evolved RNA catalysts would not have been refined by extensive selection as are modern proteins. Therefore, early RNA catalysts would still carry out their side reactions at relatively high rates. These side reactions could be subjected to selection for other activities. So evolution of any RNA transacylase would probably be closely followed by catalytic aminoacyl-RNA synthesis and by the accompanying synthesis of peptides by related catalysts. This notion is supported by similar peptide-forming reactions on recently evolved RNA and the continuing delectability of similar reactions on highly selected modern aminoacyl-RNA synthetases pointed out above.

Peptides made by this route would be easily freed from peptidyl-RNA by uncatalyzed hydrolysis, and would be able to take roles as cofactors to the dominant RNA catalysts. This could establish a functional advantage for peptide synthesis in an RNA world, and a particular advantage for the synthesis of specific, ordered RNA-stimulating peptides. Therefore, despite obvious differences in the two reactions, the kind of peptide synthesis demonstrated here as a side reaction during aminoacyl transfer could have set the stage for the evolution of a more specific, coded translation system. It has been suggested that uncoded peptide synthesis may have

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been the evolutionary antecedent of coded synthesis, and even that uncoded peptides may have been first formed as 3′ peptidyl-RNAs (Orgel, 1989). However, that such uncoded peptidyl-RNAs might follow easily from aminoacyl-RNAs, as products of the same catalyst, is a new possibility raised by this work.

MATERIALS AND METHODS

Internally labeled RNA

RNA is an in vitro transcript made using T7 RNA polymerase, uniformly labeled with [^{32}P]-GTP and purified under conditions of single-nucleotide resolution by 7 M urea/10% acrylamide gel electrophoresis (Ciesiolka et al., 1996).

RNA singly labeled at 3′ terminus

[5′ α ^{32}P]-pGp was ligated to an unlabeled transcript shortened by 1 nt at the 3′ end, using T4 RNA ligase (Illangasekare & Yarus, 1999).

Reactions of phe-AMP

Acylations have been generally described (Illangasekare & Yarus, 1997). Synthetic phe-AMP (Illangasekare et al., 1995) at several millimolar is added at 0 °C to 10 nM–4 μM RNA, 40 mM $CaCl_2$, 80 mM $MgCl_2$, 100 mM NaCl, and 0.2 M HEPES at pH 7.25. Reactions were stopped by addition of 30 vol of 0.1 M NaOAc/78% (v/v) ethanol and reduction to dry ice temperature. Alternatively, for gel electrophoresis, reactions were stopped by addition of the gel loading solution: 0.1 M NaOAc, 3 M urea, 17 mM EDTA, 0.02% xylene cyanole, 0.2% bromphenol blue. Acylations are either fractionated in 8% acrylamide gels (19:1 acrylamide: bis), 8 M urea, and 0.1 M NaOAc, pH 5, or ethanol precipitated, and incubated with 2.4 U nuclease P1 (Pharmacia, Sigma) in 3 μL 10 mM NaOAc, pH 5.2, for 30 min at 23 °C.

Phe adenylate was tested for phe-phe-AMP formation up to 40 min starting at 9 mM; $<$0.5% product was observed. Peptidylation of 29-mer RNA with phe-phe-AMP adenylate was conducted up to 60 min starting at 2.5 mM phe-phe-AMP; $<$0.05% product was observed. Limits on rate constants given in the text include the effects of reactant instability, which reduces the sensitivity of these attempts to detect products.

Thin-layer chromatography

The products are of acylation and P1 nuclease treatment are resolved on TLC using cellulose 300 DEAE plates (Whatman), silica gel 60 F254 plates (E.M. Science), or cellulose F (Baker Flex), all eluted with 1-butanol:acetic acid:water, 5:2:3 (v/v).

High-performance liquid chromatography

Acylated nucleotides were purified and characterized by reverse-phase HPLC on C4 glass. In an 0.1 M NH_4OAc:acetonitrile gradient consisting of linear segments of increasing slope (23°, pH 7.0) on a Rainin Dynamax column (25 cm, 5 μm porosity), GMP elutes at about 4% acetonitrile, guanine (observed as a result of depurination in trifluoroacetic acid) at about 6%, AMP at 7%, the ester phe-GMP at 20%, the peptidyl ester phe-phe-GMP at about 44% acetonitrile, and the adenylate phe-phe-AMP at 49% acetonitrile.

Cu^{2+} hydrolysis

Aminoacylated RNAs were ethanol precipitated, and taken up in freshly made solutions: 10 mM $MgCl_2$, 20 mM $CuSO_4$, and 50 mM NaOAc, pH 5.5.

Mass spectroscopy

Phe-GMP and phe-phe-GMP were characterized by electron spray mass spectroscopy at cone voltage of 25 V in a Fisons V.G. Quattro spectrometer (Donald Dick, Mass Spec. Laboratory, Chemistry Department, Colorado State University). Water/acetonitrile/0.1% formic acid was used as solvent for detection of the positive ion, and water/acetonitrile/0.1% NH_4OH for the negative ion.

Rates of parallel reactions

The pseudo first-order rates of Nakajima et al. (1986) for reaction of TyrRS (AMP-tyr) and leucylamide were corrected from 45 °C to 0 °C using the measured energy of activation for the reaction (18 kcal/mol) from the same authors. This is a 111-fold decrease. Because the free unprotonated amino group is the active species, the rates were also corrected for protonation in the interval pH 8.5 to 7.25, a further 17.8-fold decrease. The rate of Lewinsohn et al. (1967) for the reaction of ala-ala with ala-AMP was corrected from pH 7.5 to pH 7.25 for the same reasons; this corresponds to a 1.68-fold decrease. For correction for the temperature change from 25 °C to 0 °C, Arrhenius corrections were made as above, assuming an activation energy of 14 kcal/mol; this implies a further 8.8-fold decrease.

Markers

The aminoacyl ester phe-GMP and peptidyl ester phe-phe-GMP were synthesized by the method of Gottikh et al. (1970). Carbonylimidizole (375 μmol; Sigma) and equimolar tBOC-L-phe (Sigma) or tBOC-L-phe-phe (Bachem) were dissolved in dried tetrahydrofuran. After 10 min at 23 °C, this mixture was poured into 500 μL acetonitrile:water (1:4 v/v) containing 20 μmol of disodium 5′ GMP. After 20 min, this was precipitated by addition of 15 mL cold acetone. The dried product was deprotected by dissolving in 500 μL trifluoroacetic acid (TFA) at 0 °C and immediate evaporation under vacuum. The product was purified using TLC on Cellulose F (J.T. Baker) with *n*-butanol:acetic acid:water (5:2:3). At this point, phe-GMP was eluted with water and used. Phe-phe-GMP was eluted, then further purified using HPLC on C4 glass as above. The adenylate phe-phe-AMP was made using dicyclohexylcarbodiimide (DCC), as for other adenylates (Illangasekare et al., 1995), save that phe-phe peptide (Sigma) was the starting material.

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Modeling kinetics

Data from phosphorimages were fit to kinetic models that were numerically integrated. Rate constants were adjusted to yield the lowest squared deviations from the data, using the Simplex algorithm implemented in the simulation program Tutsim (Tutsim Products, Palo Alto; V6.06PT). Models included the successive reactions of equations 1 and 2, and simultaneous exponential decay of adenylate at a rate that was independently measured by HPLC resolution of the products. Kinetic constants quoted are for 20 fits to the same data (e.g., Fig. 2) conducted with wide variation in the starting values of rate constants. This procedure for finding the "best" model confined k_{cat}/K_m and k_{2nd} to mean values with a standard error of 2–5%.

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