3
Fitness Landscapes, Error Thresholds, and Cofactors in Aptamer Evolution

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3.1
Introduction

The idea that RNA was genetic as well as enzymatic material goes back to earlier speculations concerning the possible role of RNA in the origin of life (Woese, 1967; Crick, 1968; Orgel, 1968). Although the idea was clearly expressed, and the molecular nature of RNA – with genetically specified positioning in three dimensions of different chemical building blocks – should have convinced everybody of the potential enzymatic capacity of RNA based almost entirely on first principles, the acceptance of the idea – in line with the deeply non-theoretical nature of molecular biology – came only after the discoveries of RNA self-splicing and the enzymatic activity of RNase P RNA (Kole and Altman, 1981; Zaug and Cech, 1982). The empirical finding that RNA can be a catalyst as well as an information carrier made it strongly plausible that the first genetic systems could indeed have consisted of RNA alone (Pace and Marsh, 1985; Sharp, 1985; Orgel, 1986).

White (1976) had formerly argued that the so-called nucleotide coenzymes (like NAD, NADP, FAD, FMN, etc.) are fossils of an earlier metabolic stage when RNAs acted as enzymes. It is interesting to note that whereas in White’s paper the emphasis was on metabolism, in Gilbert’s (1986) manifesto for the “RNA world” interconversions of RNA molecules are the focus. A much lesser known – but very rigorous – attempt was that of Gánti (1979), who put early enzymatic RNAs into his minimal cell model (see Fernando et al., 2005, for a review on models of minimal cells); the so-called chemoton (see Gánti, 2003a,b). In that model it is assumed that replicative ribozymes catalyze steps of an autocatalytic metabolic cycle, surrounded by a growing membrane. This approach was complemented by a thorough confirmation of White’s insights on coenzymes as remnants of early ribozymes (Korányi and Gánti, 1981). In Gánti’s theoretical world the RNA world was in full bloom by the advent of the experimental demonstration of ribozyme activity in natural systems.

Analysis of a “bag of genes” enclosed in compartments led to the stochastic corrector model (Szathmáry and Demeter, 1987), demonstrating that selection at the
The stochastic corrector not only solves the competition problem, but is also a theoretical construct to account for selection for ribozyme functions in reproducing compartments (Fig. 3.1), as explicitly stated in the paper.

Fig. 3.1 The stochastic corrector model (Szathmáry and Demeter, 1987). Different templates (open and closed circles) contribute to the well-being of the compartments (protocells) in that they catalyze steps of metabolism, for example. During protocell growth templates replicate at differential expected rates, but stochastically. Upon division (→) there is chance assortment of templates into offspring compartments. Stochastic replication and reassortment generate variation among protocells, on which natural selection at the compartment level can act and oppose to (correct) internal deterioration due to within-cell competition. Such compartmentation selects for efficient ribozyme variants.

compartment (protocell) level is strong enough to oppose (correct) the adverse consequences of within-cell competition among replicative, unlinked genes. The difficulty of internal competition within an early genomic set was first pointed out by Eigen (1971).
However, a burning open question, also realized by Eigen (1971), still remains; namely, the problem of the error threshold of replication (that is, a sharply defined threshold beyond which heredity breaks down and evolutionary adaptation becomes impossible). This concept is the flip side of the coin on which we see “mutational load” on one side. Ever since the pioneering works of Haldane (1937) it was clear to population geneticists that too high a mutational load (the decrease in average fitness of a population due to recurrent deleterious mutations) could kill a population, but Eigen looked at this problem from another angle. If you fix the mutation rate, how long can a replicator grow before it can no longer be maintained by natural selection, despite it being a fast replicator? Early replicators must have been error prone; therefore, they could not have been very long (the size of a tRNA is usually assumed). Does the stochastic corrector model push up the error threshold so that genomes composed of several to many different genes can be maintained by selection? The answer is encouraging, but not sufficient (Zintzaras et al., 2002): the origin of a sizeable genome is still a problem (see also Santos et al., 2004).

We outline here a possible resolution to this conundrum. In the first part of this chapter we analyze existing ribozymes to obtain a “function landscape,” which assigns an activity value (ideally) to each mutant sequence. Then we use this function landscape as a proxy for the fitness landscape; the crucial assumption being that ribozyme activity affects protocell fitness, hence protocell fitness translates back to some average ribozyme fitness. In conclusion, we argue that the position of the error threshold was previously estimated to be too severe: neutral and compensatory mutations crucially modify the picture. This result strengthens the possibility of an RNA world in protocells (Szathmáry, 1990a).

In the late 1908s one of us was interested in putting the important theoretical considerations of an RNA world to experimental test. Contemporary natural ribozymes almost exclusively conform to Gilbert’s vision of an RNA world, rather than lending support to a metabolically complex RNA era, as envisaged by Benner et al. (1987, 1989). It was clear that a proof of the principle of the general enzymatic capability of RNAs should come from novel experiments. It was suggested that a protocol similar to the production of catalytic antibodies should be followed (Szathmáry, 1989, 1990b). The suggested method envisaged selection of RNAs by binding to transition state analogs of a given reaction, linked to an affinity chromatography column. Amplification would have happened at the DNA level after reverse transcription of the best-binding RNA molecules. Szathmáry (1989) also pointed out that the same protocol could possibly be used to obtain RNA “aptamers” (as we call them now) that could specifically bind amino acids, thus enabling the community to test certain ideas (primarily the stereochemical one) of the origin of the genetic code.

Following this line, in the second half of the chapter we summarize experimental work on co-ribozymes (cofactor-assisted ribozymes) and aptazymes (aptamers attached to ribozymes). Finally, we discuss ideas around how aptamers and nucleotides could have been relevant for the origin of the genetic code and translation.
3.2
Functionality Landscapes Inferred from Examples

3.2.1
Fitness Landscape

Fitness landscapes or adaptive landscapes are often used in evolutionary biology to envisage the relationship between genotypes – or phenotypes – and Darwinian success (fitness). The idea of studying evolution by visualizing the distribution of fitness values as a kind of landscape was introduced by Wright (1932). Maynard Smith (1970) was the first to coin the term “protein space” for a high-dimensional space in which each sequence of length \( N \) amino acids (out of \( 20^N \) possible sequences) represents one point and is next to \( 19N \) points representing all the one-mutant neighbors of each other. In order to produce an adaptive landscape in sequence space a fitness value has to be assigned to each sequence, and an evolving population of proteins typically climbs uphill in the fitness landscape. This concept has since been used by a number of authors (for example Eigen, 1985; Schuster, 1986, 1987).

The simplest theoretical fitness landscape is the single-peaked fitness landscape used in Eigen’s (1971) study of the error-threshold in a replicating population of RNA sequences. In this landscape one sequence (the “master sequence”) has the highest fitness value, and all other sequences have the same or lower fitness. This biologically rather unrealistic fitness landscape still attracts considerable theoretical interest, mainly because it can be tackled analytically (Drossel, 2001). In evolutionary optimization methods such as genetic algorithms (see references in Flamm et al., 1999), as well as the use of the concept of a potential or energy function in physics (for example spin-glasses; Bonhoeffer and Stadler, 1993), fitness landscapes have also been applied to the study of biological evolution. However, the structure and characteristics of these landscapes are quite unlikely to match with the fitness landscapes of biological systems.

The N-K model of Kauffman (Kauffman, 1993) describes a landscape where \( K \) out of \( N \) elements are involved in some epistatic interaction. The model produces a rugged fitness landscape which was believed to resemble molecular fitness landscapes on the basis of its ruggedness. Mutational additivity usually holds for positions in biological sequences that do not interact, and such mutational additivity has been demonstrated for several proteins (Tekada et al., 1989; Sarai and Tekada, 1989; Sandberg and Terwilliger, 1993; Serrano et al., 1993; Zhang et al., 1995; Skinner and Terwilliger, 1996; Nikolova et al., 1998; Aita et al., 2001, 2002). In these cases, the most realistic of the N-K landscapes is the Mount Fuji type fitness landscapes (also known as multiplicative fitness landscapes), where each element in a sequence individually and independently contributes to the fitness and there is a single fittest sequence.
3.2.2 Damage Selection Experiments with Ribozymes

A wealth of information has been accumulated on ribozymes since their discovery nearly 20 years ago. Most of the studies can be fitted into one of the three main lines of research: (1) characterization of known ribozymes (that is, inferring the structure and mechanisms of catalysis; Lilley, 1999); (2) modification of natural ribozymes to be used in therapeutics (Sullenger and Gilboa, 2002); and (3) \textit{in vitro} evolution of novel ribozymes (Joyce, 1998, 2002; Landweber et al., 1998; Spirin, 2002). The characterization of ribozymes frequently involved mutagenesis experiments, where the enzymatic activity of certain mutants was measured in order to get insight into either the structure of the molecule or the mechanism of catalysis. While not directed toward the study of fitness landscapes, these experiments certainly contain a wealth of empirical information necessary for assembling the \textit{realistic} fitness landscape of the studied ribozyme. Albeit all naturally occurring ribozymes are being studied extensively, there are only a few instances where the realistic fitness landscape can be conveniently investigated. Group I and group II introns, as well as the RNAase P, have to be excluded because of their rather large size. Furthermore, it is inevitable to employ an RNA folding algorithm in any sensible investigation of the fitness landscapes of ribozymes. Therefore, ribozymes with a pseudo-knot in their structure also have to be excluded because most conventional folding algorithms cannot satisfactorily cope with pseudo-knots. This requirement singles out the hepatitis delta virus, which contains such structural elements (Perrotta and Been, 1991).

On the other hand, the hammerhead, hairpin, and \textit{Neurospora} VS self-cleaving ribozymes can be separated into a substrate and a \textit{trans}-cleaving ribozyme. With respect to these three ribozymes the \textit{trans}-cleaving enzyme does not contain a pseudo-knot structure. The hammerhead can be separated into a 13-mer enzyme and a 41-mer oligonucleotide substrate (Jeffries and Symons, 1989). The hairpin can be separated into a 50-mer enzyme and a 15-mer substrate (Fedor, 2000). The \textit{trans}-acting ribozyme is 144 nucleotides long for the \textit{Neurospora} VS ribozyme (Fig. 3.2), and the substrate is 20 nucleotides long (Guo and Collins, 1995). We restrict our further analysis to the \textit{trans}-acting ribozyme, and assume that the substrate is the same as the natural one. Unfortunately for our study, many of the mutagenesis experiments have been directed towards the substrate and substrate-binding regions (Joseph et al., 1993; Joseph and Burke, 1993; Nishikawa et al., 1997; Ananvoranich and Perreault, 1998; Pérez-Ruiz et al., 1999) in order to produce new RNA- or DNA-cleaving ribozymes to be used in therapeutics (Yu et al., 1998; Andäng et al., 1999; Andäng et al., 2004; Zhang et al., 2004). Based mainly on experiments with the VS and the hairpin ribozymes the following general conclusions can be derived:

- \textit{Structure is important}. From experimental data on the VS ribozyme Lilley and co-workers (Lafontaine et al., 2002c) state that “the secondary structure of the ribozyme is important, but the nature of most individual base pairs is not. Many can be reversed...
or replaced by a different pair without major loss of activity, so long as a base pair is retained at a given position.” Similarly, in the hairpin ribozyme all base pairs can be altered (except base pair G11:C/U-2) as long as the base pairing is maintained (Fedor, 2000).

- **There are critical regions in the molecule.** For the single-stranded regions the structure has to be maintained, but at many such positions the nature of the base located there is also important. For example, most of the bases in the four loops of the hairpin ribozyme are essential, and any change in those positions severely reduces activity (Siwkowski et al., 1997; Shippy et al., 1998). For the VS ribozyme 16 such critical sites were identified (Lafontaine et al., 2002a): these sites are located around the active site, the substrate-binding region, and in the two- or three-way junctions.

- **Structure can be varied slightly.** The structure of the naturally occurring ribozymes can be slightly varied, as there are regions that are not crucial to function. For example, the stem–loop IV of the VS ribozyme is virtually completely dispensable, but the junction 3–4–5 must be formed (albeit after the complete removal of stem–loops IV and V the ribozyme still has detectable activity; Sood and Collins, 2002). Similarly, in the hairpin ribozyme the helices H1 and H4 can be shortened and greatly extended without any loss of activity (Fedor, 2000; Sargueil et al., 1995).

![Sequence and secondary structure of the enzyme part of the Neurospora VS ribozyme](image)

**Fig. 3.2** Sequence and secondary structure of the enzyme part of the *Neurospora* VS ribozyme (numbering according to Beattie et al., 1995). Roman numbers indicate the regions of the ribozyme. Capitalized nucleotides indicate positions for which mutagenesis studies are available. Bold nucleotides indicate critical sites.
While the previous general conclusions can be easily incorporated into a model of a fitness landscape, one general difficulty stills remain; namely, the combined effect of multiple mutations. Most mutagenesis experiments have investigated only single mutations (or mutations involving a base pair) in the vicinity of the wild type in sequence space and rarely report the activity of double or higher order mutants. In those few instances where the effects of multiple mutations were evaluated, the activities of the single-point mutants were not always included. The only remarkable exception is the study of Lehman and Joyce (1993) from an initial pool of the *Tetrahymena* ribozyme, where they found that in general the mutational effects were multiplicative (which implies mutational additivity for ribozyme activity).

Table 3.1 summarizes the available experimental information on multiple mutational effects for some of the known nucleolytic ribozymes. The plot of the measured enzymatic activities of the double mutants on the estimated activities from the single mutants (Fig. 3.3) clearly suggests that mutational effects are nearly multiplicative, with a slight positive synergy. Such positive synergy was also found for chemical modifications of the hairpin ribozyme (Klosermeier and Millaer, 2002). Accordingly, the fitness of a molecule containing *n* mutations (*w*_n*) could be estimated as:

\[
\begin{align*}
\text{multiplicative} & = \prod_{i=1}^{n} w_i \\
\text{fitness} & = \text{multiplicative} (ax^b)
\end{align*}
\]

where *w*_i is the fitness of a single-error variant, *w*_n multiplicative is the fitness of an *n*-error variant assuming multiplicative effects, and *a* and *b* are parameters to be fitted given the data (Fig. 3.3). We stress, however, that although the data set contains information from three different ribozymes the number of points is still quite small. Therefore, some care should be taken when translating the empirical available information to a fitness function.

Besides these synergistic effects there are also examples of mutations that “rescue” enzymatic activity to some extent. Mutations that result in the loss of catalytic activity also exist (Table 3.2). Mutants containing these and other point
mutations might or might not have detectable activity. To our knowledge these interactions are impossible to predict, thus they can only be incorporated into a definition of a fitness landscape if known from experiments. In conclusion, the easiest way to deal with multiple mutations is to assume mutational independence (multiplicative effects), although it slightly overestimates the decrease in fitness due to multiple mutations. A more realistic assumption can come from taking the synergy into account, albeit more data would be highly welcome. If rescue mutations or other such effects are known of the ribozyme, then they can also be incorporated to increase the realism of the fitness landscape.

### 3.2 Functionality Landscapes Inferred from Examples

#### Table 3.1 Available experimental data for the evaluation of multiple mutant effects on fitness

<table>
<thead>
<tr>
<th>Mutant 1</th>
<th>Activity</th>
<th>Mutant 2</th>
<th>Activity</th>
<th>Activity of the double mutant</th>
<th>Estimated activity of the double mutant</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>U39C&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.289</td>
<td>A11G</td>
<td>0.32</td>
<td>0.093</td>
<td>0.095</td>
<td>Joseph et al., 1993</td>
</tr>
<tr>
<td>G21U&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.01</td>
<td>A20U</td>
<td>0.9</td>
<td>0.01</td>
<td>0.009</td>
<td>Sargueil et al., 2000</td>
</tr>
<tr>
<td>G21U&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.01</td>
<td>A20G</td>
<td>0.2</td>
<td>0.003</td>
<td>0.002</td>
<td>Sargueil et al., 2000</td>
</tr>
<tr>
<td>G21U&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.01</td>
<td>A20C</td>
<td>0.6</td>
<td>0.15</td>
<td>0.06</td>
<td>Sargueil et al., 2000</td>
</tr>
<tr>
<td>G21U&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.01</td>
<td>A43G</td>
<td>0.4</td>
<td>&lt;0.001</td>
<td>0.004</td>
<td>Sargueil et al., 2000</td>
</tr>
<tr>
<td>G21U&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.075</td>
<td>A43G</td>
<td>0.085</td>
<td>&lt;0.001</td>
<td>0.0064</td>
<td>Siwkowski et al., 1997; Sargueil et al., 2000</td>
</tr>
<tr>
<td>G21U&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.075</td>
<td>A43U</td>
<td>0.002</td>
<td>&lt;0.001</td>
<td>0.00015</td>
<td>Siwkowski et al., 1997; Sargueil et al., 2000</td>
</tr>
<tr>
<td>A7C&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1</td>
<td>A20C</td>
<td>0.81</td>
<td>1.04</td>
<td>0.81</td>
<td>Anderson et al., 1994</td>
</tr>
<tr>
<td>A730Cb</td>
<td>0.32</td>
<td>A731C</td>
<td>0.39</td>
<td>0</td>
<td>0.12</td>
<td>Kumar et al., 1992</td>
</tr>
<tr>
<td>G726Ab</td>
<td>0</td>
<td>A730C</td>
<td>0.32</td>
<td>0</td>
<td>0</td>
<td>Kumar et al., 1992</td>
</tr>
<tr>
<td>U752Cc</td>
<td>0.80</td>
<td>U753C</td>
<td>0.42</td>
<td>0.52</td>
<td>0.336</td>
<td>Lafontaine et al., 2001b</td>
</tr>
<tr>
<td>G722C; C763Gc</td>
<td>0.81</td>
<td>C723G; G762C</td>
<td>0.84</td>
<td>0.75</td>
<td>0.680</td>
<td>Beattie et al., 1995</td>
</tr>
<tr>
<td>G716Cc</td>
<td>0.21</td>
<td>U717A</td>
<td>0.21&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.02</td>
<td>0.044</td>
<td>Beattie et al., 1995</td>
</tr>
<tr>
<td>C662Cc</td>
<td>0.23</td>
<td>A661U</td>
<td>0.23&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.06</td>
<td>0.053</td>
<td>Beattie et al., 1995</td>
</tr>
</tbody>
</table>

<sup>a</sup> Hairpin ribozyme (numbering follows Butcher and Burke, 1994a,b).
<sup>b</sup> Hepatitis delta virus (numbering according to Makino et al., 1987).
<sup>c</sup> Neurospora VS ribozyme (numbering according to Beattie et al., 1995).
<sup>d</sup> No data available. The activity of mutant 2 is assumed to be equal to the activity of mutant 1.
Construction of the Fitness Landscape

Based on the foregoing general conclusions we propose a method to generate a fitness landscape for any ribozyme for which enough mutagenesis data are available. The result of the method is an algorithm which assigns a relative activity to each of the $4^N$ possible RNA sequences of length $N$. For the sake of simplicity we restrict the sequence space to sequences of a given length; however, the method could be applied if insertions and deletions were also considered. The algorithm consists of four steps: (1) compatible structure, (2) mispairs, (3) critical sites, and (4) predicted structure. In each step we calculate an activity value pertaining to the given step. The relative activity and the fitness of the sequence ($A_{\text{sequence}}$) is the product of the individual activities calculated at each step (that is, $A_{\text{sequence}} = A_{\text{structure}} \times A_{\text{mispair}} \times A_{\text{critical}} \times A_{\text{energy}}$).

### Table 3.2 Single null and multiple mutations that in some cases “rescue” enzymatic activity to some extent

<table>
<thead>
<tr>
<th>Mutation that abolishes activity</th>
<th>Multiple mutant</th>
<th>Relative activity</th>
<th>Ribozyme</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>A43C</td>
<td>A43C; G21U</td>
<td>0</td>
<td>Hairpin</td>
<td>Siwkowski et al., 1997a; Sargueil et al., 2000</td>
</tr>
<tr>
<td>G726A</td>
<td>G726A; A730C</td>
<td>0</td>
<td>HDV</td>
<td>Kumar et al., 1992</td>
</tr>
<tr>
<td>G726A</td>
<td>G726A; G727C; A731C</td>
<td>0.02</td>
<td>HDV</td>
<td>Kumar et al., 1992</td>
</tr>
<tr>
<td>G726A</td>
<td>G726A; G728A; C729G</td>
<td>0</td>
<td>HDV</td>
<td>Kumar et al., 1992</td>
</tr>
<tr>
<td>G726A</td>
<td>G726A; G727U; C729G</td>
<td>0.01</td>
<td>HDV</td>
<td>Kumar et al., 1992</td>
</tr>
<tr>
<td>G726C</td>
<td>G726C; G728U; A730C</td>
<td>0</td>
<td>HDV</td>
<td>Kumar et al., 1992</td>
</tr>
<tr>
<td>G726U</td>
<td>G726U; G727U; A731C</td>
<td>0.04</td>
<td>HDV</td>
<td>Kumar et al., 1992</td>
</tr>
<tr>
<td>G727C</td>
<td>G726A; G727C; A731C</td>
<td>0.02</td>
<td>HDV</td>
<td>Kumar et al., 1992</td>
</tr>
<tr>
<td>G727C</td>
<td>G727C; C729A; A731G</td>
<td>0</td>
<td>HDV</td>
<td>Kumar et al., 1992</td>
</tr>
<tr>
<td>G728C</td>
<td>G727A; G728C</td>
<td>0.03</td>
<td>HDV</td>
<td>Kumar et al., 1992</td>
</tr>
<tr>
<td>G728C</td>
<td>G728C; A731U</td>
<td>0.01</td>
<td>HDV</td>
<td>Kumar et al., 1992</td>
</tr>
<tr>
<td>G728C</td>
<td>G728C; A730G</td>
<td>0.06</td>
<td>HDV</td>
<td>Kumar et al., 1992</td>
</tr>
<tr>
<td>G728C</td>
<td>G727A; G728C; C729U</td>
<td>0.04</td>
<td>HDV</td>
<td>Kumar et al., 1992</td>
</tr>
<tr>
<td>C763A</td>
<td>C763A; A766G</td>
<td>0</td>
<td>HDV</td>
<td>Kumar et al., 1992</td>
</tr>
<tr>
<td>C763A</td>
<td>C763A; G764A</td>
<td>0</td>
<td>HDV</td>
<td>Kumar et al., 1992</td>
</tr>
<tr>
<td>C763G</td>
<td>C763G; A765U</td>
<td>0</td>
<td>HDV</td>
<td>Kumar et al., 1992</td>
</tr>
<tr>
<td>C763G</td>
<td>C763G; A765U</td>
<td>0</td>
<td>HDV</td>
<td>Kumar et al., 1992</td>
</tr>
</tbody>
</table>
3.2.3.1 **Compatible Structure**

In order to have any enzymatic activity the molecule should fold into the structure of the ribozyme. Here a number of alternative structures can also be considered.

A sequence is said to be compatible with a structure if for every base pair \((i,j)\) in the structure the bases at the \(i\)th and \(j\)th positions in the sequence can form one of the allowed base pairs (that is, A:U, U:A, G:C, C:G, U:C, G:U). Enforcing strict compatibility might result in an overestimation of the negative effects of mispair mutations. Some mispair mutants retain relatively high level of activity; for example the C662G mispair mutation in the VS ribozyme decreases activity to 23% of the wild type (Beattie et al., 1995). Thus, even sequences with partial compatibility should be considered compatible in this step. The negative effect of mispairs is taken into account in the next step.

If a sequence is not compatible – even when considering the possibility of mispairs – with any of the possible structures, then it has no activity and its fitness is set to 0. The activity factor for this step \(A_{\text{structure}}\) is the activity associated with the structure to which the sequence can fold. The activities of the various possible structures can be different.

3.2.3.2 **Mispairs**

When a sequence is perfectly compatible with a structure (that is, there are no mispairs in it) then \(A_{\text{mispair}} = 1\), otherwise every single allowed mispair decreases activity to some extent. Every mispair has an associated relative enzymatic activity \(A_{\text{mispair},i}\), and the activity factor for this step is the product of the activities of the individual mispairs: \(A_{\text{mispair}} = \prod A_{\text{mispair},i}\).

If synergistic effects are taken into account, then they should be incorporated in this step and/or the next step. Some regions of the ribozyme can show different sensitivity to mispairs, thus the associated relative enzymatic activity \(A_{\text{mispair},i}\) will be different.

3.2.3.3 **Critical Sites**

The nature of nucleotides at critical sites of the molecule is taken into account in this third step of the algorithm. These sites are well studied so we can nearly assign a measured activity to every possible nucleotide at these positions. In fact, all possible single mutants of the single-stranded regions of the hairpin ribozyme have been analyzed (Siwkowski et al., 1997; Shippy et al., 1998).

As before, the product of the individual activities \(A_{\text{critical},i}\) gives the activity factor for this step \(A_{\text{critical}} = \prod A_{\text{critical},i}\). If synergistic effects are taken into account, then they should be incorporated in this step and/or the previous step. Furthermore, if other epistatic effects were present, they would likely affect positions involved in this step.
3.2.3.4 Predicted Structure

The last step in establishing the fitness of a sequence is to predict the secondary structure the sequence will fold into and contrast it with the structure resolved in the first step. The folding can be done with any available RNA folding routine, as for example the MFold (Zuker et al., 1999) or the Vienna RNA Package (Hofacker et al., 1994). It has to be noted at this stage that the predicted minimum free energy structure of the wild-type ribozyme sequence does not always correspond with the actual secondary structure. In this case that structure can also be accepted as a good structure. Furthermore, if mispairs are allowed then they have to be taken into account during structure comparisons. When the predicted and the target structure are the same \( A_{\text{energy}} = 1 \), otherwise \( A_{\text{energy}} = 0 \). This step is undoubtedly the most costly in terms of CPU time.

3.2.4 Case Study: The Fitness Landscape of the *Neurospora* VS Ribozyme

The *Neurospora* VS ribozyme (Saville and Collins, 1990) can be used as a model system to show the usefulness of the algorithm in generating the functional fitness landscape of the molecule (Fig. 3.2). There is a wealth of mutagenesis information available for this ribozyme (Beattie et al., 1995; Lafontaine et al., 2001a,b, 2002a,b,d; Rastogi et al., 1996; Rastogi and Collins, 1998; Sood and Collins, 2002): out of the 144 positions in the ribozyme (stem–loops II–VI; nucleotides 640–783 according to the conventional numbering), 87 positions have documented mutants (excluding deletions and insertions) and the total number of mutants studied so far is 183.

The ribozyme has six regions (Fig. 3.2), where stem–loop I contains the cleavage site and is the substrate of the reaction, while regions II–VI perform the catalysis. The naturally occurring VS ribozyme is self-cleaving; however, it can be divided into a trans-acting ribozyme plus a substrate system (Guo and Collins, 1995). Unlike trans-cleaving versions of the hammerhead, hairpin, and HDV ribozymes, the VS ribozyme does not use long stretches of complementary base pairing to associate with its substrate. Nonetheless, the ribozyme–substrate interaction is quite tight (Guo and Collins, 1995; Lafontaine et al., 2001b). The pseudo-knot forming between the loops of the substrate and the stem–loop V of the ribozyme is crucial for substrate binding and orientation (Rastogi et al., 1996; Andersen and Collins, 2001).

3.2.4.1 Compatible Structure of the VS Ribozyme

The length of stem–loop IV and the distal part of stem–loop VI can be varied to a great extent. The length of stem–loop V can be varied slightly (Lafontaine et al., 2002b), but the length of stem–loop III cannot be changed (Lafontaine et al., 2002b). There are no data available on stem–loop II. Furthermore, the A718 bulge in region III can be complemented (that is, replaced by a base pair, with the insertion of U after position 660) without significant loss of activity (Beattie et al., 1995; Lafontaine et al., 2002b). These changes constitute the basis of
other acceptable structures beside the wild-type structure. Table 3.3 lists various structures and their activities.

In order to maintain the wild-type total length $N = 144$ constant, all structures including deletions or insertions were slightly modified in our study. Single-stranded regions were removed from or added to the beginning and the end of the structure in all possible combinations. We allowed some mispairs in the structure, but a mispair can only be considered if the following criteria are met (in what follows all numbers refer to positions in the wild-type sequence. In the alternative structures these positions change according to the shift due to the insertion or deletion):

- Two mispairs cannot be adjacent. There are six experimentally tested mutants that have mispairs at adjacent positions. Four of them have absolutely no activity ([G727C, U728A], [G722C, C723G], [G762C, C763G], [A759U, C760G]). In the case of the remaining two mutants ([G716C, U717A], [A661U, C662G]) the activities are 0.02 and 0.06, respectively.
- The pairs [653:771], [654:770], and [655:769] in stem–loop II have to be paired. The mispair mutants of the region ([G653C], [C771G], [G655C]) do not have measurable activities, except for the mutant [C769G] whose relative enzymatic activity is 0.18.
- The base pairs adjacent to a junction have to be paired. The stable forming of the junction is critical for enzymatic activity. If the bases next to a junction do not form a base pair, then the structure of the junction changes considerably and enzymatic activity would vanish. Accordingly, the following base pairs have to be paired: [658:721], [663:715], [666:685], [687:709], [722:763].

### Table 3.3 Enzymatic activities of different structures of the Neurospora VS ribozyme

<table>
<thead>
<tr>
<th>Structure</th>
<th>$A_{structure}$</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Original structure</td>
<td>1.000</td>
<td>Beattie et al., 1995</td>
</tr>
<tr>
<td>Deletion of the A652 bulge</td>
<td>0.013</td>
<td>Lafontaine et al., 2002b</td>
</tr>
<tr>
<td>Deletion of the A718 bulge</td>
<td>0.136</td>
<td>Lafontaine et al., 2002b</td>
</tr>
<tr>
<td>Pairing of the A718 bulge</td>
<td>0.820</td>
<td>Lafontaine et al., 2002b</td>
</tr>
<tr>
<td>Length 8 stem III</td>
<td>0.087</td>
<td>Lafontaine et al., 2002b</td>
</tr>
<tr>
<td>Length 7 stem V</td>
<td>0.045</td>
<td>Lafontaine et al., 2002b</td>
</tr>
<tr>
<td>Length 6 stem IV</td>
<td>0.470</td>
<td>Lafontaine et al., 2002b</td>
</tr>
<tr>
<td>Length 4 stem IV</td>
<td>0.590</td>
<td>Lafontaine et al., 2002b</td>
</tr>
<tr>
<td>Length 8 stem VI</td>
<td>0.970</td>
<td>Lafontaine et al., 2001b</td>
</tr>
<tr>
<td>Length 6 stem VI</td>
<td>1.000</td>
<td>Lafontaine et al., 2001b</td>
</tr>
</tbody>
</table>
A stem–loop cannot contain more than two mispairs. Generally, two mispairs in the same region greatly reduce activity (0.78 [G704C, U706A], 0.54 [U670A, C672G], 0.28 [A748U, U750A], 0.25 [A735U, U737], 0.07 [A690U, C692G], and 0.02 [G716C, U717A]) or abolish it completely (see the adjacent mispairs mentioned above or the mutant [G679C, A681U]). We assume that if a stem–loop contains more than two mispairs, then the resulting molecule would not have any enzymatic activity.

3.2.4.2 Allowed Mispairs in the VS Ribozyme

We have distinguished various kinds of different mispairs according to their position. First, a mispair next to the active site (that is, at positions [731:754] and [729:758]) decreases activity to $A_{\text{mismatch},i} = 0.025 \pm 0.011$. Second, a mispair next to the loop of stem V (at positions [695:701]) decreases activity to $A_{\text{mismatch},i} = 0.05$. Thus, mutants [U695G] and [A701C] have enzymatic activities of 0.06 and 0.04, respectively. Third, a mispair inside a stem decreases activity to 0.2.

The experimentally tested mispairs that do not fall into any of the previous categories have the following activities: 0.29 [C773G], 0.23 [C662G], 0.21 [G716C], 0.12 [G650C], 0.05 [A720U], 0.00 [U659A]. The mean activity of these mutants is $0.15 \pm 0.095$, but it should be stressed that these mispairs are located in important parts of the ribozyme. There are no experimentally tested mutants in the functionally less important parts, and it would be reasonable to assume a slightly less severe decrease of activity. Finally, a mispair next to the loops of stem IV and VI will probably not decrease activity to a great extent. For these mispairs we have assumed $A_{\text{mismatch},i} = 0.80$.

3.2.4.3 Critical Sites in the VS Ribozyme

Critical sites in the VS ribozyme are located in the junctions, in the substrate binding region, and in the A730 internal loop (Table 3.4). Thus, the two junctions of the ribozyme (3–4–5 and 2–3–6) play an important role in the formation of the 3D structure of the molecule (Lafontaine et al., 2001a,b). Some critical positions exist in both junctions: positions 656, 657, 665, 686, 710, 712, 713, 767 and 768. The other nucleotides of the junctions can be changed to some other nucleotide with minor loss of activity.

The substrate interacts with the enzyme through base pairing with bases 697–699 located in the loop at the end of region V. Therefore, these positions are also considered as critical for the function of the molecule. Finally, the internal loop of region VI (positions 730, 755, 756, and 757) shows the greatest susceptibility to nucleotide substitution (Lafontaine et al., 2001b; Sood and Collins, 2002) and virtually any change in the sequence leads to severe reduction in cleavage rate. This loop is quite probably the active site of the VS ribozyme. For those positions nucleotide A756 appears to be the most important, particularly the amino group at location 6 of the purine base (Lafontaine et al., 2002d).
3.2 Functionality Landscapes Inferred from Examples

Table 3.4 Activities of the critical sites in the *Neurospora* VS ribozyme

<table>
<thead>
<tr>
<th>Critical site</th>
<th>$A_{\text{critical,i}}$</th>
<th>U</th>
<th>C</th>
<th>G</th>
</tr>
</thead>
<tbody>
<tr>
<td>656</td>
<td>1.000</td>
<td>0.002</td>
<td>0.003</td>
<td>0.061</td>
</tr>
<tr>
<td>657</td>
<td>1.000</td>
<td>0.063</td>
<td>0.063^a</td>
<td>0.063^a</td>
</tr>
<tr>
<td>665</td>
<td>0.014</td>
<td>0.01^b</td>
<td>1.000</td>
<td>0.01</td>
</tr>
<tr>
<td>686</td>
<td>0.006</td>
<td>1.000</td>
<td>0.006^a</td>
<td>0.006^a</td>
</tr>
<tr>
<td>697</td>
<td>0.000</td>
<td>0.001</td>
<td>0.000</td>
<td>1.000</td>
</tr>
<tr>
<td>698</td>
<td>1.000</td>
<td>0.000</td>
<td>0.041</td>
<td>0.000</td>
</tr>
<tr>
<td>699</td>
<td>0.006</td>
<td>0.018</td>
<td>1.000</td>
<td>0.000</td>
</tr>
<tr>
<td>710</td>
<td>0.002</td>
<td>1.000</td>
<td>0.029</td>
<td>0.002</td>
</tr>
<tr>
<td>712</td>
<td>1.000</td>
<td>0.005</td>
<td>0.005^b</td>
<td>0.006</td>
</tr>
<tr>
<td>713</td>
<td>0.019</td>
<td>1.000</td>
<td>0.136</td>
<td>0.019</td>
</tr>
<tr>
<td>730</td>
<td>1.000</td>
<td>0.036</td>
<td>0.055</td>
<td>0.011</td>
</tr>
<tr>
<td>755</td>
<td>0.840</td>
<td>0.170</td>
<td>1.000</td>
<td>0.019</td>
</tr>
<tr>
<td>756</td>
<td>1.000</td>
<td>0.001</td>
<td>0.002</td>
<td>0.002</td>
</tr>
<tr>
<td>757</td>
<td>0.044</td>
<td>0.018</td>
<td>0.014</td>
<td>1.000</td>
</tr>
<tr>
<td>767</td>
<td>1.000</td>
<td>0.047</td>
<td>0.047^a</td>
<td>0.047^a</td>
</tr>
<tr>
<td>768</td>
<td>0.573</td>
<td>0.015</td>
<td>0.573^c</td>
<td>1.000</td>
</tr>
</tbody>
</table>

^a No data available. We assume that enzymatic activity is the same as for the known mutant.

^b No data available. We assume that enzymatic activity is the same as for the known mutant with the lower activity.

^c No data available. We assume that enzymatic activity is the same as for the known mutant with the higher activity.

### 3.2.4.4 Predicted Structure for the VS Ribozyme

The minimum free energy (MFE) structure of the original sequence of the VS ribozyme is not the experimentally determined secondary structure. The two structures differ in only three base pairs in stem–loop VI, and the energy difference between the two is a mere 0.3 kcal. But it has to be noted that the nuclear magnetic resonance structure of the isolated stem–loop VI is the same as in the MFE structure (Flinders and Dieckmann, 2004). Thus both structures might play an important role in the catalysis. Accordingly, we accept the sequence if it folds either to the MFE or the experimentally predicted structure of the wild-type VS ribozyme. We have used the RNA folding algorithm (Hofacker et al., 1994) for secondary structure predictions.

As previously indicated, the enzymatic activity of the molecule is simply the product of the activity factors estimated in the above four steps. If the resulting activity was less than the lowest activity that can be reliably measured (that is, $A_{\text{sequence}} \leq 10^{-3}$), then it was set to 0.
3.2.4.5 Properties of the Estimated Fitness Landscape for the VS Ribozyme

To characterize the fitness landscape some additional analyses were made. All possible one- or two-mutant molecules around the original sequence were generated and their fitness recorded. Molecules containing more than two mutations could not be completely enumerated in reasonable time, thus only one million randomly chosen sequences with 4, 5, 6, 7, 8, 9, and 10 mutations were evaluated (Figure 3.4).

Of the 432 possible single mutants, 114 (26.4%) had the same activity as the wild-type ribozyme (that is, they are “selectively neutral” neighbors), 222 (51.4%) had an activity higher than 0.1, and 254 (58.8%) still retained “measurable” enzymatic activity. Of the 92 664 possible double mutants only 6,579 (7.1%) had the same activity as the wild type, whereas 29 096 (31.4%) still retained some enzymatic activity (that is, for 68.6% of the sequences $A_{sequence} \leq 10^{-3}$).

In addition, we generated 10 million random sequences (a very tiny fraction of the sequence space, which contains approximately $4.973 \times 10^{86}$ sequences). None of them had any enzymatic activity.

3.3 Error Thresholds Inferred from Functional Landscapes:

The “Realistic” Error Threshold of the Neurospora VS Ribozyme

As previously pointed out, the formal description by Eigen (1971) of the mutation–selection dynamics of a population of biological sequences led to the realization of one of the greatest paradox of prebiotic evolution: the error rate poses a limit to the length of the information that can be selectively maintained within the system. A stable cloud of mutants (that is, a quasi-species) can form around a master sequence as long as the maximum chain length ($N$) is below the critical error rate per site per replication ($\mu^c$) as determined by the following expression:
\[ N < \frac{\ln s}{\mu^*} \]  

(3.2)

where \( s \) is the selective superiority of the master. Very roughly then, assuming as customary that \( \ln s \approx 1 \), Eq. (2) states that the maximum selectively maintainable amount of information \( (N) \) is about the inverse of the mutation rate per base per replication. Experimental evidence suggests that without the aid of peptide enzymes the upper bound of copying fidelity per nucleotide per replication could not be higher than 0.99 (Johnston et al., 2001), and is quite likely significantly lower than this figure. Accordingly, the maximum \( N \) would be lower than 100 nucleotides.

However, Eigen's model is based on the assumption that the whole genotype – the master sequence – has to be maintained for functionality. This assumption might be justifiable in a DNA-protein world, but in the RNA world the enzymatic activity of a molecule was mainly based on its three-dimensional structure rather than on the exact order of its building blocks (except for a few critical sites). It is a well-established fact that the number of possible RNA secondary structures is considerably less than the number of possible sequences (Schuster et al., 1994; Stadler and Haslinger, 1999), and it is also possible that two molecules with completely different sequences share the same secondary structure (Huynen, 1996; Huynen et al., 1996; van Nimwegen et al., 1999). In other words, when RNA structure is considered it might be feasible to maintain ribozyme functionality (phenotype) at mutational rates that would not allow the preservation of the master sequence.

In order to determine the “realistic” error threshold for the VS ribozyme we have explored the dynamics of a population of RNA molecules with \( N = 144 \) at various mutation rates per nucleotide per replication \( (\mu) \). At the replication step a sequence is picked at random according to its fitness. Thus, the probability \( p_j \) of choosing sequence \( i \) with enzymatic activity \( A_i \) is:

\[ p_i = \frac{A_i}{\Sigma A_j} \]  

(3.3)

where \( \Sigma A_j \) is the sum of activities for all sequences in the population. The next step is to copy the chosen sequence with error rate \( \mu \) (only point mutations were considered). Because a quarter of the times (assuming equal probability for each nucleotide) no effective change will occur in the position even though there is a mutational event, the effective mutation rate is \( \mu^* = 0.75 \mu \).

The new sequence then replaces a randomly chosen sequence, which allows keeping a constant population of molecules and is also equivalent to the assumption that the rate of degradation is the same for all molecules and independent of enzymatic activity (Bonhoeffer and Stadler, 1993). As a final point we should emphasize here that the occurrence of thresholds for error propagation was originally derived as a deterministic kinetic theory that is only valid in the limited case of an infinite number of molecules. Alves and Fontanari (1998) have extended it to finite populations and found that the critical error rate per site per
replication decreases linearly with $1/N$. In our present case we extrapolated to an infinite population size by recording the time to extinction (that is, the number of generations when no functional ribozymes remained in the population) at various error rates and fitting a straight line to those last few points which still showed a downward trend. The error threshold is then the intersection of the line with the error rate axis.

As shown in Fig. 3.5 the “realistic” error threshold for the VS ribozyme was estimated to be $\mu^* = 0.052$ (this figure refers to the “effective” mutation rate; see above). To compare this figure with the error threshold that would be obtained without considering secondary structure we have considered two different fitness landscapes: Mount Fuji and single-peaked landscapes.

In Mount Fuji landscape we have assigned an activity value to all possible nucleotides at a given position, with the wild-type nucleotides at each position having a value $A_i = 1$ for the enzymatic activity. For those positions where experimental data are available we considered that value, otherwise we either used the value for the same position derived for another nucleotide (if more than one such value were available, we used the lowest of the two) or used a predefined value. In the last case we considered two scenarios: those mutants have uniformly either $A_i = 0.8$ or $A_i = 0.2$. Therefore, in the first case we assumed that those positions are not functionally important, whereas in the second case they are. In no case can the enzymatic activity of the molecule be higher than 1. The fitness value of a
sequence was then calculated as the product of the individual activities, and the resulting “Mount Fuji error thresholds” were $\mu^* = 0.032$ ($A_i = 0.2$) and $\mu^* = 0.025$ ($A_i = 0.8$), which are substantially lower than the “realistic” error threshold from the functional landscape. Mount Fuji landscape retains some characteristics of the functional landscape – the fitness effect of the single-stranded regions – but it is no longer possible to have compensatory mutations by changing one base pair into another. In fact, every mutation affecting a helical region counts as a mispair, which causes the error catastrophe to occur at lower value.

For the single-peaked landscape we used the same assumption as in Eigen’s model. Thus, the wild-type sequence has $A_i = 1$ at each position and all other sequences have $A_i = 0.217$, which is the average activity of all experimentally tested one-point mutants. The “single-peaked error threshold” was found to be $\mu^* = 0.023$, lower than in either of the previous cases. The reason is that this landscape retains no information about the structure, and no neutral mutations are possible.

By using Eq. (2) above and assuming $\ln s = 1$, the maximal error rate for the VS ribozyme would be $\mu^* \approx 0.007$. This figure is nearly an order of magnitude lower than the one we got by using a realistic fitness landscape. Furthermore, according to the Eigen’s model the error rate of 0.052 would permit a ribozyme of maximum length 20 to be maintained. In summary, it is quite obvious that the inclusion of structural information, as well as information derived from experimental data, crucially alleviates the burden imposed by Eigen’s (1971). This is the first report of a realistic error threshold calculated for an existing ribozyme. To the best of our knowledge there is only one other indication of an error threshold calculated using a structural landscape. Huynen and co-workers (1996) used the secondary structure of the phenylalanine tRNA $(N = 76)$ as their object of investigation, and assumed that the fitness decrease of a mutant is proportional to the difference between the target structure and the structure of the mutant sequence. They reported that the error threshold for the tRNA$^{Phe}$ is 0.0031. This seems to be too low, as even the Eigen’s model predicts a higher error threshold ($\mu^* \approx 0.01$). This low value might be an artefact of the folding algorithm, which for tRNAs often predicts a minimum free energy structure unlike the known cloverleaf structures.

There is as yet no reported replicase ribozyme. The most promising result thus far is a ribozyme that can extend a sequence by 14 nucleotides according to a template (Johnston et al., 2001). This ribozyme works with a 0.967 copying fidelity. If a functional replicase ribozyme had the same fidelity, then it could replicate the VS ribozyme without the threat of the error catastrophe.

### 3.4 Looking for Catalytic Partners: Cofactors and Aptamers

The discovery that present-day living cells use RNA catalysts to hydrolyze RNA molecules or to perform the complex reactions of excision, ligation, and cyclization supporting a limited catalytic diversity, raises the question of the respective domains of RNA and protein catalysis. The recent advances in RNA catalysis
using the SELEX method make it possible to enhance the catalytic capabilities of RNA with small molecules as catalytic partners. In this way some RNAs may be analogous to protein in catalytic competence.

Purine nucleotides, and in particular those containing adenine, participate in a large variety of cellular biochemical processes (Maurel and Décourt, 1999). Their best-known function is that of monomeric precursors of RNAs and DNAs. Nevertheless, derivatives of adenine are universal cofactors. They serve in biological systems as source of energy (ATP), allosteric regulators of enzymatic activity and regulation signals (cyclic AMP). They are also found as acceptors during oxidative phosphorylation (ADP), as components of coenzymes (such as in FAD, NAD, NADP, coenzyme A; Fig. 3.6), as transfer agents of methyl groups of S-adenosyl-methionine, as possible precursors of polyrenoids in C5 (adenosylhopane) (Neunlist et al., 1987), and – last but not least – adenine 2451 conserved within the large rRNA in the three kingdoms, would be involved in catalysis during the formation of the peptide bond (Muth et al., 2000, 2001; Green and Lorsch, 2002).

On the other hand, biosynthesis of the amino acid histidine, which would have appeared late in evolution, begins with 5-phosphoribosyl-1-phosphate (PRPP) that forms N’-(5-phosphoribosyl)-ATP by condensation with ATP. This reaction is akin to the initial reaction of purine biosynthesis. Finally, the ease with which purine bases are formed in prebiotic conditions (Oró, 1960) suggests that these bases were probably essential components of an early genetic system. The nucleotides that by post-transcriptional modification can acquire the majority of functional groups present in amino acids possess a great potential diversity that is expressed in the modified bases of tRNAs and rRNAs and also at the level of ribonucleotide coenzymes (several coenzymes derive from AMP; Fig. 3.7). In particular many coenzymes are nucleotide analogs and the role of these cofactors at all steps of the current metabolism, and their distribution within the three kingdoms, suggests that a great variety of nucleotides were present in the last common ancestor. It as been suggested (White, 1976; Trémolières, 1980) that coenzymes and modified nucleotides that were present before the appearance of the translation machinery may have played a prominent role in primeval catalysis.

Proteins would have appeared only at a later stage, coenzymes and ribozymes being fossil traces of past catalysts. Indeed, in the living cell, only a minority of enzymes function without coenzyme; they are mostly hydrolases, and apart from this group, 70% of the enzymes require a coenzyme. If metal coenzymes involved in catalysis are considered, the number of enzymes that depend on coenzymes increases further. Present-day coenzymes, indispensable cofactors for many proteins, would be living fossils of catalysts of primitive metabolism (Maurel and Haenni, 2005).

Most coenzymes are nucleotides (NAD, NADP, FAD, coenzyme A, ATP, etc.) or contain heterocyclic nitrogen bases that can originate from nucleotides (thiamine pyrophosphate, tetrahydrofolate, pyridoxal phosphate, etc.). Consequently the efficiency of selected ribozymes can be further expanded if coenzymes and/or nucleotide analogs containing functionalities (thiols, amino groups, imidazolyl
Fig. 3.6 Coenzyme structures.
moieties, etc.) are used. Modern selections yield various co-assisted dependent ribozymes, justifying an RNA-based metabolism.

3.4.1
Co-ribozymes (cofactor-assisted ribozymes)

Aptamers are capable of recognizing targets as small as metal ions (most RNA enzymes are metallo-ribozymes using metal as cofactors). They can interact with a wide variety of molecules that are important for metabolism, including amino acids, porphyrins, nucleotide factors, coenzymes, small peptides, and short oligonucleotides (Illangasekare and Yarus, 1997; Jadhav and Yarus, 2002a; Joyce, 2002; McGinness et al., 2002; Reader and Joyce, 2002) (Table 3.5).

The first aptamer selected for a biological cofactor was an ATP-binding RNA (Sassanfar and Szostak, 1993; Fig. 3.8) showing a change in the conformation of the RNA and number of close contacts between the ATP and RNA.

Since the ATP motif also binds adenosine and NAD⁺, the idea was that it could serve to bind adenosine-derived cofactors as well. The presence of adenosine in many common biological cofactors (ATP, CoA, FAD, NAD⁺, SAM, coenzyme B₁₂) has been postulated to reflect an evolutionary origin for modern metabolism. It is even possible to consider that catalytic groups that were part of nucleic enzymes were incorporated in specific amino acids rather than being “retained” as coenzymes. This could be the case for imidazole, the functional group of histidine, whose present synthesis in the cell is triggered by a nucleotide (Maurel and Ninio, 1987; Benner et al., 1989; Maurel, 1992). Further, the use of organic cofactors was illustrated by a DNA enzyme that requires histidine as a cofactor during RNA cleavage (Szazani et al., 2004).

A small aptamer recognizing anionic moieties has also been obtained by Szostak and co-workers (Szazadni et al., 2004). In this case the significant interactions are with the phosphate of ATP (Kₐ of 5 μmol/L compared with the AMP Kₐ of 5.5

![Fig. 3.7](image)

List of coenzymes derived from AMP.

<table>
<thead>
<tr>
<th>Coenzyme</th>
<th>R</th>
<th>R’</th>
<th>R''</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Activated methionine</td>
<td>Methionine</td>
<td>H</td>
<td>H</td>
<td>O</td>
</tr>
<tr>
<td>Amino acid adenylate</td>
<td>Amino acid</td>
<td>H</td>
<td>H</td>
<td>1</td>
</tr>
<tr>
<td>Activated sulfate</td>
<td>SO₃⁻⁻⁻⁻</td>
<td>H</td>
<td>H</td>
<td>1</td>
</tr>
<tr>
<td>Cyclic 3'-5'-AMP</td>
<td>PO₃⁻⁻⁻⁻</td>
<td>H</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>NAD</td>
<td>H</td>
<td>H</td>
<td>2</td>
<td></td>
</tr>
<tr>
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<td>PO₃⁻⁻⁻⁻</td>
<td>H</td>
<td>2</td>
<td></td>
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<tr>
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<td>H</td>
<td>H</td>
<td>2</td>
<td></td>
</tr>
<tr>
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<td>PO₃⁻⁻⁻⁻</td>
<td>H</td>
<td>2</td>
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</tbody>
</table>
### Table 3.5  Small targets of RNA aptamers

<table>
<thead>
<tr>
<th>Target</th>
<th>Minimal size</th>
<th>$K_d$ ($\mu$mol/L)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenine</td>
<td>–</td>
<td>10</td>
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</tr>
<tr>
<td>Guanine</td>
<td>32</td>
<td>1.3</td>
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<td>–</td>
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</tr>
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<td>38</td>
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<td>Jenison et al., 1994</td>
</tr>
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<td>Caffeine</td>
<td>–</td>
<td>3500</td>
<td>Jenison et al., 1994</td>
</tr>
<tr>
<td>Riboflavin</td>
<td>35</td>
<td>0.5</td>
<td>Burgstaller and Famulok, 1994</td>
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<td>NMN/NAD</td>
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<td>d-ATP</td>
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<td>14</td>
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<td>Lorsch and Szostak, 1994</td>
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<tr>
<td>Biotin</td>
<td>31</td>
<td>6</td>
<td>Wilson et al., 1998</td>
</tr>
</tbody>
</table>

**Fig. 3.8**  ATP and the corresponding aptamer.
mmol/L). This is of particular interest as two messenger RNA riboswitches from *Escherichia coli* are known to discriminate between phosphorylated small molecules.

The first self-incorporation of a coenzyme into a ribozyme was performed in 1995 by Breaker and Joyce (1995), substituting neatly alternative coenzymes into the primary structure of group I intron by replacing the guanosine substrate with natural coenzymes or analogs (Fig. 3.9a and b respectively).

Looking for significant metabolic reactions and for coenzyme-dependent ribozymes, the primary biological cofactor used in acyltransfer reactions, coenzyme A (CoA), has been the target of RNA pools leading to a 52-nucleotide minimal aptamer (Burke and Hoffmann, 1998) which recognizes the adenosine moiety of CoA and binds others ATP analogs (Fig. 3.10). The selection of coenzyme synthetase ribozymes is of particular interest in an RNA-catalyzed energy metabolism. Yarus, exploring the origin of ribonucleotide coenzymes, demonstrated the RNA-catalyzed formation of three common coenzymes CoA, NAD, and FAD from their precursors, 4'-phosphopantetheine, NMN, and FMN, respectively (Huang et al., 2000). A ribozyme capable of utilizing CoA for the synthesis of acyl-CoA was selected in *vitro*. The co-ribozyme isolated, that is an acyl-CoA synthetase, produced acetyl-CoA and butyryl-CoA (Jadhav and Yarus, 2002b).

The use of organic cofactors has been illustrated by a hairpin ribozyme that requires adenine as a cofactor during RNA reversible self-cleavage reaction (Meli et al., 2003). This may lead to a better understanding of prebiotic cofactors in primeval catalysis. Our working hypothesis is based on the demonstration of
esterase activity in a nucleoside analog, the \( N^6 \)-ribosyladenine (Fuller et al., 1972; Maurel and Ninio, 1987). The activity, due to the presence of an imidazole group that is free and available for catalysis, is comparable to that of histidine placed in the same conditions (Fig. 3.11). We have studied the kinetic behavior of this type of catalyst (Ricard et al., 1996) and have shown that the catalytic effect increases greatly when the catalytic element, the pseudohistidine, is placed in a favorable environment within a macromolecule (Décout et al., 1995). Moreover, primitive nucleotides were not necessarily restricted to the standard nucleotides encountered today, and because of their replicative and catalytic properties, the \( N^6 \) and \( N^3 \) substituted derivatives of purines could have constituted essential links between the nucleic acid world and the protein world.

Following this line of investigation we started the selection of ribozymes dependent on adenine. Starting from a heterogeneous population of RNAs with \( 10^{15} \) variants (a population of \( 10^{15} \) different molecules) we have selected five populations of RNAs capable of specifically recognizing adenine after ten generations (Meli et al., 2002). When cloned, sequenced, and modeled, the best one among the individuals of these populations, has a shape reminiscent of a claw capable of grasping adenine. Following this result we have isolated from a degenerated hairpin ribozyme, by \textit{in vitro} selection, two adenine-dependent ribozymes capable of triggering reversible cleavage reactions (Fig. 3.12). One of them is also active with imidazole alone (Meli et al., 2003).

A quarter of classified enzymatic reactions are redox reactions involved in various biological events, such as metabolism of biological molecules, detoxification, energy production, and regulation of protein functions. An RNA molecule binding hemin and exhibiting a peroxidase activity has been reported (Travascio et al., 1999). In this case, RNA and DNA of the same nucleotide sequence are capable of forming comparable cofactor-binding sites promoting catalysis.
Recently, Tsukiji et al. (2004) reported a ribozyme that exhibits activity analogous to alcohol dehydrogenase (ADH). The ADH ribozyme was selected in vitro from a pool of random RNA sequences in the presence of NAD$^+$ and Zn$^{2+}$, based on the ability to convert a benzyl alcohol derivative to the corresponding aldehyde that was covalently attached to the 5' end of the RNA pool. In fact, the ribozyme oxidizes the benzyl alcohol to benzaldehyde in an NAD$^+$ and Zn$^{2+}$-dependent manner, with a rate acceleration at least 7 orders of magnitude higher than the uncatalyzed reaction.
3.4.2

Aptazymes

Joining aptamer and ribozyme yields aptazymes. Several examples of new aptazymes have been obtained from random-sequence RNA selections. Breaker et al. merged sequences of ATP aptamers into the hammerhead ribozyme to design allosteric ribozyme controlled by ATP (Tang and Breaker, 1997). The same idea was used by Araki et al. (1998) with the hammerhead ribozyme and a flavin-specific RNA aptamer. FMN binding affects the necessary conformation for catalytic activity. The aptazymes exhibit high activation such as this ATP-dependent ligase showing 830-fold activation and a theophylline-dependent ligase that shows 1600-fold activation (Robertson and Ellington, 1999, 2000).

These allosteric ribozymes composed of two independent structural domains can activate kinetic reactivity and regulation providing molecular switches made of RNA (Soukup and Breaker, 1999). Furthermore, these kinds of selected ribozymes can have allosteric responses that are orders of magnitude greater than those seen for protein enzymes. The allosteric selection strategy also provides novel RNA molecular switches responding to cNMP targets. For instance Koizumi et al. (1999) demonstrated specific activation of ribozymes cleavage with cGMP and cAMP exhibiting an 5000-fold activation in the presence of the effector compounds.

One of the most popular biotech applications of riboswitches is developing in gene therapy, allowing patients to take pills to switch genes on or off. On the other hand, natural aptamers are involved in riboswitch regulation of transcription termination and translation initiation in bacteria. Either FMN or thiamine acts on bacterial riboswitch RNAs located within the 5'-untranslated region (5'-UTR). Binding of the target ligand alters the conformation resulting in a change in gene expression (Winkler and Breaker, 2003). Recently Winkler et al. (2004) demonstrated that glmS ribozyme responds to glucosamine 6-phosphate (GlcN6P), a crucial compound in sugar metabolism in all three kingdoms of life and in cell wall biosynthesis in Gram-positive microorganisms, for genetic purposes. Also in prokaryotes, mRNA binds coenzyme B12 to modulate gene expression involved in cobalamine transport protein. The complexity of riboswitch structures is supported by numerous short helices and conserved sequences elements, allowing a great functional diversity in modern cells as well as possible diversity of ancient RNA functions.

3.5

The Use of Coenzymes:

From the RNA World to the Protein World via Translation and the Genetic Code

Although conclusive evidence for an RNA world does not exist, there is a widely shared belief that such a world did exist. The main obstacle to the acceptance of this appealing idea is that we do not understanding the origin of nucleic acid
replication of long templates (which would give unlimited hereditary potential: Maynard Smith and Szathmáry, 1995), despite some excellent results concerning the non-enzymatic replication of short pieces of nucleic acid analogues (von Kiedrowski, 1986), and exponential, non-autonomous amplification of somewhat longer oligonucleotides (the SPREAD procedure; Luther et al., 1998). It is the successful selection for aptamers and ribozymes (see various chapters of this book), more than anything else, that convinces people of the plausibility of an RNA world. In line with an early suggestion (Szathmáry, 1989, 1990b), it now seems that transition state stabilization is a crucial component of successful catalysis by ribozymes (Rupert et al., 2002).

In the previous section we gave an overview of coenzyme usage by aptamers (co-ribozymes). In this final section we comment on the evolutionary significance of these findings. We think, in the footsteps of White (1976), Korányi and Gánti (1981), and Gánti (2003a), that coenzymes have played a crucial link between the RNA and RNA–protein worlds. If we assume that coenzymes were already of significance in primordial metabolism in the RNA world, then it is difficult to see how they could have been widely replaced by any other molecules in metabolic evolution. One can replace RNA enzymes by protein enzymes (see Maynard Smith and Szathmáry, 1995, for a discussion) through evolution one by one, but any one coenzyme takes part in so many different reactions that unless there is a very strong selective force, replacement is unnecessary and/or unimaginable. The fact that ribozymes can make use of cofactors has been shown in a number of experiments. Roth and Breaker (1998) demonstrated the use of histidine in one DNA enzyme and recently, Tsukiji et al. (2004) showed that a selected ribozyme can catalyze reduction of an aldehyde using the NADH cofactor.

The replacement of ribozymes by protein enzymes must have been driven by the higher catalytic versatility of the latter (Benner et al., 1987), given the fact that the 20 amino acids offer significantly more functional groups than the four nucleotides (Table 3.5). Thus if such enzyme takeover indeed has taken place then modern metabolism is a “palimpsest” (Benner et al., 1989). Yet, inspection of Table 3.5 reveals that when complemented by coenzymes, the catalytic versatility of ribozymes approaches that of proteins, at least based on the diversity of functional groups. But of course it makes a big difference how often an enzyme needs such an aid. Presumably proteins in a rich metabolism need coenzymes less often and/or use them more efficiently than ribozymes.

It now seems that all critical steps of protein synthesis can be catalyzed by ribozymes, including amino acid binding (Famulok and Szostak, 1992) and activation (Kumar and Yarus, 2001), peptide formation, as it happens in contemporary ribosomes; Noller et al., 1992), or in artificial systems (e.g. Illangasekare and Yarus, 1999; Sun et al., 2002). The next logical question then is how and why amino acids have been introduced into the RNA world in the first place. We cannot review the entire relevant literature here, but rather briefly present our view here that amino acids were among critical coenzymes in the RNA world (Szathmáry, 1990c, 1993, 1996, 1999), and that proteins are evolved descendants of amino acid coenzymes.
Before one accepts this line of argument another question must be considered: why did evolution not raise the number of nucleotides in replicable templates? One constraint is replicability. For example, N6-ribosyl-adenine could be a chemically feasible substitute for the amino acid histidine (Maurel and Ninio, 1987; Décout and Maurel, 1993), but it could not be inserted into RNA by conventional replication. From the chemical point of view even replicable alternative base pairs do not look unfeasible: Piccirilli et al. (1990) have shown that alternative base pairs can be designed, synthesized, and incorporated into nucleic acids. Further additions to the genetic alphabet have also been proposed (reviewed by Szathmáry, 2003). To be sure, the requirement of replicability puts a severe constraint on the diversity of these novel building blocks, but it does seem reasonable to propose that increasing the size of the genetic alphabet in templates would also increase the size of the catalytic alphabet when such molecules are used as enzymes. A theoretical analysis predicts that the increase in catalytic efficiency of ribozyme-like molecules should be slower than exponential with the number of letters in the alphabet (Szathmáry, 1991, 1992). But this advance comes at a price: copying fidelity of these molecules should decrease faster than exponential, so there must be an optimal alphabet size, where the product of catalytic efficiency and copying fidelity (a measure of fitness, cf. Eigen, 1971) is maximal (reviewed in Szathmáry, 2003). Despite the technical difficulties involved, it would be important to test this prediction in experimental systems.

Assuming that the trade-off between replicative fidelity and enzymatic efficiency holds then the canonical solution to the problem of increasing catalytic versatility should come by the addition of coenzymes to ribozymes, rather than by increasing the alphabet size of templates (cf. Szathmáry and Maynard Smith, 1997). Once again there are two options. Coenzymes can be either covalently linked to ribozymes or they can be used by reversible binding. Most experimental attempts demonstrated the first option (reviewed by Jadhav and Yarus, 2002b) but we emphasize that this is more the result of the applied selection protocol, where selection on cells is not an option. Note that it is possible to obtain nucleic acid enzymes where cofactors are reversibly rather than covalently bound (Roth and Breaker, 1998; Tsukiji et al., 2004). In general we favor the reversible alternative, for the following reason. Imagine a ribozyme (R) catalyzing a step in metabolism, using a cofactor (C). If the cofactor is bound covalently (R–C), then the following constraints hold: (1) there must be a selective charging activity, either within the ribozyme or performed by another ribozyme, specific to the first ribozyme (R + C $\rightarrow$ R–C); (2) each cofactor molecule is stuck with its own ribozyme molecule; (3) coenzymes cannot be used as cosubstrates at various points in metabolism, since they are irreversibly linked to particular ribozyme molecules. The last point is perhaps the most important one. First, it holds for the contemporary nucleotide-like coenzymes that in any particular reaction they are not catalysts but cosubstrates: only the coenzyme coupling is catalytic, when the coenzyme is recycled by other reactions restoring its original state (Gánti, 2003a). Schematically it holds that A + C $\rightarrow$ B + C* and D + C* $\rightarrow$ E + C, where C and C* are the two states of the same coenzyme (e.g. NAD and NADH), and A, B, D, and E are metabolic intermediates.
ates. Suppose that a full reaction looks as follows: \( A + R–C \rightarrow B + R–C^* \). Because of the constraints on this ribozyme \( R \), specific for the \( A \rightarrow B \) conversion, it cannot catalyze the reaction \( D \rightarrow E \) that would restore the original state of its coenzyme part \( C^* \). Therefore, the only possibility for the coenzyme coupling would be a reaction such as \( R' + D + R–C^* \rightarrow R' + E + R–C \), where \( R' \) is a different ribozyme, specific for the \( D \rightarrow E \) reaction! We conclude that efficient use of coenzymes and the realization of coenzyme coupling require in general that coenzymes be reversibly bound by most ribozymes, where the latter are specific to different individual reactions in the network. (Coenzymes in the contemporary protein world work this way.) A good question still is whether we should find coenzymes such as \( C \) mostly in free form or bound to a nucleic acid moiety \( R'' \) (which, according to the above reasoning, in general need not be a ribozyme). In the scenario of White (1976) and Jadhav and Yarus (2002a,b) the favored alternative is an \( R–C \) option. This is consonant with the fact that typically the nucleotide parts act as “handles” (\( H = R' \)) only and do not take part in catalysis (Benner et al., 1987). A good reason for this solution is that RNA handles can be more readily recognized and bound by other RNA molecules than anything else (Szathmáry, 1990c, 1993). Thus, rather than forcing all coenzyme-using ribozymes to self-charge the non-nucleic acid part \( C \), they can bind the \( H–C \) molecule reversibly, for which there is only one synthetic reaction per coenzyme: \( S + H + C \rightarrow S + H–C \), where \( S \) is a coenzyme synthetase. Occasionally it may happen that the handle is self-charging (\( H + C \rightarrow H–C \)), but in general one would favor specific but as short handles as possible, which hence could not self-charge, therefore in general one would expect synthetases (\( S \)) to catalyze the \( H + C \rightarrow H–C \) reaction.

The link between these general considerations on coenzymes and the way out of the RNA world is that, according to our favored scenario, amino acids had been utilized as coenzymes of ribozymes before the genetic code was used in the coded synthesis of proteins in translation. As emphasized before, there is evidence that histidine can complement a nucleic acid enzyme (Roth and Breaker, 1998). Furthermore, various short peptides turn out to be useful in a nucleic acid context (Bergstrom et al., 2001; Viladkar, 2002). We urge for more experiments where the use of various amino acids as coenzymes of ribozymes can be tested.

The rationale for amino acids (aa) acting alone or linked to handles (\( H–aa \)), favoring the latter, is similar to the one revealed above for coenzymes in general (Szathmáry, 1993, 1996, 1999). One can imagine that amino acids are introduced into ribozymes via specific post-replicative modification. Suppose that a ribozyme \( R \) uses two different amino acids, \( aa_1 \) and \( aa_2 \), at two different points in the molecule. This would need two modifying ribozymes \( M_1 \) and \( M_2 \), specific for \( aa_1 \) and \( aa_2 \) and for \( R \), similar to tRNA modification after transcription (Björk, 1995). Even if another ribozyme \( R' \) uses the same two amino acids \( aa_1 \) and \( aa_2 \), a different pair of modifying ribozymes \( M_1' \) and \( M_2' \) would be needed to produce the functional form of \( R' \). Introduction of more and more amino acids into more and more ribozymes by post-replicative modification is therefore an impasse (Szathmáry and Maynard Smith, 1997).
A feasible alternative is to bind amino acids reversibly. The simplest known aptamer for isoleucine (Lozupone et al., 2003) is significantly longer than a short RNA hairpin, such as the anticodon stem and loop of tRNA, although the latter would be a specific and stable handle for a linked amino acid (Szathmáry, 1999), provided it would not have to self-charge. A small ribozyme self-charging Phe is 29 nucleotides long (Illangasekare and Yarus, 1999), which is also considerably longer than an anticodon stem–loop. Thus amino acids could be bound specifically and reversibly via oligonucleotide handles by the ribozymes using them. Stability and reversible binding of such handles would have favored an RNA hairpin (Szathmáry, 1999).

A consequence of the above reasoning is that the anticodon hairpin is likely to have been the earliest adapter. A remarkable other benefit is that genetic coding pops out for free: different handles could have been bound safely to the same amino acid, but charging of different amino acids to the same handles would have been forbidden. The origin of the unambiguous but degenerate nature of the genetic code lies in the use of such coding coenzyme handles (CCHs), according to our view (Szathmáry, 1990c). The crucial difference between the CCH hypothesis and the similar, in many ways, idea of Wong (1991) about selection for RNA peptidation is that in the latter coding does not naturally emerge due to the fact that oligopeptides rather than amino acids are assumed to be linked to RNA.

As discussed above, many aptamers that bind amino acids are self-charging, but this is not necessary, as shown by a series of remarkable experiments performed in Hiroaki Suga’s lab. First, they selected for a ribozyme that self-acylates at the 3’ end of its integral tRNA part (Saito et al., 2001a). The leader sequence at the 5’ end can be cut off by RNase P from this pre-tRNA, which in turn is able to aminoacylate the tRNA part in trans (Saito et al., 2001b). The cognate amino acid Phe is recognized by a U/A-rich (codonic/anticodonic!) region, whereas the 3’ end of tRNA is recognized by a triplet in the ribozyme. Perhaps even more interesting is the next series of experiments, in which a somewhat similar bifunctional ribozyme charging Gln was selected for (Lee et al., 2000). One domain of this ribozyme recognizes an activated glutaminyl ester and charges it to its own 5’-OH group (thus forming a covalent intermediate). The other domain binds tRNA and transfers the aminoacyl group to its 3’ end. The first domain can be separated from the other and can be significantly simplified. The glutamine-binding domain can be reduced to 29 nucleotides and can aminoacylate the other domain in trans (Lee and Suga, 2001). Interestingly, a paired cognate codon:anticodon triplet is essential in this domain for the recognition of Gln.

These experiments lend support to the idea that short adapter molecules (handles) could have been specifically charged by synthetase ribozymes (Szathmáry, 1993, 1999). Where on the adapter this was carried out is an open question. Woese (1967) and Szathmáry (1999) suggested that the primordial site for amino acid linking may have been nucleotide 37, immediately adjacent to the anticodon. Not only is this position universally modified, but several amino acids play a part in the process, in a way that makes sense in the light of scenarios about evolution of the genetic code (cf. Szathmáry, 1999). As suggested by
Wong (1991), primordial amino acids may have been N-linked, because the ester bond is too labile.

Surprising suggestive evidence came to light recently. In *E. coli* the tRNA<sub>Asp</sub> molecule is modified in its wobble position. The queuosine residue is glutamylated by a truncated version of glutamyl-tRNA synthetase (Blaise et al., 2004; Salazar et al., 2004) that recognizes the anticodon stem and loop of tRNA<sub>Asp</sub>. This finding raises the possibility of a role of other synthetase-related molecules in tRNA modifications as well as the possibility of similar activity in the RNA world, catalyzed by ribozymes. Testing this idea again calls for further experimentation.

We mentioned that, perhaps surprisingly, codons and/or anticodons tend to show up in aptamers selected to bind amino acids. Does nature want to tell us something or is this merely due to chance? The most recent analysis (Caporaso et al., 2005) favors the latter view. As was noted by Szathmáry (1999), it is not only codons but also anticodons that show up more frequently than random in selected aptamers. Indeed now the data set (including aptamers for Gln, Tyr, Leu, Ile, Phe, His, Trp, Arg) seems to be biased towards anticodons, as advocated in the CCH model (Szathmáry, 1993).

We note that there are still many puzzles around tRNA to be sorted out. Krzyzaniak et al. (1994) found that Phe and Met can be specifically charged to their cognate tRNAs in the absence of enzymes at high pressure. Should this result be confirmed, then it shows that at least these two tRNAs are barophylic aptamers, which raises interesting thoughts in connection with the deep-sea hot-spring scenario for the origin of life (reviewed by Hazen et al., 2002) and the need to select for extremophilic aptamers in general. Another odd result is that histidine and its anticodon GpUpG act similarly as catalysts in certain *in vitro* metabolic reactions (Shimizu, 2004). Clearly, it would be very important to test and possibly generalize these observations. We are content that many surprising results will come to light concerning aptamers and their role in evolution.

### 3.6 Outlook

Certain stages of evolution can only be reconstructed by inference from results of comparative and experimental analysis. The ultimate goal is to construct scenarios for certain critical transitions that are plausible and preferably make some predictions that were not part of the initial assumptions and that can be tested. Analysis of present-day living systems gives us strong hints that an RNA world in some form existed for a while.

Reconstruction of that world can only be partial, and should be rather based on experiments that prove certain principles. Aptamers eminently serve this purpose for the RNA world: the experimental results of their *in vitro* evolution can be justifiably called breathtaking. In this chapter we have addressed two key issues: how the fitness of RNA replicators is affected by mutations, and how coenzymes could have complemented the RNA world and how they, in a literary sense, could have
betrayed it by paving a way out of it into the protein world. None of these issues can be taken as settled, however. Although we have put forward a detailed analysis of the function landscape of an RNA enzyme, and have used this to infer the fitness landscape of RNA replicators, many more data are needed in order to be conclusive. In a similar vein, hypotheses centered on the evolutionary role of coenzymes should be more rigorously tested. More ribozymes utilizing cofactors, including various amino acids, should be selected for. Ribozymes that charge other RNA molecules with amino acids should be analyzed to see whether codonic or anticodon triplets predominate in the critical binding sites. Ultimately, some crucial steps in the takeover of ribozymes by protein enzymes need to be re-enacted in experiments and detailed models.

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Part 2

In Vitro Selection of Target-binding Oligonucleotides