CHAPTER 4

Ribozyme-Catalyzed Genetics

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Summary

RNA World research in recent years has sought to establish whether ribozymes have the catalytic versatility and potency to transmit genetic information and to sustain a credible metabolism. At a minimum, organisms from just before the Protein Revolution would have had to catalyze nucleotide polymerization and invent the machinery for protein synthesis. There are now RNA enzymes (ribozymes) that catalyze the individual steps in each of these reactions. Some of the current challenges include increasing the vigor with which the individual reactions are catalyzed, strengthening the affinity and specificity of substrate recognition, integrating ribozymes into metabolic paths and coordinated networks of linked reactions, and deriving a ribozyme-catalyzed metabolic context to sustain the core reactions.

RNA World theories of the earliest evolution of life enjoy increasing acceptance and experimental support. The simplest statement of the RNA World theory is that our evolutionary history includes at least one organism that depended on RNA molecules both as the primary repository for genetic information and as the principle set of catalysts for cellular functions. In modern organisms, these two roles are predominantly filled by DNA and proteins, respectively. RNA World organisms are variously referred to as "ribo-organisms" or "ribocytes." The first ribocyte to make use of genetically encoded translation is called the "breakthrough organism." Far removed both from life's origins and from recognizably modern biochemistry, the descendents of the breakthrough organism are thought to have accumulated a broad diversity of proteins enzymes that took over nearly all of the functions of the cell. The transition from ribocytes to modern forms may have left traces of the ancestral state in the form of nucleotide cofactors, ribosomes built largely from RNA, and the requirement for ribonucleotides as biosynthetic precursors for deoxyribonucleotides. Speculation on how this transition may have taken place, and the relevant experimental evidence, are discussed in other chapters of this volume.

This review evaluates the degree to which ribozymes identified to date are adequate to the task of sustaining genetic information flow. Emphasis is on the reactions that underlie transmission of genetic information. The first section defines the aspects of RNA World theories that are within the purview of this endeavor. The next two sections describe the evolution, activities, and experimental challenges of ribozymes that catalyze each of the discrete reactions of replication and proteins synthesis. The fourth section addresses progress towards generating an RNA-catalyzed metabolism to support the flow of genetic information, and the fifth section offers concluding remarks. Additional reviews in related areas have been published elsewhere.[1,5-9]

Two RNA World Views

While significant challenges remain, the notion that ribozyme catalysis can sustain genetic information flow has survived the initial wave of experimental examination. By extension, the notion of RNA-based life has similarly survived. RNA World theories tend to come in two

The Genetic Code and the Origin of Life, edited by Lluís Ribas de Pouplana.

flavors, emphasizing either the abiotic chemical processes that led to the first living things (origins per se) or the early evolution of cellular organisms. The two views place different requirements on the chemical capacities of nucleic acids —which can be revealed by experimentation— without being mutually exclusive. Each is critically defined by a key, hypothesized transition:

Chemical view: Abiotic nucleotide chemistry → RNA-catalyzed biochemistry.

Biochemical view: RNA-based life → Protein/DNA-based life

The fundamental quest of the chemical approach is to connect abiotic chemistry with the first living entities, and is sometimes dubbed the "RNA early" view. Chemical reaction pathways appropriate to an early, lifeless Earth (or Mars or Europa or other world) are sought that yield self-replicating molecules —usually in the form of RNA or RNA-like polymers— or their constituent parts. Work in this area includes the prebiotic synthesis of the components of RNA (nucleobases, D-ribose sugars, nucleotides, and polyphosphorylated nucleotide monomers), the chemical properties of non-standard nucleobases and nucleotide analogs, non-enzymatic copying of monomers into random or templated polymers, and their encapsulation into proto-cells. Abiotic routes leading directly to RNA have thus far proven elusive. Alternate scenarios involving other genetic and biochemical infrastructures have been proposed as contexts for the initial appearance of RNA, and are collectively dubbed the "Pre-RNA-World".[1-4]

The fundamental quest of the early biochemical evolution approach is to trace extant biology and biochemistry backwards to an earlier, presumably simpler life form without proteins or DNA. It makes no specific claims as to the source(s) of the RNA or the mechanism by which life originated, nor does it specify the complexity of the biochemical context within which RNA arose. Work in this area addresses the chemical and biological versatility of ribozymes, the adaptive landscapes and evolutionary pathways along which new functions are derived from existing functional RNAs, the assembly of multiple ribozymes into complex metabolic pathways in vitro, the influence of engineered RNAs on the physiology of modern or artificial cells, and the roles of natural, non-protein-coding RNAs in existing cells. These questions are biochemical and biological as much as they are chemical in scope, and they seek to define the nature of life based on an RNA-catalyzed metabolism.

The ultimate test of RNA World theories lies less in establishing how an RNA World could have come into existence, and more in demonstrating whether RNA-based organisms can stay alive. One must define the requirements of life and ask whether RNA molecules have the chemical properties needed to fulfill those requirements. For the protein-catalyzed biochemistry of modern organisms to have come into existence within an RNA-catalyzed biochemistry, the ribozymes of that ancestral state must have catalyzed the transmission of genetic information through the various reactions of replication and protein synthesis. Additional ribozymes may also have been required catalyzed some elements of biosynthesis and energy extraction. Ribozymes that accelerate many of the requisite classes of reactions several orders of magnitude above background rates have been identified through in vitro selections, laying the foundation for future work to establish the validity of RNA-based life.

RNA-Catalyzed Genetics I: Nucleotide Polymerization

The Chemical Basics of Polymerization

The essence of replication is the sequential, templated addition of nucleotides onto a growing chain. A phosphate ester is formed at the expense of a phosphoanhydride when the terminal hydroxyl of the growing chain attacks the alpha phosphate of an incoming mononucleotide triphosphate to release pyrophosphate (Fig. 1). The large body of work that has gone into understanding natural ribozymes[8] creates a framework within which to understand polymerase ribozymes. Most natural ribozymes catalyze nucleophilic reactions at phosphate centers to yield the ligation of two strands, phosphoester hydrolysis, or phosphoester exchange. Three powerful catalytic strategies used by natural ribozymes include specific positioning of substrates,

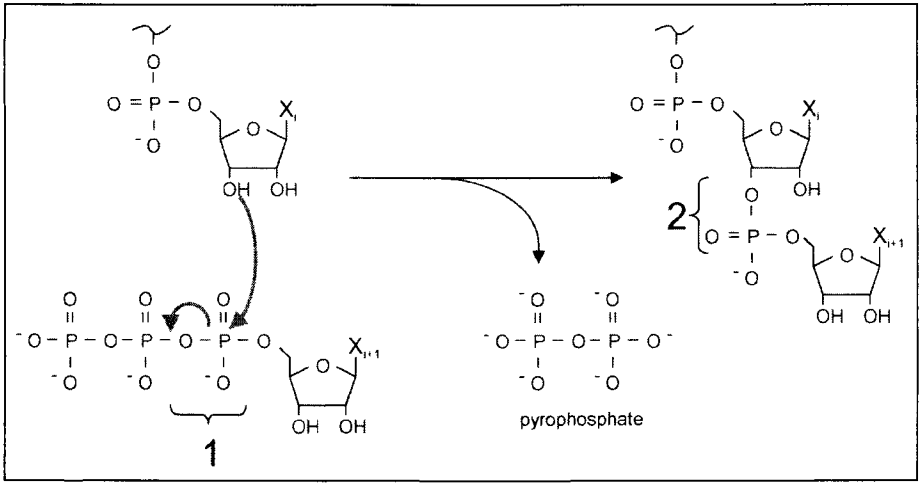

Figure 1. During nucleotide polymerization, a phosphoanhydride bond (bracket 1) is consumed in order to produce a phosphate ester (bracket 2). Electron movement in this and subsequent figures is indicated by gray arrows. Oxyanion transition state intermediates are not shown.

incorporation of metal ions into active sites, and general acid-base catalysis. The next few paragraphs outline these mechanisms and how they shape ribozyme evolution. Later sections address how these and other chemical mechanisms influence the acyltransfer reactions of protein synthesis.

Substrate Binding

The molecular details of binding interactions govern the distances and orientations among substrates and active site residues. These, in turn, dictate much of an enzyme's specificity and its ability to accelerate a chemical reaction. Thus, it will be extremely important to determine how substrate recognition contributes to catalysis in polymerases and other newly isolated ribozymes. A population of substrate-binding RNAs may even be more richly endowed with catalytic species than would be found in a random pool,[12] although such "preadaptation" has not been explored in detail. A generic polymerase must bind both a primer-template junction and activated mononucleotides. The challenges of binding to the primer-template junction represent some of the strongest limitations to existing polymerase ribozymes. Generic features of the helix, such as overall shape and hydrogen bonding to ribose hydroxyls are more important in polymerase ribozymes than are specific structural elements that stabilize large natural RNAs, such as tetraloop-receptor interactions[10] and adenosine platforms.[11] In contrast to the challenges of generic recognition of helices, nucleotide triphosphates offer many potential interaction surfaces through aromatic stacking, hydrogen bonding to sugars and bases, and metal-mediated charge-charge interactions through the phosphates. While nucleic acid aptamers that recognize nucleotides with a wide range of specificities and affinities have been identified and characterized at atomic resolution, there is little known about how polymerase ribozymes bind their respective NTPs.

Coordinated Metal Ions

Specifically bound divalent metal ions play important structural and catalytic roles in several ribozymes through inner- or outer-sphere coordination to water or to phosphate oxygens or to ribose hydroxyls.[13-15] Indeed, specific metal-binding sites have been identified in active sites for several ribozymes through crystallographic analysis, Mn(II) rescue of sulfur-substituted substituents, and other approaches. In addition to general acid/base catalysis, bound metals

can accelerate reactions by orienting substrates for attack, stabilizing the developing negative charges on the transition states and leaving groups, and providing strain by distorting a ground state structure more towards that of the transition state.[16-18] Although it was believed for some time that the natural ribozymes all required divalent metal ions for their activity, two groups have recently shown that high concentrations of monovalent ions are sufficient for near maximal activity in the hammerhead, hairpin, and VS ribozymes.[19,20] Thus, many unanswered questions remain as to how the natural ribozymes catalyze reactions at phosphates. Less still is known about metal ion utilization by the many ribozymes recently derived from in vitro selections. Elucidating their mechanisms will keep RNA biochemists busy for years to come.

Acid-Base Catalysis

Reactions at ribose-phosphate bonds require protonation and deprotonation events, and can thus benefit from acid-base catalysis. Ribose hydroxyls are poor nucleophiles unless they are deprotonated to the oxyanion. The relevant leaving groups (generally another ribose hydroxyl) are unstable until they acquire a proton. The cleavage rates for several ribozymes vary in a log-linear fashion with pH, implying a single deprotonation event at the rate-limiting step. For many years, the substituents responsible for proton transfers were thought to be metal hydrates, and the evidence is strong that this is the case for at lease some ribozymes. A metal-bound water is more acidic than free water (e.g., pKa = 11.42 for $Mg(H_2O)_6$ vs. 15.7 for H_2O), allowing proton exchange to occur more readily near neutral pH. For the hammerhead ribozyme, this relationship is retained for a variety of metal ions, with the net reaction rate shifted according to the pKa of the hydrated metal.[21] In recent years nucleotide bases have been recognized to have a role in proton transfer, even though their pKa's in solution are far from neutrality. It has long been recognized from NMR studies that specific structural contexts can markedly shift the pKa's of nucleobases.[22,23] In the hepatitis delta virus (HDV) ribozyme, the protonated N3 of an active site cytosine (C75) is in position to donate a proton as the general acid in that ribozyme's self-cleavage reaction, and a ribozyme-bound hydrated metal hydroxide is in position to abstract a proton in the basic function.[24,25] Specifically, transfer of a proton from C75 to the ribose 5' hydroxyl stabilizes the leaving group. The pKa of the cytosine N3 is normally near 4.2, but the pKa of C75 is perturbed to neutrality within the structural context of the ribozyme, making it "histidine-like".[25] Thus, ribozymes can access at least two catalytic strategies to effect proton transfers.

Evolution of a Ligase Ribozyme into a Polymerase

Polymerase ribozymes have not yet been isolated directly from a pool of random sequences, but remarkable progress has been made through step-wise evolution from ligase ribozymes isolated in the Bartel lab. The original ligases, comprising a diverse collection isolated from random sequence pools, condense a small oligoribonucleotide onto their 5' ends through attack by the 2' or 3' hydroxyl of the oligo on the 5' terminal α-phosphate of the ribozyme (Fig. 2A). Random mutagenesis and further cycles of selection of the "Class I ligase" produced ribozymes with ligation rates around 1 min^{-1}.[26,27] The oligo binds to the ligase ribozyme through base pairing to an internal guide sequence and shows optimized, templated ligation rates as high as 100 sec^{-1}.[27]

Remarkably, the ligase ribozymes also catalyzed limited template-directed primer extension (Fig. 2B). In this reaction, the primer/IGS helix is provided in trans as a primer-template junction, held in place through base-pairing to an adjacent site in the ribozyme.[28] The reaction is exactly analogous to that of the original ligation and almost certainly uses the same active site, with nucleotide triphosphates serving the same role as the 5' terminal triphosphate of the original ribozyme. The rate of polymerization is not significantly diminished compared to ligation by the non-optimal, parental ribozyme (k_{cat} = 0.3 min^{-1} for addition of GTP), although the affinity for NTP substrates is low (K_m^{GTP} = 5 mM). The ribozyme shows 85-95% fidelity when presented with competing nucleotides, depending on NTP concentration.[28] This is comparable to the fidelity of Pol η, an error-prone DNA polymerase associated with repair of

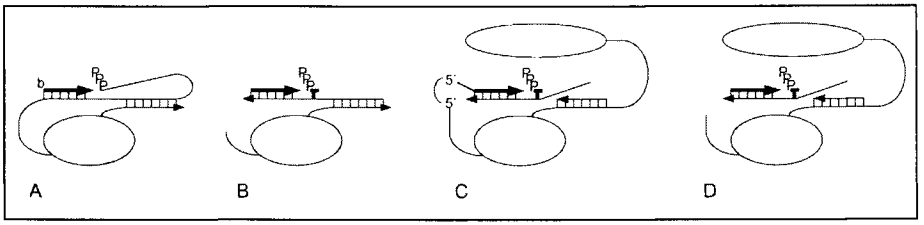

Figure 2. Strategy used in selecting a polymerase ribozyme. In each figure, the primer that is extended by the ribozyme is shown by a thick line, and the 5' triphosphate or NTP that is attacked by the primer is indicated.

DNA damage in humans.[29] The primary limitation of the ligase ribozyme as a polymerase lies in its interaction with the primer-template, which must still base pair with the enzyme, though it must be emphasized that this polymerase is really just a ligase that has been forced into an expanded role.

Johnston et al made two fundamental changes to the ligase that made possible the direct selection of a generic polymerase. First, they appended 76 random nucleotides to the 3' end of the ribozyme and inserted 8 random nucleotides at each of two locations within the ribozyme. Second, they fused the 5' end of the primer oligonucleotide to the 5' end of the ribozyme in a 5'-5' junction, so that any templated primer extension would covalently modify the ribozyme (Fig. 2C). The NTPs used in this step included affinity tags that allowed specific capture of extended species. Furthermore, the sequence of the primer was changed during each cycle of the selection to avoid a requirement for sequence-specific interactions. Finally, the original internal guide sequence was sequestered through addition of an exogenous oligonucleotide, forming a stem that had previously been shown to be required for ribozyme activity. The resulting polymerase ribozymes no longer required base pairing interactions of any kind to hold the primer-template junction onto the ribozyme (Fig. 2D), which extends the primer by more than one full helical turn with approximately 99% fidelity (12 errors observed in 100 isolates, each carrying 11 newly synthesized nucleotides).[30] Observed rates of extension for individual addition steps range from 0.004 hr^{-1} to 6 hr^{-1}, reflecting fast chemistry (on the order of 2 min^{-1} for k_{cat}) and low affinity for both NTPs and primer-template substrates (M Lawrence and DP Bartel, personal communication). Overall processivity is limited because the polymerization rate is on the order of the same as the rate at which the primer/template duplex dissociates from the ribozyme, since processivity is given by the ratio of k_{pol} to k_{off}. Future versions of this ribozymes with improved affinities for NTPs (lower K_m^{NTP}) and for the primer-template duplex (lower K_m^{duplex}) may therefore show greater processivity.

Significance of Polymerase Ribozymes with These Kinetic and Fidelity Parameters

Considerable effort, intelligence, and design have led to a halting polymerase ribozyme. Sequence requirements were carefully evaluated such that extraneous nucleotides could be pruned and new random tracts could be introduced at sites where they were most likely to be beneficial. It is a staggering accomplishment, yet it does not seem reasonable to view these ribozymes as evidence that self-replicating species could have resulted from random, non-enzymatic polymerization of prebiotic nucleotides. There is a considerable functional gap between the current polymerase ribozymes and a useful replicase.[4,31] It currently requires about 24 hours to synthesize one turn of a helix. Three weeks would be required for the nearly 200-nucleotide ribozyme to synthesize its own complement, and another three weeks to copy the complement back into the active plus strand. Still more time would be required for production of ribozymes for other functions and RNAs that serve as structural components of the cell, notwithstanding the time wasted synthesizing inactive RNAs carrying fatal errors (approximately one misincorporation

per hundred nucleotides). Hydrolysis of the RNA backbone could easily destroy the ribozyme or its incipient products faster than new ribozymes can accumulate, removing the opportunity for exponential growth and Darwinian selection. Low affinity for the primer-template duplex and for NTPs currently limits reaction conditions to unrealistically high concentrations of each. A useful replicase will require greater speed, processivity, and fidelity. Enhanced substrate affinity could allow reactivity at reasonable substrate concentrations. An active proofreading mechanism could enable the net fidelity to exceed the energetic limits afforded by Watson-Crick base pairing (approximately 99% accuracy). A useful replicase would also require a means of separating the product strands to serve other active roles within the ribocyte or to participate in further replication cycles. Engineering these or other polymerase ribozymes into credible replicases would go a long way towards solidifying theoretical models of RNA-based organisms, irrespective of the assumed providence of such cells. Efforts are no doubt underway to arrive at such improved ribozymes.

Bountiful Ligase Ribozymes As Evolutionary Fodder

Polymerization in some form might be able to evolve from other ligases along evolutionary paths similar to those outlined above. If so, then there could be many different evolutionary starting points from which a replicase could arise. The naturally occurring Group I and Group II self-splicing ribozymes use phosphoester exchange to ligate RNA strands, and were among to first to show limited polymerization activity (see discussion in ref. 31). The Group I intron from the *sunY* gene of bacteriophage T4 has been engineered to carry out template-directed polymerization of sorts. The "monomers" added during each step were trinucleotide fragments, and the leaving group for the reaction was a 5' guanosine residue, for a net reaction of $G\text{-}X_n$ + $G\text{-}Y_n \rightarrow G\text{-}X_nY_n$ + G-OH, with n=10 [32] or n=3.[33]

Even the small, classic, endonucleolytic ribozymes such as the hammerhead, hairpin, VS and hepatitis delta ribozymes, catalyze both the forward cleavage reaction and the reverse ligation reaction, using a 2',3' cyclic phosphate to activate the reaction. Freezing out large-scale RNA motions —either through compact tertiary structure or through the formation of engineered crosslinks— is thought to determine where the ligation/cleavage equilibrium lies. The ligation reaction for the hairpin ribozyme is favored 6-30 fold over the cleavage reaction,[34-36] and ligation by the HDV and VS ribozymes is also notable.[37-40] Introduction of a disulfide crosslink into the hammerhead ribozyme was recently shown to accelerate the rate of ligation without altering the cleavage rate, thereby shifting the equilibrium to favor ligation over cleavage [41]. It might be possible to re-engineer the abundant new small RNA-cleaving ribozymes into ligases by similarly freezing out their large-scale motions.

In vitro selections have yielded an abundance of bona fide ligase ribozymes. Some of these generate 2'-5' linkages (rather than 3'-5'), and nearly all bind the primer strand through an internal guide sequence rather than binding exogenous primer-template duplexes. Nevertheless, the diversity of sequences that catalyze RNA strand ligations suggest bountiful starting points for their evolutionary conversion into polymerases. Some in vitro selected ligase ribozymes are described below, and their ligation rates are summarized in Table 1.

Derivatives of the Canonical Class I Ligase

The Class I ligase that gave rise to the polymerase described above has also spawned other evolutionary derivatives. Wright and Joyce used it to develop a continuous in vitro evolution system, in which a "culture" of replicating ribozymes (aided by a few protein enzymes) can be propagated indefinitely while introducing new mutations.[42] Rogers and Joyce derived a Class I ligase variant that is devoid of any cytosine residues, forming all of its structure using A, G, and U.[43] The C-less ribozyme is 2500-fold slower than the parent from which it was derived, with k_{cat} dropping from 20 min^{-1} in the parental version to 0.008 min^{-1} in the C-less version, but these results showed that complex, functional species can arise even with a reduced alphabet of nucleotides. The activity of the original Class I ligase is sharply reduced at low pH. Miyamoto et al. optimized the low-pH activity through in vitro evolution. Their Class I variant is 250-fold

Table 1. Ligation rates of in vitro-selected ligase ribozymes

Ribozyme	k_{cat}, min^{-1}	Rate Acceleration
original Class I ligase	1	10^7
optimized Class I	100	10^9
C-less Class I	0.008	8×10^4
Class I at pH 4.0	0.0000005	[a]
optimized for reaction at pH 4.0	0.0001	[a]
Class I cogeners	1	10^7
"Spartan Spandrel"	0.0006	6×10^3
TyrS-activated ligase		
- TyrS	0.0000003	3
+ TyrS	0.035	3.5×10^5
Lysozyme-activated ligase		
- Lysozyme	0.000003	30
+ Lysozyme	0.01	10^5
Group I intron core	0.00003	3×10^3
Optimized with 85 nt insertion	0.26	2.6×10^6
Ligase with "lysine-A" analogs	0.000025	2.5×10^2
Ligation from 5'-linked AMP- or 5'-phosphorimidazolide-activated oligonucleotides	0.007	[b] 7×10^4
Uncatalyzed templated ligation	$\sim 10^{-7}$	1

[a] Background reaction at pH 4.0 not determined. [b] Background reaction assumed to be same as with triphosphate activating group.

more active at pH 4.0 than the parental sequence at that pH, but the derivative did not actually prefer acidic buffers, retaining a positive correlation between activity and pH.[44]

Congeners of the Class I Ligase

The selection that yielded the Class I ligase also yielded several other structural classes.[26] Some of these have been shown to catalyze ligations at respectable rates (approx 1 min^{-1}),[26,27] though most have not yet been subject to optimization of their ligase function.

Dual-Function Ligase/Nuclease Ribozymes: The "Spartan Spandrel"

Landweber and Pokrovskaya found a ligase ribozyme that, in the presence of Mn(II), also catalyzes self-cleavage at a site that is distinct from the ligation site. This unintended dual function is referred to by those authors as a "spandrel" to emphasize how evolution for one function can simultaneously preadapt a species for a second function. While the ligation rate is slow (0.0006 min^{-1}), its structure is among the most Spartan of the selected ribozymes, requiring only a few specific nucleotides between paired helices at the ligation junction.[45] The simplicity of the "Spartan Spandrel" suggests that sequence space may be riddled with low-activity ligase ribozymes.

Allosteric Ligases

Robertson and Ellington identified a ligase ribozyme, dubbed "L1," that is activated 1,000 to 10,000-fold by addition of an exogenous oligonucleotide (separate from the ligation substrate).[46] By mutagenizing the catalytic core and applying further cycles of selection, they identified variants of the L1 ligase that were activated nearly 10^5-fold by a tyrosyl transfer RNA (tRNA) synthetase (TyrS) encoded by the Cyt18 gene.[46] Allosteric ligases are being developed in several labs for potential biotechnology applications (e.g., sensors).

Modular Assembly of Ligase Ribozymes

Another set of structurally complex ligase ribozymes was identified by inserting 85 random nucleotides into a group I self-splicing intron core of 225 nucleotides and selecting variants that condensed an oligonucleotide onto its end at the expense of the 5' triphosphate.[48] The intron core alone catalyzed the desired ligation with a rate of 3×10^{-5} min^{-1} (300-fold above uncatalyzed rate), while after selection this rate was improved to 0.26 min^{-1}.

Ribozymes Containing Lysine Analogs

Nucleotide analogs that carry added chemical functionality offer a possible route by which to increase the catalytic activity of ribozymes in general. With this is mind, nucleotides carrying the side chains of histidine (imidazole), lysine (n-alkylamine), and other substituents (pyridine) have been used in selections for various ribozymes. In the ligase arena, Teramoto et al. identified ribozymes containing alkylamino side chains at the N6 position of adenosines to mimic the positively charged side chain of lysine.[47] However, the modification did not appear to confer any advantage to the population, as only a 250-fold rate enhancement was observed over the uncatalyzed reaction rate of approximately 10^{-7} min^{-1}.

Ligation Using Other Chemistries

Polyphosphorylated nucleotides are hypothesized to have been used by ribocytes prior to protein-catalyzed chemistry.[49] However, because polyphosphorylation of the 5' hydroxyl is challenging for prebiotic chemistries, alternative leaving groups have been explored, such as cyclic phosphates, imidazolides, and adenylates[3,50,51] (Fig. 3). Ligase ribozymes using each of these reagents have been isolated. The Szostak group described a ligase in which the 3' OH of the RNA condenses with a donor oligonucleotide activated with 5'-linked AMP[52] (the same adenylate intermediate generated by phage T4 DNA ligase). The same group later found a ribozyme that formed 5'-5' tetraphosphate, triphosphate and pyrophosphate linkages from 5'-phosphorimidazolide-activated oligonucleotides.[53] Neither reaction is rapid —maximal observed ligation rate = 0.4 hr^{-1}, or about 0.007 min^{-1}— but the two activities suggest that one ribozyme could synthesize the substrate needed by another ribozyme, constituting a coupled, ribozyme-catalyzed, two-step reaction mechanism. Along similar lines, the Breaker group has described deoxyribozymes that cap DNA with 5' adenylates, as well as other deoxyribozymes that catalyze templated ligation.[54,55] DNAzymes could therefore catalyze similar multistep reactions.

RNA-Catalyzed Genetics II: Protein Synthesis

The joining of two amino acids by a ribozyme launched the Protein Revolution and began the end of the RNA World. Protein synthesis is a progression of aminoacyl transfer reactions, wherein an amino acid (the acyl group) is passed from one acyl donor to the next (Fig. 4). In each step a nucleophile attacks a phosphate or carbonyl center with displacement of progressively less reactive leaving groups. In the first step of the cascade, the carboxylate of the amino acid joins to the α-phosphate of ATP, displacing pyrophosphate and forming a high-energy, mixed phosphoanhydride between the amino acid and AMP. In the second step, the amino acid is transferred to the 2' or 3' ribose hydroxyl of tRNA to make the less reactive ester, and displacing AMP. Both of these reactions are catalyzed by aminoacyl tRNA synthetase proteins (ARS), which are discussed in detail in other chapters of this volume. On the ribosome, the acyl group (initiator amino acid or growing peptide chain) is transferred to the alpha amino of another amino acid, forming a new peptide bond at the expense of an ester bond with concomitant displacement of tRNA. Ribozymes that catalyze each of the individual reactions of this cascade have been isolated. This section first discusses the underlying chemistry of acyltransfers, then describes the acyltransfer ribozymes that have been isolated in vitro for the three individual steps.

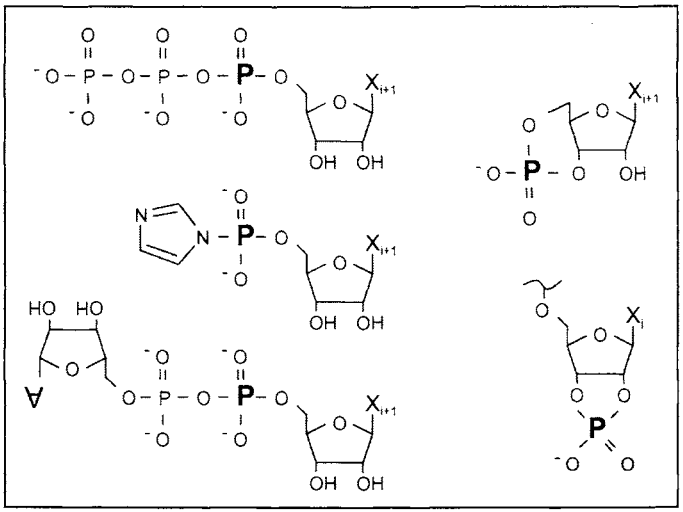

Figure 3. Alternative activated nucleotide monomers that can be used in polymerization and ligation reactions. From top to bottom, left side: triphosphate, phosphorimidazolide, adenylate; right side, 3',5'-cyclic phosphate, 2',3'-cyclic phosphate. The 2',3'-cyclic phosphate is produced upon self-cleavage by several small, natural ribozymes. Reversal of the cleavage reaction re-ligates the two termini.

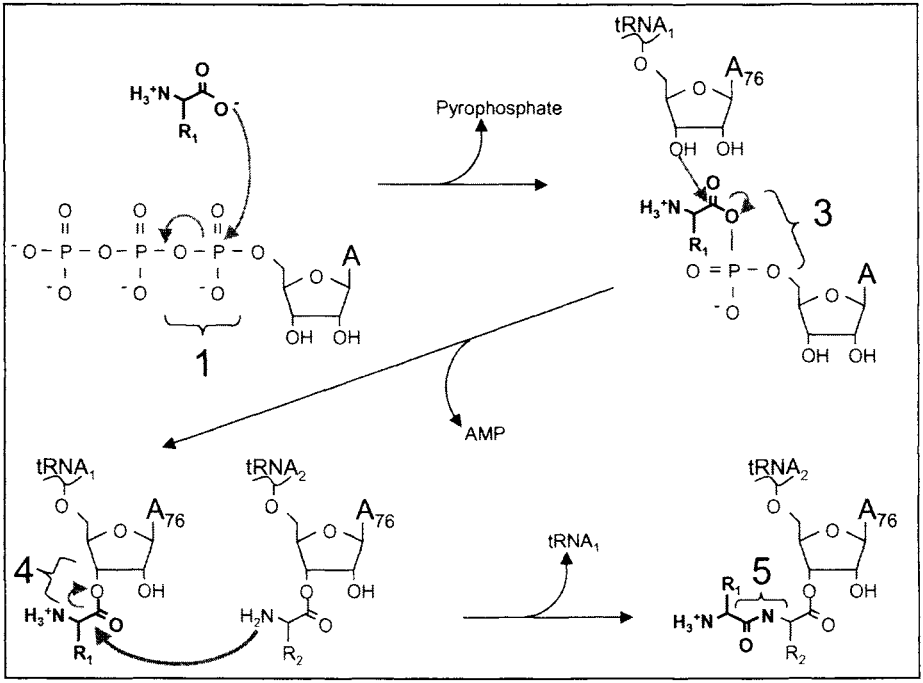

Figure 4. Stepwise acyl transfer in peptide synthesis. The initial activation step converts a phosphoanhydride (indicated by bracket 1) into a mixed carboxy-phosphoanhydride (bracket 3). Reaction with tRNA produces a ribose ester (bracket 4), then condensation with another amino acid produces the peptide product (bracket 5). A_{76} indicates the 3' terminal nucleotide of aminoacyl-tRNA.

Table 2. pKa's and relative activation of several leaving groups relevant to the reactions of transmitting genetic information

Conjugate Acid of Leaving Group	pKa	Activation
RNH_2	≈35	no
ROH	15-20	weak
H-OH	15.7	weak
ribose hydroxyls	12.35	moderate
$HP_2O_7^{-2}$	12.32	moderate
$HOCH_2CN$	11.0	good
RSH	10.6	good
RNH_3^+	≈10	good
$H_2P_2O_7^{-1}$	7.09	very good
H-AMP	6.2	very good
$H_3P_2O_7$	2.15	very good

The Chemistry and Enzymology of the Reactions of Protein Synthesis

The underlying chemistry of natural protein synthesis is dominated by leaving group reactivity, nucleophilicity, electrophilicity, acid-base chemistry, proximity effects, and charge stabilization. Although the mechanistic enzymology of aminoacyltransfer ribozymes is in its infancy, the known mechanisms of protein acyltransferase and those of phosphoesters-manipulating ribozymes let us postulate several catalytic strategies that ribozymes could use to accelerate acyltransfers.

Leaving Group Reactivity

To say that an acyl donor is highly reactive usually means that it carries a good leaving group. Leaving groups whose conjugate acids have low pKa's are generally more reactive than those with high pKa's (Table 2). This trend is part of what makes amides and peptides so stable against hydrolysis. In the absence of any additional reaction, hydrolysis of an amide or peptide bond would displace RNH^- as a leaving group. The RNH^- species has a conjugate acid, RNH_2, whose pKa is very high (e.g., ≈36 for isopropylamine), making the RNH^- a lousy leaving group. Proteases often protonate the nitrogen to facilitate peptide bond hydrolysis (Fig. 5). The resulting RNH_2 is a good leaving group because its conjugate acid, RNH_3^+, has a relatively low pKa (around 10). The significance of using one activating group vs. another takes at least three forms: the need to protect against water hydrolysis (an acyl group that is more reactive with the desired substrate is also more sensitive to spontaneous hydrolysis), the existence of any requirement to protonate the leaving group (which would increase the functional demands on the active site residues), and the geometry of the active site (vs. steric constraints and charge distribution of leaving group; e.g., AMP vs. inorganic phosphate leaving groups).

Four classes of leaving groups are especially important for discussions of RNA-catalyzed protein synthesis. ***Phosphoanhydrides***, such as adenylates, are the most reactive species used during normal protein synthesis. Spontaneous hydrolysis is especially pernicious for the aminoacyl adenylate ($t_{1/2}$ ≈ 10 min at 0°C, pH 7.0; ≈1-3 min at 37°C[56,57]). To prevent their premature hydrolysis or reaction with unintended nucleophiles, ARS enzymes keep the adenylate hidden in the active site until they react with the 2' or 3' hydroxyl of the cognate tRNA. ***Thioesters***, such as acyl coenzyme A (acyl CoA), are also highly activated, as the pKa values of sulfhydryls are near 9-10. The polarizability of the RS^- thiide ion further stabilizes it as a leaving group. Thioesters of coenzymeA and the related pantotheine are used in a plethora of acyltransfers, including non-ribosomal peptide synthesis. ***Sugar esters***, such as aminoacylated

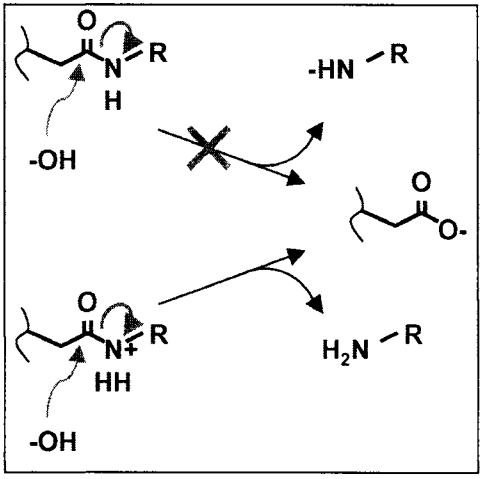

Figure 5. Effect of protonation on leaving group pKa and acyl group reactivity. Direct amide hydrolysis (top) is disfavored because the RNH^- leaving group is unstable; hydrolysis of a protonated amide (bottom) occurs much more readily with the RNH_2 leaving group.

tRNA, are considerably less reactive than phosphoanhydrides or thioesters, as the pKa's of ribose hydroxyls are 12.35.[58] Even so, the amino acid-tRNA ester bond is labile, hydrolyzing rapidly at 37°C when not shielded from solvent by elongation factor EF-Tu ($t_{1/2}$ for hydrolysis ≈20 min at 37°C, pH 7.5[59]). ***Cyanomethyl esters*** show intermediate reactivity (pKa of the conjugate acid, $HOCH_2CN$ = 11). They are more reactive than sugar esters, but less susceptible to hydrolysis than adenylates. While not employed in biological reactions, cyanomethyl esters of amino acids have figured prominently in some ribozyme-catalyzed aminoacylation studies.

Nucleophilicity of the attacking nucleophile also governs reaction kinetics. As above, the pKa of the conjugate acid of a given species is an important determinant of reactivity, since electron-rich atoms are generally better nucleophiles that electron-poor atoms, but the correlation with pKa is not precise. More accurately, good nucleophiles are good Lewis bases (electron donors). Thus nucleophilicity increases with anionic charge (RO^- > ROH) and according to position in the periodic table (RS^- > RO^-; RNH_2 > ROH). The steric environment of the attacking nucleophile is another important determinant of reactivity, as a crowded environment limits the productive angles of approach during reactive center collisions.

General acid-base catalysis serves to activate the nucleophile (deprotonation) and stabilize the leaving group (protonation). As noted above, hydrated metals and nucleobases with shifted pKa's fulfill this role in some ribozymes. For recently selected acyltransferase ribozymes, there are few mechanistic data available that would suggest specific moieties as general acids or bases.

Electrophilicity of the Carbonyl Carbon

The electronegative oxygen withdraws electrons from the carbonyl carbon atom through the double bond, creating a partial positive charge on the carbon. Hydrogen bonding to the oxygen or association with a positive charge further polarizes the C=O double bond, increasing the partial charge on the carbon and making it more reactive with electron-rich nucleophiles. RNA molecules bristle with hydrogen bond donors and acceptors, and they are adept at positioning metal ions and cofactors in specific spatial arrangements. Acyltransferase ribozymes are thus expected to incorporate electrophillic enhancement into their catalytic strategies.

Charge Stabilization

Negative charge accumulates on the carbonyl oxygen in the acyltransfer tetrahedral transition state and on the leaving group as the transition state resolves into products. Depending on the reaction and on the timing of protonation and deprotonation steps, negative charges may also occur on either the attacking or leaving groups at different times in the course of the

reaction. Neutralizing these charges could prevent unfavorable burying of charged species while helping to position them within the active site. Oxyanions are integral to the mechanisms of the naturally occurring ribozymes that act on phosphate centers, which, as noted above, are generally believed to be stabilized by specifically bound divalent cations. However, as previously mentioned, several of the natural ribozymes are now known to operate in the absence of divalent metals if provided instead with high concentrations of monovalents,[19,20] suggesting that monovalent ions may offer a functionally equivalent route to oxyanion stabilization. Monovalent-stimulated self-cleavage by the hammerhead ribozyme is log-linear with pH —a property also seen with the divalent-dependent reaction— and with the ionic radius of the cation.[20]

Proximity Effects

If both the nucleophile and acyl donor are sufficiently reactive, an increase in their respective local concentrations can yield significant rate accelerations. For an evolving population of ribozymes, juxtaposition of substrates could be attained, in principle, through recombination among substrate binding pockets.[12] This is particularly true for the highly reactive adenylates (see below). However, a higher local concentration is not always enough for acyltransfers. For example, in both chloramphenicol acetyltransferase (CAT)[60-62] and dihydrolipoyl acetyltransferase (E2p),[63] the attacking hydroxyl nucleophile is normally deprotonated by a conserved His residue. The oxyanion of the transition state in CAT is then stabilized by a hydrogen bond from a conserved Ser while the protonated His is stabilized by a conserved Asp. Mutations in the His-Asp-Ser catalytic triad reduce or eliminate acetyltransfer activity[62,63] even though Km for the substrates is unaffected. Thus, binding and juxtaposition alone may not be enough for reactivity in these enzymes. Establishing the relationships between substrate binding by RNA and the catalytic potential of ribozymes promises to stimulate mechanistic and evolutionary studies of macromolecular catalysis.

Applying These Principles to the Ribosomal Peptide Bond Formation

Proximity effects and acid-base catalysis have both been proposed to operate in ribosome-catalyzed peptide bond formation. In the 2.4 Å structure of the *Haloarcula marismortui* large ribosomal subunit, the peptidyl transferase active site is composed entirely of RNA. The nearest protein components are too far away (≈16 Å) to contribute meaningfully to catalysis.[64] The N3 of a universally conserved adenosine residue in the active site (A2451) is within hydrogen bonding distance of the alpha amino group on the A-site-bound tRNA. From chemical modification by dimethyl sulfate (DMS), the pKa of this adenosine in the E. coli ribosome appears to be shifted from the normal 3.8 (< 1 for the N3 position!) to around 7.6, possibly due to a charge relay to neutralize a buried phosphate.[65] These observations prompted models in which this adenosine acts as a general base to deprotonate the attacking amino group. However, no pKa shift is observed at this position in the ribosomes of three other bacterial species, and mutant ribosomes with base substitutions at the conserved adenosine are still functional.[66,67] These authors of these last two studies postulate that pH-dependent structural rearrangements may have accounted for the previous observations,[66] and that the primary function of the peptidyl transferase active site in ribosomes is simply to juxtapose the reactants.[67] Indeed, poly(U) can direct peptide bond formation when amino acids are supplied as 2'(3') adenosyl esters.[68] Furthermore, there is an A•C base pair immediately behind A2451 (A2450•C2063) in the high-resolution crystal structure of the *Haloarcula* large ribosomal subunit. A•C pairs are only stable when the N1 of adenosine is protonated, and the pKa of N1 in such pairs is often shifted to near neutrality. Deprotonation of the A2450•C2063 pair above neutral pH could act as a structural switch that increases the reactivity of A2451 to DMS.[69,70] The exact mechanism by which the ribosome accelerates peptide bond formation —and the precise contribution of 23S rRNA— remain controversial.

Ribozyme Catalysis of Step 1, Amino Acid Activation

Kumar and Yarus isolated ribozymes that mimic the first step of aminoacylation by forming a mixed carboxylate-phosphate anhydride at the expense of a nucleotide triphosphate. A surrogate for cysteine, 3-mercaptopropionic acid (3MPA), was used in place of an amino acid. During the selection, RNA transcripts were incubated with 3MPA. Any RNAs that joined the 3MPA to their own 5' end were recovered through their ability to form a disulfide between thiopropyl sepharose and the unique sulfur introduced by the 3MPA. Since these transcripts carry GTP at their 5' ends, formation of the mixed anhydride with the terminal α-phosphate was expected to produce a guanidylate (the formal chemical equivalent of an adenylate), with concomitant displacement of pyrophosphate. The product was confirmed by showing that when radiolabeled transcripts were incubated with various amino acids, digested to mononucleotides and separated by HPLC, the radiolabel comigrated with genuine, chemically synthesized aminoacyl guanidylate. The reaction requires Ca(II), and at pH 4.0 it proceeds at a rate of 1.1 min^{-1}, with Km =48 mM for 3MPA. (This Km value is close to the 50 mM concentration used during the selection.) The alpha amino group of an amino acid makes its aminoacyl anhydride much more labile to hydrolysis than the mixed anhydride formed from simple organic acids (such as 3MPA). As a result, the product decays rapidly ($t_{1/2}$ = minutes for aa-adenylates vs. hours for organic acid adenylates at 0°C, pH 7). Using 3MPA in place of a normal amino acid was thus a necessary precondition for success of this selection, as it greatly increased product lifetime.

This RNA catalyzes the formal equivalent of the first step of protein synthesis by loading amino acids onto a mixed phosphoanhydride. The ultimate goal of such a ribozyme is to initiate a series of reactions that result in aminoacylation of a tRNA-like species. Because of the rapid hydrolysis without stable aminoacylation, this ribozyme, for now, only provides a complex means by which to convert a 5' triphosphate into a 5' monophosphate. It is anticipated, however, that an engineered or evolved descendent of the Kumar and Yarus ribozyme will present the mixed anhydride quickly to another substrate (e.g., its own or another RNA's 3' hydroxyl) to complete the reaction, and that they may show greater substrate affinity and specificity.

The ribozymes of Kumar and Yarus are the latest in a series selected to condense various substrates onto the alpha phosphate of their 5' terminal NTP, with concomitant release of pyrophosphate. The "Iso6" ribozyme first isolated by Huang and Yarus[71] catalyzes attack by a variety of phosphorylated compounds[72] to yield transcripts capped by nucleotide cofactors, by the normal eukaryotic mRNA cap (GpppG), by expanded and contracted caps (GppppG and GppG), and by other phosphorylated organic compounds.[71-73] Iso6 also possesses decapping and pyrophosphatase activities, albeit at rates that are >1000-times slower than the condensation reaction.[74] Iso6 derivatives were the first ribozymes to demonstrate multiple-turnover kinetics between two exogenous, small-molecule substrates. When one, two, or all three 5' terminal nucleotides were omitted from the transcript and instead supplied as exogenous substrates (pppG, pppGpG, and pppGpGpG), each truncated ribozyme catalyzed the formation of (5'—>5') polyphosphate-linked oligonucleotides in trans.[75] Substrate nucleotides are bound with Km values around 10-30 μM, while the pyrophosphate product is a powerful inhibitor of the reaction with Ki around 0.2 μM. Like the amino acid activating ribozymes above, Iso6 is a Ca(II)-dependent metalloribozyme that prefers acidic pH. While Iso6 and the aminoacylguanylate-forming ribozymes share little if any sequence identity, it will be interesting to determine whether they use similar structural frameworks or catalytic mechanisms.

Ribozyme Catalysis of Step 2, Making an Ester from an Activated Amino Acid

In vitro selections can employ either the natural adenylate or some other activation strategy to aminoacylate ribose. Pre-activated amino acids or organic acids in the form of adenylates,[76,77]

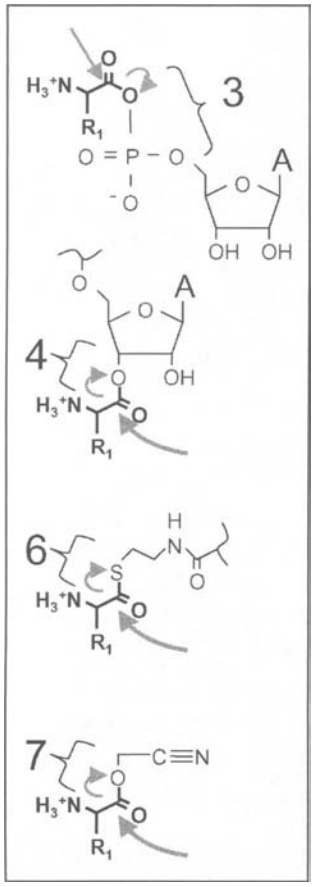

Figure 6. Activated amino acids (in bold) used in biology or in selections in vitro: Adenylate, or phosphoanhydride (bond #3), ribose ester (bond #4), the thioester of CoA (bond #6), cyanomethyl ester (bond #7). Electron movement during nucleophilic attack and displacement of the activating group is indicated by gray arrows.

3'(2') ribose esters,[78,79] and cyanomethyl esters[80] have been presented to RNA pools in the course of selecting acyltransfer ribozymes (Fig. 6).

Activation As Aminoacyl Adenylates

Illangasekare and Yarus incubated RNA transcripts with Phe-AMP, then tagged the Phe-RNA products with the N-hydroxysuccinimido (NHS) ester of naphtoxyacetate.[76] The naphtoxyacetate and the phenyl ring of Phe decreased the overall polarity of the modified RNAs and shifted their HPLC mobilities, allowing recovery of the desired products. Since HPLC is cumbersome for kinetic analysis, these authors later monitored electrophoretic gel shifts on low-pH gels. The original ribozyme, designated R29, was slow (second order rate constant of 70 $M^{-1}min^{-1}$) and non-specific, reacting equivalently with various aminoacyl adenylates.[57,76] Substrate recognition is primarily through the adenosine portion of the aa-AMP, as shown by the fact that AMP competes with the substrate, but free Phe does not.[57] These results provided the first proof that RNA could catalyze this class of condensation reaction, but also showed that naïve ribozymes can be poor reagents for rebuilding biology from scratch. However, further screens and additional engineering produced a rapid, highly specific, aminoacylating ribozyme designated RNA77.[81] This 90 nucleotide species shows second-order rate constants of around 50,000 $M^{-1}min^{-1}$ for Phe-AMP and Tyr-AMP (nearly 10^8-fold above the uncatalyzed rate), but only 1 to 5 $M^{-1}min^{-1}$ for other aa-AMP species.

Activation As Ribose Esters and Cyanomethyl Esters

The activated amino acids used by the Suga lab's ribozymes to acylate the 5' hydroxyl of a substrate RNA are cyanomethyl esters (CME). The CME group is stable enough against hydrolysis to facilitate its use in the selections. It nevertheless efficiently activates the amino acid to react with RNA substrates while offering few binding interactions that might subvert substrate specificity. Energetically neutral exchange reactions then transfer the amino acid from one hydroxyl group to another (Fig. 7). Like the Bartel polymerases, the Suga acyltransferases were built from simple ribozymes to produce successively more elegant elaborations.

The first ribozyme in this series catalyzed only the exchange reaction (the reverse of the second step shown in Fig. 7).[78] RNA pools bearing 5' hydroxyl groups were incubated with short oligonucleotides that carried the aminoacyl ester of N-biotinylated methionine on their terminal 3' oxygen. Those RNAs that catalyzed transfer of the amino acid from the oligonucleotide onto their own 5' hydroxyls were recovered by streptavidin (StrAv) affinity chromatography. These ribozymes stabilize the transition state through outer sphere coordination to a divalent metal ion in the active site.[82] Several follow-up studies defined the nucleotides required in the active structure and in forming the binding site for the metal ion.[83-85] Because the exchange reaction is energetically neutral, these ribozymes are expected to catalyze the

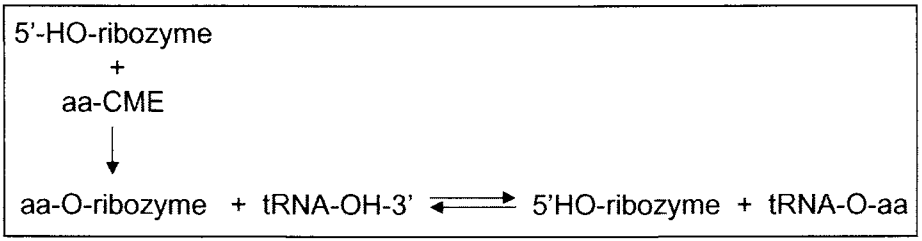

Figure 7. Ribozyme-catalyzed aminoacylation of tRNA starting from CME-activated amino acid and proceeding through an acyl-ribozyme intermediate.

forward and reverse reactions with roughly equivalent proficiency. Furthermore, the 2'(3') esters mimic the acylated 3' end of tRNAs used during peptide bond formation.

The second generation in this series added new substrate recognition activity.[80] Seventy random nucleotides were appended to the 3' end of a proficient, first-generation exchange ribozyme, and the resulting pool was subjected to selection for its ability to transfer biotinylated glutamine onto itself from a CME-Gln-bio substrate (the first step in Fig. 7). The winners from this selection were also required to retain their original exchange activity (second step) by accepting Gln from the 3' end of a small oligonucleotide. The final product RNAs are "ambidextrous" in that they can acquire Gln either from the CME-Gln-bio or from the 3' glutaminylated oligonucleotide. In a leap towards biological relevance, the "AD02" ribozyme showed the ability to perform a sequential 2-step reaction, in which it first acquires an amino acid from an activated source (CME-Gln-bio), then transfers it onto tRNA (both steps in Fig. 7). This second reaction is slow and operates at low yield (k_{cat} = 0.00195 min^{-1}; $Km^{Glu\text{-}CME}$ = 158 μM; 4% conversion of tRNA to aa-tRNA after four cycles of thermal denaturation/renaturation). However, it is highly specific for Gln over the unrelated amino acids Phe and Ala, and the product is a genuine aminoacylated tRNA. This portion of the ribozyme makes inner-sphere contacts with a required metal ion, and a 29 nucleotide stem-loop domain within the ribozyme can be provided in trans to assemble the active species.[86]

The third generation in this series partially removes the requirement for intramolecular tRNA recognition.[80] Lee and Suga appended a 70 nucleotide random segment onto the 5' end of a tRNA to generate a hybrid pool. By incubating the hybrid pool with CME-Phe-bio followed by recovery on immobilized streptavidin, they identified an RNA that directly aminoacylated the tRNA domain. The ribozyme-tRNA hybrid is a substrate for RNaseP, which cleaves off the 5' domain to release mature tRNA aminoacylated on its terminal 3' oxygen.[87] The reaction is not restricted to operating in an intramolecular fashion, as a 57 nucleotide miniribozyme derived from the 5' domain proficiently aminoacylates tRNA in trans.[88] While the ribozyme distinguishes between CME-Phe and unrelated CME-activated amino acids, it is not specific for the activating group, reacting equivalently with Phe-thioesters and Phe-adenylates. Further advances in this area may facilitate expansion of the genetic code and the incorporation of non-natural amino acids into proteins.

One additional ribozyme falls within this class, although it was intended to model a very different reaction. Jenne and Famulok sought peptidyltransferase ribozymes using a 2'(3') AMP ester of biotinylated Phe, which mimics the terminus of aminoacyl tRNA. The RNA pool they used was modified at the 5' end with the amino acid citrulline. Any species capable of condensing the alpha-amino group of citrulline with the activated Phe could have been recovered through the biotin-streptavidin (bio-StrAv) interaction; however, species that adduct the bio-Phe at any other position, such as a terminal or internal hydroxyl, could also be isolated. Indeed, the selected species was shown to self-aminoacylate at an internal 2' OH. As with the original Illangasekare and Yarus self-aminoacylating ribozyme, substrate recognition occurs largely through the AMP moiety.[79]

Ribozyme Catalysis of Step 3, Making an Amide/Peptide from Activated Amino Acids

Mimicking the third reaction of peptide synthesis was an early target of in vitro selections, and four independent sets of ribozymes have been described that form amide or peptide bonds. The first of these was the Lohse and Szostak acyltransferase described above. This ribozyme forms an amide linkage if the 5' OH of the ribozyme is replaced with a 5' NH_2, although the rate of bond formation is decreased about 3-fold by making the substitution (1.8 vs. 0.58 min^{-1} for ribozyme, 0.00083 vs. 0.00029 min^{-1} for the uncatalyzed reaction).[78] This ribozyme is the only one of the four that indisputably uses a ribose ester as the substrate (bond #4 in Figs. 4 and 6). The other three amide- or peptide-bond forming ribozymes all use (or at least appear to use) a mixed phosphoanhydride with AMP as the activated amino acid donor (bond #3 in Figs. 4 and 6).

The second species in this series is that of Wiegand, Janssen and Eaton, which forms an amide bond with biotin if the biotin is provided in a mixed anhydride as the adenylate (bio-AMP).[77] During selection, the attacking amine was on the end of an aliphatic chain covalently attached to the ribozyme. This strategy is similar to the use of the tethered citrulline noted above for the Jenne and Famulok ribozyme. The resulting catalysts are unusual among ribozymes in their requirement for Cu^{2+} ions and for their dependence on 5-imidazole-substituted uridines in place of normal U in the RNA chain. The catalyzed reaction proceeds at a rate of 0.04 min^{-1}, which is 10^4-fold above the uncatalyzed rate. Like the self-aminoacylating ribozymes of Illangasekare and Yarus, the Eaton ribozyme mimics natural tRNA aminoacylation by using AMP as a leaving group. The catalytic mechanisms employed by the Eaton and Yarus ribozymes are not yet known, but their substrates are so reactive that proximity effects may be sufficient to drive their respective reactions.

Third, a 29 nucleotide derivative of RNA29 from the Yarus lab noted above not only self-aminoacylates, it also forms di- and tripeptides from Phe-AMP as side reactions.[89] These RNAs first append an amino acid onto their 3' ends (k_{cat}/Km = 154 $M^{-1}min^{-1}$) and then add one or two additional amino acids, with k_{cat}/Km = 10-30 $M^{-1}min^{-1}$ for first addition. Amino acid adenylates condense into peptides in solution at a rate of approximately 0.3 $M^{-1}min^{-1}$, so RNA29 accelerates the condensation reaction by 30- to 100-fold[90] over the uncatalyzed rate.

Fourth, a ribozyme intended to mimic natural peptide bond formation was isolated by Zhang and Cech.[91] During the selection, the attacking amino acid was a phenylalanine attached to the 5' end of the RNA chain through a flexible linker that carried a disulfide bond. The analog of peptidyl tRNA was N-biotinylated Met, ostensibly supplied as a 3'(2') ester of AMP (but see below!). Condensation of the two amino acids allowed partition of reactive from unreactive molecules on StrAv affinity beads. Cleavage of the disulfide with dithiothreitol released the RNA into solution, providing an extra measure of specificity in recovering active species.[91] It now appears that these ribozymes use the aa-adenylate (*phosphoanhydride*) form of the substrate rather than the 3'(2') aminoacyl *esterified* adenosine. In tandem HPCL/activity assays, the adenylate and the ester are well separated. The adenylate reacted with k_{cat} close to those originally published (Km actually improved slightly), while the 3'(2') aminoacyl esterified adenosine failed to react. Some isolates from the original selection also show moderate stereopreference for the L-Met-containing phosphoanhydride substrate over the D-isomer. (RL Gottlieb, Z Cui, L Sun et al, in preparation). A family of ribozymes related to isolate 27/71 can join other pairs of amino acids into dipeptides, forming at least 30 different dipeptides from the appropriate adenylates. The majority of the catalytic rates are within 5-fold of the rate observed for the original Met-Phe combination.[92] Species RBZ180 derived from the 27/71 family is especially rapid, with k_{cat} = 4.05 min^{-1} and $Km^{aa\text{-}AMP}$ = 210 μM, for an overall acceleration of more than 10^5 over the uncatalyzed rate.

Significance of Ribozymes with These Kinetic and Fidelity Parameters

Acyltransfer ribozymes will need to become faster and more specific if they are to take over the synthesis of cellular aminoacyl tRNAs or peptides. They remain addicted to substrates that are pre-activated as esters, thioesters, or mixed phosphoanhydrides (e.g., adenylates). They are not yet able to generate and utilize the activated species from readily available, kinetically stable, thermodynamically activated reagents, such as ATP. The Kumar and Yarus ribozyme begins to break this chemical dependence by converting amino acids into the highly activated adenylates, but it does so at the expense of its own 5' triphosphate, making this RNA a single-use reagent. An alternative strategy that has yet to be realized is to utilize an exogenous NTP to generate activated amino acids in multiple turnover reactions. An obvious benefit from operating as a multiple turnover enzyme is the increase in overall efficiency for how the ribocyte's resources are used. A multiple-turnover enzyme would also be in a better position to acquire an editing function; a mis-acylated species could be hydrolyzed without requiring resynthesis of the transcript, and of course the editing function would permit increased fidelity. As they stand, ribozymes for peptide synthesis reactions show varying degrees of substrate affinity and specificity. The second-generation Bartel and Suga ribozymes demonstrate that substrate-recognition domains can be grafted onto existing acyltransferases. Similar efforts could yield ribozymes that synthesize di- and tri-peptides of specific sequence, perhaps through direct RNA-templated polymerization of activated amino acids.[93]

One additional prerequisite for inventing translation in an RNA-based metabolism is the necessity for a triplet code by which to link mRNA sequence with amino acid identity. Several mechanistic models have been suggested as to how this may have come about based on biophysical interactions between amino acids and specific binding sites in RNA.[93,94] Many aptamers that recognize amino acids contain the corresponding codons, although the significance of the correlation is controversial.[95] Various models for the origin of the Code are considered elsewhere in this volume.

These challenges aside, once activated amino acids became available to RNA World ribozymes, it would have been a small step to begin using them to make specific oligopeptides, thereby planting the seeds of the Protein Revolution. That ribozymes for uncoded peptide synthesis are relatively frequent in random populations is suggested by the successive aminoacyl- and peptidyl-RNA synthesis by Yarus's RNA77, by this ribozyme's small size, and by the diversity of dipeptides assembled by the Zhang ribozyme. Small size (low information content) implies that relatively few nucleotides need to be specified, thereby increasing the probability that such sequences are encountered in random searches through sequence space and that they could have arisen from within an RNA-catalyzed metabolism.

Towards an RNA-Catalyzed Metabolism: What's Missing?

Replication and translation are necessary components of any RNA World that might have preceded our own evolution —one to propagate the genome and one to launch the Protein Revolution— but it is difficult to envision a viable organism with only these activities. What else is required to build a cell from scratch? Biosynthesis may have been required early, before supplies of abiotically synthesized organic compounds were exhausted completely. Where biosynthesis runs counter to free energy gradients, energy extraction through catabolic reactions could have helped to power those reactions. A sufficient set of RNA-catalyzed activities, then, may need to include a minimal, recognizably modern metabolism. Benner et al used chemical intuition and the phylogenetic distribution of extant pathways to infer that the last ribocytes "had a complex metabolism that included dehydrogenations, transmethylations, C-C bond formation, and an energy metabolism based on phosphoesters," and that it synthesized porphyrins and terpenes.[49,96] Ribozymes for a constrained sort of nucleoside biosynthesis have been described,[97] and several labs are in the early stages of exploring RNA-catalyzed polyphosphorylation and the synthesis of lipids and amino acids (Table 3).

Table 3. Some targets for new ribozyme reactions and chemical activities

Biosynthetic Targets for Ribozymes:
cofactors
nucleotides
amino acids
lipids
Target Reactions for New Ribozymes:
cofactor-dependent electron transfer
Claisen condensation
hydration/dehydration
reactions with cationic intermediates
hydrogenation/dehydrogenation
radical chemistry
mutases

How many of these reactions can RNA actually catalyze? While all of the reactions in the transmission of genetic information are substitutions at esters or at phosphate esters, catalysis of additional reaction classes is required for an expanded metabolism. Confirmed ribozyme-catalyzed reactions encompass many additional reaction types (reviewed in refs. 1,6,7). Some of these include S_N1 displacement at the C1 of ribose to form a glycosidic bond,[97] S_N2 displacement of halogenated carbons to form N-C and S-C bonds,[98-100] C-C bond formation through Diels-Alder chemistry[101-103] and Michael addition,[104] porphyrin metallation,[105] and even very weak redox activity.[106,107] Many of these ribozymes may use catalytic strategies similar to those described above for reactions at carbon and phosphate esters (proximity effects, charge stabilization, acid/base catalysis, etc.). The next several years are likely to see advances in ribozyme catalysis of new classes of reactions, incorporation of nucleotide cofactors, amino acids and other small molecule prosthetic groups into ribozymes, and integration of multiple ribozymes into cells and multistep pathways.

New Classes of Reactions

The opportunities for expanding ribozyme activities are enormous (Table 3). Lipid synthesis makes use of redox reactions, dehydrations, and Claisen condensations—each of which includes an anionic intermediate, like the ester and phosphate reactions above. Some of the mechanistic strategies used in acyltransfer reactions may also be important for ribozyme-catalyzed electron transfers. Redox reactions using flavin or nicotinamide cofactors are often initiated by (de)protonation events and involve anionic intermediates. Reactions that require stabilization of cationic intermediates should be especially facile for RNA, with its polyanionic backbone, yet none of this ilk has yet been described. Ribozymes with new activities will continue to appear in the literature. Some of these activities may never have existed in an RNA World, or may not have a counterpart in extant cells, yet they could still find a niche in the metabolism of artificial cells, in the metabolic engineering of normal or diseased cells, or in practical synthetic applications.

Direct selection for catalysis requires a partition method that distinguishes active RNAs from the rest. A popular tool in recent years has been to couple a substrate covalently to an oligonucleotide, ligate this construct to the nucleic acid pool, allow the reaction to proceed, and purify active species on the basis of the chemical properties of the covalently tethered product (Fig. 8). This strategy has been fruitful for condensation with biotinylated,

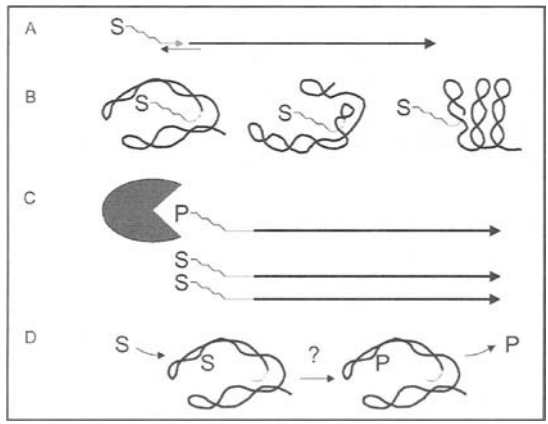

Figure 8. Ribozyme selection in vitro for reactions involving small molecule substrates. A) Substrate (S) is covalently tethered to a DNA or RNA oligonucleotide (thin gray line) through a flexible linker, such as polyethylene glycol (jagged line). The modified oligo is then ligated to each member of the RNA pool (thick line) with the help of a bridging DNA oligonucleotide. Strand polarity 5' to 3' is indicate by arrows. B) Upon folding, the sequence diversity library becomes a shape diversity library, wherein the flexible linker allows the substrate to access most of the folded RNA surface. C) Active species are recovered through the unique properties of the tethered product (P) such as through interactions with streptavidin or mercury if the product contains biotin or sulfer, respectively. D) Reactivity is often maintained when the tethered substrate is instead provided in trans, a critical feature for biological relevance, though some bind the substrate at low affinity and require high concentration.

sulphur-containing, or otherwise tagged substrates, but it does not readily extend to reduction of a C=C double bond, rearrangements (mutases), radical-mediated reactions, (de)hydrations, (de)hydrogenations, or methyltransfers. Ribozymes for these reactions are likely to exist in accessible sequence space —particularly if the RNA can exploit reactive cofactors— though their identification through in vitro selection may require highly creative new strategies.

It is our goal and that of several other labs to move ribozyme biochemistry toward biological relevance. In most cases, new ribozymes catalyze model reactions without producing biologically useful products, or they exploit biologically unreasonable reactants (such as cyanomethyl esters or tethered substrates). Some of the challenges for constructing a ribozyme-based metabolism include improving substrate recognition, exploiting reactive nucleotide cofactors and peptides, and integrating several activities into coordinated metabolic pathways.

Improved Substrate Recognition

Most new ribozymes are identified through their ability to promote a reaction between one substrate that is free in solution and another that is covalently tethered. In most cases, specific binding interactions are observed for the free substrate. In a few cases, reactivity is still notable when the tethered substrate is provided in trans at high concentrations,[75,108] but severing the attachment destroys reactivity in others.[97] Apparently the affinity for free substrate in those latter cases is insufficient to form a productive E•S (Michaelis-Menten) complex. Target recognition per se does not seem to be an intrinsic limitation, as RNA aptamers with high affinity have been isolated that recognize a wide variety of molecular targets. The Bartel polymerase described above is intermediate in this sense. While the tethered primer template duplex used during its selection can be provided exogenously, the limitations to its processivity are derived as much from rapid product dissociation as from slow polymerization chemistry. Many ribozymes bind one or more substrates non-covalently, although little is known about the details of these interactions. Small molecule binding by aptamers, on the other hand, is much better understood, and numerous aptamer modules have been described that bind nucleotide cofactors.

While ribozymes could in principle be developed from such modules, there are not yet any examples of this engineering/evolutionary path. The interrelation between small molecule binding and catalytic activities is fertile ground for future work.

A Role for Nucleotide Cofactors

The chemical versatility of unadorned RNA is generally regarded as being less than that of unadorned proteins, but this comparison is inappropriate. Protein enzymes make extensive use of prosthetic groups such as metal ions and nucleotide cofactors that ribozymes could similarly exploit. The seemingly superfluous inclusion of nucleotide components in cofactors such as CoA, FAD, NAD^+, and SAM has been interpreted as molecular fossils of an RNA world.[49,109,110] (The tRNA portion of glutaminyl-tRNA used in the C-5 pathway for porphyrin synthesis is also cited in this category as evidence that ribocytes made porphyrins,[49] although in this case it is equally probable that this reaction is not primitive; rather, a primitive cell that had already invented translation simply usurped a convenient, readily available source of activated Glu en route to inventing porphyrin synthesis.) In general, if a given cofactor can be made abiotically, or if its synthesis by ribozymes does not violate established RNA-catalyzed chemistry, it is reasonable that it should be available to ribozymes in RNA World models. Adenine forms readily upon condensation of ammonia and hydrogen cyanide.[111] The nicotinamide of NMN^+ and NAD^+ appears in some abiotic chemical reactions,[112] as does the pantotheine portion of CoA.[113-115] In vitro evolution studies of cofactor-mediated catalysis by ribozymes is therefore justified from an RNA world perspective. As of this writing, phosphoryl transfer from ATP remains the only significant nucleotide cofactor-assisted catalysis by nucleic acids,[54,116,117] though this may soon change.

Cofactor-dependent ribozymes could bind their cofactors non-covalently as in aptamer complexes, covalently through a self-capping reaction or other adduction, or by incorporating them into the RNA chain during transcription. The world of protein enzymes is replete with precedent for covalently attached cofactors. Various flavoproteins are covalently tethered to their flavins. In the fatty acid synthesis pathway, acetyl CoA carboxylase carries an attached biotin, and acyl carrier protein carries a covalently attached pantotheine. The self-capping ribozyme of Huang et al appends the phosphorylated cofactor precursors FMN, NMN^+, and phosphopantotheine onto its 5' end, releasing pyrophosphate in each case.[73] Jadhav and Yarus used this ribozyme —augmented with additional random sequence— to identify RNAs that catalyze synthesis of biotinyl CoA and acetyl CoA from the acyl adenylates and ribozyme-tethered CoA.[118] The ability to synthesize acetyl CoA would have been a seminal event in the evolution of RNA World metabolism. While covalent attachment prevents the cofactor from diffusing away, it does not obviate the need for non-covalent binding interactions, both with the substrates that react with the activated form of the cofactor (e.g., acylation substrate) and with the substrates that re-activate the cofactor (e.g., acetyl-AMP).

A Role for Amino Acids and Peptides

Amino acids are abundant in prebiotic chemical reactions. If there is an RNA World in our history, it surely arose in a chemical environment that included some amino acids and small peptides. These reagents are thus legitimate tools for augmenting ribozyme activities, either through direct chemical functionality or by allosteric activation. Indeed, a commonly cited model for takeover of catalytic functions by proteins is that the protein portions of ribonucleoprotein enzymes gradually took on greater importance while the RNA portions diminished, leaving behind only the nucleotide cofactors in the active site (Fig. 9).

Roth and Breaker isolated a deoxyribozyme that requires L-histidine or a closely related analog to catalyze RNA phosphoester cleavage.[119] The pH dependence of the reaction suggests that the rate-limiting step includes protonation of the histidine imidazole. These data are interpreted as indicating that the histidine serves as a general base catalyst similar to the first step of the reaction catalyzed by RNAseA, although it is also possible that the protonated histidine allosterically stabilizes the active structure of the ribozyme. Small peptides could also serve

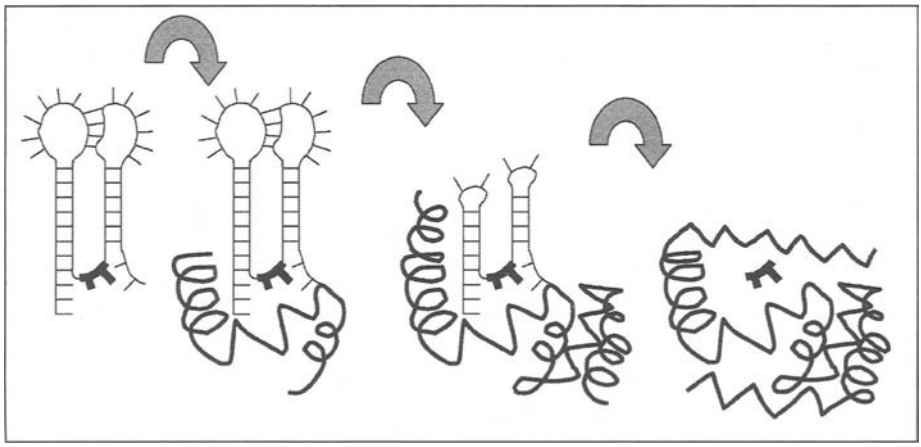

Figure 9. Protein takeover of catalytic function from a ribozyme (left side), through ribonucleoprotein enzymes (middle two), to a modern protein (right side). The dinucleotide cofactor in the active site is the only portion of the original RNA left behind in this model, making it a "molecular fossil." Redrawn based on White, 1982.

chemical roles for ribozymes. Like the nucleotide cofactors, such peptides could be bound to ribozymes through covalent or non-covalent interactions. Baskerville and Bartel found ribozymes that form a stable phosphoamide bond between their 5' termini and a specific polypeptide at the expense of pyrophosphate.[120] An optimized version of the ribozyme recognizes and reacts with the substrate even when the peptide is embedded within a fusion protein. Peptides and proteins could also augment ribozyme activity by inducing allosteric regulation; the nucleoprotein ligase enzymes of Robertson and Ellington are activated as much as 10,000-fold upon binding either the TyrS protein encoded by Cyt18 or the lysozyme of bacteriophage T4.[121] Ribonucleoprotein complexes similar to those shown in Figure 9 are therefore legitimate targets for RNA World research, and they are beginning to show up in the experimental literature. Furthermore, to the extent that small peptides were utilized by ancient ribozyme to augment their activities, any requirement for specific sequences would have provided selective pressure towards increased translational fidelity.

A Role for Modified Nucleotides

More than one hundred different modified nucleotides are found in modern rRNA and tRNA. Some of these have been proposed to have been present during the RNA World to augment stability against hydrolysis or catalytic reactivity, while others have been exploited in ribozyme selections in vitro. Ribozymes have been isolated that carry 5-pyridyl-U or 5-imidazole-U in place of normal uracils,[77,103] or that carry alkylamino side chains at the N6 position of adenosines.[47] There are at least three difficulties in applying these results to RNA World physiology. First, the current set of modified ribozymes catalyze reactions for which unmodified ribozymes have also been identified; thus, it is not inevitable that there are advantages to including such modifications. The second difficulty lies in choosing the appropriate set of modifications from among the hundreds of possibilities. As the proposed ancestral set of nucleotides becomes increasingly dissimilar from the present set of A, C, G, and U, fewer clues to the nature of the RNA World are available from modern biology. For addressing basic chemical questions, modifications can be chosen based solely on how useful they might be for selected catalysts, but some other criterion must be applied for the modification to be relevant to RNA world. Once such criterion could be the demonstration of an abiotic or RNA-catalyzed pathway to its synthesis. Messenger RNA is largely devoid of significant modifications (A→ I and C→U deaminations in double-stranded mRNA do not introduce new chemical functional-

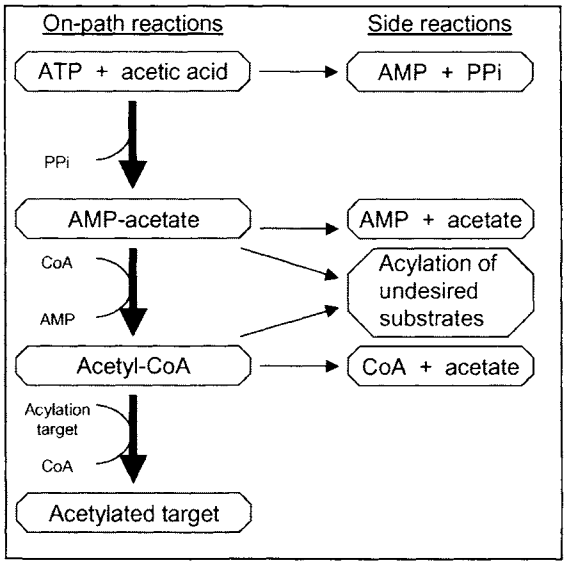

Figure 10. Kinetic control of reaction outcome, illustrated by a simple scheme for acetylating a desired substrate. A useful catalyst must accelerate the on-path reactions (thick arrows) well above the combined rates of competing reactions, such as hydrolysis and reaction with alternative substrates (thin arrows).

ity). The third difficulty lies in removing the modifications from the cellular lexicon once mRNA-encoded protein synthesis developed. It is conceivable that early ribocytes depended upon modified nucleotides, and that losing the ability to synthesize them during the Protein Revolution made the transition from RNA World to Protein World irreversible. Future developments in the theory and application of ribozymes with modified nucleotides may dispel these concerns, though for now it is difficult to know how to incorporate modified nucleotides appropriately into RNA World biochemistry.

Integration of the Individual Activities into a Working Metabolism

Achieving this objective requires both increased catalytic vigor of existing ribozymes and kinetic control of reaction outcomes. The significance of the latter is that biological catalysts are required not merely to accelerate an inevitable reaction, but also to channel reactive intermediates into particular products before they have a chance to participate in side reactions (Fig. 10). It is by guiding this kinetic competition among possible chemical outcomes that enzymes earn their keep within a cell. In principle, a set of ribozymes closely related to those currently available could attach CoA onto the 5' end of a transcript, activate an organic acid such as acetate in the form of an adenylate, transfer that acid onto the CoA to generate a thioester, and from there proceed to substrate acylation. All of these individual activities are available in the laboratory, but at present they do not adequately compete with the alternative reaction pathways, such as hydrolysis.

It would also be useful to have quantitative estimates of the affinities and kinetic rate constants needed to achieve kinetic control for each reaction, and computational models of cells may help in this regard (for review of theoretical cell models, see ref. 122). The rate enhancements of most ribozymes are many orders of magnitude below those observed for protein enzymes. The catalytic prowess of natural enzymes is a tribute to billions of years of optimization, but a more appropriate comparison might be made with catalytic antibodies, which often display rate enhancements similar to those of in vitro selected nucleic acid catalysts.[106] In this light it is not the polymer type (protein vs. nucleic acid), but the evolutionary naiveté of the catalyst, that limits the catalytic activity observed in ribozymes. Additional cycles of engineering and evolution, such as those that produced the polymerase ribozyme, may yet increase the catalytic vigor of these diverse ribozymes to levels sufficient for integration and for practical and biological application.

A cytoplasm carrying high concentrations of ribozymes would need to contend with biophysical and spatio-temporal coordination of many RNA species to avoid intermolecular aggregation. At the same time, productive intermolecular interactions as the quaternary assembly of multi-subunit enzymes could benefit metabolic integration. Substrate channeling and coordinated levels of enzyme activity, for example, could exploit RNA-RNA docking interactions, while minimizing such interactions could prevent such aggregation and keep each RNA maximally accessible to the cell's polymerases for replication. Two experimental avenues currently being explored along these lines include fabricating an artificial, multistep pathway in vitro and integrating in vitro-derived ribozymes into the physiology of modern cells. How would such a system respond and adapt to the complex demands of an intracellular environment? Success along either front, or fabrication of artificial, self-replicating cells based on ribozymes would go a long way in moving RNA biochemistry toward RNA-based life.

Concluding Remarks

Begging for Phosphates

The one essential reaction not yet demonstrated for RNA catalysis of genetic information flow is nucleotide polyphosphorylation. Given suitably activated mononucleotides, ribozymes can catalyze all of the subsequent reactions required for the transmission of genetic information, as well as a scattered sampling of other reaction types. They can bind an exogenous primer-template substrate for polymerization and extend the substrate more than one complete helical turn. They can use the energy of an NTP to activate an organic acid or amino acid, then use the activated substrate in acyl transfers to generate thioesters, ribose esters, amides and peptide bonds.

Proof of Principle?

The plausibility of RNA World theories accrues incrementally as one objection after another falls to experimental observation. Some authors now consider its plausibility to be fully established, but this is an overstatement of the case. At present none of these reactions proceeds with the vigor needed to form a working metabolism. It is tempting to wave the magic wand of Evolution and proclaim that if ribozymes can limp through a given reaction now, they could leap through it once a few favorable mutations are introduced. Experimental demonstration of this postulate will be more satisfying than a quasi-religious extrapolation. The RNA-mediated activities observed to date are to real RNA-based life what Goddard's rockets were to manned space flight: a very long way from the ultimate goal, but extraordinarily important milestones along the way. RNA World theories have risen in stature from enthusiastic conjecture to a hypothetical system that can be approached experimentally. The concept of an RNA World, whether in our own evolutionary past or in the frozen oceans of Jupiter's moon Europa, is now worth taking very seriously.

There are detractors from RNA World theories who seem to want the entire theory proven or discarded at once, with no tolerance for the intervening ambiguity. While it is not yet known whether ribozymes have what it takes to sustain life, it would be simplistic pedantry to dismiss the theory for lack of data before the requisite experiments have been carried out. The experimental goal is not to prove whether our own evolution ever passed through an era in which the world was populated with ribocytes, as this question cannot yet be addressed experimentally. Instead we seek to define both the chemical limits of catalysis by ribozymes and the inherent features of living systems built around RNA catalysis. The 21st Century will see the discussion move towards issues that link the chemical properties of individual macromolecules with the activities required to maintain cellular function. Then sophomoric rhetoric, whether from undisciplined cheerleaders or from jaded detractors, can be replaced by relevant experimental data.

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