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RNA CATALYSIS AS A PROBE FOR CHAPERONE ACTIVITY OF DEAD-BOX HELICASES

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Abstract

DEAD-box proteins are vitally important to cellular processes and make up the largest class of helicases. Many DEAD-box proteins function as RNA chaperones by accelerating structural transitions of RNA, which can result in the resolution of misfolded conformers or conversion between functional structures. While the biological importance of chaperone proteins is clear, their mechanisms are incompletely understood. Here we illustrate how the catalytic activity of certain RNAs can be used to measure RNA chaperone activity. By measuring the amount of substrate converted to product, the fraction of catalytically active molecules is measured over time, providing a quantitative measure of the formation or loss of native RNA. The assays are described with references to group I and group II introns and their ribozyme derivatives, and examples are included that illustrate potential complications and indicate how catalytic activity measurements can be combined with physical approaches to gain insights into the mechanisms of DEAD-box proteins as RNA chaperones.

Keywords

catalytic RNA; intron; ribozyme; chaperone; RNA folding; DEAD-box; helicase

1. INTRODUCTION

1.1. Large RNAs become trapped in non-native conformations and fold slowly

To carry out their cellular duties, many RNAs must fold into functional three-dimensional structures. However, the journey to the native state includes opportunities to go astray and populate inactive, misfolded structures (Russell, 2008; Treiber and Williamson, 1999). With only four standard bases, RNA is prone to forming non-native secondary structures (Herschlag, 1995), which can persist on the time scale of hours to give ‘kinetically trapped’ folding intermediates (Treiber and Williamson, 1999), and the same basic issue of local stability can apply to modular tertiary contacts (Russell, 2008). Slow folding to functional structure is a common problem among RNAs, as nearly every large RNA that has been studied misfolds into at least one inactive, non-native structure (Shcherbakova *et al.*, 2008; Treiber and Williamson, 1999). In light of these intrinsic features of RNA, it is not surprising that cells can take action to rescue incorrectly folded RNAs.

1.2. DEAD-box helicase proteins function as RNA chaperones

A major way that cells promote RNA folding is through chaperone proteins, which accelerate escape from kinetically trapped intermediates (Lorsch, 2002; Mohr *et al.*, 2002; Russell, 2008; Schroeder *et al.*, 2004; Zemora and Waldsich, 2010). DEAD-box proteins are

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a major group of RNA chaperones and comprise the largest family of superfamily 2 helicases (Fairman-Williams *et al.*, 2010; Linder, 2006). They consist of a conserved helicase core made up of two RecA-like domains that may be flanked by additional domains. DEAD-box proteins bind RNA and ATP via the helicase core and are thought to promote RNA structural transitions by using the ATPase cycle to achieve tight, yet regulated RNA binding in a mode that can disrupt secondary structure (Hilbert *et al.*, 2009; Jarmoskaite and Russell, 2011; Potratz *et al.*, 2010). These disruptions are used to rescue RNAs from misfolded non-native structures and to convert RNAs between multiple functional structures. The prominent role played by DEAD-box proteins is highlighted by the fact that essentially every process known to be carried out by a structured RNA requires at least one DEAD-box or related protein (Fairman-Williams *et al.*, 2010; Jankowsky and Bowers, 2006; Linder, 2006, 2008). Some DEAD-box proteins have been shown to act as general RNA chaperones, interacting with multiple RNA substrates nonspecifically to promote native folding (Del Campo *et al.*, 2009; Halls *et al.*, 2007; Huang *et al.*, 2005; Mohr *et al.*, 2002).

In this article we describe how to harness the power of RNA catalytic activity to study protein-assisted RNA folding. Rather than listing detailed protocols, which are likely to be different for individual proteins and RNAs, we describe the basic concepts that underlie the experimental design, and we address major issues that can complicate data analysis. For further details on basic issues that arise when using catalytic activity to measure native RNA folding, we refer readers to an earlier work (Wan *et al.*, 2010a). We also refer to examples that illustrate how catalytic activity has been used, alone and in conjunction with complementary approaches, to gain insights into the mechanisms of DEAD-box proteins as RNA chaperones.

2. CATALYTIC ACTIVITY AS A PROBE OF RNA FOLDING

To track productive folding of an RNA, a signal must exist that allows the native state of the RNA to be distinguished from all other conformations. Functional assays provide a powerful probe, because even folding intermediates with extensive native structure can be readily distinguished if they are unable to function. Catalytic RNAs are well suited for this purpose, as their native states can be readily detected by monitoring chemical conversion of a substrate to its corresponding product.

2.1. Catalytic activity distinguishes the native state from all other conformations

The central goal of using catalytic activity to monitor RNA folding is to measure the fraction of the RNA that is present in the native state unambiguously and quantitatively. This information can then be combined with other approaches to gain insights into the structural properties of folding intermediates. In the case of the ribozyme derived from a group I intron in *Tetrahymena thermophila*, a misfolded conformation exists that contains the full set of native secondary and tertiary contacts and is difficult to distinguish from the native state by physical approaches (Russell *et al.*, 2006; Wan *et al.*, 2010b). However, it is straightforward to distinguish between the two structures in a catalytic activity assay because the natively folded ribozyme, but not the misfolded ribozyme, can cleave an oligonucleotide substrate in *trans*. Using this assay, the transition from the misfolded state to the native state has been measured (Chadee *et al.*, 2010; Russell *et al.*, 2006; Russell and Herschlag, 2001; Wan *et al.*, 2010b).

2.2. Catalytic activity can be used to study chaperone-assisted folding

The ability to track native RNA folding allows the influence of chaperone proteins to be monitored. While this chapter highlights group I and group II introns and the DEAD-box

helicase chaperone proteins, the general techniques can be applied to a broad range of catalytically active RNAs and chaperone proteins. Indeed, RNA catalytic activity of the hammerhead ribozyme was used nearly two decades ago to study chaperone activities of the HIV NC protein (Herschlag *et al.*, 1994; Tsuchihashi *et al.*, 1993), and group I introns have been used to monitor *in vitro* and *in vivo* chaperone activities of a variety of RNA binding proteins (Clodi *et al.*, 1999; Rajkowitsch *et al.*, 2005; Semrad *et al.*, 2004; Semrad and Schroeder, 1998).

Group I and group II introns are mobile genetic elements that catalyze their own excision from precursor RNA via two transesterification splicing reactions (Fig. 1A). In order for these splicing events to occur, the RNA must fold to an active three-dimensional conformation. Introns can be converted to ribozymes with constructs that lack the exons (Fig. 1B,C). Ribozymes of group I and group II introns cleave oligonucleotide substrates that include a 5' splice site in reactions that mimic the first step of splicing (Kuo *et al.*, 1999; Qin and Pyle, 1997; Swisher *et al.*, 2001; Tanner and Cech, 1996; Zaug and Cech, 1986; Zaug *et al.*, 1988).

Group I and group II introns and their corresponding ribozyme constructs are well suited for studying chaperone-assisted RNA folding because they have extensive networks of secondary and tertiary structures (Fig. 1B,C), and RNAs from both groups have been shown to fold slowly and to accumulate intermediates (Emerick *et al.*, 1996; Fedorova *et al.*, 2010; Fedorova *et al.*, 2007; Karunatilaka *et al.*, 2010; Russell and Herschlag, 1999; Sinan *et al.*, 2011; Steiner *et al.*, 2008; Walstrum and Uhlenbeck, 1990; Zhang *et al.*, 2005). These RNAs are sufficiently complex to include diverse sets of intermediates and corresponding kinetic barriers during folding, allowing detailed probing of the abilities of chaperones to assist in overcoming these barriers, while remaining simple enough to deeply probe the folding processes and pathways.

3. SELF-SPLICING AS A READOUT FOR NATIVE STATE FORMATION

A straightforward way of measuring native folding is to follow self-processing of intron-containing constructs after folding is initiated by addition of Mg^{2+} . The reaction can be followed in the format of a continuous assay, in which folding and the catalytic steps take place concurrently and in the same reaction. If the rate of native RNA structure formation is slower than the subsequent catalytic steps, the observed splicing rate provides a good measure of the rate of folding to the native state.

It is common practice to body label the RNA to visualize all the products of intron splicing. This can be accomplished by including a radiolabeled nucleotide, commonly [α - ^{32}P] UTP, to label nucleotides throughout the RNA (Huang *et al.*, 2005; Mohr *et al.*, 2006; Pyle and Green, 1994). The reaction is then monitored by denaturing polyacrylamide gel electrophoresis, which allows the unspliced precursor to be separated from the splicing products (Fig. 2A). The loss of precursor RNA over time is quantitated to indicate the observed rate of splicing, and in turn folding to the native state (Del Campo *et al.*, 2009; Mohr *et al.*, 2006; Potratz *et al.*, 2011) (Fig. 2B).

3.1. Interpreting chaperone-promoted changes in observed splicing rate

A simple reaction scheme for intron folding and splicing is shown below and is instructive for understanding how a chaperone protein affects the splicing process.

To identify whether an RNA helicase influences folding, the observed rate constant ($k_{observed}$) is compared in the absence and presence of multiple protein concentrations and in the presence and absence of ATP. If the helicase increases the rate of folding to the native

state (k_{fold}), most likely by accelerating resolution of one or more intermediates (I), this increase will lead to an increase in $k_{observed}$ if the splicing rate constant (k_{splice}) is larger than the folding rate constant. For introns with robust self-splicing activity this condition is generally met because large RNAs usually fold slowly *in vitro* (Pan and Russell, 2010; Shcherbakova *et al.*, 2008).

The observed rate constant is typically plotted as a function of protein concentration to determine the efficiency with which the chaperone promotes folding (Fig. 2C). The observed rate constant may increase with protein concentration at low concentrations and then level or begin to decrease at higher protein concentrations (Fig. 2C). Possible physical sources for this inhibition are trapping of intermediate conformations in a protein-bound state (shown in Scheme 1 as a single intermediate (I) for simplicity) and chaperone-induced unfolding of the native state. Although unfolding of native RNAs may be physiologically important for RNAs that must cycle between functional structures, this inhibition complicates analysis of the effects of the chaperone on the folding process toward the native state. Thus it is most straightforward to interpret the data in terms of protein-accelerated folding at relatively low protein concentrations where these additional effects are minimized (Fig. 2C).

3.2. Potential complications

Since the chaperone is present during the splicing reaction, it could affect the catalytic steps of splicing (k_{splice}) in addition to the folding steps. If the catalytic steps (k_{splice}) are rate-limiting, influencing them will alter the overall rate constant ($k_{observed}$) even if the protein does not affect the rate of native structure formation (k_{fold}). However, this will not be an issue provided that folding remains slower than splicing. Group I introns allow the rate-limiting step to be determined. A folding incubation is performed in the absence of the exogenous guanosine cofactor and then guanosine is added to permit splicing. If this reaction gives a larger splicing rate than the reaction in which guanosine was present continuously, it indicates that folding is rate limiting.

A second issue concerns the multi-step nature of the splicing process. Although it is shown as a single step for simplicity in scheme 1, some group I and group II introns display rapid, reversible first steps of splicing, giving accumulation of intermediates in a fast phase that is followed by slower completion of the second step and formation of products (Chin and Pyle, 1995; Golden and Cech, 1996; Karbstein *et al.*, 2002; Woodson and Cech, 1989). If native folding is slower than the reversible first step but faster than the second step, the precursor will be lost with an initial rate constant that reflects folding, as in the simple case with a more rapid second step. A slow step will result in additional loss of precursor. This loss could mistakenly be assigned to an additional, slower folding pathway of the precursor, whereas it actually reflects completion of the second splicing step. This behavior can be identified by the appearance and subsequent disappearance of intermediates representing the products of the first step, *i.e.* the free 5' exon and the intron-3'-exon.

A final issue concerns the choice of RNA constructs. Although emphasis is typically on the intron for folding, exon length and composition can cause substantial effects (Nolte *et al.*, 1998; Potratz *et al.*, 2011; Zingler *et al.*, 2010). The exons may misfold themselves and/or stabilize structure within the intron, and either of these effects may be important biologically. Care must be taken when comparing results from constructs that differ in the properties of the exons.

4. SUBSTRATE CLEAVAGE AS A READOUT FOR NATIVE STATE FORMATION

Many of the potential complications associated with using self-splicing constructs in continuous assays can be avoided by using ribozyme versions. Lacking exons, ribozyme constructs allow interpretation of all folding processes to reflect the intron domains. Further, although ribozyme versions can be used in continuous assays, the fact that they cleave oligonucleotide substrates in *trans* makes them well suited for discontinuous assays, in which folding and catalytic activity are separated into two discrete stages (Fig. 3A). The main advantage of this separation is the ability to directly assess the fraction of native ribozyme during folding. This allows productive folding to be dissected and unfolding of the native structure to be probed (**Sect. 5.1**). In addition, the discontinuous assay permits the use of folding conditions that do not support robust catalysis, an option not available using a continuous assay.

4.1. Setting up a discontinuous assay: folding and catalysis stages

The discontinuous assay is composed of two stages, the folding stage (stage 1) and the catalysis (or cleavage) stage (stage 2) (Fig. 3A). The folding stage contains the ribozyme alone or with the chaperone under conditions desired for chaperone-assisted RNA folding. To prevent catalytic activity at this stage, the oligonucleotide substrate is omitted. In the catalysis stage, conditions are changed such that further folding to the native state is blocked, and radiolabeled oligonucleotide substrate is added, typically in small excess of the ribozyme (2–3-fold over the ribozyme). The cleavage reaction is allowed to proceed for various times (t_2), and the substrate and product from each time point are separated on a denaturing polyacrylamide gel (Fig. 3B). The fraction of product, normalized by the substrate and ribozyme concentrations, is plotted against cleavage time (t_2) (Fig. 3B,C). Most commonly, the cleavage stage produces a burst of product formation, with the amplitude reflecting the fraction of ribozyme in the native state (**Sect. 4.2**, below). This fraction increases as a function of time spent in the folding stage (t_1), giving a rate constant for native folding of the ribozyme (Fig. 3D). After initial experiments have been performed and the cleavage rate constant is known, a single time point in the cleavage reaction (stage 2) may be sufficient to determine the burst amplitude (see Fig. 4A). This timepoint should be chosen after completion of the burst phase, but before significant contribution from subsequent turnovers (solid symbols in Fig. 3C).

A key advantage of the discontinuous assay is that any set of conditions can be used for the folding stage. However, setting up the catalysis stage requires care, as conditions must support enzymatic activity while blocking further native folding. This is because ribozyme that reached the native state during stage 2 would produce cleavage products and cause the calculated fraction of native ribozyme to be artificially high.

The ability of the catalysis stage to prohibit folding is probed by comparing cleavage reactions from a ribozyme that has been prefolded to the native state and a ribozyme that is transferred directly to the catalysis stage, omitting the folding step. (In practice, testing for suitable conditions for the catalysis stage can be undertaken simultaneously with learning how to prefold ribozyme to the native state, as described below.) The burst amplitude of product from ribozyme that skipped the folding stage should be much less than that from the prefolded ribozyme, indicating little formation of native ribozyme during the cleavage reaction. It may not be possible to completely block native folding in stage 2, but a relatively small fraction of native ribozyme produced in this stage can be accounted for by normalization (Potratz *et al.*, 2011).

Although optimal conditions for stage 2 are likely to be different for different catalytic RNAs, some general guidelines are applicable. A study using a ribozyme engineered from the group II intron $\alpha 5\gamma$ from *S. cerevisiae*, D135 (Fig. 1C), used a high pH in the catalysis stage to accelerate cleavage, and high Mg^{2+} concentration (100 mM) and low temperature (15 °C) to enhance the arrest of folding (Fig. 4A) (Potratz *et al.*, 2011). Cleavage is not particularly fast under these conditions (10^{-2} min^{-1}). However, slow cleavage is acceptable for the catalysis stage in a discontinuous assay as long as cleavage is significantly faster than folding. In addition, proteinase K is included at the catalysis stage to ensure that protein transferred from the folding stage has no effect on substrate cleavage.

To compare a ribozyme prefolded to the native state with an unfolded ribozyme, it must be known how to fold the ribozyme to the native state. Establishing how to fold the ribozyme to the native state can be undertaken concurrently with exploring conditions to use for the catalysis stage. Folding of a ribozyme (typically by adding Mg^{2+}) is initiated and aliquots are removed at different times from the folding stage. These aliquots are transferred to stage 2 conditions in the presence of a small excess of substrate. The product burst amplitudes are plotted against folding time. When the burst amplitude no longer increases as a function of folding time, the ribozyme has been folded to the native state as fully as it can be under that set of conditions (Fig. 4B). The folding time should be varied widely to determine whether there are slower folding pathways that give additional native ribozyme formation on longer time scales.

4.2. Interpreting results from the catalysis stage

To make optimal use of the discontinuous assay, it is critical to interpret the burst amplitude quantitatively in order to determine the fraction of native ribozyme. Selected examples from work involving chaperones will be covered below, and a more thorough guide to interpreting results from catalytic rate measurements was published in this series last year (Wan *et al.*, 2010a).

The interpretation of the burst amplitudes depends on the relative rate constants of different steps in the catalytic cycle, which is shown in Scheme 2.

In general, the cleavage stage is performed under multiple turnover conditions, and the fraction of product is normalized by the substrate and ribozyme concentrations, giving the ratio of product to ribozyme. This ratio is plotted against cleavage time (t_2) (Fig. 3B,C). The reaction products are bound by base-pairing, with or without additional tertiary contacts, and for many ribozymes their dissociation rate constants ($k_{P5' \text{ release}}$ and $k_{P3' \text{ release}}$) are small and limit the rates of subsequent turnovers. In all, there are three possible regimes: (1) both products are released quickly relative to the catalytic step (k_{cleavage}); (2) both are released slowly; (3) one is released quickly and the other slowly (Wan *et al.*, 2010a). Regimes 2 and 3 result in bursts of product formation, and the amplitude of this burst can be used to calculate the fraction of native ribozyme, as described in two examples.

The D135 ribozyme was used in a catalytic reaction with three-fold excess substrate. When performed with the ribozyme prefolded to the native state, the cleavage stage resulted in a burst of product with an amplitude approximately equal to one turnover of the ribozyme (Fig. 4A) (Potratz *et al.*, 2011). This is consistent with regime 3, in which there is a burst of product formation with a rate constant equal to the cleavage rate (k_{cleavage}) and a subsequent linear phase dictated by the slow release of one of the products. Because the 5' product of the oligonucleotide substrate forms twelve Watson-Crick base pairs with the ribozyme, it is likely to be the product that is released slowly. Under this reaction regime, the amplitude of the burst phase is equal to the fraction of D135 ribozyme folded to the native state.

The ribozyme engineered from a group I intron from *Azoarcus evansii* (Fig. 1B) was used in a catalytic reaction with two-fold excess substrate (Sinan *et al.*, 2011). When the ribozyme was prefolded to the native state, the cleavage stage resulted in a burst of product with an amplitude of half to three-fourths of the ribozyme concentration, depending on solution conditions (Fig. 4B). This is consistent with regime 2, in which the slow release of both products allows equilibrium between the cleavage (k_{cleavage}) and ligation (k_{ligation}) to be reached. The burst amplitude is smaller than the fraction of native ribozyme and must be corrected by the value of the internal equilibrium to reveal the fraction of native ribozyme.

4.3. Using the discontinuous assay to probe chaperone-assisted folding

When optimal conditions for stage 2 are established and the relationship of the burst amplitude to the fraction of native ribozyme is understood, the discontinuous assay can provide important insights into chaperone-assisted RNA folding. The most straightforward experiment is to compare the folding reaction in the presence of various concentrations of chaperone, plotting the fraction of native ribozyme against time (t_1) (Fig. 3D). A chaperone may increase the rate of native state formation (Sinan *et al.*, 2011; Tijerina *et al.*, 2006), an effect that most likely arises from accelerated resolution of one or more kinetically-trapped intermediates. This interpretation is particularly clear for RNAs that are known to misfold (Pan and Woodson, 1998; Russell and Herschlag, 1999; Sinan *et al.*, 2011; Tijerina *et al.*, 2006). Further confirmation can be obtained by allowing the RNA to misfold first and then adding the chaperone (Sinan *et al.*, 2011; Tijerina *et al.*, 2006).

It is possible for a chaperone to increase the fraction of ribozyme that reaches the native state rapidly without having a significant impact on the observed rate constant (Fig. 5A,B) (Potratz *et al.*, 2011). This effect may arise from an influence of the chaperone early in folding, which decreases the probability of misfolding at later folding steps. Alternatively, resolution of an intermediate can become sufficiently fast that this pathway is then indistinguishable from pathways that avoid the intermediate.

Analogous to effects on self-splicing constructs, higher protein concentrations can inhibit folding of ribozymes to the native state (Fig. 5C). The physical processes responsible for inhibition are presumably the same for ribozymes and self-splicing constructs, but the discontinuous assay allows the origins of the inhibition to be distinguished. For self-splicing constructs, inhibition by trapping protein-bound nonfunctional intermediates and by unfolding native RNA both lead to a decrease in the observed splicing rate (Fig. 2C). In a discontinuous assay unfolding of native ribozyme decreases the endpoint of the folding curve (Fig. 5C). Trapping of folding intermediates, without unfolding of natively-folded RNA, would result in a decrease in the rate of native ribozyme formation but not a decrease in the endpoint.

5. Other applications of the discontinuous assay

The discontinuous assay is amenable to a diverse set of experiments. It can be used to monitor a decrease in the native ribozyme, and an additional stage can be included to probe the role of ATP in chaperone-mediated folding (Halls *et al.*, 2007; Potratz *et al.*, 2011). Further, the progress of native ribozyme formation obtained from the assay can also complement insight from other powerful physical approaches, and thus provide a more complete understanding of the action of RNA chaperones.

5.1. Unfolding native structure

Many chaperones function non-specifically and are capable of disrupting the native states of RNAs as well as misfolded states (Bhaskaran and Russell, 2007). For probing the mechanisms of chaperone activity in structure disruptions, it can be very useful to monitor

the native state because it is relatively homogeneous and the structure may be known. In contrast, folding intermediates may be heterogeneous and their structures poorly defined.

To monitor loss of the native ribozyme, the ribozyme is first pre-folded to the native state, and then the chaperone protein is added. Even for general chaperones, the level of activity may be reduced for the native structure because it is typically highly stable, so it may be necessary to lower the Mg^{2+} concentration and/or to use relatively high protein concentrations to detect net unfolding. A decrease in the fraction of native ribozyme over time indicates that the chaperone protein has mediated at least partial unfolding of the native ribozyme, giving intermediates that do not readily refold to the native state upon transfer to the stage 2 conditions. To ensure that the loss of native ribozyme reflects reversible unfolding, the protein should be proteolyzed and the ribozyme again folded to the native state (Fig. 5D) (Potratz *et al.*, 2011).

5.2. Integrating results with other methods

The materials and methods required to employ the use of RNA catalytic activity are standard for many laboratories, and the ease of implementing this method makes it a convenient tool for obtaining a kinetic view of the fraction of native ribozyme. Results can be highly complementary to those from methods that provide physical information on folding intermediates.

Three physical probes that have been used extensively for RNA folding studies are chemical footprinting, small angle X-ray scattering (SAXS), and single-molecule FRET. Time-resolved chemical footprinting can be performed with several different probes and provides a highly specific view of nucleotides that are engaged in secondary or tertiary contacts during a folding process (King *et al.*, 1993; Mortimer and Weeks, 2007, 2008, 2009; Sclavi *et al.*, 1997; Tijerina *et al.*, 2007). Hydroxyl radical footprinting has been particularly valuable for probing structured intermediates and elucidating folding pathways (Laederach *et al.*, 2007; Laederach *et al.*, 2006; Sclavi *et al.*, 1998). The orthogonal information provided by catalytic activity measurements – how much of the ribozyme is in the native state – is tremendously valuable because it can be used to place constraints on the folding pathways modeled from footprinting data (Laederach *et al.*, 2007; Mitra *et al.*, 2011). SAXS provides rich information on the overall size and shape of RNA as it folds, which is highly complementary to footprinting (Kwok *et al.*, 2006; Schlatterer and Brenowitz, 2009), and again coordinated activity measurements under the same conditions can assist greatly in constraining physical descriptions of intermediates (Kwok *et al.*, 2006; Laederach *et al.*, 2007; Mitra *et al.*, 2011; Roh *et al.*, 2010; Sinan *et al.*, 2011). Last, single-molecule FRET experiments are uniquely powerful for detecting and characterizing intermediates that do not accumulate in bulk experiments (Bokinsky *et al.*, 2003; Bokinsky and Zhuang, 2005; Karunatilaka *et al.*, 2010; Rueda *et al.*, 2004; Zhuang, 2005; Zhuang *et al.*, 2000), and the concurrent detection of catalytic activity can be used to tremendous advantage for distinguishing the native state from folding intermediates (Bokinsky *et al.*, 2003; Liu *et al.*, 2007; Rueda *et al.*, 2004; Zhuang *et al.*, 2000).

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Figure 1.

Group I and group II introns. **A**, Self-splicing reactions. Left panel, Group I intron splicing reaction. An exogenous guanosine attacks the 5' splice site in the first step, and the 5' exon attacks the 3' splice site in the second step. Right panel, Group II intron splicing reaction. A bulged adenosine near the 3' end of the intron attacks the 5' splice site, generating a lariat intermediate. The 5' exon then attacks the 3' splice site to ligate the exons together. **B**, Secondary structure of the *Azoarcus* group I intron ribozyme. The nine-nucleotide substrate is lowercase. The cleavage site is indicated by a thin arrow, and two tertiary contacts are indicated by thick arrows. **C**, Secondary structure of the aI5 γ group II intron from *S. cerevisiae* (Swisher *et al.*, 2001). The domains shown in black are present in the D135 ribozyme. Tertiary interactions are indicated by greek letters. Interaction sites between exon and intron sequences are indicated by the abbreviations IBS and EBS (Intron Binding Site and Exon Binding Site, respectively). The 24-nt substrate is shown at the right and the cleavage site is indicated by an arrow.

Figure 2.

Self-splicing constructs. **A**, Denaturing gel showing splicing products for a group II intron: (from top to bottom) lariat intron, unspliced precursor, linear intron, and spliced exons. **B**, Simulated plot of a splicing reaction showing the fraction of precursor as a function of time. The simulated data are fit by a single exponential curve to obtain rate constants for splicing in the presence (circles) and absence (diamonds) of protein. **C**, Simulated plot showing how the observed splicing rate varies with protein concentration. The rising linear portion of the data (circles) is fit with a line to obtain a second order rate constant for chaperone-accelerated folding. The plateau and decrease in rate (diamonds in gray area) reflect inhibition by the chaperone at higher concentrations (see section 3.1).

Figure 3.

The discontinuous assay. **A**, Reaction schematic. The ribozyme folds in the first stage, and then it cleaves the oligonucleotide substrate (S) in the second stage under reaction conditions that block further native folding. **B**, Denaturing gel showing the results of cleavage of a 5' labeled oligonucleotide substrate by native ribozyme. **C**, Simulated plot of multiple cleavage reactions representing different folding times prior to the cleavage reaction. The curves with larger bursts represent longer folding times, giving greater accumulation of native ribozyme. The amplitude values are shown with filled symbols. **D**, Simulated plot showing the fraction of native ribozyme (f_N) plotted as a function of folding time (t_1). The burst amplitudes from simulated cleavage reactions in panel C are plotted as a function of folding time. In this simulated scenario, the folding progress can be fit by a single exponential function, giving a single rate constant for native state formation.

Figure 4.

Examples of catalytic reactions with the D135 and *Azoarcus* ribozymes. **A**, Identifying conditions that block folding for the catalysis stage of the discontinuous assay. Comparing burst amplitudes resulting from D135 ribozyme placed into stage 2 conditions with (circles) or without (triangles) a prior incubation in Mg^{2+} -containing buffer to allow prefolding to the native state. The burst is much smaller without the preincubation, indicating that these conditions for stage 2 (pH 8.0, 100 mM Mg^{2+} , 500 mM KCl, 15 °C) effectively block folding. The solid circle indicates how a single time point can be sufficient to determine the fraction of native ribozyme if the kinetics of cleavage are known. **B**, Ribozyme prefolding to the native state. The burst amplitudes from cleavage reactions of the *Azoarcus* ribozyme are smaller than they would be for stoichiometric product formation, and further work showed that this results from an equilibrium between substrate cleavage and ligation (Sinan *et al.*, 2011). Note also that the bursts from ribozyme prefolded at 37 °C and 10 mM Mg^{2+} for 15–45 min are identical, suggesting that 15 minutes is sufficient for complete folding (see section 4.1). Panel A reprinted from (Potratz *et al.*, 2011) with permission from Elsevier. Panel B adapted from (Sinan *et al.*, 2011). This research was originally published in Journal of Biological Chemistry. Selma Sinan, Xiaoyan Yuan, and Rick Russell. The *Azoarcus* Group I Intron Ribozyme Misfolds and Is Accelerated for Refolding by ATP-dependent RNA Chaperone Proteins. *JBC*. 2011; 286:37304–37312. © the American Society for Biochemistry and Molecular Biology

Figure 5.

The discontinuous assay with the D135 ribozyme and the DEAD-box helicase Mss116p. **A**, Cleavage time courses initiated with ribozyme folded in the presence of Mss116p for the times indicated. The increase of the burst amplitude with folding time (t_1) indicates productive folding to the native state. **B**, Comparison of ribozyme folding in the presence (diamonds) and absence (circles) of Mss116p. The protein changes the folding profile from multi-phasic to a single exponential phase. **C**, High Mss116p concentrations inhibit the native conformation. At 500 nM (diamonds) and 1000 nM (inverted triangles) Mss116p, the fraction of native ribozyme at steady state is lower than in the presence of lower protein concentrations (gray symbols). **D**, The native conformation of D135 can be unfolded by Mss116p. Ribozyme prefolded to the native state (black/gray solid circle) is divided into reaction tubes containing a high concentration of Mss116p (black circles) or no Mss116p (gray circles). After approximately 10 min, the protein is proteolyzed and the ribozyme is treated to allow it to refold to the native state (black and gray diamonds), demonstrating that the loss of native ribozyme arises from unfolding and not an irreversible process. Panels A–D adapted from (Potratz *et al.*, 2011) with permission from Elsevier.

Scheme 1.

Scheme 2.