



First published online as a Review
in Advance on October 15, 2007

Structures, Kinetics, Thermodynamics, and Biological Functions of RNA Hairpins

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Annu. Rev. Phys. Chem. 2008. 58:79–103

The *Annual Review of Physical Chemistry* is online at
<http://physchem.annualreviews.org>

This article's doi:
10.1146/annurev.physchem.59.032607.093743

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0066-426X/08/0505-0079\$20.00

Key Words

RNA folding, tertiary structure, RNA-protein interactions

Abstract

Most RNA comprises one strand and therefore can fold back on itself to form complex structures. At the heart of these structures is the hairpin, which is composed of a stem having Watson-Crick base pairing and a loop wherein the backbone changes directionality. First, we review the structure of hairpins including diversity in the stem, loop, and closing base pair. The function of RNA hairpins in biology is discussed next, including roles for isolated hairpins, as well as hairpins in the context of complex tertiary structures. We describe the kinetics and thermodynamics of hairpin folding including models for hairpin folding, folding transition states, and the cooperativity of folding. Lastly, we discuss some ways in which hairpins can influence the folding and function of tertiary structures, both directly and indirectly. RNA hairpins provide a simple means of controlling gene expression that can be understood in the language of physical chemistry.

Ribozyme: catalytic RNA molecules; smaller ribozymes (~30–80 nt) include the hepatitis delta virus, whereas larger ribozymes (~200–400 nt) include the *Tetrahymena* ribozyme

Riboswitches: RNA molecules that bind substrates (often small molecules) tightly and specifically

1. INTRODUCTION

This article concerns the structure, kinetics, and thermodynamics of RNA hairpins with an emphasis on their biological roles. When the central dogma of molecular biology was laid out, the role of RNA was subservient to that of DNA and proteins. In the ensuing years, our understanding and appreciation of RNA biology have undergone a revolution. It is now recognized that RNA is much more than a passive intermediary between DNA and proteins. RNA can both store genetic information and perform varied functions. These discoveries have led to the notion that RNA, or an RNA-like polymer, played a central role in the emergence of life on Earth—the so-called RNA world hypothesis (1–3).

One of the early breakthroughs in understanding the functional potential of RNA came when the structure of the first functional RNA was solved (4, 5). In the early 1970s the crystal structure of transfer RNA (tRNA) was determined, which revealed that RNA can assume complex three-dimensional shapes with a solvent-protected core. Because of this structural complexity, RNA can achieve extraordinary functional complexity. It is now recognized that RNA can make and break covalent bonds, in ribozymes (6), and bind small molecules with high affinity and specificity, in riboswitches (7). The biological functions of such RNA molecules include aiding virus replication, controlling gene expression, and synthesizing proteins. More recently, investigators have discovered additional functions for RNA, including the regulation of gene expression by microRNAs (miRNAs), in which the function is mediated through simple Watson-Crick base pairing (8).

The goal of this article is to provide an introduction to the structure, kinetics, and thermodynamics of RNA hairpins and their functions in biology. Because RNA often functions in a more complicated structural context than simple hairpins—e.g., engaging in tertiary interactions and binding proteins and metabolites—we emphasize biological and structural context. We also discuss how RNA secondary structures can themselves be quite structurally complex and have profound influence on the folding and catalysis of functional RNAs.

It is our intention that this article provides an entrée to RNA hairpins that is useful to the physical chemist. The thermodynamics and kinetics of RNA structure formation are emphasized, with a brief introduction to RNA hairpin structure. We focus on the natural biological and structural contexts of RNA hairpins with the hope that this inspires new physical chemical investigations to discern how hairpins contribute to RNA function and folding in nature.

2. STRUCTURE OF RNA HAIRPINS

To set the stage for kinetic and thermodynamic analysis, we begin with an introduction to the structure of RNA hairpins. There are many RNA hairpin structures available for downloading at the Protein Data Bank (<http://www.rcsb.org/pdb/home/home.do>) (9) and the Nucleic Acid Database (<http://ndbserver.rutgers.edu/>) (10). Much of the information in these two databases overlaps; however, the Nucleic Acid Database has search features that are nucleic acid specific and aid in the retrieval of hairpin structural data. The goal of this section is not to provide a comprehensive survey of

these hairpin structures, but rather to introduce some salient features of RNA hairpin structures to facilitate discussion of the folding and function of RNA. We refer the interested reader to an excellent review on hairpin structures (11).

The hairpin is the most common of the secondary structural elements in RNA. Nearly 70% of the nucleotides in 16S rRNA are involved in hairpin structures (12, 13). For DNA, hairpin structures are much less common, with a few exceptions, such as in the structures of single-stranded DNA viruses (14).

2.1. The Stem

In its simplest form, the hairpin consists of a stem and a loop and is often referred to as a stem-loop (SL) structure (**Figure 1**). In longer RNAs with multiple stem-loops (for example, in certain viruses), the notation SL1, SL2, and so on is used (15). The stem comprises primarily Watson-Crick base pairs (bp) formed between the two antiparallel stretches of RNA. In 16S rRNA, stems range in length from 1 to 10 or more bp, with an average length of 3–4 bp (12, 13). Structures with 1 bp are not populated in isolation and are stabilized in the context of flanking sequence by coaxially stacking of the stem onto an adjacent helix and engagement of the hairpin loop in tertiary interactions with nearby nucleotides (16). Hairpin structures with 2 bp, however, have been prepared and characterized structurally, thermodynamically, and kinetically (17–19); in these cases, a UNCG tetraloop (described below) was used to stabilize the structure. Such small stem-loops provide an optimal model system for detailed mechanistic study.

The stem of an RNA hairpin, similar to double-stranded DNA, is composed mostly of Watson-Crick base pairs and therefore is largely a right-handed helix (**Figure 1**).

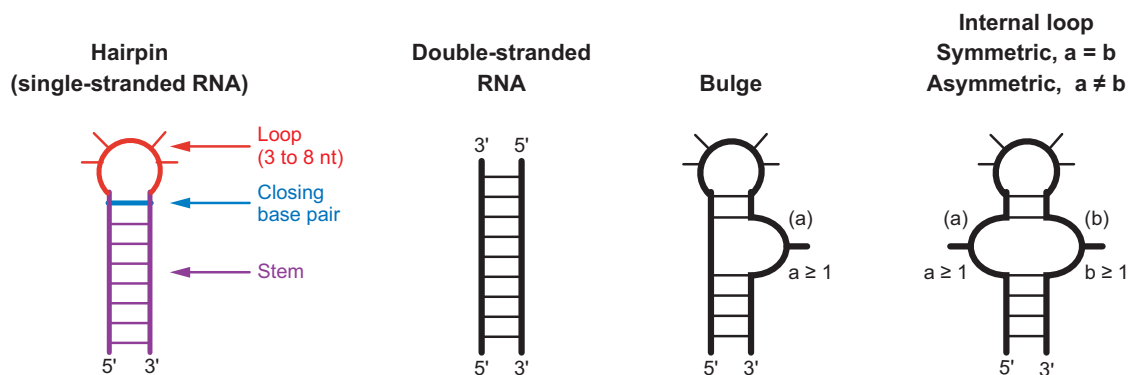


Figure 1

Components of RNA hairpins. The figure highlights the three components of hairpins: the loop, closing base pair, and stem. Also shown are common RNA secondary structure elements, which are defined by patterns of paired and unpaired nucleotides in a helical context. Hairpins are characterized by unpaired nucleotides at the tip of helical stem. Bulges and internal loops are characterized by unpaired nucleotides found in either one (bulge) or both strands (internal loop).

RNA**defects/imperfections:**

secondary structural elements other than Watson-Crick base pairing, including bulges and internal loops

Bulge: unpaired nucleotides on only one strand

Internal loop: unpaired nucleotides on both strands

RNP: RNA-protein complex

Tetraloop: a hairpin loop consisting of four non-Watson-Crick paired nucleotides

TGGE: temperature gradient gel electrophoresis

cUNCGg: example of notation for closing base pair and loop; the lowercase font denotes the closing base pair, and the uppercase the loop

N: any of the four bases (A, G, C, or U) is tolerated

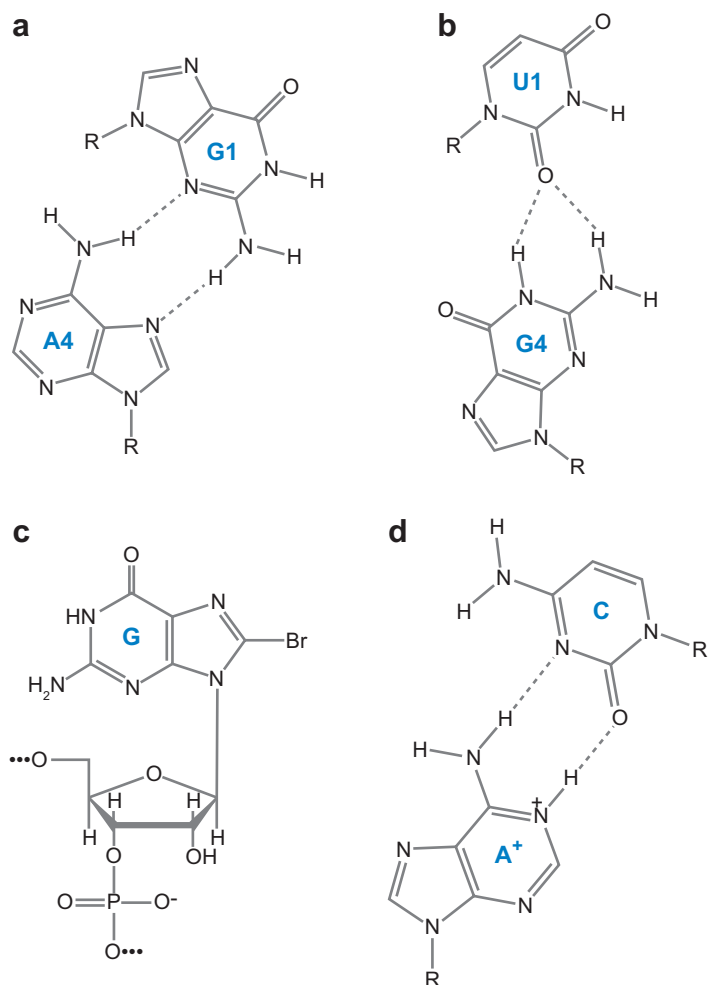
Here, however, the similarity between RNA and DNA ends. First, the geometry (A-form) of the double-stranded stem in RNA is different than in DNA (typically B-form). An RNA stem with only Watson-Crick base pairs has an accessible minor groove, containing the 2'-hydroxyl groups, and an inaccessible major groove, containing the majority of the sequence-specific information including Watson-Crick functionalities and the Hoogsteen faces of the bases (20). In more drastic contrast to double-stranded DNA, the stem of an RNA hairpin often contains non-Watson-Crick structural elements, including GU wobbles and single mismatches (21), and structural defects or imperfections, such as bulges and internal loops (**Figure 1**). A bulge contains one or more unpaired nucleotides on one strand, whereas internal loops contain one or more unpaired nucleotides on both strands. Symmetric internal loops tend to distort the helix less than bulges and asymmetric internal loops, which often kink or bend the helix (22–25). The unpaired nucleotides in the stem can engage in diverse structural interactions, such as noncanonical hydrogen bonding and stacking, which lend themselves to additional thermodynamic stability and functional diversity (26, 27). Defects also widen the major groove of the stem (28), making it accessible to drugs (29, 30), other RNA structural elements (31, 32), and proteins (33). It is the structural diversity of stems and loops that leads to complex tertiary structures, RNA-protein (RNP) assemblies, and functional diversity.

2.2. The Loop

The region of unpaired nucleotides at the apex of the structure is the hairpin loop (**Figure 1**). The most obvious property of the loop is to serve as the region in which the directionality of the backbone is reversed to afford the two antiparallel strands of the stem. Because of steric repulsion, it takes a minimum of three nucleotides to make a turn in an RNA hairpin, although loops of four nucleotides, so-called tetraloops, are much more common (12, 34, 35).

Certain triloop and tetraloop sequences are especially thermodynamically stable (32, 36–38), and these often correlate with sequences conserved in evolution, as determined through phylogenetic studies (34). In some cases, researchers identified stable RNA loop sequences by combinatorial approaches using temperature gradient gel electrophoresis (TGGE) (32, 38, 39). Stable RNA triloops and tetraloops are generally U rich, whereas C-rich RNA tetraloops are especially unstable (38, 40; P.C. Bevilacqua & M. Hauser, unpublished observations). Poor stacking in stretches of U residues and good stacking in stretches of C residues in the reference state (41) account for these effects.

Stable and phylogenetically conserved tetraloops include cUNCGg, c/gGNRAg/c, and gCUUGc (**Figure 2a,b**). Structures have been solved for these loops and reveal extensive stacking and hydrogen bonding between loop residues, as well as unusual conformations of certain nucleotides such as a *syn* conformation about the glycosidic bond for the G in the UNCG loop (42–46) (**Figure 2b**). In the case of UNCG and GNRA tetraloops, the second loop nucleotide (N) does not make contacts in the loop—hence its lack of conservation—but is required to relieve steric clashes. GNRA tetraloops form a sheared base pair between the first and last positions of the loop

**Figure 2**

Molecular interactions in RNA hairpins. (a) Sheared GA base pair formed in a GNRA loop. (b) Bifurcated hydrogen bonding in a UUCG loop. (c) 8-bromoguanosine conformer. Note that the base is in the *syn* conformation over the ribose ring to eliminate steric clashes with the bulky Br substituent. (d) Structure of protonated AC base pair.

(Figure 2a) and are especially adept at making tertiary interactions with other RNA motifs—so-called tetraloop receptors (31, 47)—and with certain proteins (48). TGGE selections identified an extended family of UNCG tetraloops referred to as YNMG (32, 39). Further studies revealed that YNMG loops form similar structures as UNCG tetraloops, although with reduced stability as compared to the UNCG subfamily (32, 49–52). This reduced stability appears to be essential for tertiary interactions with GNRA-like loops, possibly because certain YNMG loops have greater flexibility than UNCG loops (32, 53, 54).

Tetraloops are so stable and well-structured in RNA that other loops are often built from tetraloop platforms. For example, GNRA and UNCG tetraloops can be expanded at any position other than before the first nucleotide of the loop (55, 56; J.M. Blose & P.C. Bevilacqua, unpublished observations). In these cases, the structure

R: a purine, either A or G

Y: a pyrimidine, either C or U

M: either A or C

of the tetraloop remains largely intact with the expanded nucleotides extruded and available for interaction with proteins or other parts of a larger RNA molecule.

Lastly, it is worth noting that DNA hairpins can adopt especially stable triloops. This is likely because the sugar of DNA, without the 2' hydroxyl, prefers the C2'-endo conformation, which allows for tighter turns and different stacking interactions of the bases (57). In particular, the d(cGNAg) triloop is especially stable (58), forming a sheared GA pair between the first and last nucleotides of the loop, much like in the GNRA tetraloops in RNA (44). In addition, the d(GNA) loop can be expanded at any position other than before the first nucleotide in the loop (59–61), much like in RNA. Similarities exist between stable tetraloop sequences in DNA and RNA as well, as revealed by TGGE selections on DNA tetraloops (61).

2.3. The Closing Base Pair

The first base pair next to the loop is referred to as the closing base pair (**Figure 1**). Certain hairpin loop sequences prefer certain closing base pairs. For example, UNCG tetraloops prefer a CG closing base pair over a GC by approximately 2 kcal mol^{-1} in ΔG°_{37} (32, 36, 37), which is much greater than expected from nearest-neighbor effects in a standard A-form helix (62, 63). The GNRA tetraloop also prefers a CG closing base pair, but here the margin is only $1.3 \text{ kcal mol}^{-1}$ (56), in line with the common occurrence of a GC closing base pair for this loop sequence (34). Preference for a CG closing base pair over a GC holds for certain RNA triloops ($2.1\text{--}2.5 \text{ kcal mol}^{-1}$) (38), as well as for d(GNA) triloops and expanded triloops, in which the contribution can be especially large (3 kcal mol^{-1}) (59, 61, 64). Given the parallels in closing base pair preferences in RNA and DNA, as well as similarities in loop preferences, the molecular basis for closing base pair stability in the two nucleic acids is likely similar. It should be noted that only certain loops have closing base pair preferences, with many less structured loops, such as UUUU and AUAAUA, displaying a weak or nonexistent preference (36, 65).

Although more is known about stable hairpin sequences, there has been some investigation into unstable hairpins as well. Combinatorial TGGE selections revealed that unstable triloops tend to have UG and AU closing base pairs, as well as loop sequences poor in uracil (38). Melting experiments demonstrated that RNA hairpins with the loop sequence AUAAUA were least stable with UG, AU, and UA closing base pairs (65). Because G, A, and C tend to stack better than U (41; P.C. Bevilacqua & M. Hauser, unpublished observations), residual stacking in the reference state may be responsible for less favorable folding thermodynamics. Such unstable loop and closing base pair sequences could be important determinants of instability in RNA messages, as discussed below.

3. FUNCTIONS OF RNA HAIRPINS IN BIOLOGY

Our primary motivation behind studying RNA folding is to elucidate its role in biology. In this section, we address some of the functions that RNA hairpins perform in nature. A thorough review of the function of RNA hairpins in biology appeared

recently, and we refer the interested reader to it (66). Our chosen perspective is a structural one, moving along the hierarchy of RNA folding from unstructured, to base paired (hairpin), and finally to globular tertiary structures, which typically contain multiple hairpins. Because RNA molecules (as they occur in nature) range in size from ~20 nucleotides (nt) to thousands of nucleotides, the focus in each subsection below therefore pertains to the local structure of a given segment of a much longer RNA.

3.1. Functions of Unstructured RNA

RNA can function in biology when it has little or no self-structure. For example, noncoding RNAs, such as mature miRNAs, control gene expression by base pairing to complementary coding and noncoding regions on mRNA (8, 67). In these cases, function is mediated through simple Watson-Crick base pairing between two different strands of RNA.

In addition to base pairing with other RNAs, much of the unstructured RNA found in nature is complexed with proteins (68–70). In particular, unstructured RNA molecules can bind proteins in a sequence-specific fashion to effect biological functions such as splicing, development, and gene regulation (71–75). However, RNAs, or segments of RNA, that are unstructured in RNP complexes are not necessarily unstructured in the absence of their protein partners (73, 76), and such RNA unfolding events can play important roles in modulating gene expression, as discussed below.

3.2. Functions of Isolated RNA Hairpins

Although RNA can be found in nature without significant secondary structure, most RNA strands spontaneously fold into structures that offer additional layers of biological function and regulation (62, 63, 77, 78). Indeed, the local interactions in RNA are sufficiently stable that most RNAs contain substantial and quite stable secondary structures under physiological conditions (79). For example, the 4-bp hairpin, ggacU-UCGgucc, has a ΔG_{37}° of almost -5 kcal mol $^{-1}$ (32), whereas a longer 8-bp hairpin having a UUUU loop and a stem with approximately equal GC and AU content has a ΔG_{37}° of -9.4 kcal mol $^{-1}$ (80). Of course, longer hairpins with stable loop sequences have proportionately greater stability.

Some RNA molecules exert their biological functions when they contain only secondary structural elements such as hairpins, for example, in controlling transcription termination and gene expression (73). In these cases, it is often the relative free energies of overlapping and mutually exclusive hairpins on a nascent transcript that determine whether transcription of a given RNA is terminated or continued, and therefore whether a gene is expressed (81–84). One can compute free energy values for folding from an unstructured state to a given final hairpin structure using nearest-neighbor rules (77) and use a partition function analysis to calculate the relative populations of the hairpin states (85, 86).

As an added level of complexity, the relative population of these secondary structures can be kinetically determined in some instances (87–89). For example, during transcription the 5' end of the RNA exists prior to synthesis of the 3' end. As such,

kinetically stable but thermodynamically inferior hairpins can form and exert biological control even after more stable downstream RNA elements are transcribed. This is often found in the case of transcription attenuation (73). In such instances, binding to the RNA of allosteric effectors, such as small molecule-bound proteins or small molecules themselves (as in the case of riboswitches), is needed to switch the RNA conformation into that of the more stable terminator; moreover, such binding generally has to occur within a limited window of time to control gene expression (73, 87–89).

RNA molecules with secondary structures can also bind proteins in a sequence-specific (90) or nonspecific fashion, as in the case of the double-stranded-RNA-binding domain, which binds to the stem of hairpins (91). One important way in which RNA hairpins can exert their biological effects is by modulating the chemical stability of the inherently unstable RNA transcript.¹ Such RNA hairpins often participate in the regulation of mRNA levels by either preventing or, more rarely, promoting mRNA degradation. For example, investigators observed resistance to nuclease cleavage *psbA* mRNA transcripts in spinach chloroplasts. Mutants possessing 3' hairpins with a stable cUUCGg loop structure were more stable than their wild-type counterparts (92). In addition, exonuclease resistance was observed for transcript messages of the photosynthetic bacterium *Rhodobacter capsulatus*, in which short and long mRNA messages (decay products of the full-length *puf* operon) were protected from exonucleolytic cleavage by the presence of an intercistronic hairpin at the 3' portion of the short messages (93, 94). These observations have led to the design of synthetic oligonucleotides that contain 3'-terminal hairpins or form hairpins upon binding mRNA targets, protecting messages from exonuclease degradation (95). Hairpins can also protect mRNA transcripts when positioned 5' of the message. For example, a hairpin in the 5' UTR of the *ompA* transcript, which codes for a major outer membrane protein, serves to stabilize the mRNA, allowing it to be one of the most stable transcripts in *Escherichia coli* (96). Although the above examples present hairpins as stabilizing factors for mRNA, there is evidence in the literature that certain hairpins act in ways to promote the correct cleavage of mRNA. For instance, processing of the mRNA of ribosomal protein S20 in *E. coli* requires a hairpin to lock local structures and present the correct single-stranded regions for processing by RNase E (97).

In summary, RNA hairpins are especially important in controlling gene expression. Fundamentally, this is because RNA can switch between two conformations, one of which allows gene expression and one of which blocks it.

3.3. Functions of Hairpins in RNA Tertiary Structures

As we might expect, the RNA molecules that perform the most complex cellular functions—such as small molecule binding, protein synthesis, splicing, and tRNA maturation (6, 33, 98, 99)—typically adopt tertiary structures in which secondary

¹RNA molecules are inherently unstable because of the presence of the 2'-OH, which acts as an internal nucleophile to cleave the backbone locally. This strand scission can be promoted by high pH, high temperature, and divalent ions, as well as the presence of ribonuclease proteins.

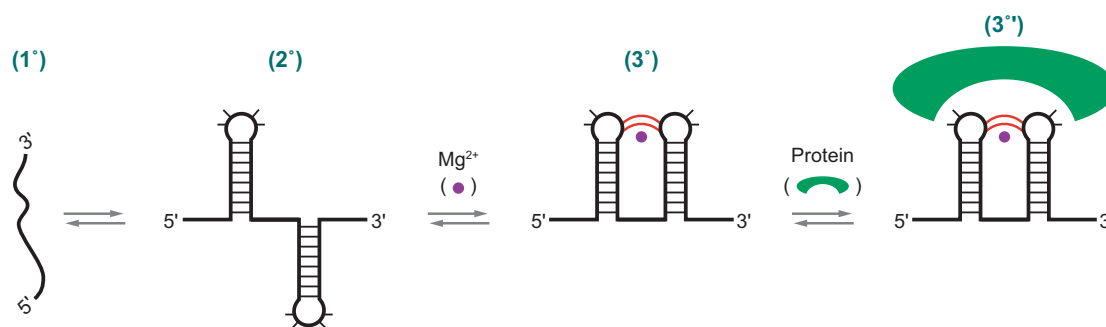


Figure 3

Hierarchical folding of RNAs with multiple hairpins. Multiple secondary structural elements can form in the folding of primary structure to secondary structure. In some cases the isolated hairpins may perform some biological function. In the presence of divalent metal ions (in particular Mg²⁺), secondary structural motifs can interact to form tertiary structures such as a loop-receptor interaction or this putative kissing-loop interaction. Finally, RNA tertiary structures can interact with other biological molecules such as proteins, as shown in the folding of the 3° complex. Folding tends to be hierarchical, as shown, with secondary structures forming first, and more complex structures assembling out of these.

structures interact with each other through space to give compact, solvent-excluded structures with binding pockets and active sites (100). In general, such RNAs fold in a hierarchical fashion, with tertiary structure assembling in an Mg²⁺-dependent fashion out of preformed secondary structures, including hairpins (101, 102) (**Figure 3**). These RNAs can interact with proteins as well, and often do so in a sequence-specific fashion in, for example, the ribosome and spliceosome RNP machinery (33, 103). Thus, it is important to understand the folding and function of RNA hairpins in these more complex systems as well. As described below, RNA hairpins can exert biological control in systems with tertiary structure through multiple means.

4. KINETICS OF RNA HAIRPIN FOLDING

The deepest mechanistic insight into RNA hairpins comes from understanding their folding kinetics. We begin with kinetics because this provides the most comprehensive description of hairpins. Once the kinetics of hairpin folding is understood, the thermodynamics, in principle, is known as well. In addition, because the folding of many RNAs is dominated by misfolding and trapping in which the minimum free energy is often not reached (104, 105), it is important to understand RNA folding kinetics. In this section, we provide an overview of how hairpins fold and some of the methods and models used to study and describe their kinetics.

As the majority of naturally occurring RNA comprises just one strand,² secondary structural elements typically form when an RNA molecule engages in intramolecular

²We refrain from referring to RNA as single stranded because it is composed of single-stranded and double-stranded regions. RNAs composed of two strands include double-stranded RNA viruses, such as reovirus and duplexes formed between miRNAs and their RNA targets.

RNA folding problem:
understanding and
predicting how RNA folds
from a random coil into its
native structure

interactions by folding back on itself—so-called fold-back structures. Understanding how RNA folds from a random coil to its native structure is referred to as the RNA folding problem. Although RNA molecules can adopt a wide array of secondary structures (including helices, hairpins, kissing loops, bulges, internal loops, hairpin loops, pseudoknots, and multistrand junctions), in almost every case the structural element either contains a hairpin or is contained within a hairpin. Thus, understanding the kinetics of hairpin folding is central to advancing our understanding of the overall RNA folding problem.

4.1. Models for RNA Hairpin Folding

The helix-coil transition is typically modeled by statistical mechanics. The three most common models, in order of increasing complexity, are the all-or-none model, the aligned (or zipper) model, and the staggered (or Zimm-Bragg) model (106). Using a definition of cooperativity as absence of well-populated intermediates (107), one can view the all-or-none model as the most cooperative and the staggered model as the least cooperative. For short RNA molecules that have one stable native state, the all-or-none model works quite well (18, 19) and forms the basis for the nearest-neighbor model of free energy prediction (see Section 5). For longer RNA molecules, especially those with long segments of AU base pairing interspersed with GC base pairs and structural defects, more states populate than the hairpin and random coil, necessitating the aligned or staggered models (108).

4.2. Measurements of RNA Hairpin Folding Kinetics

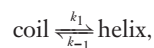
Many studies have been conducted on the process of double-helix formation of RNA and DNA, as summarized in References 106, 109, and 110. Because the kinetics tend to be very fast, a rapid kinetics approach is generally needed. A large number of studies have been conducted using temperature-jump kinetics, using a laser or discharge of a capacitor to rapidly heat the water (111–113).³ The laser method offers typical detection times on the order 10 ns, whereas the capacitor method has a detection limit near 1 μ s. Temperature jump is a perturbation method in which the system starts at equilibrium at a certain temperature (T_{init}) controlled by a thermostatted bath and ends at equilibrium at a higher temperature (T_{final}). Methods of duplex detection include absorbance and the detection of the hyperchromicity of unstacking, as well as fluorescence, optical activity, scattering, and conductivity (113).

The success of the method depends on the system having a nonzero enthalpy change over the temperature range of interest. RNA hairpin folding is well suited to this technique because enthalpy changes are typically large (114). It is the relaxation of the system in going from T_{init} to T_{final} that is observed. Typical association rate constants for duplex formation are on the order of 10^5 – 10^7 $\text{M}^{-1} \text{s}^{-1}$ and are fairly independent of duplex length and sequence composition (109, 110). The thermodynamic

³Rapid mixing techniques can be used for duplexes as well, but not for hairpins, so we do not discuss them here.

effects of temperature, oligonucleotide length, and sequence composition are primarily manifested instead in the rate of strand dissociation, which can be quite slow (on the order of days or years) for stable sequences. This property of duplexes (and hairpins) makes the prediction of the dissociation rate fairly reliable (41, 110).

In the simplest case of two-state kinetics for hairpin formation (19),



the observed relaxation rate, $1/\tau$, is given by the equation (112)

$$1/\tau = k_1 + k_{-1}. \quad (1)$$

Because there is no concentration dependence to the relaxation rate of hairpins, relaxation data alone are not enough to determine the values of the individual rate constants. Typically, the equilibrium constant for the helix-coil transition, K_1 , is determined using calorimetry or the temperature dependence of spectroscopic properties (so-called optical melting),

$$K_1 = k_1/k_{-1}, \quad (2)$$

which, in combination with Equation 1, then affords both rate constants. Relaxation lifetimes are generally on the order of 1–10 μs . These are sufficiently short that secondary structure forms before tertiary structure (milliseconds to minutes) in the folding of complex RNAs (102).

Estimates of the local concentration of one end of the RNA hairpin in the presence of the other are on the order of 0.1 M (106). This consideration, in combination with typical association rate constants for duplex formation of 10^5 – $10^7 \text{ M}^{-1} \text{ s}^{-1}$, offers a qualitative explanation for the observed relaxation rates in hairpins. As with duplexes, the effects of sequence and length on hairpin kinetics are manifested primarily in the dissociation rate constant.

Although the lifetimes of hairpin formation are short, they are actually much longer than the theoretical limit, which is on the order of 10 ns (110). In fact, this slow effect is manifested in nucleic acid systems as simple as the stacking of the bases in an RNA single strand, in which unfavorable activation entropies of -10 to -30 eu have been reported. These entropic effects likely represent the penalty of ordering the backbone in the nucleation step, although solvation and diffusion effects are possible as well (110, 115). Studies on a conformationally restricted hairpin—gcUUCGgc in which the *syn* conformation at the fourth position in the loop was constrained by substituting 8-bromoguanisine for G (**Figure 2c**)—led to fourfold faster rates of folding (18, 19), consistent with expectations for reduced entropy loss in this context (116). Folding of RNA and DNA hairpins with eight or more nucleotides appears to be slowed even further because stable nonnative structures of the loop and stem form along the reaction pathway (19, 117, 118).

Folding of hairpins in the context of larger functional RNAs is slower still. For example, folding of the hairpins in yeast tRNA^{Phe} is on the order of 10^3 – 10^4 s^{-1} (109). This may arise because the greater number of nucleotides allows for more alternative states to be explored at the secondary and tertiary structural levels. Single-molecule force pulling experiments have been applied to hairpins in the context of large RNAs

Optical melting: technique using the temperature dependence of spectroscopic parameters to determine the thermodynamic parameters of a folding process

as well, including the ~400-nt *Tetrahymena* intron and an ~80-nt subdomain (119, 120). These studies revealed multiple two-state transitions, intermediate states, and hysteresis effects, consistent with increased folding complexity in larger RNAs.

Stable loop sequences affect hairpin folding and unfolding rates. In the case of the UNCG tetraloop, researchers found that the CG closing base pair favored the native state by accelerating folding and decelerating unfolding, 5.2-fold and 3.2-fold, respectively (18). On the basis of these observations, it appears that certain stable tetraloop-closing base pair combinations could affect RNA folding by nucleating folding and steering it along certain pathways.

For the GAAA tetraloop, investigators substituted the fluorescent base 2-aminopurine into the loop at several positions and studied the loop dynamics. It was concluded that loop dynamics is slowed because of the restricted mobility of loop residues (121). The folding of GNRA tetraloops has also been approached computationally using stochastic dynamics simulations, and a three-state model for folding was advanced in which formation of the stem preceded loop formation (122). Recent studies in which the relative rates of hairpin formation are determined by a kinetic trapping method (123) concluded that stable GNRA tetraloops do not determine the pathway of RNA folding (124), although the hairpins studied had longer stems, up to 13 bp in this case. Thus, the effect of stable tetraloops on directing RNA folding may be dependent on the loop sequence or stem length and composition. In line with this notion, Woodside and coworkers (125) have demonstrated via pulling experiments that the transition state's position in the folding of DNA hairpins with long (20–30-bp) stems is dependent on local GC content and that intermediates populate upon the introduction of a single mismatch into the stem. Thirumalai and coworkers (126, 127) have used a coarse-grained off-lattice model to simulate the force-induced unfolding and force-quench refolding of RNA hairpins. They find that the position of the transition state is a function of the force of stretching and the rate of loading. Further experimental and theoretical studies are needed to understand fully the effect of hairpin loop and stem length and composition on hairpin folding dynamics.

As mentioned above, larger RNAs often switch between two hairpin conformations to control gene expression. Each hairpin can be quite stable, making the time for switching too slow to be on the biological timescale. However, in some instances it appears that intermediate structures transiently form that facilitate the formation of the final state. In two cases, the stems of two RNA hairpins have been proposed to coexist in a pseudoknot topology (82, 83), whereas in another case RNA hairpin intermediates, so-called guide helices, have been shown to form that are absent in the native state (128, 129).

4.3. Implications of Intramolecular RNA Hairpin Folding on Functional Complexity

We close this section with a brief consideration of the intramolecular nature of RNA hairpin folding. When one considers the enormous functional complexity of RNA as compared to DNA, a major contributing factor is that RNA is composed of just one strand that folds back on itself to form hairpins. Indeed, proteins, which have the

greatest functional diversity of all biopolymers, fold in a similar intramolecular manner, whereas DNA, which has no known enzymatic properties *in vivo*, does not. The vast majority of DNA in the cells comprises two strands engaged in Watson-Crick base pairing, which sequesters the majority of the functional groups and limits structural and functional diversity—a desirable property for a molecule whose function is to store genetic information.

RNA's one-stranded composition is arguably more important to its functional diversity than the presence of the 2' hydroxyl of the ribose sugar. Indeed when composed of just one strand in the laboratory setting, DNA has functional properties that rival those of RNA. For instance, single-strand DNA has been evolved to adopt complex structures that catalyze chemical reactions (130) and bind biological targets including thrombin and ATP (131, 132).

5. THERMODYNAMICS OF RNA HAIRPIN FOLDING

5.1. Models and Thermodynamic Parameters for Folding

The thermodynamics of RNA hairpin folding is generally ascribed to the cooperative two-state all-or-none model described in the previous section. This model ignores any intermediates in the folding process, which is reasonable thermodynamically if intermediates do not populate appreciably. This is probably a good assumption for most short hairpins wherein the entropic contribution of loop formation to folding free energy is so dominant near the melting temperature that intermediates with bulges, internal loops, or large hairpin loops are too unstable to populate (106). However, for hairpins with AU-rich helical termini (which tend to fray) or very long stems (>20 bp), a partition function approach that considers aligned and staggered populated intermediates is necessary (108, 125).

The thermodynamics of hairpin folding has been empirically determined largely by the optical melting of short oligonucleotides. A nearest-neighbor model has been applied to parse out thermodynamic terms for loop size, loop sequence, and closing base pair identity (62, 133–135). In some cases, the thermodynamic contributions of certain mismatches are explicitly accounted for, whereas in others a cassette free energy bonus is assigned, for example, in specific loop-closing base pair sequences such as cUNCGg and c/gGNRAg/c. It should be noted that even for favorable loop-closing base pair combinations, the contribution of the loop to overall folding is unfavorable (63). The nearest-neighbor model for hairpin formation has good predictive ability, generally estimating the correct stability to within 5%–10% of the measured value (63, 134).

5.2. Cooperativity in Hairpin Folding

Another issue that arises in the folding of hairpins is the cooperativity of loop and stem folding. DNA loops, which tend to have relatively fewer molecular interactions than their RNA counterparts, possess greater folding cooperativity, as determined from double and triple mutant cycles (56). High folding cooperativity is consistent with a

model wherein loop formation in DNA is a concerted process linked through a conformational change (60, 136, 137). The folding of related loops in RNA, conversely, is not concerted, which may be related to the much greater number of intramolecular contacts in most RNA loops, often mediated by the 2' hydroxyl (56). The cooperativity of stem folding does not apply to DNA only, however. Investigators have found intermediate-to-high folding cooperativity for 8-bp RNA stems in both internal and external contexts (i.e., near the middle and base of the stem, respectively), whereas folding cooperativity in DNA was high only in the external context (80). Thus, for RNA the stem appears to fold more cooperatively than the loop (56, 80).

5.3. Competing Structures

Lastly, we note that folding of hairpins, especially those with long stems or self-complementary loops, can populate states other than native and random coil, including self-complementary duplexes. This can complicate any attempted thermodynamic, kinetic, or structural studies, especially at the high strand concentrations required for crystallography (138) and nuclear magnetic resonance (32). Lowering the salt concentration in the solution can help favor the hairpin state, but this is not always possible or desirable. An alternative approach to favor population of the hairpin state is to destabilize the duplex state. The conformationally restricted nucleotide 8-bromoguanisine has been substituted for the *syn* G in the loop, which disfavored the competing duplex by 2.4 kcal mol⁻¹ and populated the hairpin conformer (139). Other structures can compete with the hairpin as well, including stacked helices, slipped base pairs, and misfolded loops (19, 76, 105, 117, 118).

6. INFLUENCE OF RNA HAIRPINS ON TERTIARY STRUCTURE

As discussed above, RNA folding generally occurs in a hierarchical fashion (101, 102) (**Figure 3**). This is a consequence of both the ~1000-fold faster rate of hairpin folding than tertiary structure formation and the fact that tertiary interactions typically require between one to two prefolded RNA structural elements. Because complex RNA function, such as catalysis, requires complex tertiary structure, it is reasonable to ask whether the structurally simpler RNA hairpins play any important roles in these more complex folds. We present three ways in which hairpins influence the activity of such functional RNAs: (*a*) by driving alternative folding of functional RNAs to affect the population of the native state, (*b*) by p*K_a* perturbations to provide enhanced functionality of nucleic acids, and (*c*) by affecting the thermostability of tertiary structure via thermodynamic linkage between the steps in hierarchical folding.

6.1. RNA Hairpins Can Drive Folding of Nonnative States

As discussed above, RNA is especially prone to adopting conformations other than the native state (105). Often such alternative or misfolded states are a nuisance to the experimentalist, especially when investigating the structure or properties of one

particular state (104, 105, 139). However, alternative pairings can also be desirable, and a number of RNAs appear to have evolved such pairings as a way to regulate biological activity. In the self-splicing *Tetrahymena* ribozyme, two mutually compatible RNA hairpins form upstream of the ribozyme and act to misfold the ribozyme and turn self-splicing off, whereas another mutually exclusive upstream hairpin competes with the other hairpins to switch the ribozyme back to the native fold (140, 141). Similar competition between upstream hairpins occurs in the hepatitis delta virus ribozyme (142). Likewise, many riboswitches involve terminator and antiterminator RNA hairpins in their expression domains that fold in ways to disfavor small molecule binding by the aptameric domain (7).

6.2. RNA Hairpins Can Form Motifs with Protonated Base Pairs

Another way in which hairpins can influence functional RNA activity is by presenting motifs with shifted pK_a values. The stems of RNA hairpins often contain AC mismatches (Figure 2d). These can form wobble pairs upon protonation of the N1 of A. Such bases typically have pK_a values near 6.5 (143, 144), although optimal nearest neighbors can shift the pK_a to neutrality (145). The primary driving force for pK_a shifting in systems as simple as hairpins appears to be linkage between protonation and folding of the stem (146, 147). The hairpin in these cases can present the protonated base as a possible proton transfer agent, or as a oxyanion hole (148). Stems in similar contexts may play important roles in RNA catalysis of the peptidyl transfer reaction by the ribosome (33, 148).

6.3. RNA Hairpin Stability Can Alter the Thermostability of Tertiary Structure

A third way that hairpins can affect RNA function is through thermodynamic linkage between the two steps in the hierarchy of folding. It has long been known that secondary and tertiary structure can form either cooperatively or separately, depending on solution conditions (149). If the folding of secondary and tertiary structure is cooperative, hairpins in the context of the native tertiary structure can be highly populated at temperatures well above their isolated melting temperatures. Under such conditions, strengthening secondary structure (by changes such as AU to GC base pairs) leads to a higher melting temperature of tertiary structure, or greater thermostability, as demonstrated in intramolecular folding triplex model oligonucleotides (150). This finding suggests a simple means by which greater thermostability can evolve in functional RNAs.

7. CONCLUSIONS AND OUTLOOK

Hairpins are the most common motif in RNA. They occur in virtually every RNA molecule and are often conserved throughout evolution. Although structurally simple in comparison to ribozymes and riboswitches, RNA hairpins encompass significant structural diversity in their own right. They can possess diverse loop and stem

structures and assemble into tertiary structures with active sites. RNA hairpins can regulate biological activity through the sequestration and exposure of recognition elements for proteins or small molecules, switching on or off functional elements, presenting protonated base pairs, or affecting the thermostability of tertiary structure. The folding of small hairpins is reasonably well-described by a cooperative two-state model, whereas more complex hairpins often have intermediates in their folding pathways, as well as transition states whose positions are determined by sequence content. Switching between two hairpin conformations often involves states with transient base pairing, which lower the activation energy of switching. Future studies are needed to fully understand these myriad processes in terms of reaction intermediates, energy barriers, and transition-state structures. Such physical studies will provide a deeper understanding of RNA's role in biology.

SUMMARY POINTS

1. RNA hairpins form when a single strand of RNA folds back on itself. Hairpins comprise three parts: a stem, which can contain non-Watson-Crick elements; a loop, which often has noncanonical pairing interactions involving hydrogen bonding and stacking; and a closing base pair, which can lead to extra stability for certain loop-closing base pair combinations.
2. Hairpins are most stable for loops of four, so-called tetraloops, especially for cUNCGg, c/gGNRAg/c, and gCUUGc. These loops dominate the stable hairpin sequences, with many larger loops simply being expansions of tetraloops. Such loops often engage with receptors composed of other hairpins or internal loops, which help build up large complex RNA structures.
3. The kinetics of RNA folding is fast, with assembly typically complete in 1–10 μ s. The folding of small hairpins is largely cooperative and can be described near the melting temperature with a two-state model. The kinetics of folding of larger hairpins can be non—two state, especially when defects are introduced into the helix. The transition state for the folding of such hairpins can also move along the helix depending on the pattern of GC and AU base pairing.
4. The thermodynamics of hairpin folding can be described by a nearest-neighbor model. Certain loop-closing base pair sequences are given a bonus free energy, although their overall contribution to folding is still unfavorable. RNA hairpin loops tend to fold less cooperatively than their DNA counterparts, although RNA stems fold as or more cooperatively than DNA stems.
5. Hairpins can make diverse and important contributions to the function of tertiary structures in RNA. Folding of hairpins can modulate the population of ribozymes and riboswitches, altering the expression of a given gene. The presence of AC mismatches can lead to protonated base pairs at neutral pH that can contribute to RNA catalysis. Finally, the strength of RNA hairpins can affect the thermostability of RNA tertiary structure.

FUTURE ISSUES

1. Little is known about how RNA folds in the cell. Folding during transcription can determine whether folding is kinetically or thermodynamically controlled. In addition, cellular conditions of crowding and RNA chaperones may have an effect on the folding landscape for RNA hairpins.
2. The kinetics of how RNA switches between two conformations is not well understood. Limited studies suggest the existence of transient structures with intermediate or alternative base pairing that facilitate switching. Little is known about how cellular conditions affect conformational switching.

DISCLOSURE STATEMENT

The authors are not aware of any biases that might be perceived as affecting the objectivity of this review.

ACKNOWLEDGMENT

We thank Dan Herschlag, Lois Pollack, Nate Siegfried, and Doug Turner for comments on the manuscript and helpful discussions. Funding was provided by NSF grant MCB-0527102. In addition, acknowledgment is made to the Donors of the American Chemical Society Petroleum Research Fund for partial support of this research.

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