Published in final edited form as:

Methods Enzymol. 2007 ; 425: 121–137. doi:10.1016/S0076-6879(07)25005-0.

Probing the Intermediacy of Covalent RNA Enzyme Complexes in RNA Modification Enzymes

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Abstract

Within the large and diverse group of RNA-modifying enzymes, a number of enzymes seem to form stable covalent linkages to their respective RNA substrates. A complete understanding of the chemical and kinetic mechanisms of these enzymes, some of which have identified pathological roles, is lacking. As part of our ongoing work studying the posttranscriptional modification of tRNA with queuine, we wish to understand fully the chemical and kinetic mechanisms involved in this key transglycosylation reaction. In our previous investigations, we have used a gel mobility-shift assay to characterize an apparent covalent enzyme-RNA intermediate believed to be operative in the catalytic pathway. However, the simple observation of a covalent complex is not sufficient to prove intermediacy. To be a true intermediate, the complex must be both chemically and kinetically competent. As a case study for the proof of intermediacy, we report the use of this gel-shift assay under mildly denaturing conditions to probe the kinetic competency of the covalent association between RNA and the tRNA modifying enzyme tRNA-guanine transglycosylase (TGT).

1. Introduction

Given the abundance and structural diversity of modified nucleosides that have thus far been identified within all types of RNA, it can be hypothesized that they serve important molecular roles in many cellular processes. Although most of these roles remain unknown, it is becoming clear that certain modified nucleosides are involved in the pathogenesis of human disease (Bjork *et al.*, 1999; Jacobs, 2003; Kwak and Kawahara, 2005), whereas others are critical for the virulence of pathogenic organisms such as *Shigella flexneri* (Bjork *et al.*, 1999; Durand and Bjork, 2003). As such, the enzymes responsible for the biosynthesis of modified nucleosides may present unique therapeutic targets for rational drug design.

The characterized catalytic mechanisms of RNA-modifying enzymes are diverse, reflecting the varied structural nature of the modified nucleoside products (Garcia and Goodenough-Lashua, 1998). Within this diverse group, the reaction mechanisms used by a number of enzymes appear to proceed through a stable covalent linkage to their respective RNA substrates (Fig. 5.1). For example, intermediate 1 is postulated to form on the reaction pathway of tRNA U54 methyltransferase (RUMT) (Kealey and Santi, 1995), a member of the m⁵U RNA methyltransferase family (similar intermediates have been identified in the related m⁵C RNA methyltransferases; Kealey and Santi [1995] and King and Redman [2002]). The covalent intermediate is proposed to form by Michael addition of the cysteine nucleophile to the C6 position of the uracil base. The histidine-linked phosphoamide intermediate 2 has been observed in the action of tyrosyl-DNA phosphodiesterase (TDP) on RNA substrates (Interthal

et al., 2005) and the related lysine-linked intermediate 3 has been observed in the action of mRNA-capping enzyme guanylyltransferase (Huang *et al.*, 2005). Pseudouridine synthase is thought to use an aspartate residue to form a covalent complex with its tRNA substrate (Hamilton *et al.*, 2006). In this case, it is not yet clear whether the aspartate nucleophilically attacks the C6 position of the uracil base, resulting in the carbon-oxygen-linked intermediate 4 or if the aspartate attacks the 1' position of the ribose yielding intermediate 5. Finally, and most relevant to ongoing investigations in our laboratory, the base-exchange reaction that is catalyzed by the tRNA-modifying enzyme tRNA-guanine transglycosylase proceeds through the ribosyl ester linkage that is present in intermediate 6.

A clear understanding of the mechanisms of RNA-modifying enzymes that may operate by formation of a reaction intermediate such as those described previously requires more than the mere observation of an enzyme-substrate complex. To prove the existence of a reaction intermediate, the following three criteria must be met.

1. Is the intermediate isolable, detectable or trappable? Rigorous characterization of a putative enzyme-RNA covalent intermediate first requires that the intermediate can be isolated. In the best case, the suspected intermediate can be isolated in quantities sufficient for structural analysis (e.g., by X-ray diffraction). In the absence of this, detection of the intermediate by SDS-PAGE analysis of reaction mixtures or by spectroscopic methods (e.g., NMR, MS) can be suggestive of its existence in the reaction mechanism. However, such analyses can prove difficult to obtain given the technical difficulties associated with analysis of large and highly charged (i.e., RNA phosphodiester backbone) enzyme-RNA complexes. As an alternative approach, the intermediate can be trapped with an appropriately reactive compound, the product of which would provide putative evidence for the intermediate's existence (Hamilton *et al.*, 2006; Redman, 2006).
2. Is the intermediate chemically competent? After the successful isolation or detection of a putative enzyme-RNA intermediate, it is requisite to demonstrate its chemical competence to ensure that the detected intermediate is not an artifact of the experimental conditions. Specifically, the isolated enzyme-RNA intermediate should react to give the same products as does the overall reaction of enzyme and substrate under the identical reaction conditions.
3. Is the intermediate kinetically competent? Finally, and perhaps most experimentally challenging, the kinetic competence of the putative enzyme-RNA intermediate must be demonstrated. No individual step on the reaction pathway can be slower than the overall rate of the reaction (k_{cat}). Therefore, the rates of formation and breakdown of the suspected enzyme-RNA intermediate must be equal to or faster than k_{cat} . The kinetic assessment of the intermediacy of a complex can be complicated by the lack of a suitable assay to quantify the amount of intermediate complex present in the reaction mixture. Here we report the use of a simple gel-shift assay to probe the kinetic competency of the covalent association between RNA and an RNA modifying enzyme.

2. A Case Study for Probing Reaction Intermediacy: The Covalent RNA Complex of tRNA-Guanine Transglycosylase (TGT)

Our laboratory's interest in the catalytic mechanism of tRNA-guanine transglycosylase (TGT) led us to develop and refine methods for the characterization of these stable covalent intermediates to address these criteria for intermediacy. TGT catalyzes the posttranscriptional replacement of guanine with the modified nucleoside queuosine in eukaryotes and the queuosine precursor preQ₁ in eubacteria (Noguchi *et al.*, 1982; Okada and Nishimura, 1979;

Okada *et al.*, 1979). This base substitution (Fig. 5.2), in which the wobble position guanine is replaced, has been observed in four tRNAs that code for aspartate, asparagine, histidine, and tyrosine. Each of these cognate tRNAs share the common anticodon sequence of G₃₄U₃₅N₃₆. The TGT reaction proceeds by means of ping-pong kinetics, whereby the tRNA substrate binds first with concomitant loss of guanine leading to the proposed TGT-RNA covalent intermediate (Goodenough-Lashua and Garcia, 2003). The incoming modified base then binds in the active site and nucleophilically displaces the enzyme, resulting in the formation of a new glycosidic bond and the release of the modified RNA product.

The occurrence of an intermediate in catalytic mechanism of TGT was supported by the report of the solution of the X-ray crystal structure of the *Z. mobilis* TGT bound to a minihelical RNA substrate (Xie *et al.*, 2003). Importantly, those results clearly demonstrated that the proposed intermediate was indeed isolable, satisfying criterion 1 (see earlier). The X-ray crystal structure revealed that active site Asp264 (*E. coli* numbering) forms a covalent ribosyl ester linkage with the 1' ribosyl carbon of the wobble position guanosine of tRNA. From this and other biochemical data (Kittendorf *et al.*, 2001, 2003; Romier *et al.*, 1996a; Romier *et al.*, 1996b), a catalytic mechanism in which Asp264 functions as the enzyme nucleophile leading to the covalent enzyme-RNA intermediate was considered likely (Fig. 5.3).

3. Denaturing Gel Electrophoresis: A Tool to Probe Enzyme-RNA Complexes

In vitro biochemical studies of the TGT-catalyzed reaction have been aided by an extremely facile assay to probe the existence of stable TGT-RNA complexes. Romier and colleagues (1996b) were the first to report the use of a denaturing gel electrophoresis assay to study intermediate components resulting from the incubation of wild-type and mutant *Z. mobilis* TGT with substrate tRNA. The authors observed that denaturing PAGE analysis of equilibrium mixtures contained protein bands of higher apparent molecular weight than monomeric TGT and presumed these bands to be stable *covalent* enzyme-RNA complexes. We routinely use this gel-mobility shift assay to probe the ability of wild-type and mutant *E. coli* TGT to form stable RNA complexes with cognate tRNA (Curnow and Garcia, 1994; Goodenough-Lashua and Garcia, 2003; Kittendorf *et al.*, 2001, 2003), as well as with novel RNA structures (Kung *et al.*, 2000; Nonekowsky and Garcia, 2001). Although the observation of these bands is not sufficient to argue for a true *covalent* TGT-RNA association, we have observed a correlation between the ability of TGT to form the complexes and enzyme activity. With similar reasoning, Xie and colleagues (2003) used the band-shift assay to investigate the chemical competence of a 9-deazaguanine (9dzG)-stabilized TGT-RNA-9dzG ternary complex, and most recently, Meyer and collaborators (2006) probed the ability of small molecule inhibitors to stabilize a tRNA-TGT intermediate in a ternary complex.

3.1. PAGE band-shift analysis of reaction mixtures

- 5× Bicine reaction buffer: Bicine (250 mM, pH = 7.7), MgCl₂(100 mM), DTT (25 mM).
- Native-PAGE loading buffer: TRIS HCl (60 mM, pH = 7.0), glycerol (50%), bromothymol blue (0.02%).
- Native-PAGE electrophoresis conditions: native buffer strips (Amersham), 5°, 400 V, 10 mA, 2.5 W, 250 AVH.
- SDS-PAGE loading buffer: TRIS HCl (60 mM, pH = 7.0), glycerol (10%), SDS (2%), bromothymol blue (0.02%).
- SDS-PAGE electrophoresis conditions: SDS buffer strips (Amersham), 15°, 250 V, 10 mA, 3 W, 200 AVH.

Equilibrium mixtures were prepared by incubating enzyme TGT (10 μ M) with substrate RNA (10 μ M) in 1 $\times$ Bicine reaction buffer at 37° for 20 min.

3.1.1. Native-PAGE—An aliquot (10 μ l) of the reaction mixture was combined with native-PAGE loading buffer (10 μ l). An aliquot (1 μ l) of that combination was loaded onto an 8–25% gradient polyacrylamide gel (PhastGel, Amersham) and electrophoresed under native conditions (see earlier) on a PhastSystem unit.

3.1.2. SDS-PAGE—An aliquot (10 μ l) of the reaction mixture was combined with SDS-PAGE loading buffer (10 μ l) and allowed to incubate at room temperature for 1 h. An aliquot (1 μ l) of that combination was loaded onto an 8–25% gradient polyacrylamide gel (PhastGel, Amersham) and electrophoresed under denaturing conditions (see earlier) on a PhastSystem unit.

Notes: (1) The incubation of the SDS-quenched reaction mixtures on ice or for periods of <1 h resulted in two bands corresponding to TGT-RNA complexes, apparently reflecting different TGT-RNA conformers present in solution. However, after 60 min at room temperature, the upper band coalesced with the lower as shown in Fig. 5.4, suggesting that the band was incompletely denatured complex. (2) This method does not require the use of the PhastSystem electrophoresis equipment. Equivalent results have been obtained with other electrophoresis units and gels (e.g., Bio-Rad).

3.2. Band quantification by fluorescent detection

The plastic gel backing was removed from the polyacrylamide gel with the aid of a gel backing remover wire (Pharmacia) and the gel stained with 1 $\times$ Spyro Red (Invitrogen) in 7.5% acetic acid for 1 h with shaking. The stained gel was washed with 7.5% acetic acid (2 $\times$ 5 ml), followed by water (1 $\times$ 5 ml). The bands were visualized by green laser excitation (532 nm) and fluorescent detection by use of a Typhoon 9200 gel imaging system (Molecular Dynamics). The band volumes (created by a 3D plot of pixel locations and intensities) were quantified using the ImageQuant software package (Molecular Dynamics). In summary, rectangles of identical size and shape were drawn around bands in the same lane to encompass each band with proportionately equal background space (Fig. 5.5). A report of volumes within each defined rectangle using the “local average background” subtraction method was generated. To calculate the percent of the protein in the upper band of each lane, the volume of the upper rectangle was divided by the sum of the volumes of the upper and lower bands.

Note: For kinetic studies, an enzyme concentration in the final quenched sample of 1.9 μ M was sufficient to visualize the resulting bands with the Spyro Red protein stain. Greater final concentrations would be required for Coomassie detection, less for more sensitive detection methods (e.g., radiochemical detection).

3.3. Detection of biotinylated-RNA containing bands by colorimetric assay

- Phosphate-buffered saline-T (PBS-T): NaCl (155 mM), Na₂HPO₄ (2.97 mM), KH₂PO₄ (1.06 mM), Tween-20 (0.05%).

After electrophoresis, polyacrylamide gels were blotted onto nitrocellulose membranes (Bio-Rad) according to vendor's protocols. The blots were incubated with horseradish peroxidase-conjugated streptavidin (2 μ g/ml) (Pierce) in PBS-T for 30 min and washed with PBS-T (2 $\times$ 10 ml). The HRP-conjugated bands were detected with the chromogenic substrate CN/DAB (Pierce) that forms a dark precipitate visible in ambient light.

In our hands, equimolar recombinant *E. coli* TGT (45 kDa) incubated with *E. coli* tRNA^{Tyr} (ECY) or a minihelical analog consisting of the anticodon stem and loop substituted with a 2'-

deoxyguanosine located at position 34 (dG₃₄ECYMH) (Nonekowski *et al.*, 2002) forms a stable TGT-RNA complex of apparent molecular weight greater than monomeric TGT as assessed by SDS-PAGE analysis (Fig. 5.6A). (Note: The 2'-deoxyguano-sine modification was designed to probe the role of the 2' hydroxyl in the TGT reaction.) However, it was unclear whether these higher molecular weight bands represented true covalent complexes or a mixture of covalent and noncovalent (but stable to the denaturing conditions used) associations.

The crystal structure of TGT bound to substrate RNA revealed that under the crystallization conditions, Asp264 could nucleophilically attack the RNA ribose, displacing guanine (Xie *et al.*, 2003). On the assumption that this occurs under normal reaction conditions, we reasoned that a mutation of Asp264 would render TGT unable to form the covalent ribosyl ester linkage; however, the noncovalent association between the enzyme and RNA substrate should be maintained. Moreover, if this noncovalent interaction persisted under mildly denaturing conditions, it should be detected in gel mobility-shift experiments that use the mutant TGT. To probe this hypothesis, aspartate 264 of the *E. coli* TGT was mutated to asparagine (Kittendorf *et al.*, 2003). SDS-PAGE analysis of reaction mixtures containing TGT(D264N) and RNA demonstrated that, even under mild denaturing conditions at decreased temperatures, no TGT(D264N)-RNA complex was formed (Fig. 5.7). By native-PAGE analysis, TGT (D264N) was observed to exist as a mixture of multimeric forms. TGT(D264N) that was incubated with RNA contained a mixture of monomeric TGT(D264N) and a new band of slightly lesser mobility that we have assigned to be the noncovalent TGT-RNA complex (Fig. 5.8A). The suspected TGT-RNA bands that had been observed by SDS-PAGE and native-PAGE analyses were probed with the nucleic acid intercalating agent, ethidium bromide (data not shown); however, no increase in staining was observed relative to the protein bands. Presumably this is due to the cumulative effect of the small size of the RNA mini-helix substrate and of the ability (not widely recognized) of ethidium bromide to stain protein (Csapo *et al.*, 2000; Vincent and Scherre, 1979). To selectively visualize RNA-containing bands, we designed an RNA mini-helix substrate in which the 5' end contained a biotinylated guanine base, enabling facile detection by Western blot with streptavidin-linked horseradish peroxidase (HRP). After native-PAGE separation, Western blot analysis of wild-type and TGT(D264N) reaction mixtures containing the biotinylated RNA revealed that both wild-type and mutant TGT form complexes with RNA (Fig. 5.8B). However, SDS-PAGE separation followed by Western blot analysis indicated that only the wild-type TGT remained associated with RNA under denaturing conditions (Fig. 5.6B). Importantly, these experiments provide direct evidence that under native conditions, both noncovalent and covalent TGT-RNA associations are maintained, whereas under denaturing conditions, the noncovalent association is ablated, while the covalent interaction remains intact.

4. Analysis of the Chemical Competency of Covalent Enzyme-RNA Complexes

With the successful isolation of the *Z. mobilis* TGT-RNA crystal, Xie and coworkers were able to probe the chemical reactivity of the intermediate with the heterocyclic base substrate preQ₁ (Xie *et al.*, 2003). The authors treated the complex with molar excess of preQ₁ and found, as assessed by gel-shift analysis, that the complex band disappeared, presumably forming preQ₁-substituted RNA. In our hands, a histidine-tagged *E. coli* TGT-RNA complex purified from free RNA components by nickel chelation chromatography similarly reacts with excess preQ₁ to yield free TGT (Fig. 5.9). Independently, each of these experiments demonstrates the chemical competence of the proposed enzyme-RNA intermediate.

5. Analysis of the Rate of Formation of Covalent Enzyme-RNA Complexes

Finally, the kinetic competence of the proposed enzyme-RNA intermediate remained to be substantiated. The denaturing gel mobility-shift assay provides a convenient method for measuring the observed first-order rate constant ($k_{\text{formation}}$) for formation of covalent enzyme-RNA intermediates. Such analysis is absolutely necessary for assessing the kinetic competence of any proposed intermediate. As proof of concept, we interrogated the kinetics of formation of the intermediate that is observed on incubation of wild-type TGT with the mini-helical RNA, dG₃₄ECYMH. By use of a KinTek rapid quench flow apparatus equipped with a circulating water bath, reaction mixtures containing wild-type TGT and RNA were sampled and quenched over a time course ranging from 0.1 sec to several minutes. After SDS-PAGE separation of the reaction mixtures, the protein bands were stained with a fluorescent protein dye and subsequently visualized by laser excitation, enabling direct protein quantification on the basis of the fluorescence signal (Fig. 5.10).

5.1. Kinetic studies that use rapid quench flow

- Drive buffer: Bicine (50 mM, pH = 7.7).
- SDS quench buffer: TRIS HCl (60 mM, pH = 7.0), glycerol (10%), SDS (2%).

A KinTek RQF-3 rapid quench flow apparatus was charged with drive buffer and SDS quench buffer and equilibrated to 37°. Detailed specifications of the apparatus are available at the KinTek Corp. web site: www.kintek-corp.com. A simplified schematic of the apparatus appears in Fig. 5.11 The sample port A was loaded with a solution of enzyme, TGT (15 μM), in Bicine reaction buffer, and sample port B was loaded with a solution of RNA substrate (15 μM) also in Bicine reaction buffer. Aliquots (15 μl) of each reagent were incubated in the mixing chamber for various periods (0.1–120.0 s) and quenched automatically with SDS buffer (90 μl). The mixtures incubated for an additional hour at room temperature before PAGE band-shift analysis.

Note: The quenching agent must be sufficiently denaturing to stop enzymatic function yet sufficiently mild to not disrupt the covalent linkage of the intermediate. Particularly in the case of enzymes that form relatively labile ribosyl ester intermediates, the conventional acidic quench is deleterious to the stability of the covalent bond. A discussion of alternative quenching agents appears in a recent review (Barman *et al.*, 2006).

The ratio of the amount of the TGT-RNA covalent complex to free enzyme was computed by dividing the band volume of the complex band by the total volume of the complex band and free enzyme band. A plot of the percent of complex formed versus time gave a first-order rate of formation of the TGT-RNA covalent complex of 0.684 sec^{-1} (Fig. 5.12). It should be noted that the reaction proceeds to approximately 30% completion. Under the conditions used (e.g., 10 μM each, enzyme and RNA) approximately 3 μM free guanine will be generated. The reaction generating the covalent complex is reversible, and the concentration of free guanine generated is approximately $10 \times K_M$ (Goodenough-Lashua and Garcia, 2003). Therefore, the reaction actually proceeds to an equilibrium point, which seems to be approximately 30% completion.¹

6. Summary

A gel-mobility shift assay has been used to probe the occurrence of noncovalent and covalent complexes that form on incubation of RNA and some RNA modifying enzymes. This

¹A report on the kinetic competence of the covalent intermediate in the TGT reaction is in preparation.

denaturing band-shift assay is amenable to quantitative analysis and, most importantly, provides data regarding the rate of covalent complex formation. Furthermore, this assay may presumably be extended to determine the rate of covalent complex breakdown, provided that the enzyme-RNA intermediate is stable to isolation. Given the difficulties associated with obtaining crystallographic data and the hurdles associated with analysis of protein-RNA by mass spectrometry, this method offers a relatively simple assay that uses standard PAGE analyses to probe complex protein-RNA interactions.

Acknowledgments

We are grateful to Professor Carol Fierke for the use of the rapid quench flow apparatus, to Professor Ronald Woodard for use of the electroblotting apparatus, and to Professor Kyung-Dall Lee for use of the Typhoon imaging system. Work in the Garcia laboratory has been generously supported by grants from NIH (GM065489 & GM45968), NSF (9720139), and the University of Michigan, College of Pharmacy, Vahlteich & Upjohn Research Funds.

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Figure 5.1.

Chemical structures of known RNA-enzyme covalent complexes. The shared covalent bond is highlighted with an arrow. Abbreviations: GT, Guanylyl-transferase; Ψ, pseudouridine synthase; RUMT, tRNA U54 methyltransferase; TDP, tyrosyl-DNA-phosphodiesterase (shown working on RNA); TGT, tRNA-guanine transglycosylase.

Figure 5.2.

The eubacterial TGT-catalyzed reaction exchanging guanine for preQ₁ by formation of a covalent enzyme-RNA intermediate.

Figure 5.3 .

Mechanism of the formation of glycosyl ester intermediate 6. Asp264 serves as the catalytic nucleophile that displaces the guanine base. Asp89 is proposed to serve as a general acid/base, possibly through an intermediary water molecule (Kittendorf *et al.*, 2003).

Figure 5.4 .

Time course of incubation times of TGT-RNA reaction mixtures in SDS loading buffer. Lane A, 5 min; lane B, 15 min; lane C, 30 min; lane D, 45 min; lane E, 60 min; lane F, 75 min; lane G, MW ladder. Protein bands are detected by Spyro Red fluorescent stain.

Lane	Rectangle	Volume $\times 10^6$
A	1	4.445
A	2	29.370
B	3	5.073
B	4	17.143
C	5	6.248
C	6	23.864
D	7	8.573
D	8	28.637

Lane	% upper band
A	13.2
B	22.8
C	20.8
D	23.0

Figure 5.5 .

Representative band volume determination. (A) SDS-PAGE of TGT-RNA reaction mixtures. (B) The same SDS-PAGE of TGT-RNA reaction mixtures with rectangles (white) drawn to encompass gel area for volume calculation. The tables show the individual volumes and derived covalent complex percentages.

Figure 5.6 .

SDS-PAGE of the TGT-RNA covalent complex. (A) *E. coli* TGT or TGT (D264N) (10 μ M) was incubated with either full-length tRNA (ECY, 10 μ M) or biotinylated mini-helical substrate (dG₃₄ECYMH, 10 μ M) in Bicine (pH 7.7) buffer at 37° for 20 min. An aliquot (10 μ l) was quenched with SDS loading buffer (10 μ l) and incubated at room temperature for 1 h. Lane A, MW ladder; lane B, TGT; lane C, TGT + dG₃₄E-CYMH; lane D, TGT + ECY; lane E, TGT (D264N); lane F, TGT(D264N) + dG₃₄E-CYMH; lane G, TGT(D264N) + ECY. Protein bands are visualized by Coomassie stain. (B) Western blot of the TGT-RNA covalent complex. SDS-PAGE was performed as in (A). Proteins were blotted onto a nitrocellulose membrane, and RNA-containing bands were identified with a streptavidin-conjugated HRP-based colorimetric

stain. Note, uncomplexed RNA substrate runs with the electrophoresis front (not shown). Lanes as indicated for gel A.

Figure 5.7 .

SDS-PAGE of TGT(D264N) incubated with RNA. TGT(D264N) ($10\ \mu\text{M}$) was incubated with mini-helical substrate ($10\ \mu\text{M}$) in Bicine buffer (pH 7.7) at 37° for 20 min. An aliquot ($10\ \mu\text{l}$) was quenched with SDS loading buffer ($10\ \mu\text{l}$) and incubated for 1 h at various temperatures. Lane A, MW ladder; lane B, 37° ; lane C, 22° ; lane D, 10° ; lane E, 4° . Protein bands are detected by Spyro Red fluorescent stain.

Figure 5.8 .

Native-PAGE of the TGT-RNA complex. Wild-type TGT and TGT (D264N) ($10 \mu M$) were incubated with biotinylated mini-helical substrate (dG₃₄ ECYMH, $20 \mu M$) in Bicine (pH 7.7) reaction buffer at 37° for 20 min. An aliquot ($10 \mu l$) was treated with native loading buffer ($10 \mu l$) and analyzed immediately by native-PAGE. (A) Coomassie-stained native polyacrylamide gel. Lane A, TGT; lane B, TGT + RNA; lane C, TGT(D264N); lane D, TGT(D264N) + RNA. (B) Streptavidin-conjugated HRP-based colorimetric stain of nitrocellulose blot of native polyacryl-amide gel to detect RNA-containing bands. Note, uncomplexed RNA substrate runs with the electrophoresis front (not shown). Lanes as indicated for gel A.

Figure 5.9 .

SDS-PAGE of TGT-RNA complexes in the absence of and presence of substrate preQ₁. Lane A, MW ladder; Lane B, TGT-dG₃₄ECYMH and TGT mixture purified from reaction mixture by chelation of histidine-tagged TGT by nickel chromatography; lane C, purified TGT-dG₃₄ECYMH intermediate mixture treated with excess preQ₁.

Figure 5.10 .

SDS-PAGE of the time course of the formation of the TGT-dG₃₄ECYMH covalent intermediate. TGT and dG₃₄ECYMH were incubated at 37° and quenched at intervals from 0.1–120.0 sec with the aid of a rapid-quench device. Protein bands are detected by Spyro Red fluorescent stain. The amount of intermediate formed at each time point was quantified by use of fluorescence detection. Lane A, MW ladder; lane B, TGT; lanes C–L, 0.1, 0.5, 1.0, 3.0, 5.0, 10.0, 20.0, 40.0, 60.0, 120.0 sec, respectively.

Figure 5.11 .
Simplified schematic of KinTek RQF-3 Rapid Quench Flow apparatus.

Figure 5.12 .

Percent covalent complex formation versus time. Each point represents the average of 4–7 independent measurements ($\pm$ SEM). The inset is an expansion of the earliest time points.