ABSTRACT:
The application of real-time NMR experiments to the study of RNA folding, as reviewed in this article, is relatively new. For many RNA folding events, current investigations suggest that the time scales are in the second to minute regime. In addition, the initial investigations suggest that different folding rates are observed for one structural transition may be due to the hierarchical folding units of RNA. Many of the experiments developed in the field of NMR of protein folding cannot directly be transferred to RNA: hydrogen exchange experiments outside the spectrometer cannot be applied since the intrinsic exchange rates are too fast in RNA, relaxation dispersion experiments on the other require faster structural transitions than those observed in RNA. On the other hand, information derived from time-resolved NMR experiments, namely the acquisition of native chemical shifts, can be readily interpreted in light of formation of a single long-range hydrogen bonding interaction. Together with mutational data that can readily be obtained for RNA and new ligation technologies that enhance site resolution even further, time-resolved NMR may become a powerful tool to decipher RNA folding. Such understanding will be of importance to understand the functions of coding and non-coding RNAs in cells. © 2007 Wiley Periodicals, Inc. Biopolymers 86: 360–383, 2007.

Keywords: NMR spectroscopy; RNA folding; time-resolved NMR; photolabile caged compounds

INTRODUCTION

RNA Structural Transitions

RNA structural transitions are implied in a number of cellular events: Large amplitude structural transitions occur during RNA folding itself, i.e. the transition from an unstructured polynucleotide chain to its functional three-dimensional structure. Similarly, structural transitions are observed for the refolding of RNA structures involving a so-called conformational switch of one particular RNA conformation into another different one.
Such refolding includes changes on the level of secondary and tertiary structure as well as a change in function or the functional state of the molecule. Another type of RNA structural transitions involves the assembly of supra-molecular RNA structures such as RNA-small ligand and RNA-protein complexes as found in ribosomes or spliceosomes, in riboswitch RNAs and aptamers.

All types of RNA folding events occur on a wide range of timescales. The RNA folding events from unstructured states to folded structures are the fastest processes. The folding of secondary structure elements occurs between $\tau \sim 10$ and 100 $\mu$s. In contrast, the folding of tertiary structure elements is slower and has been reported to occur on a timescale of milliseconds ($\tau \sim 10$–100 ms). Generally, the kinetics of helix formation seems to be only marginally dependent on the exact nucleotide sequence. In thermal denaturation studies of tRNAs, the fastest monitored process was the folding-unfolding transition of the T-stem with a time constant $\tau \sim 10 \mu$s, followed by the folding of the anticodon helix that takes place with $\tau \sim 100 \mu$s. The formation of the tertiary contact between the D-loop and the T-loop occurred on a timescale differing by two orders of magnitude ($\tau \sim 10$ ms). The dissociation of the acceptor stem was found to take place within $\tau \sim 1$ ms. Though this event happens on the secondary structure level, it nevertheless represents an unfolding event.1

In contrast, RNA refolding events are slower and characterized by strong temperature dependence, in agreement with a high energy barrier between the two folded states. In the first kinetic studies of such processes, structural transitions were monitored between folding intermediates, also referred to as folding traps, into the fully folded forms of the RNA. For example, a partially folded tRNA$^{\text{ile}}$ folds upon addition of Mg$^{2+}$ towards the native conformation via a folded but non-native intermediate ($F_{\text{part}} \rightarrow F_{\text{inter}} \rightarrow F_{\text{native}}$).

At 10°C, the two processes revealed time constants of $\tau(F_{\text{part}} \rightarrow F_{\text{inter}}) \sim 15$ s and $\tau(F_{\text{inter}} \rightarrow F_{\text{native}}) \sim 150$ s with activa-
tion energies of \(E_A(F_{\text{part}} \rightarrow F_{\text{inter}}) = 27\ \text{kcal mol}^{-1}\) and \(E_A(F_{\text{inter}} \rightarrow F_{\text{native}}) = 33\ \text{kcal mol}^{-1}\), respectively.2

The structural transition of the equilibrium between two alternative pseudo-knotted forms of a sequence from the \(E.\ coli\ z\ \text{mRNA (}F_A \rightarrow F_B\) proceeds with time constants of \(\tau(F_A \rightarrow F_B) \sim 4\ s\) and \(\tau(F_B \rightarrow F_A) \sim 8\ s\) with activation barriers of \(E_A(F_X \rightarrow F_Y) = 34\ \text{kcal mol}^{-1}\) and \(E_A(F_Y \rightarrow F_X) = 46\ \text{kcal mol}^{-1}\) (measured at 41°C).3

The folding of RNase P,4 a multiple turnover ribozyme that cleaves off a precursor sequence of tRNA molecules, also populates a folded intermediate conformation \((U \rightarrow F_{\text{inter}} \rightarrow F_{\text{native}})\) along the folding pathway. The transition between the intermediate and the native conformation is described by time constants ranging from \(\tau(F_{\text{inter}} \rightarrow F_{\text{native}}) \sim 0.1\ s\) at 37°C up to \(\tau(F_{\text{inter}} \rightarrow F_{\text{native}}) \sim 63\ s\) at 3°C.5 The energy barrier between the folded intermediate and the native structure has been determined to be \(E_A(F_{\text{inter}} \rightarrow F_{\text{native}}) = 32–36\ \text{kcal mol}^{-1}\).

Model RNA Systems to Study RNA Folding

Ribozymes. Ribozymes, RNA catalysts, have been widely used as model systems to investigate RNA folding. Processes such as strand association, conformational reorientation of structural elements or of entire RNA domains, chemical reactions and strand dissociation are part of each chemical reaction catalyzed by ribozymes. Studying ribozymes in the context of RNA folding is advantageous, first, because the starting and the product state are distinguishable and, second, because the rate of the ribozyme catalyzed reaction can be readily measured as a function of external parameters including inhibitors. This strategy is reminiscent of studies in the field of protein folding, in which early studies were also carried out on enzymes.6

Riboswitches. Riboswitches are highly structured RNA genetic control elements found in the 5′-untranslated region (5′-UTR) of mRNA that influence transcription or translation by a conformational rearrangement of the RNA in response to direct metabolite binding. The riboswitch molecules consist of two domains, the so-called aptamer domain and the expression platform. After binding of a small molecule to the aptamer domain, the riboswitch changes its conformation. This refolding affects the conformation of the expression platform and thereby regulates gene expression. While the primary sequences of the aptamer domains are conserved, the expression platforms vary widely in sequence and structure.7,8 In fact, ligand binding can either lead to transcription termination by the formation of transcription terminating hairpins, abolishing ligand binding by sequestering the ribosome binding site or induce self-cleavage (→metabolite-responsive ribozyme).9

In the guanine-sensing riboswitch RNA for example, the structural transitions of the aptamer domain induced by binding of the ligand can be analyzed by high-resolution NMR spectroscopy.10,11

Two pronounced regions with distinct tertiary interactions could be observed. While the long-range base pairing interactions are already present in the free RNA and preorganizes its global fold, the ligand binding region is lacking hydrogen-bonding interactions in the free RNA and is therefore likely to be unstructured in the absence of ligand. Guanine mediates tertiary contacts and forces the RNA molecule to fold into a compact structure.12 Experiments on ligand-induced folding of the aptamer domain of the structurally homologous adenine-sensing riboswitch RNA reveal that folding in the presence of 10 mM Mg2+ (representing a 105 excess compared to the RNA concentration) occurs with half-life times between ~5 s (ligand/RNA ~ 5/1) and ~16 s (ligand/RNA ~ 0.25/1). The riboswitch folding reaction is determined to be exergonic \((\Delta G = -10.73\ \text{kcal mol}^{-1})\) and exothermic \((\Delta H = -40.3\ \text{kcal mol}^{-1})\) with a considerable loss of entropy \((\Delta S = -97.6\ \text{kcal mol}^{-1})\). In addition, FRET studies suggest that the control of gene expression depends both on the rates of the ligand-assisted folding and on the rate of RNA synthesis.13

RNA Folding in the Cellular Context

Within the cell, RNA folding and refolding events often take place co-transcriptionally. Structures can be formed that differ from the native fold through interactions of sequentially nearby nucleotides. Those non-native structures may constitute folding traps (Figure 2).

Transcription is divided into three distinct steps, initiation, elongation, and termination. Initiation includes binding of the polymerase to the promoter region of the DNA template strand. During elongation, RNA is synthesized in a processive manner. Transcription is then halted in the termination step and the newly synthesized RNA is released from the DNA template after dissociation of the transcription complex. During elongation, the newly synthesized RNA strand folds. Depending on cellular function, a RNA may have to undergo conformational changes in order to adopt
its native conformation. This may be especially important for non-coding RNAs, as has been shown for tRNAs. The rate of elongation, which mainly determines the transcription rate, therefore also affects RNA folding.

The rates of transcription and translation are synchronized, as the nascent RNA chain is directly screened and processed by ribosomes. The rate of transcription has been determined to be between 20–80 nucleotides (nt) s⁻¹ (for bacterial polymerases in general, and ~ 50 nt s⁻¹ for E. coli) and the rate of ribosomal protein synthesis has been found to be 10 peptide-bonds (pep-bnd) s⁻¹. The rate-limiting step of protein synthesis at the ribosome is the dissociation of EF-Tu·GTP from the ribosome. Simulation of the underlying kinetics depicted in Figure 3 shows that polynucleotides of considerable length, representing autonomously foldable RNA motives, are transcribed but not yet translated and can potentially fold into different non-native conformations (Figure 3).

RNA folding is not only dependent on the rates of translation and transcription but, in addition, other factors such as transcriptional pause sites affect the timing of the folding process. The nascent RNA chain can interact with proteins influencing the folding, such as RNA chaperones or the polymerase itself.

How pause sites and RNA binding proteins can alter the folding pathway of RNAs has been investigated for the folding of RNase P and the NusA protein (Figure 4). The folding of this RNA with its two domains (domain 1 is the specificity domain consisting of 154 nucleotides and domain 2 is the catalytic domain with 255 nucleotides) in the absence of the protein follows a two-step process, in which an intermediate is formed first. This intermediate consists of a natively folded catalytic domain and an unfolded specificity domain. While the entire folding event is completed after ~ 400 s, the intermediate is maximally populated at ~ 100 s. The folding rate is independent of the general rate of transcription when compared to the rate of transcription.

FIGURE 3 Simulation of the free mRNA during transcription and translation: The transcription length (dashed lines) and the length of free nucleotides (solid lines) are given assuming a gene of a total length of 1000 nucleotides and a transcription rate of 20 nucleotides (nt) s⁻¹ (green), 50 nt s⁻¹ (blue), and 80 nt s⁻¹ (red), the rate of translation at the ribosome is fixed to be 10 peptide-bonds (pep-bnds) s⁻¹ (= 30 nt s⁻¹). For transcription rates of 20 nt s⁻¹, the translation is no longer rate limiting and the newly synthesized RNA is directly translated into peptides.

FIGURE 4 Schematic representation of the folding pathways of RNase P in the absence (upper panel) and presence (lower panel) of the NusA protein. The domain architecture (C, catalytic domain; S, specificity domain) of the RNase P is symbolically represented with circles and squares (circles represent unfolded and squares folded domains). The time course of the population for each conformation (U: unfolded (red line); I₁,₂: intermediates (green line); F: native/ folded (blueline)) is given according to a simple two-step process and calculated for the experimentally derived rates as indicated.

FIGURE 5 Initiation and detection techniques that cover (partially) the range of the RNA folding timescales.
Biophysical Methods to Study RNA Folding

RNA folding occurs over a wide range of timescales and therefore also a wide range of methods have to be applied in order to study these processes (Figure 5). For the initiation of the folding reaction mainly three methods are applied: changes in pH,\textsuperscript{21} in temperature\textsuperscript{22,23} or in ionic strength.

For the very fast processes (in $\mu$s-scale) such as hairpin formation, only the temperature-jump (T-jump) method is fast enough to allow for a kinetic characterization. Folding events on slower timescales of milliseconds to seconds can be initiated by salt- or pH-changes.

Methods for the detection of RNA folding include UV-folding pathway; these methods are time-resolved OH- footprinting,\textsuperscript{25} chemical base modification\textsuperscript{26} and UV-crosslinking.\textsuperscript{27} While the first two methods probe the accessibility of the RNA backbone is determined from its susceptibility to phosphodiester bond cleavage in the presence of hydroxyl radicals. The hydroxyl radicals are generated by 10–30 ms exposures to a synchrotron X-ray beam. Sequencing allows then to determine the fragmentation pattern of the RNA. This provides information about the regions of the RNA that have been folded in the elapsed interval since the start of the folding reaction.

With a temporal resolution of 0.5–1 min, competitive DNA hybridization, designed to bind to single-stranded RNA and subsequent RNase H digestion, allows monitoring of structural transitions during RNA folding.\textsuperscript{28,29}

If the structural transition is very slow (in tens of minutes to hours), native gel electrophoresis is applicable. Nowadays, temperature gradient gels represent a further development of this technique.\textsuperscript{30} FRET is an additional method to measure the structural dynamics of RNA during folding in real-time\textsuperscript{31}; this technique provides temporal resolution sufficient to monitor events that happen on a millisecond timescale. The energy transfer efficiency between the donor and the acceptor dye covalently attached to target RNAs depends on their intermolecular distance and orientation, making FRET sensitive to conformational changes of the RNA molecule.\textsuperscript{32}

TIME-RESOLVED NMR SPECTROSCOPY

Nuclear magnetic resonance spectroscopy (NMR) is a powerful technique to study reversible and irreversible reactions as well as conformational transitions. The fastest timescales for such irreversible reactions that have been successfully characterized by NMR had reaction rates of $k \sim 10 \text{ s}^{-1}$,\textsuperscript{33–35} The combination of atomic resolution applicable to relatively fast kinetics cannot be achieved by any other biophysical method. Dynamic events of biomacromolecules in this time range are large scale conformational transitions, folding events (for proteins) and macromolecular assembly.

In order to monitor these events, two conditions have to be fulfilled; (i) the initiation of the conformational transition needs to be fast and (ii) NMR techniques need to be optimized to achieve maximum time resolution.

In this review, we will describe the investigation of irreversible conformational transitions in RNA systems. In these experiments, a conformational nonequilibrium is rapidly generated and recovery of the RNA system towards equilibrium is monitored by NMR spectroscopy.

The current developments in NMR spectroscopic methodology to study RNA folding benefit from prior developments in the field of protein folding. In protein folding, information under equilibrium conditions have been derived from native state hydrogen exchange,\textsuperscript{36} line-shape analysis,\textsuperscript{37,38} and relaxation dispersion experiments.\textsuperscript{39,40} However, these methods are restricted to the study of very fast folding transitions occurring on the $\mu$s-ms timescale. Additional experimental approaches similar to stopped-flow experiments studying protein folding under nonequilibrium conditions have been developed. Important experiments include muni-
monitoring the folding transition by NMR methods either indirectly using hydrogen exchange pulse labelling or even directly in real-time one-dimensional (1D), two-dimensional (2D), and three-dimensional (3D) NMR experiments.

**Folding Initiation**

**Photolabile Caged Compounds.** Using photolabile, so-called “caged”, compounds a particular conformational state of a RNA can be trapped. Upon illumination, the photosensitive “cage” can be released and a cascade of conformational transitions can be initiated.

In the case of RNA folding, the following caging strategies have been exploited thus far to generate a trapped state (see Figure 6):

1. of the entire RNA (see Caging RNA section) (Figure 6a)
2. of a reactive functional group of the RNA, e.g. the 2'-OH (see Caging RNA 2'-OH section) (Figure 6d)
3. of a ligand that binds to RNA (see Caging of low-molecular-weight ligands section) (Figure 6b)
4. of divalent ions, required for RNA folding (see Caging of divalent metal ions section) (Figure 6c)

**Caging RNA.** In proteins, generally the native conformation is significantly more stable than alternative conformations. In contrast, a given RNA sequence can adopt several conformations of similar free energies. Equilibria between

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**FIGURE 6** Examples of different protecting strategies: (a) the Watson–Crick side can be masked by photolabile groups either of the RNA or of ligands binding to the RNA (b). In addition, metal ions inducing RNA folding can be caged (c), and reactivity exerted by the 2'-OH group of the RNA as in the case of ribozymes can be protected (d) (for further explanations, see text).

**FIGURE 7** Example of bistable RNA sequences which exist in two conformations at room temperature. By introduction of a photolabile protecting group (Ghv or Uhv) at a proper sequence position, exclusively one conformation can be stabilized. Irradiation with laser light releases the photolabile protecting group in a traceless manner, and the equilibrium between conformation “A” and conformation “B” can be reestablished.
hairpin and duplex folds, which often depend on the absolute RNA concentration as well as on the salt concentration, are a common source of conformational heterogeneity in RNA. RNA sequences that can adopt two conformations with small differences in free energy have been coined bistable RNAs. In bistable RNAs, one of the two possible conformations can be caged. The idea relies on the concept that a photolabile protecting group can be introduced by chemical synthesis at any sequence position that is critically involved in the selective stabilization or destabilization of one of the two bistable RNA folds.

As a first example to study RNA folding by time-resolved NMR, such an idea was followed by the groups of Pitsch and Schwalbe. Here, single nucleotides have been substituted with the photolabile protecting group 1-(2-nitrophenyl)ethyl (NPE), caging the Watson–Crick site of the nucleotides in order to impair the formation of selected base pairs (Figure 7). The difference in free energy of both conformations in the equilibrium between conformations “A” and “B” of the 20mer RNA sequence is much smaller ($\Delta G = -0.6$ kcal mol$^{-1}$) than in the case of the photo-protected sequences “caged A” and “caged B”. In the latter case, the introduction

FIGURE 8 Structural representation of (left panel): guanosine nucleotide carrying the NPE photolabile protecting group that masks the Watson–Crick side; (right panel): loop nucleotides of GNRA tetraloop, imposing that at the position of the apical nucleotide N the introduction of the protecting group does not perturb the three-dimensional structure of the loop.

FIGURE 9 Structure selection by introduction of a photolabile protecting group monitored by 1D $^1$H NMR spectra: The imino region of the bistable 20mer RNAs exhibits the signals of both conformations (upper panel), whereas upon introduction of the photolabile protecting group the sequences are exclusively stabilized in conformation “A”.

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of the photolabile protecting group increases the free energy of conformation “B” so that conformation “caged A” is exclusively populated. Disruption of the base pair G6-C19 destabilizes conformation “caged B” by $|\Delta G| = 3.4$ kcal mol$^{-1}$.

The bulky and photolabile NPE group disrupts Watson–Crick base pairs by steric effects, as it completely camouflages the Watson–Crick side and changes the hydrogen-bond donor and acceptor pattern (Figure 8). Similar photolabile protecting groups have been introduced for U-nucleosides, for A- and C-nucleosides and for DNA molecules.

Figure 9 shows the NMR spectra of the bistable RNA sequences (introduced in Figure 7) in their caged conformation and in the conformation after photolytic cleavage, respectively. A rapid and traceless removal of the photolabile protecting group allowed initiation of the refolding process.

The concept of “caged RNA” can also be extended to larger systems. NMR studies of the P5abc system revealed that folding occurs only in the presence of 5 mM Mg$^{2+}$ and the crystal structure identified the exact binding sites of the ions. Hence, the bulky photolabile protecting group NPE was introduced at a sequence position involved in Mg$^{2+}$-binding (B. Fürtig, P. Wenter, S. Pitsch, H. Schwalbe, unpublished results) (Figure 10).

**FIGURE 10** Left panel: secondary structure of the magnesium induced folding event of the P5abc RNA molecule. Addition of Mg$^{2+}$ (yellow balls) leads to a change in tertiary as well as secondary structure; introduction of a photolabile protected nucleotide ($G_{hv}$; shown in red) at a position close to a magnesium binding site (see right panel: crystal structure of the folded P5abc element; again yellow balls represent Mg$^{2+}$; red nucleotide G at position, where $G_{hv}$ was introduced) should arrest the molecule in the “unfolded” state.

**FIGURE 11** Imino proton region of 1D $^1$H spectra of the 56mer P5abc RNA. The spectrum of the RNA containing the photolabile protecting group without Mg$^{2+}$ (293 K) displays the signature of the “unfolded” conformation (indicative for this conformation is the strong signal of U142 at 14.2 ppm) (left panel); after addition of 20 mM Mg$^{2+}$ the spectrum changes, but does not exhibit the typical reporter signals for a complete conformational transition of the “folded” state (empty circle) (mid panel); after photolytic cleavage of the protecting group, the spectrum shows the complete signature of the “folded” conformation (here the indicator is the now shifted signal U142 at 13.9 ppm—signal in the red circle) (right panel).
Since the magnesium-free and -bound states of the RNA show significantly different NMR spectra, comparison of the photo-protected RNA sequence and the free form revealed that the structural transition cannot be inhibited completely. As apparent in the imino-spectral region (Figure 11), the structural regions in the periphery of the molecule seem to adopt already the “folded” conformation. In contrast, the structural parts in the proximity of the photolabile protected nucleotides seem to adopt still the ‘unfolded’, Mg\(^{2+}\)-free conformation. Upon photolytic cleavage, the system fully relaxes into the metal-bound conformation.

Caging RNA 2'\(^{-}\)OH. As reported in biochemical studies,\(^{72}\) the hydrolysis reaction catalyzed by the hammerhead ribozyme can be controlled by the site-specific modification or caging of the 2'\(^{-}\)OH hydroxyl nucleophile in the ribose moiety of nucleotides. In the latter case (Figure 12), rapid laser photolysis of the photolabile protecting group initiates an efficient and accurate hammerhead catalyzed cleavage of the substrate RNA under native conditions. RNAs in which reactive functionalities or recognition elements are caged in this manner are useful tools to probe RNA reactivity by time-resolved NMR methods.

Caging of Low-Molecular-Weight Ligands. The concept of caging can be extended, if ligands of low molecular weight are involved in RNA structural transitions. The rationale behind this method is to interfere at the binding interface of the small ligand and thereby stabilize the apo-state of the RNA receptor in the presence of the caged small ligand.

This concept is, for example, applied to the guanine-sensing riboswitch RNA (see earlier section). Hypoxanthine and guanine are able to bind this RNA receptor. Ligand-binding induces a major structural reorganization, both on the level of secondary as well as on the level of tertiary structure. The recognition of the small metabolite is based on the formation of a base triple (Figure 13), involving the interaction of the Watson–Crick side of the purine ligand as well as its N3/N9 edge. As revealed by X-ray crystallographic structural studies and by photo-CIDNP NMR experiments (see later section) of the RNA bound state, hypoxanthine is enclosed in a tight and solvent-protected binding pocket. The introduction of a photolabile protecting group at the Watson–Crick side of the ligand therefore prevents binding of the ligand and the subsequent folding of the RNA molecule by disrupting the formation of

FIGURE 12 Photolysis of a caged oligonucleotide hammerhead substrate to initiate the cleavage reaction catalyzed by the hammerhead ribozyme. Upper panel displays the primary structure of the active site of the ribozyme. The photolabile protecting group is introduced at the 2'\(^{-}\)-OH and prevents the nucleophilic attack towards the phosphodiester backbone. When the free 2'-OH is released by a short laser pulse, the reaction can proceed and the formation of the 2',3'-cyclic phosphate can be monitored (lower panel).
the crucial base triple. In addition, the bulky photolabile protecting group excludes the ligand from the binding pocket by increasing the size of the molecule nearly by a factor of two.

**Caging of Divalent Metal Ions.** Besides small ligands such as nucleobases, metal ions, especially divalent Mg²⁺ or Ca²⁺ ions are often required for the initiation of RNA folding. Consequently, photolabile metal chelators can be used to cage a RNA conformation, too. As in the case of protein folding studies, metal ions are often needed for the proper formation of the RNA structure (in the most cases Mg²⁺). The metal ion can be caged by a chelator, which shows a higher affinity to the metal ion than the RNA. Upon photolytic cleavage, the chelator is decomposed and since the photoproducts exhibit now a lower affinity to the metal ion, it is bound by the RNA molecule and thereby induces the folding event.

As an example, the strategy for the investigation of secondary and tertiary structure folding of the Diels-Alderase ribozyme is outlined (Figure 14). This in vitro selected catalytic active RNA folds into its active pseudo-knotted RNA structure upon addition of divalent metal ions (Mg²⁺ or Ca²⁺) and its low-molecular-weight ligand. The educt, product, or transition state analogue of the catalyzed Diels-Alder reaction can be used as a ligand. In order to observe the conformational transitions from its prefolded form towards the pseudo-knotted structure, the molecule is prepared in a solution containing a high-affinity product-like ligand, and a photolabile caged form of Ca²⁺ (DMN (Dimethoxy-nitrophen), ‘caged’ Ca²⁺). A short laser pulse can decompose the caging compound and the metal ions are consequently released. The binding affinity of the DMN photoproducts to Ca²⁺ ions is lower ($K_D = 3 \times 10^{-3} \text{M}$) than the binding affinity of the RNA to Ca²⁺ and much lower than the one of the DMN itself to Ca²⁺ ions ($K_D = 5 \times 10^{-9} \text{M}$). Upon binding of the released divalent ions, the RNA folds into its active conformation. This transition can be followed by real-time NMR methods (Figure 15).

**Caging Protons.** Structural transitions of RNAs can also be pH-dependent. In the case of the minimal hammerhead ribozyme, the rate limiting step of the reaction is a pH-dependent conformational change. The iron responsive element that regulates the expression of several mRNAs, coding for proteins important for iron import, also undergoes a conformational transition upon changes in pH. A detailed description of such pH-dependent rearrangement is given for the U6 RNA intramolecular stem-loop. Here, upon pH change, base flipping alters the orientation of helices within the RNA structural motif. If the timescales of such transitions would allow examination by RT-NMR, an initiation method has to be applied that allows fast generation of pH-jumps. Such pH-jumps were recently reported to lead to a structural transition in i-motif DNA and can be facilitated by irradiating a sample containing a light-inducible hydrox-
ide ion emitter (molecular malachite carbinal base). Although the read-out in this study was done by CD-spectroscopic methods and the validity of the method at high concentrations is so far not proven, the technique may allow laser-assisted RT-NMR experiments of pH-dependent conformational transitions.

The advantages and disadvantages of three of the above discussed caging techniques are summarized in Table I:

**Mixing Techniques.** As first described for organic reactions in the early 1970s, rapid mixing of reactants and monitoring of the reaction by NMR spectroscopy is feasible and allowed study of non-equilibrium reactions between different states in real-time. Essentially, two strategies for concentration-jump techniques are possible: the first represents mixing of the solutions outside the NMR probe (as equivalent to optical stopped-flow spectrometers) and the subsequent rapid delivery of the mixture into the radio frequency coil-region; the second uses the NMR tube itself as the mixing chamber. For the latter experimental setup, there are also two possibilities amenable: in the first method one solution is injected into another inside the NMR tube, so that after the injection the turbulences cause homogeneous and rapid mixing. The second method uses mobile separators of the two solutions that can be removed rapidly and thereby facilitate mixing.

For the very early mixing instruments, already after a dead time of $\tau_{\text{dead}} \sim 0.2\ s$ a spectrum with sufficient resolution could be recorded. One of the first kinetic studies conducted on biomacromolecules using a NMR mixing device was a study of the alkaline denaturation of hemoglobin. Here, the NMR dead time was already reduced to $\tau_{\text{dead}} \sim 0.06\ s$ and detection of bulk protein signals was possible.

Nowadays, after a typical dead time of approximately 0.05 s, mixing is completed and a sufficient field-homogeneity is achieved. If applying such methodology to RNA folding experiments, the effect of shearing forces to larger RNA molecules has to be considered; however, the NMR size limitation should be below the critical cut-off value that would make usage of rapid mixing devices impossible.

**Temperature-Jump Methods.** A possible method to induce conformational changes with different amplitudes in RNAs is to rapidly increase temperature. If the temperature increase is significantly faster than the system response, kinetics of the formation of the new conformation can be extracted. Currently, there are three different strategies published to perform fast temperature-jump experiments in high-resolution NMR spectroscopy: (a) CO$_2$ laser heating applied in solid-state MAS
NMR spectroscopy,86,87 (b) microwave-assisted heating,88,89 and (c) conductive heating using probe inserts applying radio-frequency (rf) heating in the interior of the NMR tube.90 Most successful and easiest to implement is rf-heating, since the design can be coupled to any probe including cryoprobes.

**NMR Detection**

**1D NMR Methods.** The fundamental idea of real-time NMR (RT-NMR) techniques is to monitor dynamic processes during the time course of e.g. a folding reaction. Real-time NMR experiments discussed here consist of consecutive 1D spectra (Figure 16) and have been designed as pseudo-2D or pseudo-3D experiments (Figure 17).

The dimensions of the experiment are the chemical shift ($\delta^1$H along $\omega_2$ (in 2D)/$\omega_3$ (in 3D)) and the digitized time domain (RT along $t_1$ (in 2D)/$t_1$ and $t_2$ (in 3D)). The first plane in a pseudo-3D experiment is recorded before application of the first trigger, whereas all following planes are recorded after a trigger event or the reaction initiation that could be realized by any of the described methods in 2.1. All 1 + i planes comprise therefore a single kinetic trace. If a single trigger event is sufficient to induce the folding event, then the pseudo-dimensionality can be reduced to two and the trigger is then located in the midpoint of the pseudo-second dimension.

The advantage of the 1D method is its intrinsic high sensitivity at highest temporal resolution. Normally, $^1$H, $^{15}$N, and $^{31}$P are the feasible nuclei due to their high natural abundance (99.9885%, 100%, 100%, respectively) and their high sensitivity (2.79, 2.62, 1.13, relative value of $z$ component of nuclear magnetic moment in units of nuclear magneton, respectively). However, despite the high sensitivity, the spectral resolution of the 1D method is limited. It is best applied to spectral regions of high intrinsic resolution, as for example the imino signal region of RNAs.

The restricted chemical shift resolution can, at least in part, be overcome by using selectively labeled compounds to allow application of NMR filter experiments. The resolution can be increased, for example by using a selectively $^{15}$N-uridine labeled RNA which allows varying detection of either the $^{15}$N-bound imino protons (in this example: uridine imino protons) or the imino protons bound to the $^{14}$N-unlabeled nucleotides (in this example: guanosine nucleotides) in this RNA. Two spectra recorded simultaneously can be reconstructed after the measurement, in which either only uridine or guanosine residues are visible. In order to do so, selective filter techniques in HMQC-fashion are applied (Figure 18). In such an experiment, the phase of the first $^{15}$N
A $90^\circ$-pulse that acts as the discriminator is incremented interleaved in m, where m is the number of subsequent 1D spectra. This setup results in inverted signs of the coupled ($^1$H-$^{15}$N) and uncoupled ($^1$H-$^{14}$N) spin systems in the first data point, while the signs of both are the same in the second one. By adding the two, one can extract the kinetic traces of the $^{14}$N-bound proton signals while subtraction leads to analysis of the $^{15}$N-bound proton signals.

By chemical synthesis, selectively labeled nucleotides can be prepared. $^{13}$C, $^{15}$N, and/or $^{19}$F labeling appears to be most promising under these conditions.

**Ernst-Angle Excitation.** Although 1D methods have a high temporal resolution, the accumulation of signal is limited by the relaxation delay that is needed to (re-)establish Boltzmann polarization. For time-resolved NMR experiments either requiring or permitting more than a single experiment, the signal-to-noise ratio (S/N) per unit measurement time has to be optimized. The methodology of choice to tweak this parameter is Ernst-angle excitation to achieve maximum signal amplitude for fast repetitive pulse experiments.

The optimal flip angle ($\beta_{\text{opt}}$) is determined to

$$\beta_{\text{opt}} = \arccos\left(\exp\left(-\frac{T}{T_1}\right)\right)$$

The shorter the experiment time ($T$, time of acquisition + time of recycle delay) becomes at a constant longitudinal relaxation time $T_1$, which means that the recycle delay is decreased, the smaller the flip angle has to be, in order to gain maximum sensitivity.

**NMR Using Non-Boltzmann Excitation.** Generally, the sensitivity of an NMR experiment is determined by the population ratio $N_\beta / N_\alpha$ of the spin states $\alpha$ and $\beta$. This ratio is described by a Boltzmann distribution according to

$$\frac{N_\beta}{N_\alpha} = \exp\left(\frac{\gamma B_0 h}{kT}\right)$$

where $\gamma$ is the gyromagnetic ratio of the excited spins, $B_0$ is the magnetic field, $h$ is the Planck constant, $k$ is the Boltz-
mann constant, and $T$ is the temperature. Methods that dramatically increase the sensitivity of NMR experiments therefore utilize the excitation or transfer of non-Boltzmann polarization, e.g. electron polarization, to the nuclear spins. Among the different NMR methods that exploit excitation of non-Boltzmann polarization probably the most widely used methods are CIDNP\textsuperscript{95} or DNP.\textsuperscript{96,97} In the following, we wish to comment on CIDNP experiments, since they have been successfully used in the field of protein folding and their transfer to the field of RNA folding seems straightforward.

CIDNP. Chemical induced dynamic nuclear polarization (CIDNP) induces a non-Boltzmann nuclear spin state distribution in thermal or photochemical reactions, usually from collision and diffusion or disproportion of radical pairs, and is detected by NMR by enhanced absorption or emission signals. It allows large improvements of sensitivity and reduction of spectral crowding. In the field of biomolecular NMR, photo-CIDNP experiments provide insight into the solvent accessibility of aromatic residues. The polarization of aromatic residues in proteins is achieved by photosensitizers such as flavins and aza-aromatics. Early studies\textsuperscript{98,99} demonstrated that also for RNA the solvent accessibility of adenosine and guanosine residues can be monitored.

As described before, X-ray structures showed that ligand binding by the guanine-sensing riboswitch RNA is associated with the formation of a tight binding pocket (see earlier section). Comparison of $^1$H-NMR and photo-CIDNP spectra of the free ligand hypoxanthine (in the presence of the photosensitizer flavin mononucleotide (FMN) before and after a laser irradiation of 0.5 s) revealed enhanced signals for its protons H2/H8 (Figures 19a and 19b). In contrast, no enhancement effect on the signals of the free RNA could be detected (Figures 19c and 19d).

Hence, the accessibility of the ligand complexed with the RNA in solution was studied by NMR using photo-CIDNP (Figure 20). While the signals at 8 ppm, corresponding to the free hypoxanthine still showed a CIDNP effect that increased as the ligand-RNA ratio increased, no effect could be
obtained for signals of bound hypoxanthine, confirming the lack of accessibility of the ligand in complex with the RNA also in solution.

Therefore, it should be possible that a RNA folding reaction can be monitored by this methodology. This could be further improved by the utilization of fluorinated nucleotides, which in close analogy to the protein case might show higher CIDNP intensities.

Multidimensional (nD) NMR Methods. The advantage of 1D real-time NMR techniques is their high time resolution, however, they are limited by their spectral resolution. To overcome this limitation, multidimensional NMR experiments can be applied (Figure 21). They provide a higher spectral resolution to resolve additional nuclear sites in RNAs by correlating frequencies of different nuclei and spreading these correlations over several frequency axes. However, these methods are time consuming and consequently reduce the time resolution of the observed folding event. Generally, the sensitivity of the experiment determines the time required for its acquisition. Nowadays, advances in spectrometer and probe technology (e.g. the implementation of cryoprobes) have increased the general sensitivity and decreased the amount of experimental time needed. Therefore, the experimental time is determined by the time required to record a scan, which in turn is determined by the phase cycle, the spectral width and the number of points recorded. To facilitate kinetic studies with nD NMR experiments, the problem of sampling the indirect dimension has to be overcome.

Accordion/Modulated 2D NMR Spectroscopy. Accordion spectroscopy and modulated 2D experiments are techniques to reduce the dimensionality of spectra and are used to analyze dynamic processes. The accordion experiment was developed in order to measure build-up curves of exchange peaks, where normally a 3D experiment (composed of 2D-EXSY spectra at multiple mixing times) is required. The
The resulting signals depend on three variables $S(x_1, s_m, x_2)$, and a third Fourier transformation (FT) leads subsequently to a new frequency domain. To simplify the experiment, one can reduce the dimensionality by coupling $t_1$ and $s_m$ in a skew projection. Therefore, $s_m$ is incremented concomitantly with $t_1(t_m = t_1)$. Instead of a 3D spectrum, now a 2D spectrum is obtained, where the line-shape of the signal along $x_1$ is modulated by the rate of the dynamic process.

In close analogy to this method, 2D techniques are developed that report on a dynamic process by recording a spectrum while the process proceeds, resulting in a modulation of the line-shapes. In order to obtain such a spectrum, the time of the process and the overall time of the experiment have to match. The reaction is initiated as soon as recording of the experiment starts. The spectrum contains the resonances of the different states modulated by their respective kinetics. For faster processes, recording of a sensitive experiment with high chemical shift dispersion along the indirect dimension, such as a HSQC experiment is possible, for slow processes, also the application of experiments is suitable that require longer total experimental time, such as NOESY experiments.

The resulting line-shapes are modulated by the result of the FT of the kinetic process along the indirect dimension.

**SCOTCH.** SCOTCH (spin coherence transfer in chemical reactions) is a 2D NMR experiment, developed to correlate the nuclei in the starting compound with the nuclei in the reaction product of a photochemical reaction, based on a combination of radio frequency and light pulses. When laser light is applied to magnetization created by an rf-pulse, partial conversion of the starting compound into the reaction product takes place. After this conversion, the magnetization precesses at a frequency dependent on the product environment and therefore different from the original frequency of the starting compound. Double FT of the resulting FID results in cross peaks between the two frequencies, thus mapping the product to its reactant compound. The resulting spectrum is asymmetric with respect to the diagonal, since it is an irreversible reaction. SCOTCH is similar to 2D exchange spectroscopy, except that it manipulates transverse magnetization rather than longitudinal. This method was developed to study photo-induced chemical reactions of the form $A \xrightarrow{hv} C_0 \rightarrow B$. The study of the blue-shifted intermediate pB in the photocycle of photoactive yellow protein is another example of the application of SCOTCH, where a $^1$H-$^{15}$N-HSQC-based SCOTCH experiment was developed.

**Resolution Enhancement in 2D NMR Experiments.**

**Hadamard NMR Spectroscopy.** In conventional multidimensional NMR experiments, frequencies of various nuclei evolve during one or more successive evolution periods after excitation. Usually, the frequencies are uniformly sampled and the measurement is not only governed by the desired signal-to-noise ratio but also by the resolution required to resolve NMR resonance frequencies especially in the indirect frequency dimension(s). To suppress axial peaks and to achieve pure in-phase spectra without loss in signal-to-noise, a minimal phase cycle is often applied which also contributes to the increase of experimental time. Although all frequencies are monitored, the obtained resolution is too large if frequencies were known a priori.

If instead the actual frequencies are known, uniform sampling is no longer necessary. Approaches to reduce the experimental time are Hadamard NMR experiments, in which frequency domain excitation, using polychromatic pulses on multi channels, with a Hadamard encoding scheme is applied. In the processing of those experiments, the resonances become disentangled by reference to the encoding scheme.

The encoding of the rf-channels is realized by the signs of a Hadamard matrix. A Hadamard matrix of grade $n$ is a...
$n \times n$ matrix, solely composed of $-1$ and $+1$. All rows and columns are orthogonal to each other. A Hadamard matrix is therefore defined by

$$\tilde{H} \tilde{H}^T = \tilde{H}^T \tilde{H} = n \tilde{I}$$

($H$: Hadamard matrix; $H^T$: transposed Hadamard matrix; $I$: identity matrix). Hadamard matrices are assumed to exist for all $n = 4k$ if $k$ is an integer. The largest matrix found so far is of grade $n = 668$ (Figure 22).

A pulse sequence for such an experiment is very similar to the conventional type of pulse sequence (Figure 23). In the case of a HSQC experiment, instead of the free evolution period $t_1$, a list of shaped 180° pulses is applied to the $^{15}$N channel simultaneously, but modulated with the encoding matrix. This enables the necessary polychromatic excitation and decoding of the signals according to the frequency of the shaped pulses. The sign of the resulting signals in each FID is therefore identical to the sign of the respective position in the Hadamard matrix (as illustrated in Figure 22).

The construction of the 2D spectrum is the combination of the FT of the direct dimension plus the disentanglement according to the matrix form and the chemical shift information given by the respective selective pulses. As shown in Figure 24, a Hadamard spectrum contains only the resonances that have been irradiated by the selective pulses. Therefore, the spectrum contains as many columns containing peaks along the indirect dimension as selective pulses ($\sim$number of rows/columns in the matrix) were applied. The line width in the indirect dimension is identical to the spectral width of a single selective pulse. As illustrated by the spectrum of the 14mer RNA, Hadamard methods allow the recording of a 2D HSQC spectrum in several seconds rather than several minutes. One drawback is the case of overlapping signals along the indirect dimension. Those resonances become excited to the same extent by the band selective pulse and give rise to signals that are at the identical chemical shift value. This is the case for the resonance of G10 that becomes excited by the band selective pulse which should ideally just act on resonance of G12.

As depicted in the case of the 14mer RNA, a subset of the FT HSQC spectrum containing selected resonances can be recorded with the Hadamard methodology in a time fraction of $3/100$ (see Figure 25).

**SOFAST NMR spectroscopy.** In 2005, the idea of reducing the total amount of experimental time by shortening the recycle delay was resuscitated and used to record a series of 2D $^1$H- $^{15}$N-HMOC experiments that report on hydrogen exchange processes, now with a temporal resolution of seconds compared to the former resolution of minutes. The idea behind the technique that is called SOFAST-NMR (for band-selective optimized flip-angle short transient NMR), is to apply a HMOC pulse sequence that uses solely band-selective excitation for protons and has an optimized flip-angle according to the principles of Ernst-Angle excitation (see earlier section).

The application of band selective pulses is required to facilitate water suppression by not exciting the water resonance, and, more importantly, to shorten the longitudinal relaxation

FIGURE 22 Processed subsequent FIDs of a Hadamard HSQC experiment recorded on a 14mer RNA. The signals are modulated by the signs of the Hadamard matrix (as indicated by the signs in the grey circles). Disentanglement results in a Hadamard 2D spectrum that is shown in figure 24.

FIGURE 23 Pulse sequence of a Hadamard $^{15}$N-HSQC; the indirect time period is replaced by an array of selective pulses that are modulated by a Hadamard matrix. All gradients have a SINE.100 shape and are applied with strength of gp1:50%, gp2:80%, gp3:30% and gp4:40% of 50 G/cm. The $^{15}$N selective 180° pulses with a Gaussp.100 profile are applied simultaneously at the respective frequency of the resonances of interest. Nitrogen decoupling is realized by a GARP decoupling sequence during acquisition. Proton decoupling is achieved in the first “dimension” by a waltz16 decoupling sequence during application of the polychromatic pulses. All pulses if not stated explicitly are applied with phase $\phi_{rec} = (x, -x, x, -x, x, -x, x, x, x, x)$.
time by reducing the amount of total excited protons. In the case of selective excitation of a subset of proton spins in a macromolecule, while leaving all other protons unperturbed, significantly enhanced $R_1$ relaxation rates are observed. The HMQC pulse sequence is therefore of advantage, as it has a limited number of proton pulses compared to the HSQC experiment that contains the same information content. Besides the shape of the band selective pulses (in the case of the initial publication a PC9 shape), no additional technical feature is needed. Thus, this technique can be applied on any spectrometer and the data can be processed by regular FT techniques. In the case of RNA, the limited excitation bandwidth of 1-1-echo derived pulse sequences leads inherently only to inversion of the imino proton signals of interest.

**Ultrafast-NMR spectroscopy.** The group of Frydman recently introduced the technique of ultrafast-NMR spectroscopy. With this technique, single scan acquisition of a multidimensional spectrum becomes feasible. The basic idea of the concept is to replace the incremental evolution of the indirect time domain, as used in standard NMR experiments that follow the PEMD scheme, by a spatial encoding procedure. The sample as an entire ensemble of spins evolves

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**FIGURE 24** Comparison of conventional FT 2D $^{15}$N-HSQC (left) and Hadamard 2D $^{15}$N-HSQC spectrum (right). Both spectra were recorded at 293 K on a Bruker AV600 MHz NMR system. The FT spectrum was recorded within 2291 s (2048 pts × 256 pts along F2 and F1, respectively; 8 scans and 8 dummy scans, relaxation delay 1 s); whereas the Hadamard spectrum was recorded in 72 s (2048 pts along F2; Hadamard matrix of $n = 8$; 6 scans and 8 dummy scans, relaxation delay 1 s). All proton pulses were applied with a field strength of 24.39 kHz on resonance with the HDO signal, water suppression was achieved with a hard WATERGATE sequence. The selective $^{15}$N pulses have field strength of 100 Hz and a Gaus.1000 shape (the bandwidth of the selective pulses is indicated by the grey lines crossing both spectra).

**FIGURE 25** Kinetic trace (left panel) extracted of an array of 2D Hadamard $^{15}$N-HSQC spectra recorded during the conformational switching reaction of a single G10 $^{15}$N-labeled bistable 20mer (as introduced earlier) induced by laser irradiation of a photo-protected RNA sequence. Overlay of a regular FT $^{15}$N-HSQC (grey) and a Had-$^{15}$N-HSQC spectrum displaying the peak of G10 (H1/N1) in the first experiment recorded.
chemical shift along the indirect dimension in parallel, the sample is split up into independent subensembles that evolve chemical shifts in a subensemble-dependent manner. After an independent mixing procedure (so far TOCSY, COSY, and HSQC among others have been successfully applied\textsuperscript{121}) again subensemble specific acquisition is conducted, now in the physical, second time domain.

The partitioning of the sample into subensembles is carried out by a spatial encoding scheme that uses gradients— the spins are discriminated by their position (spatial coordinate $r$) according to the additional applied field gradient $G = ∂B_0/∂r$. In other words, the indirect time is now expressed as a function of the spatial position of the spin $t_i(r)$. The resulting signal is then no longer a function of two different times—$S(t_1,t_2)$—but depends on a space variable $k$ and the acquisition time $t_2 = S(k,t_2)$. The variable $k$ is encoding the spatial distribution of the respective spin along the $z$-axis of the gradient—$k = ∫G(t)dt$. Subsequent Fourier transformation leads to the final 2D spectrum.

This technique is capable of monitoring dynamic processes in biomacromolecules as demonstrated for an ubiquitin sample on which H/D exchange processes were monitored with a resolution of 4 s per 2D $^{15}$N-HSQC spectrum.\textsuperscript{122} An inherent drawback of this technique is that in contrast to the conventional 2D methods, the signal of a single scan is spread over the whole two dimensional space so that the resulting S/N ratio in the spectrum is compromised. The ubiquitin sample used in the above mentioned study had a concentration of 3.2 mM and is of low molecular weight. This high concentration might be suitable for this case, but is currently above the preparation yields and precipitation borders for more sophisticated and larger biomacromolecules. Moreover, the enhanced relaxation of larger macromolecules may also hamper the application of this technique, regarding the decreased signal intensity.

Very recently, a combination of the ultrafast and SOFAST NMR approaches was published as ultraSOFAST-NMR.\textsuperscript{122} The authors argue that the complementary techniques—the first accelerates acquisition by reducing the number of scans and the second reduces the delay between consecutive scans—enable the acquisition of 2D spectra at Hz rates. The example given is the characterization of H/D exchange of ubiquitin.

**INFORMATION DERIVED FROM KINETIC NMR DATA**

The information derived from kinetic NMR data spans from the bulk kinetic information, reporting how the whole system behaves during the time course of a reaction to residue specific characterization of the folding kinetics. While the first kind of information can also be achieved by other methods, the latter is only obtained by time-resolved NMR methodology, gaining an atomistic insight into the molecular system. Therefore, the description of the system can be achieved with highest spatial resolution, allowing deciphering atomic mechanisms of the reactions.

**Free Energy, Activation Energy**

In addition to the kinetic rate constants, thermodynamic parameters for the reactions can be derived that give further insight into the processes and mechanisms of RNA conformational switching events. When performed at different temperatures, the results of the kinetic measurements can be analyzed according to Eyring, enabling determination of the thermodynamic parameters that characterize the energy of the transition state. In the light of the known structures of the start and the end state, the resulting activation energy may be used to gain insight into the pathway of the reaction.

In this context, the parameters of refolding kinetics of the bistable 20mer RNA sequence 5’r[GACCGGAAGGUCCG CCUUCC]-3’ (see Figures 7 and 8) were determined to range from $k_{A→B}$ of 0.0136 \pm 0.001 s\(^{-1}\) at 283 K to 0.131 \pm 0.024 s\(^{-1}\) at 298 K; whereas the rate constants $k_{B→A}$ ranged between 0.0019 \pm 0.002 s\(^{-1}\) at 283 K and 0.031 \pm 0.006 s\(^{-1}\) at 298 K (Figure 26). The activation energy for the forward process was $E_{A→B} = 26$ kcal mol\(^{-1}\) with a frequency factor of $A = 10^{18}$ and for the backward process $E_{B→A} = 31$ kcal mol\(^{-1}\) with $A = 10^{21}$.

For the second, small bistable system with the sequence 5’-r[GAGGGCAACCUCUCCGGGUUG]-3’, the directly determined rate constants $k_{A→B}$ ranged between 0.005 \pm 0.001 s\(^{-1}\) at 283 K and 0.091 \pm 0.020 s\(^{-1}\) at 298 K; and the rate constants $k_{B→A}$ ranged between 0.005 \pm 0.002 s\(^{-1}\) at 283 K and 0.076 \pm 0.020 s\(^{-1}\) at 298 K. Here, the Arrhenius analysis revealed an activation energy of $E_{A→B} = 33$ kcal mol\(^{-1}\) ($E_{B→A} = 31$ kcal mol\(^{-1}\)) and a frequency factor of $A_{A→B} = 10^{23}$ ($A_{B→A} = 10^{23}$).

In both studies, these results are in agreement with a transient disruption of half of the base pairs. The activation energies of $E_a = 25–32$ kcal mol\(^{-1}\), required for the refolding of those two 20mer RNA sequences are well in the range of the activation energies determined for the refolding of larger sequences, representing substructures of biologically relevant RNA species ($E_a = 20–38$ kcal mol\(^{-1}\)). This similarity of kinetic parameters suggests that the structural transitions of complex RNA folds are based on the refolding of minimal secondary structure motives.

**Mechanisms of RNA Folding**

**Associative and Dissociative Mechanisms.** For small bistable RNA molecules, it could be shown that the refolding mechanisms may differ for different systems (Figure 27). The
conclusions were derived from the measured high activation energy and from the fact that the global refolding dynamics do not affect the local base pairing geometry. Additional static NMR analysis supports the theory of two complementary mechanisms\(^{66,67}\) that are dependent on the interconverting conformations.

For a bistable RNA system that is composed of two conformations with equal topologies (Figure 7, right side), a dissociative mechanism could be derived. Towards the transition state of the reaction, the base pairs of the stems have to dissociate partially. This dissociation precedes the formation of the base pairs that are present in the alternative conformation. This is different to the second example (Figure 7, left side), where the two conformations interconvert by an associative mechanism. Here, few base pairs present in the alternative conformation are formed, before most of the existing ones are broken. This led to the general conclusion, that the mechanism for the specific refolding reaction of a bistable RNA is strongly dependent on the structural context of the two interconverting structures.

**From Kinetics to Structure.** As derived by RT-NMR methods (see earlier section), the residue specific analysis of the ligand-induced folding of the guanine-sensing riboswitch RNA led to detailed structural information about this folding event. Here, a three-step mechanism for the formation of the native fold could be dissected. While a first low-affinity initial encounter complex could be characterized by NMR line widths analysis, the final native conformation is subsequently adopted over two steps of specific ligand binding. Residues

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**FIGURE 26** Results of RT-NMR experiments for the refolding kinetics of 5'-r[GACCGGAAG-GUCCGCCUUCC]-3'; Exemplaric kinetic traces for two imino resonances reporting on the two respective conformations, experiments were conducted at four different temperatures (283, 288, 293, and 298 K), allowing a subsequent Arrhenius analysis depicted in the most right panel.

**FIGURE 27** Schematic representation of the two mechanisms derived for the two small bistable systems by RT-NMR methods in combination with static NMR data. The nucleotides composing the loops in the respective conformations are color coded. The first steps (association: left panel and dissociation: right panel) are indicated by arrows. As indicated, the transition state is characterized by a reduced number of base pairs (indicated in grey).
with identical time constants within experimental error are clustered around distinct regions within the RNA aptamer. Incorporation of this time-dependent information into experimentally restrained molecular dynamics simulations yielded the characterization of the structural ensembles involved during the folding event (Figure 28). The structural inhomogeneity, characteristic for the “unfolded state,” is reduced gradually and accompanied by an increase of order of the structural motives. \(^{123}\)

**FUTURE DIRECTIONS**

Initial examples of NMR-spectroscopic investigations of RNA folding focused on the observation of chemical shifts. Changes in chemical shifts of the imino resonances in RNAs contain considerable information, since an imino resonance can only be observed in states protected against exchange. Most often, this exchange protection is due to the formation of a hydrogen-bonded base pair between non-neighboring nucleotides. However, in other spectral regions, such chemical shift analyses fail.

Possible additional NMR parameters complementing the chemical shift information are scalar \(J\) couplings. However, the disadvantage of measuring scalar \(J\) couplings is the fact that only small differences are observed for different conformations and therefore, \(J\) couplings need to be measured with high spectral resolution. The second disadvantage is that \(J\) coupling constants reveal information only on local conformational properties.

Residual dipolar couplings are possible parameters to describe the local as well as the global changes of the molecular conformation during the time course monitored by RT-NMR. The advantage is that the resolution of the recorded spectra may be sufficient to gain insightful parameters. The disadvantage is that the RT-NMR experiments have to be conducted twice—once for an unaligned and once for an aligned sample using alignment media such as phages or bicelles. Any interaction between all conformational states, populated transiently during the reaction time course and the alignment medium has to be excluded, as this would otherwise lead to a distortion of the reaction path and consequently a kinetic analysis would fail.
Initial experiments in our group conducted on solutions containing phages show that kinetic refolding experiments can in general be undertaken in such alignment media (Figure 29). The reaction shows the same kinetics as without alignment medium and furthermore, as a useful side effect, the deprotection yield of the photolabile protecting group is higher in the presence of phages.

In summary, the application of real-time NMR experiments to study RNA folding is relatively new. For many RNA folding events, current investigations suggest that the timescales are in the second to minute regime. In addition, the initial investigations indicate that different folding rates are observed for one structural transition, maybe due to the hierarchical folding units of RNA. Many of the experiments developed in the field of NMR of protein folding cannot directly be transferred to RNA: hydrogen exchange experiments outside the spectrometer cannot be applied, since the intrinsic exchange rates in RNA are too fast and relaxation dispersion experiments require faster structural transitions than those observed in RNA. On the other hand, information derived from time-resolved NMR experiments, namely the acquisition of native chemical shifts, can be readily interpreted in light of formation of a single long-range hydrogen-bonding interaction for RNA systems. Together with mutational data that can readily be obtained for RNA and new ligation technologies that enhance site resolution even further, time-resolved NMR may become a powerful tool to decipher RNA folding. Such information will be of importance to understand the functions of coding and non-coding RNAs in cells.

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REFERENCES