REVIEW

Ribozyme Catalysis of Metabolism in the RNA World

by **Xi Chen**, **Na Li**, and **Andrew D. Ellington***

Department of Chemistry and Biochemistry, Institute for Cell and Molecular Biology, University of Texas at Austin, Austin, TX 78712, USA (e-mail: andy.ellington@mail.utexas.edu)

In vitro selection has proven to be a useful means of explore the molecules and catalysts that may have existed in a primoridal 'RNA world'. By selecting binding species (aptamers) and catalysts (ribozymes) from random sequence pools, the relationship between biopolymer complexity and function can be better understood, and potential evolutionary transitions between functional molecules can be charted. In this review, we have focused on several critical events or transitions in the putative RNA world: RNA self-replication; the synthesis and utilization of nucleotide-based cofactors; acyl-transfer reactions leading to peptide and protein synthesis; and the basic metabolic pathways that are found in modern living systems.

Introduction. – While the '*RNA world*' hypothesis in which nucleic acid catalysts preceded protein catalysts is now well-established and widely accepted, it is also frequently referred to as though this period of evolution was somehow biochemically homogenous, with all possible ribozymes coexisting at the same time and in the same organism. This is, of course, extremely unlikely. Rather, there was a huge difference between the 'early' RNA world in which self-replicating nucleic acids arose from prebiotic chemical reactions or cycles, and the 'late' RNA world in which an extremely complex ribozyme-based metabolism just preceded the invention of translation. The events in the early RNA world are open to speculation and experimentation, and several reviews have been recently put forth on this period [1] [2]. The late RNA world, while also a matter of some speculation, is somewhat more grounded in reality, as it can be reasonably assumed that biochemical reactions common to the three domains of modern life would also have been carried out by the last common ancestor (LCA) of life. The LCA was, in turn, likely far removed from the first invention of translation, but it was nonetheless the lineal inheritor of ribozyme-based metabolism. Therefore, whatever protein-mediated biochemical reactions were present in the last common ancestor can also reasonably be assumed to have potentially been catalyzed by ribozymes in the RNA world. Indeed, it can be proposed that many modern biochemical pathways are the exact duplicates of those in the RNA world, with only the nature of the catalysts having changed, from ribozymes to proteinaceous enzymes. We will review work in the general area of *in vitro* selection and directed evolution in order to examine the status of this hypothesis. In short, we will ask what experimental evidence supports the plausible existence of what reactions in the late RNA world?

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Natural ribozymes are largely relegated to the rearrangement of bonds involving phosphate and its esters and anhydrides. Selected ribozymes, on the other hand, have proven themselves capable of a wide variety of chemistries (*cf. Table 1*). These reactions vary in their chemical complexities and relation to basal metabolism. We have arbitrarily organized these reactions based on their perceived complexity and/or hypothesized appearance in a RNA world, with the first ribozymes presumably carrying out phosphodiester bond rearrangements involved in self-replication, later ribozymes adopting cofactors to assist catalysis, and ribozymes ultimately creating activated chemical intermediates that could participate in entirely new pathways.

Self-Replication in a RNA World. – It has been assumed that one of the first reactions that would have arisen in a putative RNA world is the ability of a primordial nucleic acid enzyme to reproduce itself from simpler components. In this regard, the replication of short hexamer oligonucleotides has been observed from trimer and smaller 'foodstuffs' by *von Kiedrowski*, *Orgel*, and their co-workers [3][4]. Such nucleic acid enzymes are catalytic, but only insofar as they provide a templating function. Presumably more advanced catalysts could have arisen from such simple precursors, and would have also been able to significantly enhance the rate of replication by methods other than mere templating.

Some hints about the chemical possibilities that may have been available to early nucleic acid replicators can be garnered from those few ribozymes that remain in nature. For example, the versatile Group-I self-splicing intron can be engineered to extend its 3′-end through a limited polymerization reaction in which GpN dinucleotides are substrates [5]. The Group-I ribozyme bound the guanosine in an organized active site and used magnesium ions to polarize the attacking 3′-OH group during bond formation. However, the efficiency and accuracy of this side reaction would have vastly limited any ancient polymerase that utilized a similar mechanism.

Other researchers have used directed evolution (rather than rational engineering) to explore the chemical mechanisms that may have been available to ribozymes. *Bartel* and *Szostak* selected a complex ribozyme RNA ligase that utilized a 5′-triphosphate-RNA as a substrate and that formed a 3′-5′ phosphodiester bond at the ligation junction (*Fig. 1*,*a*, *top*) [6]. This reaction mechanism is similar to that of modern protein polymerases, and differs from that of the Group-I intron (which uses guanosine rather than pyrophosphate as a leaving group; *Fig. 1*,*a*, *middle*) and from other selected ribozymes (such as a 5′ → 3′ ribozyme ligase selected by *Hager* and *Szostak* [7] (*Fig. 1*,*a*, *bottom*), a 5′-5′ ligase ribozyme that ligates 5′-phosphorimidazolide-activated and 5′-triphosphate-activated oligonucleotides, forming 5′-5′ tetraphosphate linkage [8], or other ligases that form 2′-5′ phosphodiester bonds).

The complexity, speed, and basic chemical mechanism of the Class-I *Bartel* ligase seemed to make it an excellent avatar for adaptation to polymerization reactions, and indeed this ribozyme exhibits limited polymerization activity using nucleoside triphosphates [9]. A RNA primer can be extended by up to six nucleotides in a 4-day incubation, with an optimized fidelity of 0.92. Like the Group-I polymerase, this polymerase acted on a substrate annealed to itself. It seems unlikely that such a catalyst could have been an early self-replicator, since it could not have readily changed the register of the active site without unwinding itself. Therefore, the *Bartel* group

Table 1. *Examples of Chemical Reactions Catalyzed by Ribozymes Selected from Random Pools.* Newly formed bonds are shown in red.

Bond formed	Reaction	Rate enhancement	Reference
	RNA Cleavage		[10–12]
	2′,3′-Cyclic-phosphate hydrolysis		[10]
	5′→3′ RNA Ligation (leaving group = pyrophosphate)	7×10^6	[6] [13] [14]
	5′→5′ RNA Ligation (leaving group = imidazole)	10^3–10^4 (over templated reaction)	[8]
	AMP-Capped 5′→3′ ligation (leaving group = 5′-phosphate of AMP)		[7]
	RNA Phosphorylation		[15]
	5′→5′ Self-capping (leaving group = pyrophosphate moiety)		[16] [17]
	Acyl activation (leaving group = pyrophosphate)		[18]
	Polymerization (leaving group = pyrophosphate)		[9] [19] [20]
	RNA Branching		[21]
	Aminoacyl-RNA synthesis (leaving group = 5′-phosphate of AMP)	$\geq 10^5$	[22]

Table 1 (cont.)

Bond formed	Reaction	Rate enhancement	Reference
O–C(=O)	Acyl transfer (leaving group = 3′-OH group of RNA)	160	[23]
O–C(=O)	Acyl transfer (leaving group = 2′-OH group of AMP)		[24]
O–C(=O)	Acyl transfer (leaving group = Sulfur)		[25][26]
N(H)–C(=O)	Amide bond formation (leaving group = 3′-OH group of RNA)		[23]
N(H)–C(=O)	Amide bond-formation (leaving group = 5′ phosphate of AMP)	10^4	[27]
N(H)–C(=O)	Peptide bond-formation (leaving group = 5′ phosphate of AMP)	10^6	[28 – 30]
HC–N	Glycosidic bond formation	10^7	[31]
H_2C–N	RNA Alkylation	3×10^6	[32]
H_2C–S	Thio alkylation	2400	[33]
HC–S	*Michael* addition	3×10^5	[34]
S–C(=O)	Thiol ester formation	> 3400 ([35])	[35][36]
C–CH–CH–C or C–CH–CH–C	*Diels–Alder* (modified RNA is used in [37])	800 [37]	[37][38]

Table 1 (cont.)

Bond formed	Reaction	Rate enhancement	Reference
HC–CH(H)	Aldol reaction	4300×	[39]
H_2C–C=O	*Claisen* condensation		[40]
HC=O	Redox reaction	$>10^7$	[41]
	Porphyrin metalation	460	[42]
	Isomerization	*ca.* 100	[43]

attempted to further evolve the polymerase to recognize a detached template:primer substrate [19][20]. Using a complicated selection that involved a tethered (but not hybridized) substrate, the researchers selected a variant of the original ribozyme that could operate fully *in trans* and that was capable of extending the primer by up to 14 nucleotides in a 24-h incubation with a fidelity elevated to 0.985.

Unfortunately, the shortest version of this ribozyme RNA polymerase was 165-nucleotides. This leads to a conundrum: if longer ribozymes are necessary to catalyze more efficient and faithful reactions, and yet even these longer ribozymes cannot polymerize nucleic acid strands that are similar in length to themselves, then how was anything in the RNA world replicated?

One answer is that early nucleic acid replicases did not use the same advanced chemistries that protein polymerases use today, but rather were largely dependent upon simpler templating mechanisms and utilized oligonucleotides rather than mononucleotides as substrates. From this vantage, there may have been a series of catalysts extending from the earliest replicators (similar to those that have been experimentally observed by *von Kiedrowski* and *Orgel*) through slightly more complex ligases to mononucleotide polymerases (a 'march of progress' envisioned in [44]).

The notion that oligonculeotide ligases could have supported early self-replicators is abundantly supported by the literature. By mimicking the reverse reaction of the first step of Group-I intron-catalyzed splicing, the Group-I intron was engineered and selected to be a template-dependent ligase (*Fig. 1*,*a*, *middle*) [45–47]. Using oligonucleotide substrates of *ca.* 10-nt in length, this ribozyme achieved the synthesis of a complementary strand of RNA as long as *ca.* 200 nt. Much shorter ribozyme ligases have been selected from random sequence pools [6][13][48], and some of these ribozymes have satisfyingly been shown to self-replicate using oligonucleotide substrates [49][50]. While this was a very brief treatment of the experimental support for early self-replicators that catalyzed oligonucleotide ligation and perhaps mononucleotide polymerization, the existence of such ribozymes would likely have set the stage for the further evolution of metabolism in the RNA world. In particular, the use of nucleotide substrates could quickly have morphed into the use of nucleotide cofactors.

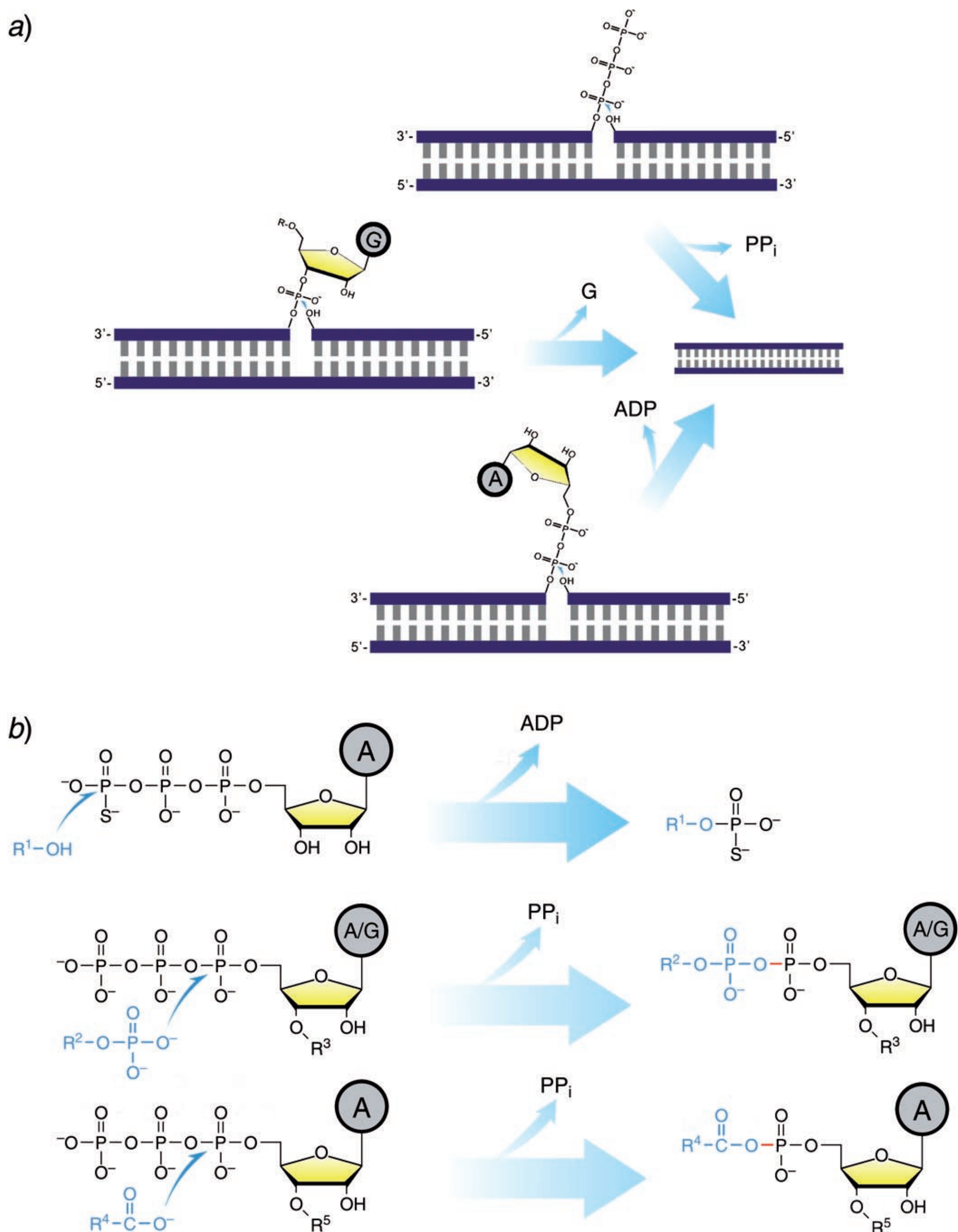

Fig. 1. *Enzymatic manipulations of phosphate chemistry. a*) RNA-Catalyzed RNA ligation: reverse reaction of the first step of group-I intron-catalyzed RNA splicing (*top*); nucleic-acid ligation by many protein enzymes (*middle*); and ligation using pyrophosphate as leaving group (*bottom*). *b*) RNA-Catalyzed RNA phosphorylation (*top*); 5′-5′ capping reaction (*middle*); and mixed anhydride formation and acyl activation (*bottom*).

Cofactors in a RNA World. – *Why Nucleotide Cofactors?* The functional groups on amino acid side chains enable protein enzymes to catalyze the various chemical reactions that make up cellular metabolism. However, even with a large array of chemical functionalities, more than half of the protein enzymes incorporate cofactors to facilitate and further diversify their catalytic activities [51]. Given the limited chemistries available to the five canonical nucleotides, cofactors would have been even more necessary in the RNA world.

The necessity for cofactors in biology turns out to be one of the primary rationales for inferring the existence of an ancient RNA world. Many of the cofactors still used by protein enzymes look like they were first invented by ribozymes; that is, they are based largely on nucleotides rather than, say, amino acids. We have nicotinamide adenine dinucleotide and *Rossman* folds, not nicotinamide amino acid, although the latter should be roughly equally functional in electron transport.

The prevalence of RNA-like cofactors (ATP, NAD, FAD, even molecules that, at a first glance, do not necessarily appear to be related to nucleotides, like folate) has been attributed to what has been called 'the principle of many users' [51]. Multiple different biochemical reactions may require the same cofactor; for example, many cellular transformations rely upon ATP as an energy source and precursor of leaving groups. If ATP were to suddenly change its form to, say, 2-aminopurine riboside triphosphate, cellular metabolism would cease: some critical enzymes might be able to deal with the change, but not all. In other words, once a 'critical mass' of enzymes uses a cofactor in a given form, the structure of that cofactor cannot readily change without the organism going through a severe fitness nadir. If this hypothesis is true, then the structures of cofactors are 'stuck' in evolutionary time in a way that is far more stringent than biochemical reactions or the catalysts that support them. Indeed, from this vantage cofactors may be the oldest metabolic fossils within a cell.

Following this analysis through to its logical conclusion, if most cofactors are old and most cofactors are based on nucleotides, then the raw material for the production of the earliest cofactors (nucleotides) must have been available at or near the origin of the RNA world. The possibility that RNA cofactors may have been available in even an early RNA world is plausible. Both pantetheine (CoA precursor) and nicotinamide (NAD precursor) could possibly have been synthesized from precursors that may have been present in the primordial soup [52][53]. If even small amounts of cofactors or related molecules were available to early catalysts, then self-sustaining metabolic reactions and pathways may have arisen *via* the further enzymatic synthesis of these cofactors. It is, therefore, notable that *in vitro* selected ribozymes are able to combine ATP with pantetheine or nicotinamide to synthesize both CoA and NAD, respectively [16].

Of course, there would also have been numerous amino acids available at or near the origin of the RNA world, as amino acids are also readily created by prebiotic chemistries or can be delivered from extraterrestrial sources. If both types of molecules were available, how then did nucleotides come to be utilized preferentially to amino acids as cofactors? It is possible that nucleotides are somehow uniquely suited to adaptation as cofactors, but this statement can be immediately seen to be false by merely examining the many amazing transformations that amino acids routinely undergo in secondary metabolic pathways. An alternative explanation, which we will

examine in greater detail below, is that, while nucleotides and amino acids are equally suitable as cofactors for protein enzymes, nucleotides are much more suitable than amino acids as cofactors for ribozymes.

Given this hypothesis and attendant analysis, then the scenario for the evolution of and evidences for a RNA world would go something like this: Self-replicating nucleic acids may have had a distinct advantage over other types of biopolymers in early evolution. While both nucleotides and amino acids may have been present prebiotically, nucleotides would have been preferentially utilized by early ribozymes as metabolism, and its attendant cofactors were being invented. Conversely, the use of nucleotides as cofactors would have simplified the task of inventing metabolism, as only pathways for nucleotide biosynthesis and elaboration would have originally been necessary; the biosynthetic pathways for the 20 amino acids would not have become necessary until much later. Below, we will suggest three steps in the evolution of cofactor utilization: *i*) adapting cofactors to important biochemical reactions; *ii*) ensuring a ready supply of cofactors through biosynthesis; and *iii*) inventing and dispersing cofactor binding sites to enable new reactions to occur. Since evolution tends to be incremental and to utilize pre-existing complexity, early metabolic pathways would likely have survived to modern times. However, at some point the ribozymes and attendant cofactors that catalyzed the individual reactions making up a pathway would have been supplanted by protein enzymes, perhaps utilizing the same, structurally invariant cofactors. Like the Cheshire cat, the ribozymes would have faded away, leaving only the cofactor smile. From this vantage, many of the metabolic pathways that we see today have their roots in a RNA world populated with ribozyme rather than protein catalysts.

Utilizing Cofactors in Catalysis. Given that ribozymes used nucleotide cofactors to invent metabolism, one interesting question that can be asked is how did they use their cofactors? There are two basic choices: non-covalent binding or covalent attachment. To assess these mechanistic possibilities, directed evolution has been used to reinvent RNA dublegangers of protein-catalyzed reactions. Both mechanisms have now been successfully demonstrated.

Given that the manipulation of phosphoryl bonds had proven possible by both natural ribozymes and selected ligases, *Lorsch* and *Szostak* [14] attempted to select ribozymes that utilized ATP-γS as a substrate and that could phosphorylate themselves (*Fig. 1,b*, *top*; R^1 = ribozyme). Initially, the anti-ATP aptamer motif (see below) was embedded in the random pool to increase the chance of identifying self-kinases that could utilize ATP. The transfer of the γ-thiol allowed capture of any self-kinases on a sulfhydryl affinity column. Among the resultant seven classes of selected ribozymes, five classes acted as 5′ kinases while the other two classes catalyzed the phosphorylation of an internal 2′-OH group. However, the anti-ATP aptamer motif was conserved in only two classes, and only one of these had detectable ATP affinity. In other words, the ribozyme actually eliminated the pre-formed ability to bind ATP in order to make a functional kinase!

Redox ribozymes that utilize NAD^+ as a cofactor have also been selected [41]. In this selection scheme, RNA transcription was initiated by guanosine-5′-monophosphorothioate (GMPS), and the thiol group was then used as a handle for the conjugation of a benzyl alcohol derivative. The conjugated pool was incubated with

biotin hydrazide in the presence of NAD^+. If the alcohol group was oxidized by NAD^+, the resultant aldehyde could be coupled to biotin and the self-tagging catalysts separated by streptavidin capture (see *Fig. 4,a*; as we shall see, this strategy will be used repeatedly in the isolation of ribozymes with a variety of activities). Two selections were carried out, one with Mg^{2+} as sole divalent cation, the other with both Mg^{2+} and Zn^{2+}. Interestingly, catalysts were only obtained with the Zn^{2+} enriched selection, consistent with the fact that some natural alcohol dehydrogenases use Zn^{2+} as a catalytic metal. Based on the estimated background rate, the selected ribozyme achieves a rate enhancement of $>10^7$-fold. Subsequently, the same ribozyme was shown to catalyze the reverse reaction – reduction of benzaldehyde by NADH [54]. Moreover, the oxidation of alcohol still progressed when the reaction was supplemented with NADH and FAD, relying on the uncatalyzed hydride transfer from NADH to FAD. These results suggest that redox couples would have easily arisen in the earliest metabolic pathways.

Taken together, these two demonstrations are especially significant for the evolution of metabolism in the RNA world. The use of adenosine as part of cofactors is ubiquitous in modern metabolism, and presumably would have been equally widespread in the RNA world. The adenosine-derived nucleotide ATP is the general cellular energy coin, while the adenosine-derived nucleotides NAD and FAD are cellular redox coins. The facile evolution of nucleic acid catalysts that utilize such adenosine-based cofactors implies that the early paths to the establishment of these adenosine-based structures in biology would have been relatively facile. As described above, once adenosine-based cofactors were established, their use would have been almost immutable, due to the principle of many users.

Cofactor Biosynthesis. The covalent attachment of cofactors to ribozymes is also possible, just as with protein enzymes. For example, *Breaker* and *Joyce* [55] showed that the Group-I ribozyme can covalently self-incorporate cofactors (NAD^+) and cofactor analogues (dephosphorylated CoA) *via* the same mechanism that is normally used for the insertion of guanosine into the RNA during the initiation of splicing.

Similar cofactor immobilizations have emerged from a quite versatile ribozyme 'cappase' selected by *Huang* and *Yarus* [17]. Originally, a RNA pool was incubated with a phosphate column, and variants that could append themselves to the column were selected. These ribozymes proved capable of not only utilizing phosphate as a nucleophile to catalyze the rearrangement of phosphoanhydride bonds, but proved to be remarkably substrate non-specific. Virtually any molecule that contained a phosphate could attack the α-phosphate at the 5′ termini of the ribozyme and thereby transfer itself to (cap) the ribozyme (*Fig. 1,b, middle*; R^2 can be virtually any moiety) [56].

Although this capping reaction happens in a different manner from the pre-mRNA capping of eukaryotic cells, it is strikingly similar to the adenylation step in the biological synthesis of several cofactors, *e.g.*, nicotinate mononucleotide $\rightarrow$ nicotinate adenine dinucleotide, FMN $\rightarrow$ FAD, and 4′-phosphopantetheine $\rightarrow$ dephospho-coenzyme A. However, one aspect that differs from cofactor biosynthesis was that the 5′-terminal nucleotide of RNA was G, due to the characteristics of the canonical T7 RNA polymerase promoter, while, in these natural cofactors, the nucleotide moiety is AMP. This incongruity was successfully resolved by carrying out another selection in which the RNA pool was initiated with a class II T7 RNA polymerase promoter, which starts

transcripts with A rather than G [16]. Selections were carried out with a mixture of N30, N60, N100, and N140 pools, but only the shorter pools yielded catalysts. The A-initiating cappase that was selected was different than the previously selected G-initiating cappase, but was equally robust in terms of its ability to use multiple different substrates.

Subsequent engineering and selections based on the remarkable cappase resulted in more general cofactor synthetase ribozymes that can synthesize RNA-linked NAD, FAD, and CoA from their precursors, and the 5′-terminal ATP of the ribozyme [16]. This allowed the construction of RNA pools that contained the catalytically useful thiol moiety. Such pools were subsequently used by both *Huang* and *Yarus* for the selection of ribozymes that could form activated acyl groups. These group transfer reactions will be discussed in greater detail below (see *Fig. 2*, *Arrow 2*).

These results support the alternative hypothesis that cofactors may have initially been attached directly to replicating RNAs, perhaps at their termini. As metabolism was further elaborated, the need to synthesize diffusible cofactors became greater, and biosynthetic pathways for the cofactors evolved. This scenario is similar to that proposed by *Yarus*, *Huang*, and their co-workers [16][35] in which the moieties that could chemically augment ribozymes (4′-phosphopantetheine, NMN, FMN) were initially conjugated to the 5′ terminus of adenosine-initiating RNAs, forming the diphosphate linkage seen in CoA, NAD, and FAD. During the further evolution of metabolism the cofactors became diffusible but kept the initiating adenosine residue and disphosphate linkage. It remains an open question whether the very first cofactors were diffusible or immobilized, and whether the first diffusible cofactors already contained nucleotide 'handles'. The relative ease with which nucleotide binding sites can be evolved, the subject of the next section, supports either conjecture.

Binding Sites for Cofactors. In addition to using directed evolution to select catalytic nucleic acids, as described in the previous sections, *in vitro* selection can be used to select nucleic acid-binding species (*aptamers*). A variety of aptamers have been isolated during the past decade that bind to small organic molecules such as amino acids, nucleotides, and (most relevantly) cofactors (for recent reviews, see [57 – 59]). The interaction between these binding sites and cofactors might, therefore, reveal how early ribozymes could have augmented their catalytic activities. For example, a number of aptamers have now been isolated that bind to adenosine or its analogues (*Table 2*). The first anti-ATP aptamer isolated by *Sassanfar* and *Szostak* [60] contains an asymmetric internal loop flanked by two double-strand RNA regions and has a binding constant of *ca.* 10 μM. The three-dimensional structure of this aptamer has been determined [61][62]. The structure forms a GNRA tetraloop-like motif with AMP contributing the A; binding primarily involves the nucleobase. Aptamers later isolated against NAD^+ [63] and SAM (*S*-adenosyl methionine) [64] share the same sequence and structural motif for adenine binding, just as many different ATP binding proteins share the *Rossman* fold. Indeed, a closer structural examination reveals many similarities between the ways in which RNA molecules bind adenosine, and proteins bind adenosine [65]. In each case, H-bonding and stacking are the two dominant factors for affinity and discrimination. This similarity is perhaps not surprising, given the relative simplicity of both the sequence and structural motifs. Given these results, it is highly likely that early adenosine- or cofactor-binding ribozymes contained a quite

Table 2. *Aptamers That Bind to Adenosine and Its Analogues*

Ligand	Aptamer structure	Selection Target (K_D)	Ref.
		Adenine (10 μM)	[66]
		ATP, NAD, SAM (10 μM)	[60][63] [64]
		CoA (2 μM)	[67]
		cAMP (10 μM)	[68]
		ATP (10 μM)	[69]

similar motif. In other words, despite *Stephen Gould*'s assertion that, if the tape of life were replayed the results would be different, at least some of the molecular events in any tape that involves RNA may be quite deterministic.

While there is a simple and common motif for adenine-binding, other sequences and structures are also possible (just as there are other ways that proteins can interact with adenosine other than the *Rossman* fold). An anti-CoA aptamer isolated by *Burke* and *Hoffman* also primarily recognizes adenosine, but the binding sequence for binding is different from the anti-ATP aptamer [66]. Even though both aptamers bind adenosine, the anti-ATP aptamer does not recognize CoA, likely because this aptamer cannot accommodate the 3′-phosphate of CoA. Aptamers have also been isolated that specifically target adenine (as opposed to adenosine), and again have a different sequence and structural motif (an interrupted three helix junction) than the anti-ATP aptamer. Anti-adenine aptamers have a binding affinity for adenine of *ca.* 10 μM, but bind adenosine much more weakly (K_d *ca.* 500 μM; [67]). Aptamers selected to bind cAMP [68] or to the triphosphate of ATP [69] also have sequence and structural motifs that differ from those of the anti-ATP aptamer. While it might be supposed that these sequence differences reflect specific contacts with the sugar or phosphate, in fact the anti-cAMP aptamer seems to primarily recognize adenine but forms a binding pocket that is sterically compatible with the cyclic phosphate. In contrast, the anti-triphosphate aptamer truly does recognize the β- and γ-phosphates, and can also bind to GTP, UTP, and CTP.

While adenine is clearly a preferred target, other cofactor chemistries are also highly compatible with nucleic acid-binding pockets. Anti-FMN and anti-FAD aptamers were first isolated by *Burgstaller* and *Famulok* [63], while *Lauhon* and *Szostak* later isolated additional flavin-binding aptamers [70]. An additional class of anti-FAD aptamers was isolated by *Burke* and co-workers [71]. These aptamers all recognize riboflavin with K_d values ranging from 0.5 to 50 μM but have different sequences, and adopt different secondary and tertiary structures: the anti-FMN and anti-FAD binding aptamers found by *Burgstaller* and *Famulok* have simple loop structures, the anti-riboflavin aptamers isolated by *Lauhon* and *Szostak* fold into intramolecular G-quartets, and the anti-FAD aptamer isolated by *Burke* and co-workers contains three helices interrupted by several internal bulges. While the first anti-NAD aptamer also turned out to be an adenosine binder, as mentioned above, *Lauhon* and *Szostak* subsequently isolated anti-NMN aptamers [70]. The anti-NMN aptamers were able to discriminate between NAD and NADH in solution by over an order of magnitude (K_d values of 2.5 μM *vs.* 37 μM). This result is especially encouraging, as it implies that such discrimination could assist hydride acceptance or transfer during cofactor-mediated ribozyme catalysis.

The relationship between nucleotide-binding sites and cofactor-binding sites is further supported by directed evolution experiments in which one is converted to the other. Aptamers against FMN and FAD were evolved to bind GMP by partially randomizing the parental aptamers and selecting for binding to the new ligand [72]. The new anti-GMP aptamers could no longer bind FMN or FAD even though no negative selections against FMN or FAD were carried out. While the sequence differences between the parents and the new aptamers ranged from 20 to 57%, as few as three nucleotide substitutions were found to alter specificity from FAD to GMP.

While all of these results support the RNA-world hypothesis, this is not to say that RNA is a magical substance with pluripotent binding properties. Indeed, as previously stated, it is precisely because *a*) most cofactors are nucleotide-derived heterocycles and *b*) RNA is quite good at binding nucleosides or heterocycles that the RNA world hypothesis is strongly supported. In those instances where a cofactor or moiety is not a nucleoside or heterocycle, RNA has a much more difficult time with binding and recognition. For example, even when additional selections against CoA avoided identifying anti-adenine aptamers, the selected binding pockets appeared to recognize the 3′ phosphate of CoA rather than the extended and somewhat aliphatic pantotheine arm [73].

Group Transfer Reactions and the Evolution of Translation. – One of the primary uses of cofactors is not only to effect a chemical transformation, but to provide a convenient and consistent handle for further reaction of a metabolic intermediate in a coordinated anabolic or catabolic pathway. To demonstrate how this might have occurred in a putative RNA world, we will focus on how a series of ribozyme-catalyzed group transfer reaction may have set the stage for the origin of translation. In addition, other ribozyme-catalyzed C–C bond-forming reactions may have set the stage for the initiation of other major metabolic cycles, including the utilization of lipids (*Claisen* condensation) and sugars (aldol reactions).

While it is hard to know how translation arose, it is likely that many of the steps in the process today are at least chemically similar to those that would have been present initially. Amino acids would likely have been activated by forming aminoacyl adenylates, a high-energy mixed anhydride between the carboxy group of the amino acid and the phosphate of AMP (*Fig. 2*, *Arrow 1*; R = amine, R′ = adenosine). In

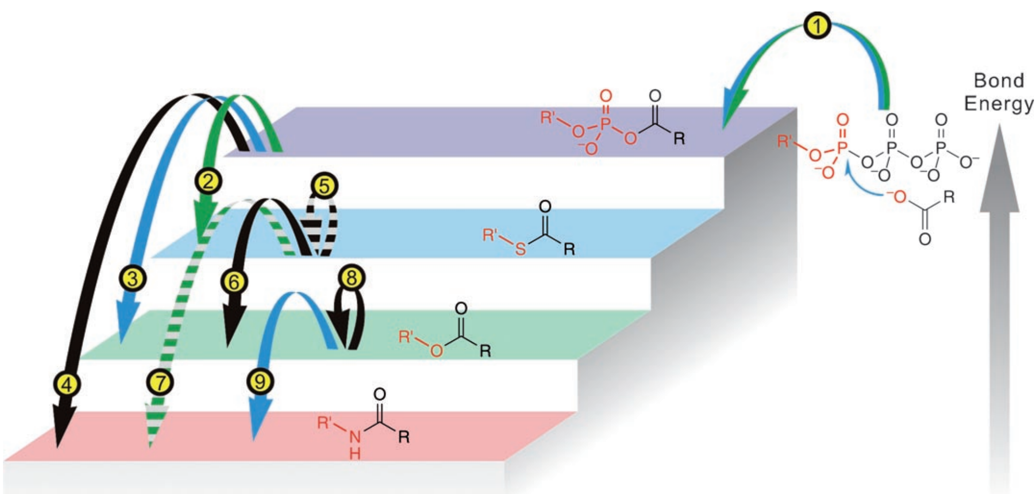

Fig. 2. *RNA-Catalyzed acyl activation and acyl transfer during peptide-bond formation.* Dotted arrows represent reactions that are catalyzed by proteins in modern life, but for which ribozyme counterparts have not yet been demonstrated. Acyl-transfer reactions in ribosomal peptide synthesis are shown in blue; those in non-ribosomal pathways are shown in green.

ribosome-mediated protein synthesis, this reaction is the first-step of tRNA amino-acylation catalyzed by amino-acyl tRNA synthetase (aaRS). Non-ribosomal peptide synthesis is also possible (for example, peptide antibiotics), and it is possible that translation originally arose from similar ribozyme-catalyzed systems. In non-ribosomal synthesis, the amino acid charging reaction is catalyzed by A-domain of non-ribosomal peptide synthatase (NRPS).

In ribosomes, the aminoacyl is then transfered to the 2′- or 3′-OH group of the 3′-terminal nucleotide of tRNA by aaRS (*Fig. 2*, *Arrow 3*; in product, R′ = tRNA). Charged tRNAs bind to the ribosome, and the amino group in the A-site aa-tRNA attacks the carbonyl group of the peptidyl-tRNA (or aminoacyl-tRNA) in the P-site, forming a new peptide bond (*Fig. 2*, *Arrow 9*; in product, R′ = A-site aa-tRNA subtract α-amino group). In non-ribosomal peptide synthesis, the aminoacyl of the aminoacyl adenylate is transferred to the thiol group of pantetheine covalently linked to the PCP domain of NRPS, forming a thioester bond (*Fig. 2*, *Arrow 2*; in product R′ = PCP-domain subtract thiol group of pantetheine). The peptide bond is then formed between two PCP-linked aminoacyl groups, catalyzed by the C-domain of NRPS (*Fig. 2*, *Arrow 7*; in product, R′ = PCP-linked aminoacyl subtract α-amino group, R = upstream amino acid subtract α-carboxy group).

From a purely chemical point of view, the synthesis of a peptide is essentially the down-hill transfer (*Table 3*) of the aminoacyl between different carriers: phosphate (anhydride) → hydroxy → amino in the ribosomal pathway (*Arrow 3* followed by *Arrow 9* in *Fig. 2*) *vs.* phosphate (anhydride) → thiol → amino in the non-ribosomal pathway (*Arrow 2* followed by *Arrow 7* in *Fig. 2*).

Table 3. *Standard Free Energies of Hydrolysis of Esters and Adenylates*[a])

Type	Example	$\Delta G^{0\prime}$ [cal/mol]
Carboxylate-phosphate mixed anhydride	Acetyl phosphate	−10300
Phosphate anhydride	ATP (→ ADP + P_i)	−7300
Phosphate anhydride	P_i	−8000
Thio-ester	Acetyl CoA	−7100
Oxygen ester	Ethyl acetate	−4720
Dipeptide	Benzolytyrosyl-glycinamide	−420

[a]) Source: [74].

To better show this putative path to translation, we will explore the experimental evidence that each of the chemical reactions might have once been catalyzed by ribozymes.

Arrow 1: *Acyl Activation.* The initial activation of the acyl group as a mixed anhydride starts the process (*Fig. 1*, *b*, *bottom*; R^4 = amine; also see *Fig. 2*, *Arrow 1*). An acyl-activating ribozyme has been selected [18]. These experiments utilized a carboxylic acid (3-sulfanylpropanoic acid, 3MPA) rather than an amino acid as the nucleophilic substrate and were conducted at lower pH in order to diminish the inherent instability of the amino adenylate product. For those catalysts in which the carboxy group of 3MPA attacked the 5′-α-phosphate of the 5′ triphosphorylated RNA,

a mixed anhydride would be formed, and the resultant thiolated RNA sulfanylpropanoic acid could be captured *via* disulfide bond-formation to an activated support. One selected variant, KK13, was shown to be able to use various amino acids and even H_2O (resulting in 5′-pyrophosphatase activity) as nucleophiles. When leucine was used as substrate, the k_{cat} was 1.1 min^{-1} at pH 4.

Arrow 2: *Acyl-CoA Synthesis.* Extensive biological evidences suggest that, as active acyl-transfer intermediates, CoA and its thioesters are synthesized and utilized by all three kingdoms of living organisms, and their origins can be traced back to the last common ancestor of life [75]. The thioester bond is of high-energy yet sufficiently stable to make a CoA an ideal acyl carrier for transacylation reactions. CoA and its thioesters are found at the center of many metabolic pathways and play essential roles in biological systems. For example, acetyl CoA is one of the primary intermediates in the catabolism of carbon compunds; it is synthesized from pyruvate and CoA by an oxidative decarboxylation reaction that feeds the ubiquitous citric acid (*Krebs*) cycle. The same basic reaction allows other acyl derivatives to be generated; succinyl CoA is synthesized by the oxidative decarboxylation of α-ketoglutarate. Beyond its involvement in the citric acid cycle, acetyl CoA also participates in amino acid degradation and biosynthesis, ketogenesis, fatty acid degradation and biosynthesis, cholesterol biosynthesis, and the glyoxylate shunt.

Interestingly, there are exceptions to the use of CoA as a thioester carrier. In many ways, these exceptions strengthen the argument that CoA was a prevalent cofactor in the RNA world. For example, in acyl-carrier protein (ACP) and peptidyl-carrier protein (PCP), which are acyl carriers in fatty acid synthesis and non-ribosomal peptide synthesis, respectively, the functional thiol group comes from covalently linked 4′-phosphopantetheine, the non-nucleotide 'arm' of CoA. Given that proteins could have used any of a number of thiols for acyl transfer (cysteine, lipoic acid), the simplest explanation for the use of pantetheine in these non-CoA acyl carriers is that they were derived from more ancient metabolism already centered on CoA.

The ready use of CoA by selected ribozymes also points to its possible antiquity. Ribozymes responsible for CoA thioester synthesis, RNA aminoacylation from an aminoacyl-CoA donor, and even *Claisen* condensation have been selected. We will first look at the possible roles of CoA in RNA-world peptide synthesis, then examine the *Claisen* condensation and fatty-acid biosynthesis in the next section.

Acyl-CoA-synthesizing ribozymes, which convert high-energy adenylate intermediates to slightly less energetic thioesters, were independently selected by two groups, using slightly different methods [35][36]. In both selections, activated biotin (biotin-AMP) was the acyl source and served as an affinity tag to capture self-biotinylated ribozymes. In the selection done by the *Yarus* group, CoA was incorporated at the 5′-termini of a pool of RNA using a transacting version of the previously described capping enzyme and the 3′-phosphate of the AMP portion of CoA as a nucleophile. The most abundant sequence from the selection, acs1, catalyzed CoA-acylation reaction. When biotin-AMP was used as the substrate kinetic constants of $k_{cat} = 0.026$ min^{-1} and $K_M = 2.2$ mM were observed.

Researchers in the *Huang* group instead utilized a 3′-dephosphorylated CoA as the initiating nucleotide for transcription [76]. Selections were initiated with a mixture of pools (as was the case with selections for an A-initiating cappase [16]), and again only

the shorter pools yielded catalysts. This illustrates a potential problem with experiments that recapitulate the RNA world: shorter motifs and shorter pools typically have an inherent advantage either because of the greater abundance of the shorter motif or the greater replicability of the shorter pool. Unless large pools and stringent selection conditions are used (as was the case with the selection of the *Bartel* Class-I ligase), researchers may preferentially identify ribozymes that are mechanistically or structurally simplistic. TES1 and TES33, ribozyme representatives from the N30 and N60 pools, respectively, were further studied. The k_{cat} and K_M values of TES1 for biotin-AMP were 0.29 min^{-1} and 1.3 mM, while those for TES33 were 0.40 min^{-1} and 0.35 mM.

Arrow 3: *Hydroxy-Group Acylation from an Adenylate.* The ribozyme catalyzing the reaction that mimics the second step of aaRS was actually the first selected ribozyme capable of catalyzing aminoacyl transfer [22]. In this selection, a RNA pool was incubated with phenylalanyl-AMP as an amino-acid donor. Some catalysts proved capable of transferring phenylalanine to a OH-group. The self-modified RNA now contained an amino group and could be reacted with the *N*-hydroxysuccinimide (NHS) ester of naphthoxyacetic acid, dramatically increasing the hydrophobicity of the RNA and thus allowing its separation from unreacted RNA by HPLC. One clone, isolate 29, was chosen for further study and the aminoacyl acceptor of this ribozyme was proved to be the 2′- or 3′-OH group of the 3′-terminal G.

Another aminoacylating ribozyme was selected by *Suga* and co-workers [25]. In this case, though, the selection was explicitly designed to be relevant to modern translation. A randomized region was appended to the 5′-end of tRNA, and the selected ribozyme catalyzes the aminoacylation of real tRNA on its amino-acid acceptor arm. Even when the ribozyme domain and tRNA were separated by RNase P cleavage, the ribozyme could still function *in trans*, just as protein aaRS does. The ribozyme could use various aminoacyl donors, including adenylates, thioesters, and oxygen esters.

Arrow 4: *Peptide Bond Formation from an Adenylate.* The first reported ribozyme in this category was selected by *Eaton* and co-workers, and catalyzed amide-bond formation between an amino group tethered to RNA (through a flexible PEG linker) and biotin (again from a biotin adenylate) [27]. 48 clones were sequenced, and 16 distinct sequences were found, 13 of which shared a 13-nt conserved motif. The k_{cat} and K_M values of the most active clone were 6.6×10^{-4} s^{-1} and 2.3 μM.

Other selected peptide bond-forming ribozymes also utilize adeylate as an acyl source. First, a peptide bond-forming ribozyme was selected that supposedly utilized an aminoacyl linked to the 3′-OH group of AMP [28], but the real substrate was later identified as a contaminating aminoacyl adenylate [29] (*Fig. 3,a*). Second, during further engineering of the previously described aminoacylating ribozyme [22], a second reaction between the product, aminoacylated RNA, and free aminoacyl adenylate was observed, resulting in the formation of a dipeptide-RNA adduct [30] (*Fig. 3,b*). Following up on these results, *Zhang* and co-workers intentionally selected ribozymes that could act as general dipeptide-synthesis catalysts by using aminoacyl adenylates as substrates [29].

Arrow 5: *Acyl Transfer between Thiol Groups.* Ribozymes in this category have not yet been selected, although ribozymes that catalyze the chemically similar transfer between OH groups have been selected (*Arrow 8*). It seems likely that many of these ribozymes might also work with thiols rather than OH groups.

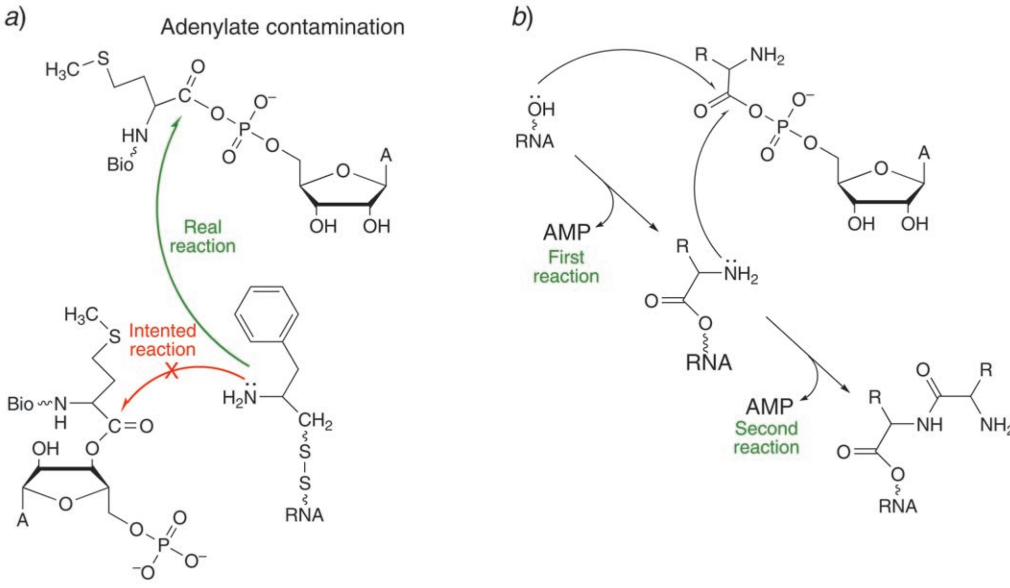

Fig. 3. *Ribozyme-catalyzed peptide bond-formation from an adenylate precursor. a*) A ribozyme selected by *Zhang* and *Cech* [28]. Reaction was observed with an aminoadenylate contaminant (*green arrow*) rather than with the intended substrate, an ester (*red arrow*). *b*) A ribozyme engineered by *Illangasekare* and *Yarus* [30]. The ribozyme first aminoacylated itself [22], then carried out a second reaction using the amino group of the newly conjugated amino acid. Thus, the ribozyme is capable to attack amino-adenylates using both oxygen and nitrogen as nucleophiles.

Arrow 6: *Hydroxy-Group Acylation from a Thioester.* Two types of ribozymes that could transfer aminoacyl groups from a thioester donor to the ribozyme or a RNA substrate were selected independently by *Suga* and co-workers, or by *Li* and *Huang* [26]. The ribozyme selected by *Suga* and co-workers has already been described (see *Arrow 3*, above). The ribozyme selected by the *Li* and *Huang* group used aminoacyl CoA (biocytin-CoA) as the acyl donor. Upon aminoacylation, RNA catalysts became covalently attached to biotin and CoA was released. After 11 rounds of selection, all clones were found to lie within a single sequence family. The consensus ribozym used an internal (2′) OH group as a nucleophile and had a k_{cat} value for biocytin-CoA of 1.2 min^{-1} and K_M of 0.05 mM.

Arrow 7: *Peptide Bond Formation from a Thioester.* This arrow is similar to *Arrow 6*, but for non-ribosomal peptide formation. A ribozyme catalyzing this reaction would support the possibility of peptide synthesis *via* the NRPS mechanism in a putative RNA world, but no such ribozyme has yet been selected.

Arrow 8: *Acyl Transfer between Hydroxy Groups.* While the major task of the ribosome is to form peptide bonds using the amino group of an aminoacylated tRNA as the nucleophile, the nucleophile can also be amino group of puromycin or OH group of hydroxypuromycin in a process called the 'fragment reaction.' A ribozyme catalyzing a reaction similar to the fragment reaction was first selected by the *Lohse* and *Szostak*

group [23]. These researchers intended to select an acyl transfer ribozyme that could move an aminoacyl group at the 3′-end of a 6-nucleotide RNA oligonucleotide to a 5′-, 3′-, or internal 2′-OH group of the ribozyme. After eleven rounds of selection, one variant predominated, which had strikingly evolved a way to juxtapose the acyl group of the RNA oligonucleotide substrate and the 5′-OH group of the ribozyme by simply using a 13-nt fragment template to align and juxtapose the two functionalities. As expected, the nucleophile was identified as the template-aligned 5′-OH group of the ribozyme. Astoundingly, when the 5′-OH group was substituted with an amino group, the aminoacyl was transfered to the ribozyme at a rate comparable to that of the original ribozyme (amide bond formation; *Arrow 9*). The fact that amide-bond formation was as competent as the selected acyl-transfer activity further emphasized the role of templating in the mechanism.

A similar selection was carried out by *Jenne* and *Famulok*, using 2′-aminoacyl-AMP as the aminoacyl donor [24]. However, the nucleophile on the selected ribozyme proved to be an internal 2′-OH group. In this instance, though, substitution of the OH for an amino group did not result in the amide-bond formation, possibly due to the differing geometries of the nucleophile (C(3′)-*endo* conformation for the 2′-OH group *vs.* C(2′)-*endo* conformation for the 2′-amino group). Acyl-transfer activity was also achieved by the ribozyme selected by *Suga* and co-workers [25][77], as mentioned previously (*Arrow 6*).

Arrow 9: *Peptide-Bond Formation from an Oxygen Ester.* This is the reaction utilized by the ribosome during peptide-bond formation. As mentioned above, ribozymes have been selected that utilize 5′-amino-modified termini as nucleophiles and 3′-*O*-aminoacyl RNAs as acyl donors for the formation of amide bonds [23]. While this reaction is chemically similar to that which occurs on the ribozyme, the generic use of amino acids as both amine nucleophiles and esterified acyl donors would better mimic the natural process. While one peptide-bond-forming ribozyme that uses the amino group of an amino acid as the nucleophile has been reported [28], the acyl donor was later identified to be a mixed anhydrate with AMP rather than an ester (*Fig. 3,a*). So far, the only ribozyme that can catalyze authentic peptide bond-formation seems to be the ribosome itself [78].

Ribozymes in Other Metabolic Pathways. – A self-sustainable organism should not only be able to replicate its genetic information but should also be capable of replenishing obtaining the energy and replenishing the building blocks necessary for replication. The acyl-group transfer reactions already described could have played a key role in many of these pathways; for example, acyl transfer from thioesters to oxygen esters (*Arrow 6*) is an essential step in phospholipid biosynthesis. However, early organisms would have also required the capacity for synthesizing and breaking C–C bonds, especially during the anabolism and catabolism of nucleotides, fatty acids, and sugars.

The first self-replicating nucleic acids and their descendents would have likely scavenged the environment for available foodstuffs. However, the material built up by prebiotic chemistry would likely have been quickly exhausted as exponentially replicators asserted themselves. Therefore, the invention of nucleotide metabolism would have been high on the list of necessary metabolic requirements for early ribo-

organisms. The phosphoryl- and other group transfer reactions described above would have been very important in the synthesis of nucleotides, but, in addition, other types of bond-forming reactions would have been necessary. In particular, the formation of the glycosidic bond is problematic by prebiotic mechanisms, and enzymatic catalysis of this reaction would have likely been greatly advantageous to whatever organism first invented it. *Unrau* and *Bartel* have shown that ribozymes can in fact catalyze the formation of nucleotides from phosphoribosyl pyrophosphate (PRPP) and nucleobases as substrates [31]. The glycosidic bond-forming ribozyme was selected from a pool in which PRPP was conjugated to the 5′-end. The pool was mixed with 4-thiouracil, and those ribozymes that catalyzed addition of the nucleobase could be isolated by separating the thione-containing product from unreacted RNA on a gel containing mercury (which retards the migration of thiones). Three families emerged from this selection. The fastest family showed a rate enhancement of at least 10^7-fold; further optimization increased this value to 10^8-fold [79]. Catalysis occurred through a novel stepwise dissociative mechanism with an oxocarbenium-ion intermediate, which was likely stabilized by the negative charge of the phosphodiester backbone [80]. A similar selection also gave rise to ribozymes that could catalyze glycosidic-bond formation with purine nucleobases [81].

Another key reaction in nucleotide biosynthesis is the formation of the components for DNA, including thymidine. The enzyme involved in the addition of the 5-methyl moiety to uridine, thymidylate synthase, utilizes an interesting *Michael* addition as part of its catalytic mechanism. In thymidylate synthase, a cysteine thiol acts as the *Michael* donor and attacks the C(6)-position of dUMP. *Famulok* and co-workers have now also selected ribozyme that catalyzes *Michael* additions [34]. The 5′-end of a RNA pool was linked to a fumaramide moiety, as a *Michael* accpetor, *via* a flexible alkyl linker containing a photocleavable group. A biotinylated cysteine was used as the *Michael* donor. After 17 rounds of selection, only one sequence survived. This RNA was shown to catalyze the predicted *Michael* addition by mass spectrometry, and showed a rate enhancement of 3×10^5 over the uncatalyzed background.

Beyond the requirements of nucleotide biosynthesis, if units of life in the RNA world were membrane-coated cells, the synthesis of fatty acids with long hydrophobic tails would have been necessary. In modern life, fatty-acid biosynthesis consists of two stages: *i*) a decarboxylative *Claisen* condensation between the thioesters malonyl-CoA and acetyl-ACP, and *ii*) a multistep reduction of the β-carbonyl group. Ribozymes have been shown to be capable of at least the chemistries required for the first stage.

We have previously mentioned that ribozymes catalyzing acyl activation (adenylate formation) and thioester formation have been selected. For example, the ribozyme selected by *Yarus* and co-workers, acs1, was also able to catalyze the synthesis of the important metabolic intermediates acetyl-CoA and butyryl-CoA from the appropriate mixed anhydride substrates, and similar catalysts could potentially have converted acetate and malonate to their corresponding thioester intermediates for fatty-acid biosynthesis in the RNA world. In addition, a ribozyme catalyzing the decarboxylative *Claisen* condensation has also been selected [40]. The selection scheme is depicted in *Fig. 4,b*. Claisen condensation between malonyl-PEG-RNA and biotinylated acyl-CoA allows the covalent linkage of RNA and biotin. The RNA was eluted with DTT,

Fig. 4. *Biotin conjugation in ribozyme selection.* Biotin conjugation as a result of *a*) a redox reaction [41], *b*) a decarboxylative *Claisen* condensation [40], and *c*) an aldol reaction [39]. Newly formed bonds are shown in *red*.

breaking a disulfide bond embedded in the PEG linker. The newly formed C–C bond was confirmed by mass spectrometry.

The reactions in the second stage of fatty-acid biosynthesis are of great interest because they represent a recurring 'leitmotif' in metabolism: reduction of β-carbonyl to hydroxy → dehydration of hydroxy to α,β-(E)-ene → reduction of an alkenyl group to an alkyl group (or the reverse, oxidation → hydration → oxidation again). As we mentioned above, while ribozymes that can reduce aldehydes to alcohols using NADH as a cofactor have been selected, no ribozymes have yet been shown to act on ketones. Moreover, no ribozyme has been selected to catalyze the interconversion between alkanes and alkenes.

The reactions involved in sugar metabolism are many and varied, but aldol condensations and cleavages are found throughout. In this regard, it is significant that *Famulok* and co-workers have selected an additional class of C–C bond-forming ribozymes, aldolases [39]. In nature, there are two major classes of aldolases. In Class-I aldolases, an internal lysine residue and carbonyl substrate form a *Schiff*'s base which acts as electron-sink. In contrast, Class-II aldolases require Zn^{2+} as a *Lewis* acid to polarize the carbonyl O-atom of the substrate and stabilize its enolate form. Considering the low nucleophilicity of nucleobase amino groups, aldolase ribozymes

that resemble Class-I aldolases might not be readily identified. Therefore, Zn^{2+} was supplemeted into the selection buffer to facilitate the evolution of a Class-II-like ribozyme. In the selection, the RNA pool was modified at its 5′-end with levulinic amide as an aldol donor. The amide was linked *via* a flexible linker and a photocleavable *o*-nitrobenzyl residue. The aldol receptor was a *N*-biotinylated 4-formylbenzamide (*Fig. 4,c*). As expected, Zn^{2+} was required by the selected ribozyme for catalysis. However, unlike some protein Class-II aldolases, Zn^{2+} could not be replaced by other cations such as Ca^{2+}, Sr^{2+}, Ba^{2+}, or Mn^{2+}. A 174-nucleotide truncated version of this ribozyme was used in kinetic analysis, and demonstrated a 4300-fold rate enhancement relative to the background.

Conclusions. – We set out to determine whether a late RNA world with a diverse metabolism could potentially have been supported by ribozymes. Upon examination of the available evidence, it appears not only would such a ribozyme-mediated metabolism have been possible, but, in many ways, the ease with which simple but robust ribozymes and cofactor-binding sites can be selected argues strongly in favor of the evolution of complex ribo-organisms. The appearance of stromatolites early in the fossil record has led to the speculation that protein-based organisms have dominated Earth's landscape for well over 3 billion years. If so, this further emphasizes the remarkable capabilities of nucleic acids to quickly evolve multiple reaction mechanisms and sequential pathways.

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