Aminoacyl Esterase Activity of the *Tetrahymena* Ribozyme

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Several classes of ribozymes (catalytic RNA's) catalyze reactions at phosphorus centers, but apparently no reaction at a carbon center has been demonstrated. The active site of the *Tetrahymena* ribozyme was engineered to bind an oligonucleotide derived from the 3′ end of *N*-formyl-methionyl-tRNA^{fMet}. This ribozyme catalyzes the hydrolysis of the aminoacyl ester bond to a modest extent, 5 to 15 times greater than the uncatalyzed rate. Catalysis involves binding of the oligonucleotide to the internal guide sequence of the ribozyme and requires Mg^{2+} and sequence elements of the catalytic core. The ability of RNA to catalyze reactions with aminoacyl esters expands the catalytic versatility of RNA and suggests that the first aminoacyl tRNA synthetase could have been an RNA molecule.

Several structurally and mechanistically distinct classes of catalytic RNA's have been discovered (*1*). In every case, the substrate for these ribozymes is RNA or DNA (*2*), and the reactions they catalyze are limited to transesterification or hydrolysis of phosphate diesters or phosphate monoesters (*3*). Observations that RNA can form a specific binding site for an amino acid (*4*) and various organic dyes (*5*) have suggested that RNA might catalyze chemical reactions in which the substrates are not nucleic acids.

Theories of the origin and evolution of life postulate that RNA played a significant role as both information carrier and catalyst (*6*, *7*). It has even been proposed that a metabolic system composed of RNA catalysts could have existed before the advent of ribosomal protein synthesis (*8*). More information about the catalytic versatility and substrate repertoire of RNA is needed before the plausibility of such scenarios can be evaluated.

The nuclear ribosomal RNA precursor of *Tetrahymena thermophila* contains a 413-nucleotide intervening sequence (IVS) that can splice itself from the larger RNA. Shortened forms of this IVS catalyze sequence-specific cleavage of exogenous oligonucleotide substrates with either guanosine (G) or water as the nucleophile (*9*–*11*). The sequence specificity of the reaction is determined by base pairing between an internal guide sequence (IGS) on the ribozyme and a complementary sequence on the substrate (*9*, *12*). A saturable binding site for guanosine (G site) is located in the catalytic core of the ribozyme (*13*). The rate constants for the individual steps in the reaction pathway for RNA cleavage have been described (*11*).

Because the ribozyme from *Tetrahymena* contains a catalytic core for reactions of phosphate diesters, we hypothesized that this core might contain a favorable arrangement of catalytic groups for analogous reactions at carbon centers. Our approach was to target a carboxylate ester to the catalytic core of the ribozyme, by covalent attachment of an amino acid to an oligonucleotide for which the ribozyme contains a binding site.

Carboxylate ester hydrolysis. We initiated study with an aminoacyl derivative of the hexanucleotide CAACCA, esterified with *N*-formyl-L-methionine at the 2′ or 3′ position of the terminal ribose (CAACCA$_{fMet}$). Use of this particular aminoacyl ester offers several advantages in that (i) it is easily prepared by ribonuclease T1 digestion of *N*-formyl-L-methionyl-tRNA^{fMet} (*14*), (ii) it can be ^{35}S-labeled in the amino acid portion in vivo, and (iii) *N*-formylation makes it more stable toward spontaneous hydrolysis than an aminoacyl oligonucleotide with a free α-amino group (*15*). A ribozyme was constructed in which the IGS was modified from GGAGGG to GGGUUG (*16*–*19*) to allow base pairing with the aminoacylated oligonucleotide (Fig. 1, A and B). The guanosine at the 5′ end (G22) was left unaltered even though it was not complementary to the A in the substrate, because a G in this position is conserved and contributes to reactivity (*20*).

This newly engineered ribozyme was tested in the endoribonuclease reaction (50°C, 10 mM $MgCl_2$, pH 7.0) with RNA substrate CAACCUAAAAA, which forms a "matched" duplex with the IGS. Cleavage occurred at the expected site (the UA bond) (*21*) with the second-order rate constant for reaction of E · G (the ribozyme · guanosine complex) and free substrate $(k_{cat}/K_m)^S = 2 \times 10^7\ M^{-1}min^{-1}$ (*22*). This value is comparable to $(k_{cat}/K_m)^S = 9 \times 10^7\ M^{-1}\ min^{-1}$ for the endonuclease reaction studied previously (*11*). Thus, these base changes within the IGS do not severely impair ribozyme folding or catalytic activity. A second substrate, CAACCAAAAAA was also examined because, like CAACCA$_{fMet}$, it would form a terminal G · A mismatch when bound to the IGS (Fig. 1A). This substrate was also cleaved at the expected site after CAACCA (*21*); the rate of the actual cleavage step was reduced by at least 30-fold for the guanosine-dependent reaction and 10-fold for the hydrolysis reaction, compared to those for the "matched" substrate (*23*).

CAACCA$_{fMet}$ was incubated with ribozyme at 30°C in the absence of guanosine. When the ^{32}P-labeled oligonucleotide moiety was monitored, the *pCAACCA$_{fMet}$ (*24*) was converted to a product of greater electrophoretic mobility (Fig. 2A) (*25*) that comigrated with authentic *pCAACCA (*21*). When the ^{35}S-labeled aminoacyl moiety was followed, [^{35}S]fMet was released from CAACCA$_{[^{35}S]fMet}$ (Fig. 2B) (*26*). In both assays, the amino acid was hydrolyzed from the oligonucleotide with first-order kinetics about five times faster in the presence of ribozyme than in its absence (Fig. 2C) (*27*). In the presence of guanosine, guanosine analogues, L-arginine, and arginine analogues (*28*), hydrolysis of the amino acid persisted with no evidence of transfer of *N*-formyl-methionine to a nucleoside or of dipeptide formation (*29*).

The ribozyme is a metalloenzyme, requiring magnesium ion both for tertiary structure formation and for RNA cleavage activity (*30*, *31*). When CAACCA$_{fMet}$ was incubated with ribozyme in the absence of Mg^{2+}, no enhancement of hydrolysis over the uncatalyzed reaction was observed. Neither Zn^{2+} nor Ca^{2+} substituted for magnesium ion in the esterase reaction. Although Ca^{2+} promotes ribozyme tertiary structure formation, this structure has no measurable catalytic activity in the endonuclease reaction (*30*). Because the Ca^{2+}-stabilized ri-

Table 1. Deletions that destroy ribozyme activity with RNA also eliminate aminoacyl-esterase activity.

RNA*	Concentration (μM)	k_{obs} (hour^{-1})†
Ribozyme	1	1.00
L-42 Sca I	1	0.21
L-21 Fok I	10–50	0.21
L-21 Mbo II	10–50	0.21
No RNA	—	0.21

*RNA secondary structures are shown in Fig. 3. †Determined by TLC as described in Fig 2B; the variation was 10%.

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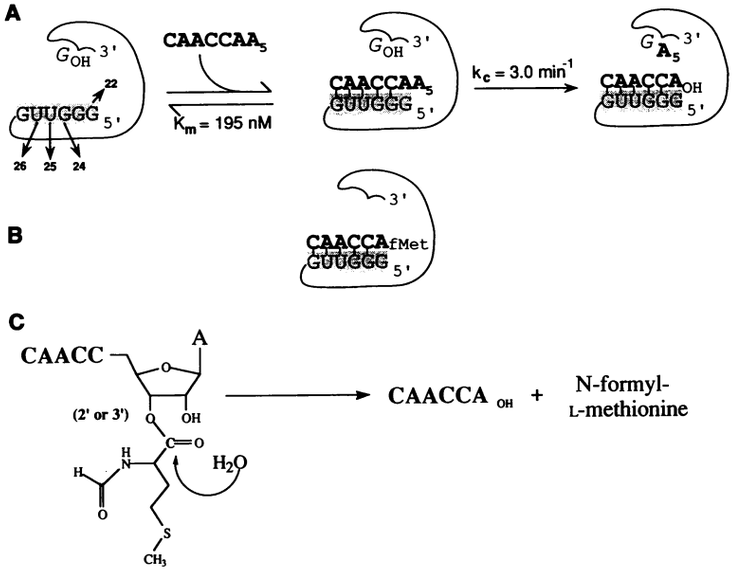

Fig. 1. Endoribonuclease and aminoacyl esterase activities of a ribozyme designed to bind the 3′ end of $tRNA^{fMet}$. (**A**) Endoribonuclease reaction. The ribozyme is a shortened version of the *Tetrahymena* IVS RNA missing 21 nucleotides from the 5′ end and one nucleotide from the 3′ end. This ribozyme was engineered to bind the 3′-terminal sequence of $tRNA^{fMet}$ CAACCA by alteration of the IGS at positions 24, 25, and 26 (*16*). The conserved G at position 22 was left unaltered. The ribozyme binds the oligonucleotide substrate S (CAACCAAAAAA, bold uppercase letters) by base pairing to the IGS (shaded). The Michaelis constant (K_m) for the reaction was determined under single turnover conditions from a plot of k_{obs} as a function of ribozyme concentration (30°C, 50 mM $MgCl_2$, pH 7.0). The rate constant for the central step (k_c) is the observed rate constant in the presence of saturating enzyme and guanosine. (**B**) Model for ribozyme-aminoacyl oligonucleotide complex. The substrate, derived from ribonuclease T1 digestion of aminoacylated $tRNA^{fMet}$, is complementary to the IGS (shaded). The usual phosphoryl group at the cleavage site is replaced with an aminoacyl group derived from *N*-formyl-L-methionine (fMet). Binding of the aminoacyl oligonucleotide to the IGS is expected to target the carboxylate ester to the ribozyme active site. (**C**) Aminoacyl ester hydrolysis, showing nucleophilic attack by H_2O (or OH^-) to release the *N*-formylmethionyl group from the oligonucleotide. The oligonucleotide product of this reaction is identical to that of the endonuclease reaction shown in (A). The substrate is a mixture of two isomeric forms, the 2′-*O*-aminoacylated and 3′-*O*-aminoacylated isomers, that interconvert in aqueous solution (*47*).

bozyme is also inactive in the esterase reaction, Mg^{2+} appears to be directly involved in the chemical step in this case as well.

Requirement for catalytic core and substrate binding site. To ascertain what sequences and structural elements of the ribozyme contribute to its esterase activity, we tested IVS transcripts with different deletions that render the enzyme inactive in the endonuclease reaction (*32*) for their ability to catalyze ester hydrolysis (Fig. 3). IVS transcripts with 3′ deletions were made by cutting the DNA template at two restriction sites, producing RNA truncated within the catalytic core. Neither the L-21 Fok I nor the L-21 Mbo II versions of the ribozyme promoted esterase activity, although these RNA constructs still contain the guide sequence. With 50 μM L-21 Mbo II RNA, a concentration that should assure binding of the oligonucleotide based on thermodynamic parameters and that was shown to give greater than 80 percent binding of CAACCAAAAAA by native gel electrophoresis (*33*), no catalysis of aminoacyl bond cleavage was observed (Table 1). These results suggest that catalysis was not simply a consequence of Watson-Crick base pairing of $CAACCA_{fMet}$ to the IGS, but required additional elements of the ribozyme's tertiary structure.

An L-42 Sca I version of the ribozyme, which lacks part of the 5′ end including the IGS, was also incapable of catalyzing ester hydrolysis (Table 1). The catalytic core of this RNA should be correctly folded (*34*). Thus, some portion of the 5′ region missing in L-42, presumably the IGS, was essential for hydrolysis of the ester linkage, which is

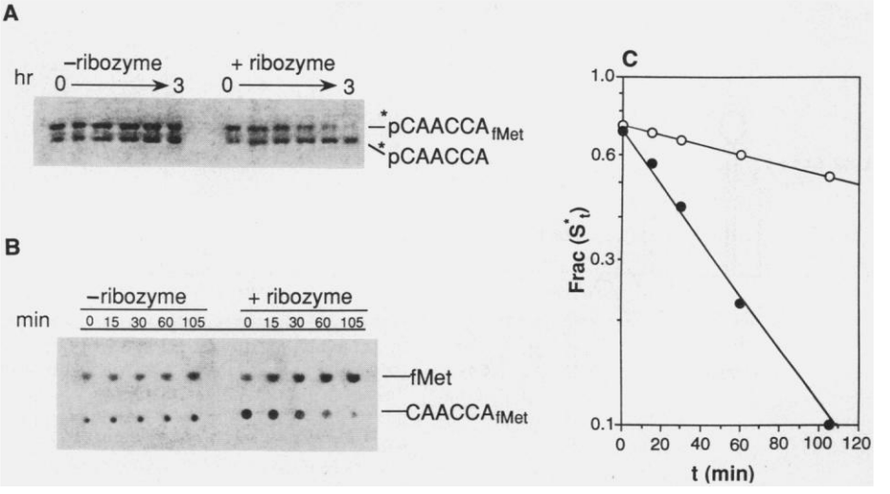

Fig. 2. Hydrolysis of aminoacyl oligonucleotide linkage catalyzed by the ribozyme. (**A**) Reaction of labeled oligonucleotide moiety analyzed by gel electrophoresis and scanned with a PhosphorImager (Molecular Dynamics). The ribozyme was first incubated in reaction buffer (20 minutes; 50°C). The temperature was reduced to 30°C and the ribozyme was added to $p^*CAACCA_{fMet}$. Reaction conditions were 1 μM ribozyme, ~0.1 nM substrate, 50 mM MES buffer [2-(*N*-morpholino)ethanesulfonic acid], pH 7.0, and 50 mM $MgCl_2$. As a control, ribozyme was omitted (left set of lanes). Reactions were quenched with EDTA and urea (buffered to pH 6.0 with potassium phosphate), placed immediately on solid CO_2, and subjected to electrophoresis on a 20 percent denaturing polyacrylamide gel (*25*). (**B**) Labeled amino acid moiety analyzed by thin-layer chromatography (TLC) and scanned with a PhosphorImager (Molecular Dynamics). $CAACCA_{[^{35}S]fMet}$ (~50 nM) was incubated with 1 μM ribozyme (or without ribozyme as indicated). The reaction was carried out as described above. Portions were removed at intervals and placed immediately on solid CO_2. After the last sample was removed (105 minutes), the portions were thawed and immediately applied to a cellulose TLC plate. The plate was dried at reduced pressure for about 30 seconds and eluted with *n*-butanol:acetic acid:water (5:3:2). Regardless of the assay used, there is some product visible at zero time. This is presumably the result of hydrolysis that takes place during substrate preparation. (**C**) Semilogarithmic plot of the data from (B). k_{obs} = 1.0 $hour^{-1}$ (±10 percent) for hydrolysis in the presence of ribozyme (closed circles) and 0.2 $hour^{-1}$ in the absence of ribozyme (open circles) (*48*). Frac S^*_t is the fraction of aminoacyl oligonucleotide remaining at time t.

consistent with a requirement for base pairing of the substrate to the IGS to fix the aminoacyl ester at the active site.

When CAACCAAAAAA was assayed as an inhibitor, the rate of ester hydrolysis decreased with increasing concentration of the RNA substrate (Fig. 4A). The concentration dependence was consistent with the measured K_i of 200 nM for CAACCAAAAAA in the endonuclease reaction. The "matched" oligo CAACCUAAAAA inhibited completely at 1 μM concentration, as was expected because of its dissociation constant (~5 nM) (*21*). This inhibition provided evidence that CAACCA$_{fMet}$ and CAACCAAAAAA bind to the same site, that is, the IGS, as implied by the diagrams in Fig. 1.

The rate of ester hydrolysis shows a hyperbolic dependence on the concentration of ribozyme (Fig. 4B). At low concentration of ribozyme the rate of hydrolysis was first order with respect to ribozyme concentration, while at high ribozyme concentration the reaction rate appeared to become independent of ribozyme concentration. Such Michaelis-Menten reaction kinetics are typical of enzyme-catalyzed reactions in which substrate and catalyst form a complex. The K_m is ~450 nM. For this reaction, we would expect K_m to be equal to the dissociation constant, K_d (*35*). This value of 450 nM is ~10 to 15 times higher than the K_d of non-aminoacylated CAACCAAAAAA (*35*), suggesting an effect of the amino acid moiety on binding.

As mentioned above, the reaction was examined in the presence of guanosine and some guanosine analogues. Measurement of ester hydrolysis in the presence of saturating concentrations of guanosine or guanosine-5′-monophosphate (GMP) showed ~25 percent inhibition compared to reactions in the absence of guanosine, while addition of uridine 5′-monophosphate had no effect. 3′-Deoxy-3′-amino-GMP inhibited the reaction, like guanosine. 2′-Deoxy-2′-aminoguanosine stimulated the rate about threefold, increasing the overall rate enhancement to 15 (Fig. 4C). Both of these guanosine analogues occupy the G site (*36*). The sensitivity of the hydrolysis reaction to G-site occupation is further evidence that the reaction was occurring at the active site described previously. Changes in solvation or conformation that occur when guanosine binds, or direct interaction of the nucleotide with the aminoacyl group are possible ways in which the nucleotides could affect hydrolysis.

Transfer of the aminoacyl group to guanosine or its analogues was not observed, in contrast to nucleotidyl group transfer observed in the endonuclease reaction. This may seem surprising in view of the fact that in the RNA endonuclease reaction, the rate of the chemical step is 1000 times faster with G as the nucleophile than with water as the nucleophile (*11*). However, closer analysis suggests that, although the aminoacyl group and the guanosine may be proximal, they may not be appropriately oriented for reaction, as described below.

Comparison of esterase and endonuclease mechanisms. We found that the hydrolysis of CAACCA$_{fMet}$ to *N*-formyl-methionine and CAACCA was accelerated 5-fold by the ribozyme and 15-fold in the presence of one particular guanosine analogue. This modest rate acceleration required magnesium ion and specific sequence elements of the RNA; both of these properties correspond to the endonuclease reaction, suggesting that a similarly folded tertiary structure is necessary for catalysis. Several observations indicate that the reaction occurs at the active site with substrate bound to the IGS: (i) a deleted ribozyme lacking the IGS but having the core sequence elements is inactive, (ii) the endonuclease substrate CAACCAAAAAA is an inhibitor, (iii) the reaction kinetics show saturation behavior, and (iv) the reaction rate is sensitive to bound guanosine or guanosine analogues. Although simple buffers and small molecules are known to catalyze the hydrolysis of carboxylate esters (*37*), these reactions are not likely to have the sequence specificity inherent in the ribozyme-catalyzed reaction.

By protein enzyme standards and by comparison to RNA-catalyzed reactions with natural substrates, the ribozyme is a modest aminoacyl esterase. The rate advantage obtained in the endonuclease reaction is six to seven orders of magnitude larger

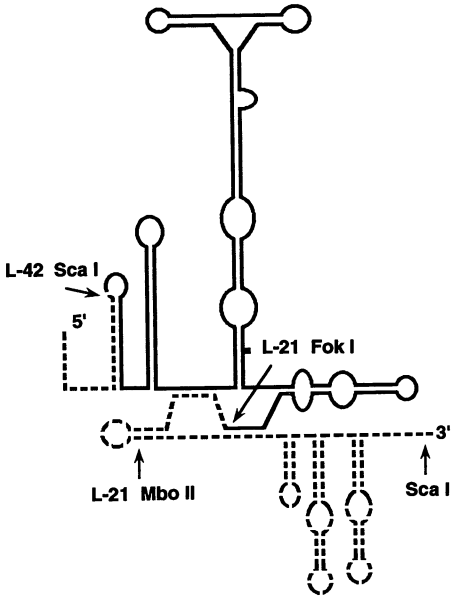

Fig. 3. Outline of secondary structure of the *Tetrahymena* ribozyme showing deleted sequence elements (dashed lines). The standard ribozyme includes the entire outline. L-42 lacks the 5′ dashed region and extends to the Sca I site. L-21 Fok I includes the 5′ dashed region and the solid region only. L-21 Mbo II is like L-21 Fok I but extends to the Mbo II site.

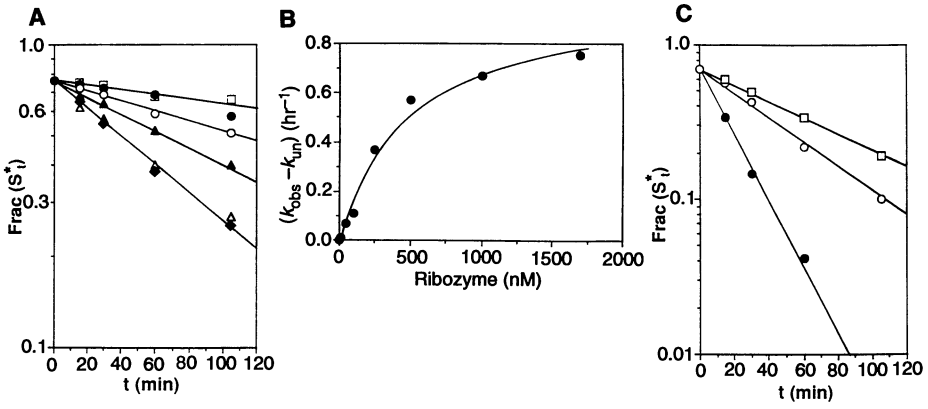

Fig. 4. (**A**) Esterase activity requires pairing of substrate to IGS. Semilogarithmic plots for hydrolysis of *pCAACCA$_{fMet}$ catalyzed by the ribozyme with CAACCAAAAAA at 23 μM (closed circles), 2.7 μM (open circles), 1.3 μM (closed triangles), and 0.7 μM (open triangles). Closed diamonds represent the ribozyme-catalyzed reaction in the absence of CAACCAAAAAA, and open squares represent the uncatalyzed reaction. Reaction protocol and data analysis were carried out with the gel electrophoresis assay (Fig. 2A). (**B**) Ester hydrolysis follows Michaelis-Menten kinetics. $k_{obs} - k_{un}$ plotted as a function of ribozyme concentration; k_{obs} is calculated from the slope of graphs such as those of Figs. 2C and 4, and k_{un} is the uncatalyzed rate of ester hydrolysis. The reactions were done with ~0.1 nM *pCAACCA$_{fMet}$ as indicated in Fig. 2A. The solid line represents the fit to a theoretical curve with K_m = 450 nM and k_c = 1.2 hour^{-1}. (**C**) Sensitivity of ester hydrolysis rate to G-site occupation. Semilogarithmic plots for the hydrolysis of CAACCA$_{f^{35}S]fMet}$ catalyzed by the ribozyme in the absence of guanosine (open circles) or in the presence of 2 mM guanosine (open squares) or 2 mM 2′-deoxy-2′-aminoguanosine (closed circles) (*36*). Other reaction conditions and quantitation were as in Fig. 2B. Substrates and products were analyzed by TLC and quantitated with the PhosphorImager (Molecular Dynamics).

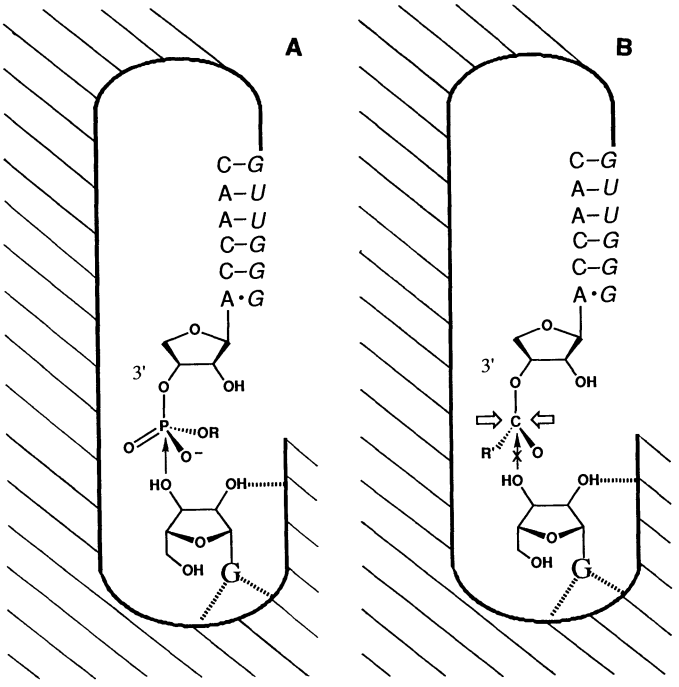

Fig. 5. Alignment of substrate and guanosine on the ribozyme in (**A**) the RNA endonuclease reaction and (**B**) the esterase reaction. The three-dimensional ribozyme surface is represented by the hatched pattern outside the dark outline. The unhatched interior represents the ribozyme active site. The substrate is bound to the IGS (represented by uppercase italic letters). Guanosine is bound by at least three separate interactions (*13*). The closed thin arrow in (A) represents a favorable pathway for the nucleophile, in-line with the 3′-oxygen-phosphorus bond (*40*). For nucleophilic attack on a carbonyl carbon, shown in (B), this direction of attack is not favorable as indicated by the x through the arrow. For carboxylate esters, a more favorable direction of nucleophilic attack is orthogonal to the pi face (*42*), shown by the thick open arrows. Assuming free rotation about the 3′ oxygen–carbonyl carbon bond, the open arrows define a plane perpendicular to the surface of the page in this model.

than that obtained in the esterase reaction (*38*). However, in the esterase reaction the natural G·U at the cleavage site is replaced with a G·A mispair because the 3′ terminal nucleotide of $CAACCA_{fMet}$ is an A. The RNA substrate containing an A at the cleavage site reacts 30 times more slowly than the corresponding U-containing substrate in the guanosine-dependent reaction (*23*). Thus, the ribozyme-substrate combination tested here may not be optimal for the esterase reaction (*39*).

In addition, the intrinsic differences in mechanism between phosphate diester and carboxylate ester reactions may limit the efficiency of aminoesterolysis with this ribozyme. In reactions of phosphate diesters in solution, the nucleophile attacks the phosphorus atom in-line with the labile phosphorus-oxygen bond (*40*). On the ribozyme, nucleophilic attack by the 3′-hydroxyl of guanosine is an in-line, S_N2 (P) reaction as judged by its stereochemistry, that is, inversion of configuration at phosphorus (*41*). Carboxylate esters undergo nucleophilic attack by a different mechanism (*42*). In the first step the nucleophile is added to the carbon-oxygen double bond by attack orthogonal to the pi face, giving a tetrahedral intermediate. In the second step the leaving group is expelled. If the relative orientations of $CAACCA_{fMet}$ and guanosine in the active site are the same as in the endoribonuclease reaction, then the reactants will not be aligned appropriately for acyl group transfer (Fig. 5).

The *Tetrahymena* ribozyme has been selected to splice itself out of pre-ribosomal rRNA, not to catalyze the hydrolysis of aminoacyl ester linkages. In fact, the ribozyme is highly specific for the phosphate at the cleavage site in the RNA substrate. For example, stereospecific substitution of a sulfur for one of the nonbridging phosphate oxygen atoms at the cleavage site reduces activity 1000 times or more (*43*). Thus, it may be possible to create mutant versions of the *Tetrahymena* ribozyme or to find other RNA's that are considerably better at catalyzing this esterase reaction.

Nevertheless, some rate acceleration might be expected because a phosphate diester is thought to mimic the transition state for ester hydrolysis. Antibodies that bind tetrahedral phosphate analogues catalyze reactions at carbon centers that proceed through tetrahedral transition states such as ester or amide hydrolysis (*44*). Since the ribozyme has an active site for a phosphate diester, the ground state or transition state interactions that occur with the phosphate might also stabilize a tetrahedral carboxylate ester transition state.

In particular, a candidate for such an interaction is the ribose 3′ oxygen of the substrate (Fig. 5), the leaving group in both the esterase and endonuclease reactions. There is evidence that this oxygen atom is directly coordinated to Mg^{2+} in the transition state of the endonuclease reaction (*31*). Thus, the 3′-oxygen of the aminoacyl ester could be coordinated to a magnesium ion serving to activate the ester toward nucleophilic attack.

The discovery of catalytic RNA supported earlier speculation regarding a self-replicating system based solely on RNA. The demonstration of catalysis of a reaction at a carbon center by an RNA enzyme suggests that the RNA world could have expanded to include reactions of amino acid and other non-nucleic acid substrates prior to the involvement of proteins. More specifically, RNA might be able to act as an aminoacyl tRNA synthetase. Since RNA can break acyl bonds to oligonucleotides, then, by the principle of microscopic reversibility, RNA must also be able to charge oligonucleotides with amino acids. The substrate for the esterase activity is derived from the acceptor stem of an aminoacyl tRNA. These observations, coupled with the observations of specific amino acid binding by RNA, provide evidence for the chemical plausibility of earlier speculation (*4*, *45*) that the first aminoacyl RNA synthetase was an RNA molecule.

Extensively deproteinized large ribosomal subunits retain peptidyl transferase activity (*46*), suggesting that the catalytic activity resides in the RNA component. With tRNA's, mRNA, and ribozymes as tRNA synthetases and peptidyl transferases, it is possible to conceive of information-driven protein synthesis being carried out exclusively by RNA.

REFERENCES AND NOTES

1. S. Altman, *J. Biol. Chem.* **265**, 20053 (1990); T. R. Cech, *Annu. Rev. Biochem.* **59**, 543 (1990); F. Michel, K. Umesono, H. Ozeki, *Gene* **82**, 5 (1989); N. R. Pace and D. Smith, *J. Biol. Chem.* **265**, 3587 (1990); R. H. Symons, *Crit. Rev. Plant Sci.* **10**, 189 (1991).
2. D. Herschlag and T. R. Cech, *Nature* **344**, 405 (1990); D. L. Robertson and G. F. Joyce, *ibid.*, p. 467; A. C. Forster and S. Altman, *Science* **249**, 784 (1990).
3. T. R. Cech, *Science* **236**, 1532 (1987).
4. M. Yarus, *ibid.* **240**, 1751 (1988).
5. A. D. Ellington and J. W. Szostak, *Nature* **346**, 818 (1990).
6. C. Woese, *The Genetic Code: The Molecular Basis for Genetic Expression* (Harper and Row, New York, 1967), p. 186; F. Crick, *J. Mol. Biol.* **38**, 367 (1968); L. E. Orgel, *ibid.*, p. 381.
7. N. R. Pace and T. L. Marsh, *Origins Life* **16**, 97 (1985); L. E. Orgel, *J. Theor. Biol.* **123**, 127 (1986); T. R. Cech, *Proc. Natl. Acad. Sci. U.S.A.* **83**, 4360 (1986); W. Gilbert, *Nature* **319**, 618 (1986); F. H. Westheimer, *ibid.*, p. 534; G. F. Joyce, *ibid.* **338**, 217 (1989).
8. S. A. Benner, A. D. Ellington, A. Tauer, *Proc. Natl. Acad. Sci. U.S.A.* **86**, 7054 (1989); C. M. Visser, *Origins Life* **14**, 291 (1984); _____ and R. M. Kellogg, *J. Mol. Evol.* **11**, 171 (1978); H. B. White III, *ibid.* **7**, 101 (1976); _____, in *The Pyridine Nucleotide Coenzymes*, J. Everse, B. Anderson, K.-S. Yu, Eds. (Academic Press, New York, 1982).
9. A. J. Zaug, M. D. Been, T. R. Cech, *Nature* **324**, 429 (1986).
10. A. J. Zaug, C. A. Grosshans, T. R. Cech, *Biochemistry* **27**, 8924 (1988)
11. D. Herschlag and T. R. Cech, *ibid.* **29**, 10159 (1990), *ibid.*, p. 10172.
12. R. W. Davies, R. B. Waring, J. A. Ray, T. A. Brown,

C. Scazzocchio, *Nature* **300**, 719 (1982); R. B. Waring, P. Towner, S. J. Minter, R. W. Davies, *ibid.* **321**, 133 (1986); M. D. Been and T. R. Cech, *Cell* **47**, 207 (1986).
13. B. L. Bass and T. R. Cech, *Nature* **308**, 820 (1984); F. Michel *et al.*, *ibid.* **342**, 391 (1989).
14. R. E. Monro, *Methods Enzymol.* **20**, 472 (1971).
15. R. Wolfenden, *Biochemistry* **2**, 1090 (1963).
16. The *Tetrahymena* ribozyme containing an altered guide sequence GGGUUG was prepared by phagemid mutagenesis (*17*) of plasmid pTZ-21H2 (*18*) and characterized by dideoxy sequencing (*19*). Plasmid was cut with Hind III and transcribed with phage T7 RNA polymerase at 30°C. The $MgCl_2$ concentration was then raised from 15 mM to 50 mM, the temperature was raised from 37° to 50°C, and incubation was continued for 2 hours to promote processing of the 3′-terminal hammerhead ribozyme and thereby generate a precise 3′ end (*18*).
17. T. A. Kunkel, J. D. Roberts, R. A. Zakour, *Methods Enzymol.* **154**, 367 (1987).
18. C. G. Grosshans and T. R. Cech, *Nucleic Acids Res.* **19**, 3875 (1991).
19. F. Sanger, S. Nicklen, A. R. Coulson, *Proc. Natl. Acad. Sci. U.S.A.* **74**, 5463 (1977).
20. E. T. Barfod and T. R. Cech, *Mol. Cell. Biol.* **9**, 3657 (1989); J. A. Doudna, B. P. Cormack, J. W. Szostak, *Proc. Natl. Acad. Sci. U.S.A.* **86**, 7402 (1989).
21. J. A. Piccirilli, T. S. McConnell, T. R. Cech, unpublished data.
22. The second-order rate constant for reaction of E · G and S, $(k_{cat}/K_m)^S$, was determined in single turnover experiments with saturating guanosine and 0.5 nM 5′-[^{32}P]-CAACCUAAAAA at 50°C, pH 7.0, 10 mM Mg^{2+}. The value of k_{obs} increased linearly with the concentration of E (1 to 5 nM, 1 mM G).
23. The rate constants (k_c) for the central step representing the conversion of E · G · S ternary complex to E · G · P are 200 min^{-1} and 6 min^{-1} for CAACCUAAAAA and CAACCAAAAAA, respectively. These values might represent the actual chemical conversion, as in the case of the ribozyme with the natural IGS, or could represent an accompanying conformational change or binding of G. The value of k_c = 200 min^{-1} for CAACCUAAAAA was calculated as described in (*11*) from $(k_{cat}/K_m)^G = 2.0 \pm 0.5 \times 10^5\ M^{-1}\ min^{-1}$ with the assumption that K_d for guanosine = 1 mM (50°C, 10 mM $MgCl_2$, pH 7.0). The value of k_c = 6 min^{-1} for CAACCAAAAAA was the observed rate of single turnover cleavage in the presence of excess ribozyme and 1 mM G. The values of k_c (-G), the rate of the central step in the guanosine-independent hydrolysis reaction, were 0.2 min^{-1} and 0.02 min^{-1} for CAACCUAAAAA and CAACCAAAAAA, respectively. The second-order rate constant for reaction of E · S and G, $(k_{cat}/K_m)^G$, was determined in single turnover experiments with 200 nM ribozyme and 1 nM 5′-[^{32}P]-CAACCUAAAAA at 50°C, pH 7.0, 10 mM Mg^{2+}. Guanosine concentration was varied from 1 to 5 μM.
24. Because of the hydrolytic instability of the ester linkage in $CAACCA_{fMet}$, the following procedure was used for 5′ end-labeling and subsequent isolation of 5′-[^{32}P]-$CAACCA_{fMet}$ (*pCAACCA$_{fMet}$). The kinase reaction was performed (37°C, 5 minutes) in 5 μl of buffer [50 mM MES (pH 7.0), 10 mM $MgCl_2$, 0.2 mM spermidine, 0.1 mM EDTA], and contained $CAACCA_{fMet}$ (1.2 pmol), [γ-^{32}P]ATP (adenosine triphosphate) (3 pmol), and polynucleotide kinase (50 units). The reaction was quenched by the addition of an equal volume of sample buffer [90 percent formamide, 20 mM sodium phosphate, 16 mM borate, 20 mM EDTA, 0.02 percent bromophenol blue, and 0.02 percent xylene cyanol (pH 6.0)] and stored on solid CO_2 prior to gel electrophoresis. The 5′-labeled oligonucleotide was purified by electrophoresis on a 20 percent polyacrylamide–3.2 M urea gel that had been equilibrated at 20 W for 2 hours. Electrophoresis was then continued for 4 to 7 hours at 4°C. The buffer was 100 mM sodium phosphate, 83 mM boric acid, and 1 mM EDTA (pH 6.0). *pCAACCA$_{fMet}$ was visualized by autoradiography, excised, and eluted for 1 hour in buffer containing 10 mM ammonium acetate, pH 4.5, and 1 mM EDTA. The eluted aminoacyl oligonucleotide was stored at −70°C.
25. Polyacrylamide gel electrophoresis was carried out on slab gels of 20 percent polyacrylamide and 7 M urea. The buffer was 100 mM tris(hydroxymethyl)aminomethane, 100 mM boric acid, 2 mM EDTA; pH 8.3. Gels were first run at 4°C (cold room) at 15 W before use and then run for ~3 hours after application of the samples. The gels were dried, and radioactivity was quantitated (PhosphorImager).
26. The [^{35}S]-aminoacyl oligonucleotide was isolated by the procedure of Monro (*14*). Treatment of the oligonucleotide with CHES buffer (pH 9.0) hydrolyzed the acyl linkage. The product did not comigrate with authentic *N*-formyl-methionine (Sigma) on TLC (silica gel 60; butanol:acetic acid:H_2O, 4:1:1). Treatment of this product with 10% HCl for 10 min at 95°C (conditions of deformylation) gave a new product that comigrated with authentic methionine sulfoxide (Sigma). These results suggest that sulfur was oxidized during preparation of $CAACCA_{fMet}$. Nevertheless, the carboxylate ester linkage remained intact, and the same material was active in the puromycin reaction with large ribosomal subunits. Treatment of 5′-[^{32}P]-$CAACCA_{fMet}$ with CHES buffer (pH 9.0) converted the aminoacyl oligonucleotide to a product of greater electrophoretic mobility that comigrated with authentic 5′-[^{32}P]-CAACCA.
27. The absolute values for hydrolysis rates obtained using the gel assay were slightly lower than those obtained by TLC, but the relative rate enhancement by ribozyme was the same.
28. L-Arginine binds at the G site with its guanidino group interacting with G-264 in the ribozyme, the same nucleotide that interacts with the guanidino group of G (*4*). The α-amino group might lie in the portion of the site normally occupied by the 2′ or 3′ OH of the ribose on G. Thus, the α-amino group on arginine might attack the carbonyl carbon of the ester to give peptide bond formation. Arginine analogues tested were arginamide, homoarginine, agmatine, glycylarginine (Research Plus), and glycylglycylarginine (Sigma).
29. If transesterification with guanosine or its analogues were occurring, an aminoacylated nucleoside or dipeptide should appear as a new radioactive spot on TLC or paper electrophoresis.
30. C. A. Grosshans and T. R. Cech, *Biochemistry* **28**, 6888 (1989); D. W. Celander and T. R. Cech, *Science* **251**, 401 (1991).
31. J. A. Piccirilli, J. S. Vyle, M. H. Caruthers, T. R. Cech, in preparation.
32. W. D. Downs and T. R. Cech, *Biochemistry* **29**, 5605 (1990); J. W. Szostak, *Nature* **322**, 83 (1986).
33. The calculated free energy for the duplex $^{CAACCA}_{AGUUGGG}$ at 30°C was determined by $\Delta G° = \Delta H° - T\Delta S°$, with $\Delta H°$ and $\Delta S°$ values (S. M. Freier *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **83**, 9373 (1986)). The terminal A · G mismatch and the first unpaired A of the ribozyme were included in the calculation. Gel binding experiments were performed at 30°C and 15 mM $MgCl_2$ with 5′-[^{32}P]CAACCAAAAAA as described previously [A. M. Pyle, J. A. McSwiggen, T. R. Cech, *Proc. Natl. Acad. Sci. U.S.A.* **87**, 8187 (1990)].
34. A mutant RNA missing the entire sequence upstream of nucleotide 46 can promote specific hydrolysis at the 3′ splice site; B. Young, thesis, University of Colorado, Boulder (1990).
35. Assuming a two-step model $E + S \leftrightarrow E{\cdot}S \rightarrow E{\cdot}P$, $K_m = (k_{-1} + k_c)/k_1$ where k_{-1} is the rate constant for $E \cdot S \rightarrow E + S$, k_1 is the rate constant for $E + S \rightarrow E \cdot S$, and $K_d = k_{-1}/k_1$. If $k_c << k_{-1}$, then $K_m = K_d$. In the esterase reaction k_c = 0.02 min^{-1} is expected to be slow compared to k_{-1} (the rate constant for the dissociation of $CAACCA_{fMet}$ from the ribozyme · substrate complex). The rate constant for dissociation of the non-aminoacylated product CAACCA is 0.8 min^{-1}. For the RNA substrate CAACCAAAAA, k_c = 3.0 min^{-1}, K_m = 195 nM, and if k_{-1}(CAACCA) = k_{-1}(CAACCAAAAAA) = 0.8 min^{-1}, k_1 can be calculated from the relationship for K_m. This gives K_d = 45 nM. The assumption that $k_{-1}(P) = k_{-1}(S)$ is true for the reaction studied previously (*11*). K_d = 45 nM is consistent with preliminary data that give K_m = 30 nM for CAACCAAAAAA in the hydrolysis reaction where $k_c << k_{-1}$, and $K_m = K_d$.
36. The L-21 Sca I ribozyme is more than 90 percent saturated at the concentrations (2 mM) of 2′-deoxy-2′-amino-guanosine and 3′-deoxy-3′-amino-GMP in these experiments (D. Herschlag and T. R. Cech, unpublished results). This should also be true for the ribozyme used in our study because guanosine binding is not expected to be affected by alteration of the IGS.
37. W. P. Jencks, *Catalysis in Chemistry and Enzymology* (Dover, New York, ed. 2, 1987).
38. $k_c/k_u \cong 10^8$ for hydrolysis of CAACCUAAAAA or $\cong 10^7$ for hydrolysis of CAACCAAAAAA, compared to 5 to 15 for the hydrolysis of $CAACCA_{fMet}$; k_c represents the observed pseudo first-order rate constant for reaction of substrate bound to ribozyme with water, and k_u represents the pseudo first-order rate constant for reaction of free substrate with water (estimated for phosphate diesters as described) (*11*).
39. An L-18 ribozyme, containing a U at position 22 instead of G, converts the terminal G · A mismatch to an U · A base pair in the ribozyme substrate complex. However, both ribozymes catalyzed the deacylation of $CAACCA_{fMet}$ at the same rate. This does not provide the G · U combination that is important for the RNA cleavage reaction.
40. For reviews see: S. J. Benkovic and K. J. Schray, in *Transition States of Biochemical Processes*, R. D. Gandour Ed. (Plenum, New York, 1978), pp. 493–527; in *The Enzymes*, P. D. Boyer Ed. (Academic Press, New York, 1973), vol. 8, pp. 201–238.
41. J. A. McSwiggen and T. R. Cech, *Science* **244**, 679 (1989); J. Rajagopal, J. A. Doudna, J. W. Szostak, *ibid.*, p. 692.
42. P. Deslongchamps, P. Allani, D. Fre'hel, A. Malaval, *Can. J. Chem.* **50**, 3405 (1972); H. B. Buergi, J. D. Dunitz, E. Shefter, *J. Am. Chem. Soc.* **95**, 5605 (1973).
43. The *pro*-Sp oxygen atom; J. A. Piccirilli and T. R. Cech, in preparation.
44. P. G. Schultz, R. A. Lerner, S. J. Benkovic, *Chem. Eng. News*, 26 (28 May 1990); R. A. Lerner and S. J. Benkovic, *BioEssays* **9**, 107 (1988).
45. A. Weiner and N. Maizels, *Proc. Natl. Acad. Sci. U.S.A.* **84**, 7383 (1987).
46. H. F. Noller, V. Hoffarth, L. Zimniak, *Science* **256**, 1416 (1992).
47. M. Taiji, S. Yokoyama, T. Miyazawa, *Biochemistry* **24**, 5776 (1985); the transacylation rates of 2′-(3′)-*O*-(*N*-acetyl-L-phenylalanyl-L-phenylalanyl)-adenosine were obtained as $k(2' \rightarrow 3')$ = 0.41 min^{-1} and $k(3' \rightarrow 2')$ = 0.18 min^{-1} at pH 7.0, 0°C.
48. The activity was also independent of buffer concentration in the range of 10 to 50 mM.
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