Mutations in the *Tetrahymena* Ribozyme Internal Guide Sequence: Effects on Docking of the P1 Helix into the Catalytic Core and Correlation with Catalytic Activity†

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Received February 28, 1996; Revised Manuscript Received June 7, 1996⊗

ABSTRACT: Binding of substrate by the ribozyme derived from the self-splicing intron of *Tetrahymena thermophila* involves at least two steps. In the first step, base pairing between the ribozyme internal guide sequence (IGS) and the substrate forms a helical duplex (P1). Through specific tertiary interactions between P1 and the ribozyme core, P1 is then docked into the ribozyme active site. We have investigated the effects of compensatory mutations in positions 2−6 of the P1 helix on docking of P1 into the ribozyme core. Equilibrium binding of matching oligonucleotides by catalytically active IGS mutant ribozymes was evaluated by gel-shift analysis. While the strength of base pairing changed with base composition as expected, the strength of tertiary interactions between P1 and the ribozyme core was not affected by the P1 mutations. These results support a model in which efficient docking of P1 is determined by P1 structure and the presence of a conserved G-U pair. Determination of the rate of dissociation of matching oligonucleotides from each ribozyme revealed that mutations in the IGS change the tightness of binding by increasing or decreasing the dissociation rate. Surprisingly, dissociation rates determined in this fashion were 20−900-fold less than the values of the multiple-turnover rate constant for these ribozymes, initially suggesting that turnover did not require product dissociation. A more detailed analysis for the wild-type ribozyme defined two distinct product dissociation rates. The slower rate equaled that determined under the conditions used for the equilibrium binding studies. The weighted average of the two dissociation rates equaled the multiple-turnover rate constant. These results are explained by a model in which ribozyme preparations consist of two ribozyme conformers: one with tight docking of P1 and another with weaker docking of P1.

The 5′ splice-site specificity of the self-splicing intron from *Tetrahymena thermophila* is determined by base pair interactions between the intronic internal guide sequence (IGS)[1] and the 5′ splice site to form a stem−loop structure, P1 (Been & Cech, 1986; Waring et al., 1986). Substrate specificity for the ribozyme form of this intron, L-21 *Sca*I, is also determined by the IGS (Zaug et al., 1986, 1988). The wild-type ribozyme (IGS = GGAGGG) cleaves its substrate 3′ to the sequence CCCUCU. The underlined bases form a G-U wobble pair that is conserved among group I introns from phylogenetically diverse sources. The substrate specificity of the ribozyme can be altered by changes in the IGS as long as the phylogenetically conserved G-U pair between G22 of the ribozyme and position −1 of the substrate is maintained. Thus, changes in the ribozyme IGS could allow cleavage 3′ to any N_5U.

Binding of RNA substrate by the ribozyme is a two-step process (Bevilacqua et al., 1992, 1993; Herschlag, 1992; Herschlag & Khosla, 1994). First, the six- nucleotide ribozyme IGS base pairs with complementary bases in the substrate to form a duplex analogous to the P1 duplex involved in self-splicing. Second, tertiary interactions between the ribozyme core and 2′-hydroxyls within the P1 duplex result in docking of P1 into the ribozyme active site. The stability of docked P1 is the sum of the free energies of base pairing and P1 helix docking. E·P is more stable than E·S, the complex that precedes cleavage, because of substrate destabilization in the active site (Narlikar et al., 1995).

The tertiary energy for docking results from contacts between the J8/7 region of the ribozyme core and 2′-hydroxyls at positions −3U of the substrate and 25G of the ribozyme IGS, and presumably between J4/5 of the ribozyme core and G22 of the IGS (Pyle et al., 1992; Strobel & Cech, 1993; Wang et al., 1993; Downs & Cech, 1994). Efficient

† This work was supported by a Physician Scientist Award to T.B.C. (PHS 1K11AI01159) and a NCDDG-HIV grant to T. R.C. (PHS AI35226). T.R.C. is an investigator of the Howard Hughes Medical Institute and an American Cancer Society Professor.

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⊗ Abstract published in *Advance ACS Abstracts,* August 15, 1996.

[1] Abbreviations: IGS, internal guide sequence; K_m, the Michaelis constant; $(k_{cat}/K_m)^S$, the second-order rate constant for reaction of the ribozyme−substrate complex under single-turnover conditions; k_{cat}(mt), the first-order rate constant for reaction of ribozyme with saturating substrate under multiple-turnover conditions; S, oligonucleotide substrate for ribozyme cleavage; P, the 5′ product of substrate cleavage by ribozyme; E, RNA enzyme or ribozyme; E·P, ribozyme−product complex; k_{off}^P, the first-order rate constant for dissociation of product from the ribozyme−product complex; P1, the duplex formed by base pairing of the ribozyme IGS to a reaction product P or substrate S; EDTA, ethylenediaminetetraacetic acid; PCR, polymerase chain reaction; wild-type (WT) ribozyme, *Tetrahymena* ribozyme with a GGAGGG internal guide sequence; dNTPs, deoxynucleoside triphosphates; K_d^P, the equilibrium dissociation constant for the ribozyme−product complex; HH, hammerhead ribozyme; *R*, the gas constant (1.987 cal mol^{-1} K^{-1}); *F*, fraction of the total ribozyme; $\Delta G°_{37}$, the standard free energy change at 37 °C.

S0006-2960(96)00510-7 CCC: $12.00

P1 docking also requires the conserved G-U wobble pair at the first base pair (Pyle et al., 1994; Knitt et al., 1994; Strobel & Cech, 1995, 1996), but base pair substitutions at other positions in P1 still allow intron self-splicing and ribozyme catalysis (Davies et al., 1982; Been & Cech, 1986; Waring et al., 1986; Doudna et al., 1989). Furthermore, isolated changes in the product strand of P1 that produce a mismatched duplex do not affect P1 docking (Herschlag & Cech, 1990a). These findings suggested a model in which docking of P1 is determined by P1 structure and the G-U wobble pair at the first base pair. However, more extensive base pair changes in P1 had larger effects on activity (Murphy & Cech, 1989), leaving open the possibility that these changes had altered the thermodynamics of substrate binding or P1 docking.

The kinetics of interactions between the wild-type ribozyme with matched and mismatched oligonucleotide substrates and products has been well characterized. Under single-turnover conditions ([S] << K_m); the reaction of the wild-type ribozyme with the matched substrate is limited by the association rate of E and S and is described by the second-order rate constant $(k_{cat}/K_m)^S$ (Herschlag & Cech, 1990b). Under multiple-turnover conditions ([S] >> K_m), after an initial burst of product formation that is stoichiometric with the ribozyme concentration, reaction of ribozyme and substrate is described by the first-order rate constant k_{cat}(mt). Under multiple-turnover conditions, product release is rate-limiting, and $k_{cat}(mt) = k_{off}^P = 0.1\ min^{-1}$ (Herschlag & Cech, 1990b).

Previously, we identified active ribozymes within a library of IGS mutants on the basis of their ability to cleave a long (818-nucleotide) RNA substrate (Campbell & Cech, 1995). Although identified ribozymes had similar rates of substrate association [$(k_{cat}/K_m)^S$], the rate of multiple turnover for these IGS mutant ribozymes differed up to 10-fold. Since, for the wild-type ribozyme, the rate of multiple turnover [k_{cat}(mt)] is equal to the dissociation rate for product (k_{off}^P), these findings suggested that the IGS mutations in these ribozymes affected product dissociation. Since product dissociation requires both undocking of P1 from the ribozyme core and the disruption of base pairing within the P1 helix, the differences in the multiple-turnover rates for the IGS mutant ribozymes could be caused by changes in the strength of tertiary interactions or simply by changes in the strength of duplex formation. Therefore, the present study was undertaken to investigate the effects of the IGS mutations in these ribozymes on P1 docking and to correlate thermodynamic binding with ribozyme catalysis.

MATERIALS AND METHODS

Preparation of Oligonucleotide RNA. Oligonucleotide RNA substrates were synthesized on an Applied Biosystems 394 DNA/RNA synthesizer. Oligonucleotides were deprotected in concentrated NH_4OH/ethanol (3:1) overnight at 55 °C. After drying in a vacuum, silyl protecting groups were removed by resuspending the pellet in 40 equiv of tetrabutylammonium fluoride (TBAF) per equivalent of silyl (400 μL of 1 M TBAF in THF) and incubated at room temperature overnight, in the dark. Following deprotection, the mixture was brought to 1.6 mL with 0.01 M Tris (pH 7.5) and 0.001 M EDTA and adjusted to 0.5 M NaCl. Oligonucleotides were ethanol precipitated, dried, and purified by electrophoresis on a denaturing 20% polyacrylamide gel. Excised gel slices were eluted with H_2O and eluents desalted by chromatography on C-18 Sep-Pak columns (Waters). Concentrations of purified RNA oligonucleotides were determined by absorption spectrophotometry.

Transcription and Purification of Ribozyme RNA. Mutant and wild-type ribozymes used in kinetic analyses were prepared as previously described by transcription of polymerase chain reaction (PCR) product (Campbell & Cech, 1995). PCR mixtures contained 0.5 μg of pT7L-21 linearized with *Pvu*I, a 0.5 μM sense primer specific for the IGS of each ribozyme, 0.5 μM antisense primer 3′L-21, 1.5 mM $MgCl_2$, 10 mM Tris (pH 8.3), 50 mM KCl, 0.2 mM dNTP's, and 2.5 of Taq polymerase (Boehringer Mannheim) in a final volume of 100 μL. The PCR mixture was thermally cycled in a Perkin-Elmer 9600 instrument for 35 cycles: 94 °C for 30 s, 50 °C for 30 s, and 72 °C for 1 min, with an additional 8.5 min of incubation at 72 °C after the last cycle. PCR products were separated by electrophoresis on a 1% agarose gel, and the 400 bp PCR product was isolated and eluted with Geneclean (Bio101). Gel-purified PCR product was digested with *Sca*I, extracted with phenol/chloroform, ethanol precipitated, and used as a template for T7 RNA polymerase. Transcription reaction mixtures consisted of ~10 μg of DNA template, 40 mM Tris (pH 7.5), 12 mM $MgCl_2$, CTP, UTP, ATP, and GTP (1 mM each), 4 mM spermidine, 10 mM dithiothreitol, and 50 u of T7 RNA polymerase in a 500 μL final volume. Transcription reaction mixtures were incubated at 37 °C for 2 h, the reactions terminated by addition of EDTA to a final concentration of 50 mM, and the mixtures loaded directly on a denaturing 4% polyacrylamide gel. The 388-nucleotide RNA was localized by UV shadowing, excised, and eluted in 5 mL of 0.01 M Tris (pH 7.5), 0.001 M EDTA, and 0.25 M NaCl at 4 °C overnight. RNA was precipitated with 2.5 volumes of 100% ethanol, washed twice with 70% ethanol, and resuspended in 100 μL of H_2O. The RNA was then adjusted to 2.5 M ammonium acetate, precipitated a second time with 100% ethanol, washed with 70% ethanol, and resuspended in 50 μL of H_2O. Purified ribozyme RNA was quantitated spectrophotometrically (Zaug et al., 1988).

Alternatively, the wild-type ribozyme was also prepared by T7 RNA polymerase transcription of pT7L-21 as previously described (Zaug et al., 1988) or by transcription of pG22C (gift of S. Strobel). The plasmid pG22C encodes a self-processing hammerhead ribozyme, with the hammerhead GUC cleavage sequence immediately 5′ to the L-21 ribozyme IGS. Transcription reaction mixtures employing pG22C contained 18 mM $MgCl_2$ but were otherwise identical to reaction mixtures described above. Under these conditions, self-processing occurred in the transcription reaction and precursor was not detected after electrophoresis of transcription reaction mixtures on preparative denaturing 4% polyacrylamide gels. The processed L-21 (5′-OH) RNA was purified exactly as described above.

Labeling of RNA Molecules. RNA molecules with 5′-OH were 5′ end labeled in a 10 μL reaction mixture that contained 25 pmol of RNA, 150 uCi [γ-^{32}P]ATP (6000 Ci/mmol, New England Nuclear), 10 u of T4 polynucleotide kinase (New England Biolabs), 70 mM Tris (pH 7.6), 10 mM $MgCl_2$, and 5 mM dithiothreitol. Reaction mixtures were incubated for 30 min at 37 °C. Following end labeling, all RNAs were gel purified as described above and quanti-

tated by scintillation counting. Prior to end labeling, ribozymes transcribed from PCR, pT7L-21, or pG22C templates were treated with calf intestine alkaline phosphatase, extracted with phenol/chloroform, and ethanol precipitated.

Determination of K_d^P. The dissociation constant for the ribozyme−product complex was determined by gel-shift analysis of mixtures of ribozyme and product at equilibrium. Ribozyme was renatured in 18 mM $MgCl_2$ at 50 °C for 10 min and cooled to 37 °C for 2 min. Final mixtures (11 μL) contained 0.01−360 nM ribozyme, 9 pM end-labeled P (oligonucleotide product), 1 mM GTP, 50 mM Tris (pH 7.5 at 37 °C), 10 mM $MgCl_2$, 10 mM NaCl, 140 mM KCl, and 3% glycerol. For ribozymes GGGGCU and GGCUCC, mixtures contained 1 pM product. For each K_d^P determination, nine to ten different ribozyme concentrations were used. Mixtures were incubated at 37 °C for a time 2τ (wild-type ribozyme for 8 h, mutant ribozymes for 0.25−88 h) estimated to allow for at least 88% association at the lowest ribozyme concentration, as given by $\tau^{-1} = k_{on}([E] + [P]) + k_{off}$ (Fersht, 1985), assuming that for each ribozyme $k_{on} = (k_{cat}/K_m)^S$ and $k_{off} = k_{cat}(mt)$ (Herschlag & Cech, 1990b). During incubation, mixtures were centrifuged every 6−8 h to minimize evaporative losses. At the end of the incubation, the entire mixture was loaded directly onto a nondenaturing 20% polyacrylamide gel equilibrated to 37 °C. Bound and free product were separated by electrophoresis at 10 W and 37 °C for 6 h and quantitated with a Molecular Dynamics PhosphorImager. The gel and electrophoresis buffers contained 34 mM Tris, 66 mM Hepes (pH 7.5), 0.1 mM EDTA, and 10 mM $MgCl_2$ (Pyle et al., 1990).

Determination of k_{off}^P. The rate of product dissociation from the ribozyme−product complex was determined by gel-shift analysis of pulse−chase experiments. Ribozyme was renatured in 10 mM $MgCl_2$ at 50 °C for 10 min and cooled to 37 °C for 2 min. During the pulse, renatured ribozyme was bound to 5′ end-labeled P in mixtures containing 200 nM ribozyme, 1 or 1000 nM P, 1 mM GTP, 50 mM Tris (pH 7.5 at 37 °C), 10 mM $MgCl_2$, 10 mM NaCl, and 140 mM KCl in a final volume of 2 μL. Following incubation at 37 °C for 10 min, the ribozyme−product complex was chased with 1 μM S and 3% glycerol in a final volume of 200 μL, and 10 μL aliquots were removed at specified times and loaded directly onto a nondenaturing 20% polyacrylamide gel equilibrated to 8 °C. Other electrophoresis conditions were exactly as described for determination of K_d^P. Bound and free product were quantitated with a phosphorimager. The effectiveness of the chase was determined in control experiments in which renatured ribozyme was added directly to an incubation mixture that contained 1 μM S and 0.02 nM 5′ end-labeled P.

Kinetics. The rate constant for multiple turnover of the wild-type ribozyme, $k_{cat}(mt)$, was determined in the presence of excess substrate (CCCUCUAA) with near-saturating concentrations of GTP. Ribozyme was renatured in 10 mM $MgCl_2$ at 50 °C for 10 min and cooled to 37 °C for 2 min. Final reaction mixtures (50 μL) contained 0.1−40 nM ribozyme, 6−400 nM end-labeled substrate (ratio of substrate to ribozyme 10:1 or greater unless otherwise specified), 1 mM GTP, 50 mM Tris (pH 7.5 at 37 °C), 10 mM $MgCl_2$, 10 mM NaCl, and 140 mM KCl. Reactions were initiated by addition of substrate and GTP (prewarmed to 37 °C for 2 min), and 7 μL aliquots were removed at specified times and mixed with an equal volume of stop solution (82% formamide, 50 mM EDTA, 0.04% xylene cyanol, 0.04% bromophenol blue, and 2 X Tris-borate EDTA). Substrate and product were separated by denaturing gel electrophoresis on a 20% polyacrylamide sequencing-type gel and quantitated with a phosphorimager. Electrophoresis was conducted under conditions that allowed single-nucleotide discrimination, and the identity of the expected cleavage product (CCCUCU) was confirmed by comigration with a synthetic 5′ end-labeled CCCUCU oligonucleotide. Reactions were followed after the stoichiometric burst to 10−40% product formation. Postburst rates of reaction were determined by first-order plots of data from at least six time points, and $k(mt) = k_{obs}[S]/[E]$.

Analysis of Ribozyme 5′ Ends. The 5′ terminal nucleotide of 5′ end-labeled RNA was determined by complete digestion with the base-specific ribonuclease U2. Reaction mixtures (final volume of 5 μL) contained 3 pmol of 5′-labeled RNA, 2 u of ribonuclease U2 (United States Biochemical), 27 mM sodium citrate (pH 3.5), 9 M urea, 1.4 mM EDTA, 0.04% bromophenol blue, and 0.04% xylene cyanol. Reaction mixtures were incubated at 50 °C for 60 min and analyzed by electrophoresis on a denaturing 20% polyacrylamide gel. All analyses included a control which lacked enzyme. An alkaline hydrolysis ladder was generated by incubation of 5 pmol of 5′-labeled RNA in 50 mM $NaHCO_3$ (pH 9.0) and 2 mM EDTA (final volume of 4.5 μL) at 94 °C for 12 min.

Native Gel Analysis of Ribozymes. Approximately 5 pmol of 5′ end-labeled ribozyme was incubated in either 50 mM Tris (pH 7.5 at 37 °C), 10 mM $MgCl_2$, 10 mM NaCl, and 140 mM KCl or 50 mM MES (pH 7.0 at 25 °C) and 10 mM $MgCl_2$ in a final volume of 50 μL. After incubation at 50 or 65 °C for specified times, 10 μL aliquots were removed, incubated at 37 °C for 2 min, and loaded directly onto a nondenaturing 4% polyacrylamide gel equilibrated to 37 °C. Electrophoresis conditions were exactly as described under Determination of K_d^P.

RESULTS

Effect of the Altered P1 Sequence on the Tightness of Product Binding. To determine the effect of mutations in the ribozyme IGS on the tightness of product binding, equilibrium binding of the matching product to the wild-type ribozyme and to ribozymes with mutant IGSs was studied (Table 1, Figure 1A). These mutant ribozymes were chosen for study because previous kinetic characterization under single- and multiple-turnover conditions showed that they were catalytically active ribozymes with rates of catalysis similar to or faster than that of the wild-type ribozyme (Campbell & Cech, 1995).

The results of a single equilibrium binding study for each ribozyme are shown in Figure 1B. In each case, the data fit well to the binding equation. At least 85% of the product was bound at the maximum ribozyme concentration except for ribozyme GGCUCC, for which a maximum of only 55% of the product was bound. Increasing the concentration of ribozyme GGCUCC, the time of incubation, or the use of different oligonucleotide preparations did not increase the maximum amount of product bound. Thus, product binding studies for this ribozyme reflect the interaction of ribozyme with only half of the product molecules. The incubation time for binding ranged from 15 min for ribozyme GGAGAU to

Table 1: Binding of Matched Oligonucleotides to Wild-Type and Mutant Ribozymes

ribozyme IGS[a]	product	ribozyme binding[b] K_d (nM)	ribozyme binding[b] $-\Delta G°_{37}$ (kcal/mol)	duplex formation[c] $-\Delta G°_{37}$ (kcal/mol)	tertiary[d] $-\Delta G°_{37}$ (kcal/mol)
GGAGGG	CCCUCU	0.1 ± 0.04	14.3 ± 0.3	9.4	4.9 ± 0.3
GGAGUC	GACUCU	0.7 ± 0.2	13.1 ± 0.2	8.6	4.5 ± 0.2
GGGGCU	AGCCCU	0.01 ± 0.01	15.6 ± 0.5	10.1	5.5 ± 0.5
GGAGAU	AUCUCU	14 ± 3	11.1 ± 0.1	6.4	4.7 ± 0.1
GGCUCC	GGAGCU	0.004 ± 0.002	16.3 ± 0.3	10.5	5.8 ± 0.3
GUGGCU	AGCCAU	2.4 ± 0.5	12.3 ± 0.2	8.1	4.2 ± 0.2

[a] Internal guide sequence for ribozymes transcribed from PCR-derived templates. [b] Binding of ribozymes to product oligonucleotide evaluated by gel shift. $\Delta G°$ is calculated from $\Delta G° = -RT \ln(1/K_d)$. Each value is the mean of two independent determinations ± the range. Data for a single K_d determination for each ribozyme are shown in Figure 1. [c] Free energy change for duplex formation was calculated as previously described (Turner et al., 1990; He et al., 1991) assuming $\Delta G°_{37}$ for initiation of helix formation was 3.4 kcal/mol. [d] Free energy of tertiary interactions is the free energy of ribozyme binding minus the free energy of duplex formation. Values are the mean of duplicate determinations ± the range.

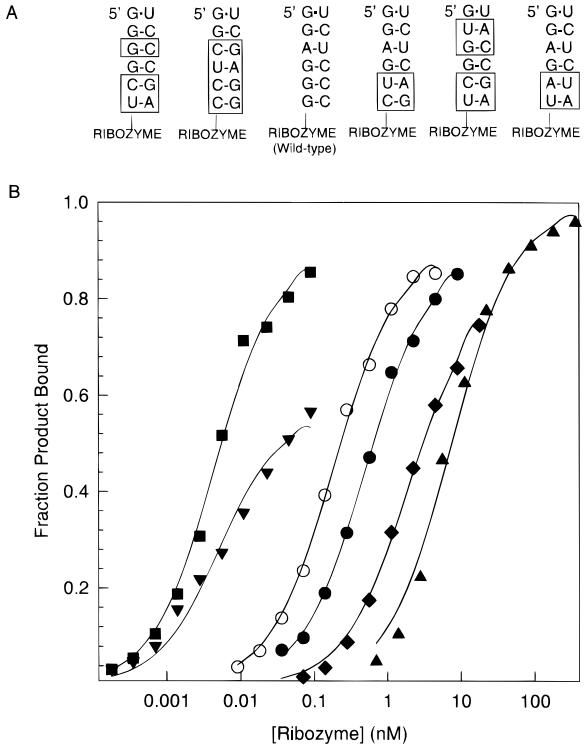

FIGURE 1: Effect of ribozyme internal guide sequence (IGS) mutations on equilibrium binding of oligonucleotides of complementary sequence. (A) IGS−oligonucleotide combinations studied herein, shown in order of increasing K_d. The left strand of each pair is the IGS of ribozyme, and the right strand is oligonucleotide product. Boxed sequences differ from wild type. (B) Wild-type and mutant ribozymes were bound to 1−10 pM matching oligonucleotide product. Ribozymes are (by internal guide sequence) GGGGCU (■), GGCUCC (▼), GGAGGG (○), GGAGUC (●), GUGGCU (◆), and GGAGAU (▲). For ribozymes GGAGGG, GGAGUC, GUGGCU, and GGAGAU, [P] << K_d^P and data were fitted to fraction product bound = (maximum fraction of product bound)[E]/(K_d + [E]) to obtain K_d^P. For GGGGCU and GGCUCC, [P] ≈ K_d^P and data were fitted to fraction product bound = (maximum fraction of product bound)[K_d + [E] + [P] − [(K_d + [E] + [P])^2 − 4[E][P]]$^{1/2}$]/2[P] to obtain K_d^P. Curves represent least squares fits of the data to these equations.

76 h for GGCUCC. In repeat experiments, increasing the incubation time for each ribozyme did not significantly change the binding curves. The data from two independent binding studies were used to determine the K_d^P for each ribozyme (Table 1). It is not known if cleavage of the product oligonucleotides occurred during these incubations. If cleavage did occur, these values for K_d^P would be upper limits. Our value of K_d^P = 0.10 ± 0.04 nM for the wild-type ribozyme determined by gel-shift analysis at 37 °C is comparable to the values determined by equilibrium dialysis (K_d = 0.2 nM) or gel-shift analysis (K_d = 0.3 ± 0.1 nM) at 42 °C (Pyle et al., 1992, 1994; Strobel & Cech, 1993).

Table 2: Discrepancy between k_{cat}(mt) and k_{off}^P for Wild-Type and Mutant Ribozymes

ribozyme[a]	$(k_{cat}/K_m)^S$ (10^8 M^{-1} min^{-1})[b]	k_{cat}(mt) (min^{-1})[b]	k_{off}^P (min^{-1})[c]	k_{cat}(mt)/k_{off}^P	k_{on}^P (10^8 M^{-1} min^{-1})[d]
GGAGGG	0.5	0.3	0.004	75	0.4
GGAGUC	0.5	0.5	0.01	50	0.1
GGGGCU	1.8	0.8	0.0009	889	0.9
GGAGAU	0.5	2	0.06	33	0.04
GGCUCC	0.8	0.2	0.001	200	3
GUGGCU	0.5	0.6	0.03	20	0.1

[a] Internal guide sequence for ribozymes transcribed from PCR-derived templates. [b] Values for the rate constants for cleavage of matching substrate under single-turnover, $(k_{cat}/K_m)^S$, and multiple-turnover conditions, k_{cat}(mt), were published previously (Campbell & Cech, 1995). [c] Values of the rate constant for product dissociation, k_{off}^P, are from the data in Figure 3A. [d] Values of the rate constant for product association, k_{on}^P, are calculated from $k_{on}^P = k_{off}^P/K_d^P$.

All five ribozymes with P1 alterations had binding curves (and K_d values) that were shifted from those of the wild-type ribozyme (Figure 1, Table 1). For ribozymes GGAGUC, GGAGAU, GGCUCC, and GUGGCU, the shift in the binding curve was related to the change in the value of k_{cat}(mt) for these ribozymes (Table 2). An exception to this was ribozyme GGGGCU which previously was found to have enhanced multiple turnover compared to the wild-type ribozyme [k_{cat}(mt) = 0.8 versus 0.3 min^{-1}] but has a 10-fold tighter product binding relative to the wild type (K_d^P = 0.01 versus 0.1 nM).

Effect of the Altered P1 Sequence on Tertiary Interactions between the P1 Helix and the Ribozyme Core. For the wild-type ribozyme, the tightness of product binding reflects the free energy of duplex formation plus the free energy of tertiary interactions between the P1 helix and the ribozyme core (Pyle et al., 1992). For the IGS mutant ribozymes, the free energy of binding ranged from −11.1 to −16.3 kcal/mol (Table 1). To assess the free energy of tertiary interactions for the ribozymes with altered P1 sequences, the free energy of duplex formation between the IGS and the corresponding product was calculated by nearest neighbor parameters for each ribozyme (Turner et al., 1990; He et al., 1991). By subtraction, the tertiary free energy for the wild-type ribozyme binding to CCCUCU is calculated as $\Delta G°_{37}(3°)$ = −4.9 ± 0.3 kcal/mol (Table 1). The average

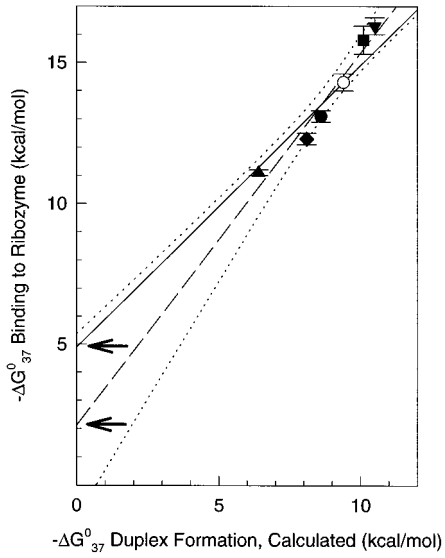

FIGURE 2: Changes in the base composition of P1 do not affect tertiary interactions. Ribozymes are (by IGS) GGGGCU (■), GGCUCC (▼), GGAGGG (○), GGAGUC (●), GUGGCU (◆), and GGAGAU (▲). The measured $\Delta G°$ for binding of the matching oligonucleotide product to the ribozyme and the calculated $\Delta G°$ for duplex formation between the IGS and the oligonucleotide are given in Table 1. The broken line is linear regression by a least squares fit to the data: slope = 1.3, *y*-intercept = −2.2 kcal/mol, and the coefficient of determination (r^2) = 0.96. Dotted lines show the 95% confidence intervals for the least squares fit. The solid line is the model where the *y*-intercept = −4.9 kcal/mol and the slope = 1. The *y*-intercepts (indicated by horizontal arrows) are the tertiary interaction energies for the two fits. Error bars represent the range of two independent determinations.

$\Delta G°_{37}(3°)$ for the five mutant ribozymes was also −4.9 kcal/mol. The model that $\Delta G°(\text{total}) = \Delta G°(\text{duplex}) + \Delta G°(3°)$, with $\Delta G°(3°) = -4.9$ kcal/mol, gives a reasonable fit to the data for all the mutant ribozymes (solid line in Figure 2). Long extrapolation outside of the range of the data by a model-independent least squares fit (broken line in Figure 2) gives a $\Delta G°(3°)$ of −2.2 kcal/mol (*y*-intercept) that is not significantly different from the model value of −4.9 kcal/mol (see dotted lines in Figure 2).

The value of $\Delta G°(3°) = -4.9$ kcal/mol for the wild-type ribozyme is about 1 kcal/mol greater than that obtained by equilibrium dialysis and thermal denaturation analysis of duplex stability [$\Delta G°_{42}(3°) = -14.0$ kcal/mol − −10.1 kcal/mol = −3.9 kcal/mol; Pyle et al., 1994]. Some of this difference can be accounted for by a previous finding that the free energy of duplex formation for CCCUCU binding to GGAGGGAAA measured by thermal denaturation at 42 °C is 0.7 kcal/mol more favorable than predicted by the nearest neighbor model (Pyle et al., 1994). Assuming that any error in estimation of duplex formation free energy is uniform for the wild-type and mutant ribozymes, ribozyme IGS mutations do not significantly affect the free energy of tertiary interactions (Table 1, solid line in Figure 2). One exception may be ribozyme GGCUCC [$\Delta G°_{37}(3°) = -5.8 \pm 0.3$ kcal/mol]. Although the tighter than expected binding seen with ribozyme GGCUCC was reproducible, it may be an overestimate of the true tightness of binding since K_d^P for this ribozyme was determined with [P] ≈ K_d^P and at equilibrium a maximum of only 55% of P was bound to ribozyme (Figure 1).

Effect of the Altered P1 Sequence on the Dissociation Rate of the Ribozyme−Product Complex (k_{off}^P). If $k_{cat}(\text{mt}) = k_{off}^P$ and $(k_{cat}/K_m)^S = k_{on}^P$, then for the wild-type ribozyme the expected value of the multiple-turnover rate constant was $k_{cat}(\text{mt}) = K_d^P[(k_{cat}/K_m)^S] = (0.1\text{ nM})(5 \times 10^7\text{ M}^{-1}\text{ min}^{-1}) = 0.005\text{ min}^{-1}$. Thus, even though the tightness of product binding by the wild-type ribozyme correlates well with measurements made by others, the previously reported multiple-turnover rate of 0.3 min^{-1} (Campbell & Cech, 1995) is 60-fold faster than predicted by K_d^P. The multiple-turnover rates for the mutant ribozymes are also 3−667-fold faster than predicted by K_d^P, if $k_{off}^P = k_{cat}(\text{mt})$. To test whether the values of k_{off}^P were really as predicted from K_d^P, we directly determined the rate of dissociation of product from the enzyme−product complex (k_{off}^P) for the wild-type and mutant ribozymes under conditions similar to those used for determination of K_d^P ([E] >> [P]).

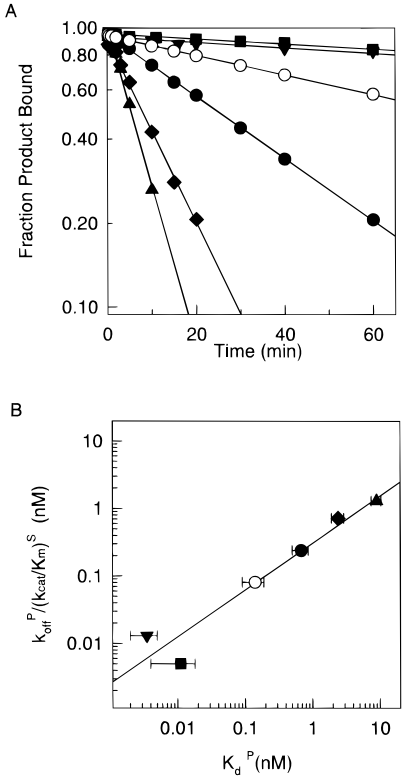

FIGURE 3: Dissociation rate of matching product from wild-type and mutant ribozymes under conditions of ribozyme excess correlates with K_d. Ribozymes are (by IGS) GGGGCU (■), GGCUCC (▼), GGAGGG (○), GGAGUC (●), GUGGCU (◆), and GGAGAU (▲). Nucleotide sequences of products are given in Table 1. (A) Effect of internal guide sequence mutations on dissociation of the matching product from the *Tetrahymena* ribozyme. Dissociation rates for ribozyme−product complexes for mutant and wild-type ribozymes were determined by binding ∼1 nM product to 200 nM ribozyme in pulse−chase experiments. Unlabeled substrate and near-saturating GTP concentrations were used in the chase. Lines represent linear regression by a least squares fit to data, and the slope of these lines equals $-k_{off}^P$. (B) Relationship of k_{off}^P and $(k_{cat}/K_m)^S$ to K_d^P for mutant and wild-type ribozymes. Lines represent linear regression by a least squares fit to data: slope = 0.7 and the coefficient of determination (r^2) = 0.93. Error bars represent the range of two independent K_d^P determinations.

Product dissociation for the wild-type and mutant ribozymes was first-order and linear with time on a semilogarithmic plot (Figure 3A). In control experiments to determine the effectiveness of the chase in these pulse−chase experiments for each ribozyme, bound product was not detected. The use of either excess unlabeled product or excess unlabeled substrate in the chase did not affect the rate of

dissociation of bound labeled product. Similarly, the rate of product dissociation was not affected by the presence or absence of near-saturating concentrations of GTP in the chase.

The Product Dissociation Rate (k_{off}^P) Correlates with K_d^P but Not with the Multiple-Turnover Rate. From the slope of each line in Figure 3A, the rate constant of product dissociation from the enzyme−product complex (k_{off}^P) was determined (Table 2). Since for the wild-type, GGGGCU, and GGCUCC ribozymes product dissociation was followed only to 70−80% of the product bound, the product dissociation rates determined for these ribozymes represent initial rates of dissociation under these conditions. The rates of dissociation followed the hierarchy predicted by K_d^P (compare Figures 1B and 3A). For the wild-type ribozyme, $k_{off}^P = 0.004$ min^{-1}, a value within error equal to that predicted by K_d^P (0.005 min^{-1}). Since the ratio of $k_{off}^P/(k_{cat}/K_m)^S$ approximated K_d^P for the wild-type and mutant ribozymes (Figure 3B), the rate of product association is approximated by $(k_{cat}/K_m)^S$. In the case of ribozyme GGAGAU, the calculated value of k_{on}^P is 10-fold less than $(k_{cat}/K_m)^S$ (Table 2). This discrepancy may be the result of error in measurement of a weak K_d^P by gel-shift analysis. Thus, $(k_{cat}/K_m)^S$ approximates k_{on}^P for these ribozymes, but the dissociation rates of product for the wild-type and mutant ribozymes do not equal the values of k_{cat}(mt) for these ribozymes.

Effect of the Ribozyme Preparation Method on the Multiple-Turnover Rate. The wild-type and mutant ribozymes used in the studies described above were prepared by transcription of a PCR-derived template. Previous kinetic and binding analyses of the wild-type ribozyme used ribozymes transcribed from plasmid templates (Herschlag & Cech, 1990b; Pyle et al., 1992).

To assess the effect of different ribozyme preparation techniques on the ribozyme multiple-turnover rate, the value of k_{cat}(mt) for the wild-type ribozyme prepared by three different methods was determined (Figure 4, Table 3). Under the conditions used for these determinations, only a single product was observed when reaction mixtures were analyzed to single-nucleotide resolution on denaturing polyacrylamide gels. This single product comigrated with an authentic CCCUCU oligonucleotide. The value of k_{cat}(mt) for a ribozyme transcribed from a plasmid template was within error identical to the value measured for a ribozyme that was post-transcriptionally processed at the 5′ end by a hammerhead ribozyme. The value of k_{cat}(mt) for the PCR-prepared ribozyme was approximately 2-fold greater than the values for the plasmid- and hammerhead-prepared ribozymes. Thus, the method of ribozyme preparation had only a minimal effect on the value of k_{cat}(mt), and the values of k_{cat}(mt) for the wild-type ribozyme (0.06−0.16 min^{-1}) were 15−40-fold greater than the value of k_{off}^P (0.004 min^{-1}).

The values for k_{cat}(mt) in the present study were determined under the same conditions as the library screening in which the mutant ribozymes were first identified [50 mM Tris (pH 7.5), 10 mM $MgCl_2$, 10 mM NaCl, and 140 mM KCl at 37 °C; Campbell & Cech, 1995]. Collectively, these values of k_{cat}(mt) for the wild-type ribozyme prepared by three different methods (Table 3) are in agreement with previously reported values for plasmid-transcribed wild-type ribozyme under slightly different conditions [in 50 mM MES (pH 7) and 10 mM $MgCl_2$ at 50 °C, k_{cat}(mt) = 0.1 or 0.3 ± 0.1 min^{-1}; Herschlag & Cech, 1990b; Strobel & Cech, 1993].

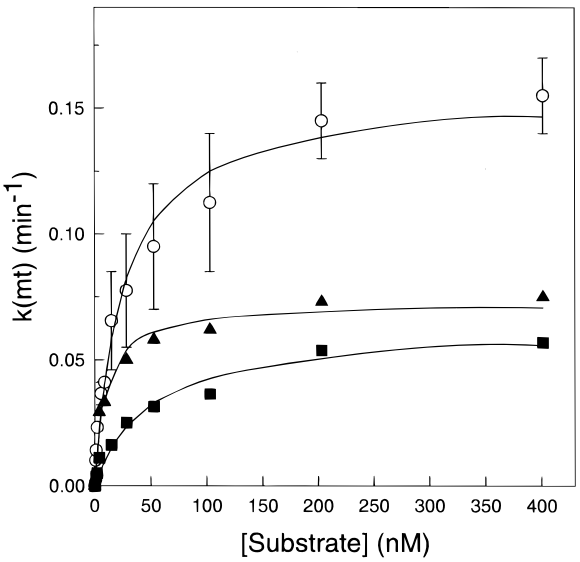

FIGURE 4: Ribozyme preparation method has a modest effect on k_{cat}(mt) for the wild-type ribozyme. The wild-type ribozyme transcribed from templates derived from the PCR product (WT-PCR, ○), the plasmid (WT-pT7, ▲), or the plasmid containing a gene for a self-processing hammerhead ribozyme 5′ to the *Tetrahymena* ribozyme (WT-HH, ■) was incubated with the matching substrate (CCCUCUAA) under multiple-turnover conditions ([S]/[E] = 10). The observed rate of multiple turnover was determined from the postburst slope of first-order plots of the data, and k(mt) = k_{obs}[S]/[E]. Curves show least squares fits of data obtained at each substrate concentration to the Michaelis−Menten equation. Data for WT-PCR are the mean of two determinations on independently prepared ribozyme transcripts ± the range.

Table 3: Ribozyme Multiple-Turnover Rate Constant Equals the Weighted Average Product Dissociation Rate Constant for Two Ribozyme Populations

		phase 1[c]		phase 2[c]		weighted
ribozyme[a]	k_{cat}(mt) (min^{-1})[b]	k_{off}^P (min^{-1})	F_1	k_{off}^P (min^{-1})	F_2	average[d] k_{off}^P(min^{-1})
WT-PCR	0.16 ± 0.02	0.3	0.21	0.006	0.79	0.07
WT-pT7	0.07	0.5	0.18	0.005	0.82	0.09
WT-HH	0.06	0.2	0.14	0.02	0.86	0.05

[a] Wild-type ribozymes were transcribed from a PCR-derived template (WT-PCR), from a plasmid template (WT-pT7), or from a plasmid template that contained a gene for a self-processing hammerhead ribozyme at the 5′ end of the *Tetrahymena* ribozyme (WT-HH). [b] The first-order rate constant under conditions of substrate saturation was determined from the data in Figure 4. The value for the WT-PCR ribozyme is the mean of two independent determinations ± the range. [c] Dissociation rate constant for product oligonucleotide (CCCUCU) for each phase of dissociation and the fraction of the total in each phase (F) were determined from the data in Figure 5. [d] The weighted average dissociation rate constant = $(k_{off}^P)_1(F_1) + (k_{off}^P)_2(F_2)$.

The presence or absence of monovalent salt at 37 °C did not significantly change the value of k_{cat}(mt) for the WT-pT7 ribozyme [k_{cat}(mt) = 0.06 min^{-1} in 50 mM Tris (pH 7.5), 10 mM $MgCl_2$, 10 mM NaCl, 140 mM KCl, and 1 μM S versus 0.05 min^{-1} in 50 mM MES (pH 7), 10 mM $MgCl_2$, and 1 μM S].

Evidence for Two Different Product Dissociation Rates for the Wild-Type Ribozyme. Values of K_d^P and k_{off}^P (determined under conditions of ribozyme excess, [E] >> [P]) did not correlate with the values of k_{cat}(mt) (determined under conditions of substrate excess, [E] < [S]). This discrepancy could be explained by the presence of two or more kinetically distinct populations of ribozymes in our

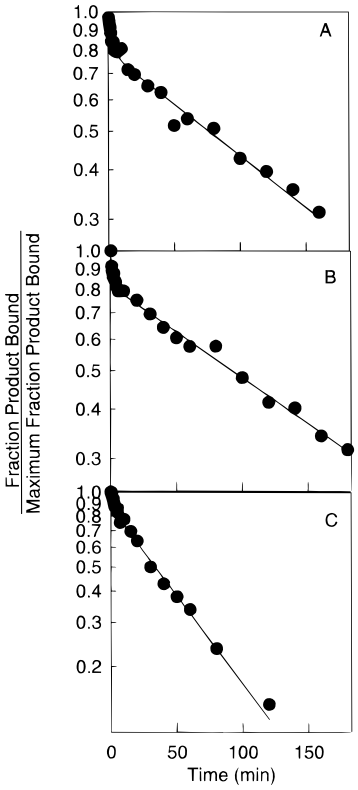

FIGURE 5: Dissociation of the matching product from wild-type ribozymes under conditions of product excess is biphasic. Dissociation rates for enzyme−product complexes for wild-type ribozymes were determined by binding 1 μM product to 200 nM ribozyme in pulse−chase experiments: (A) WT-PCR, (B) WT-pT7, and (C) WT-HH. Curves show least squares fits of data to the equation for double-exponential decay, fraction bound $= F_1e^{-k_1t} + F_2e^{-k_2t}$. In this analysis, it was assumed that ribozyme preparations consist of two populations, each with a distinct rate of product dissociation. F_1 and F_2 are the fractions of ribozymes in populations 1 and 2, and $F_1 + F_2 = 1$. The values of k_1 and k_2 are the dissociation rate constants for product bound to these ribozyme populations. Since $F_1 + F_2 = 1$, data were normalized to the maximum fraction product bound.

preparations: one population that binds product tightly (E_{tight}) and a second population that binds product weakly (E_{weak}). Because of competition between E_{tight} and E_{weak} for binding of free P, measurements of K_d^P and k_{off}^P made with excess ribozyme [where $(K_d)_{weak} >> (K_d)_{tight} >>$ [P]] would be expected to assess only ribozyme populations that bind product tightly. In the case of measurements made with excess substrate [e.g., k_{cat}(mt) where [S] >> [E] >> $(K_d)_{weak} >> (K_d)_{tight}$], both E_{tight} and E_{weak} are saturated with S, which is then cleaved to give P. Since $k_{cat}(mt) = k_{off}^P$, if ribozyme preparations are a mixture of E_{tight} and E_{weak}, the value of k_{cat}(mt) would be expected to be a weighted average of $(k_{off}^P)_{tight}$ and $(k_{off}^P)_{weak}$.

To determine if populations of ribozymes with different rates of product dissociation were present in our ribozyme preparations, the rate of product dissociation was determined after binding excess labeled product to the wild-type ribozyme in pulse−chase experiments (Figure 5). Wild-type ribozyme, prepared either by transcription of PCR or plasmid templates or by post-transcriptional hammerhead processing, exhibited two distinct rates of product dissociation (Table 3). The fraction of ribozyme with fast and slow product dissociation rates was similar for all three ribozyme preparations. For the wild-type ribozyme prepared by transcription

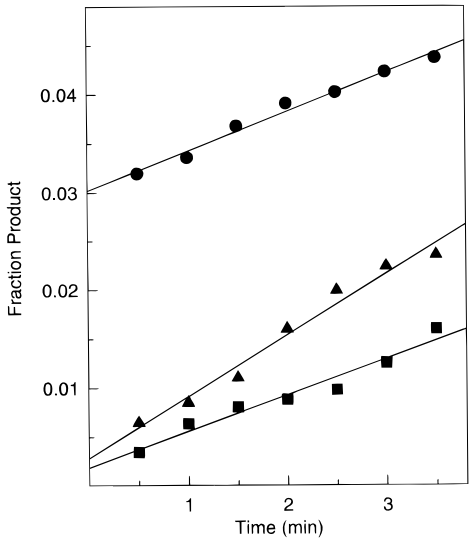

FIGURE 6: Effect of prebound product oligonucleotide on substrate cleavage by the wild-type ribozyme. Ribozyme (150 nM) was incubated with either buffer alone or 600 nM CCCUCU or CCCUCC at 37 °C for 10 min in a final volume of 20 μL. Reactions were initiated by addition of 30 μL of 5′ end-labeled substrate (CCCUCUAA; final concentration of 1.3 μM). Buffer, salt, and GTP concentrations were as described in Materials and Methods: buffer alone (●), prebound CCCUCU (■), and prebound CCCUCC (▲). Data were fitted by least squares linear regression. The slope of these lines is k_{obs}, and k(mt) $= k_{obs}$[S]/[E].

of a PCR template, the value of k_{off}^P for the slow phase (0.006 min^{-1}) was within error equal to the value determined under ribozyme excess conditions (0.004 min^{-1}). Furthermore, the weighted average k_{off}^P for each ribozyme preparation was similar to the value of k_{cat}(mt) for that preparation and similar to a value of k_{off}^P (0.06 min^{-1}) determined kinetically (Herschlag & Cech, 1990b). Thus, ribozyme multiple turnover reflects two different product dissociation rates.

One possible explanation for the biphasic rates of product dissociation is the binding of product in an alternative register with subsequent cleavage to form P′ (CCCUC) or P″ (CCCU) (Herschlag, 1992). Analysis of ribozyme−product mixtures after the binding incubation found that only 0.7% of the product oligonucleotide was cleaved to form a second product (presumably P″ on the basis of relative mobility). These results suggest that only a small portion of the fast phase of product dissociation may be due to dissociation of P″.

Effect of Prebound Product on Ribozyme Catalysis. The discrepancy between the values of K_d^P and k_{off}^P (determined under conditions of ribozyme excess) and the values of k_{cat}(mt) could also be explained by the seemingly unlikely possibility that the ribozyme might be capable of substrate cleavage without product release. To more directly determine the effect of product release on ribozyme catalysis, cleavage of substrate by wild-type ribozyme bound to product was determined during a single-ribozyme turnover in the presence of saturating substrate. If substrate cleavage requires product release, then the rate of substrate cleavage under these conditions would be expected to equal the rate of product release. Wild-type ribozyme was preincubated with either buffer alone or a molar excess of CCCUCU or CCCUCC oligonucleotide (Figure 6). For ribozyme that was preincubated with buffer alone, there was a burst that was stoichiometric with the ribozyme concentration followed by substrate cleavage at a rate [k(mt) $= k_{obs}$[S]/[E] $= 0.1$ min^{-1}]

that within error is equal to k_{cat}(mt) (Table 3). Prebound product (CCCUCU) eliminated the stoichiometric burst, but the rate of substrate cleavage was unchanged [k(mt) = 0.1 min^{-1}].

Replacement of the G-U pair between the ribozyme 5′ terminal G and product −1U with a G-C pair results in loss of tertiary interactions between the P1 helix and the ribozyme core, such that the K_d^P for binding of CCCUCC to the wild-type ribozyme is 6.4 nM (Pyle et al., 1994). Assuming that $(k_{on}^P)^{CCCUCC} = (k_{on}^P)^{CCCUCU}$, the estimated k_{off}^P for CCCUCC bound to the wild-type ribozyme is 0.3 min^{-1}. If $k_{off}^P = k_{cat}$(mt), then ribozyme prebound with product that dissociates rapidly (e.g., CCCUCC) should give a faster rate of single-turnover substrate cleavage relative to ribozyme prebound with a more slowly dissociating product (e.g., CCCUCU). As was the case for prebinding of CCCUCU, prebinding of CCCUCC to wild-type ribozyme also eliminated the stoichiometric burst (Figure 6). Moreover, as predicted, the rate of substrate cleavage [k(mt) = 0.2 min^{-1}] was faster than the rate observed for ribozyme prebound with CCCUCU (Table 3). These results confirm that the rate of multiple-turnover catalysis is equal to the rate of product release, and they further suggest that the fast phase of product release in Figure 5 might be due to a ribozyme population that has decreased tertiary interactions between the P1 helix and the ribozyme core.

Analysis of Ribozyme 5′ Ends. The finding of similar multiple-turnover rates and similar rates of product dissociation for wild-type ribozymes prepared by different methods suggested that the kinetically different ribozyme populations were produced post-transcriptionally. Exogenous exonuclease activity or self-hydrolysis of the 5′ terminal G of the ribozyme, perhaps during preincubation, could in principle give a subpopulation of ribozyme that released RNA product more quickly. To test for sequence heterogeneity in the ribozyme IGS, 5′ end-labeled wild-type ribozymes (PCR, pT7, and HH) and control oligonucleotides (GGAGCU, GACUCU, and AUCUCU) were completely digested with adenosine-specific ribonuclease U2. If exogenous exonuclease activity or ribozyme self-hydrolysis did in fact occur, one result would be a subpopulation of ribozymes with a GAGGG internal guide sequence. To determine the ability of this analysis to detect ribozymes with shortened IGS's, an L-22 ribozyme (IGS = GAGGG) was also evaluated. Analysis of these digests by electrophoresis on a denaturing polyacrylamide gel (data not shown) revealed that greater than 98% of the wild-type ribozymes had a 5′ terminus of GGA. Additionally, loss of the ribozyme 5′ terminal G was not observed after incubation of 5′ end-labeled ribozymes under conditions used for ribozyme renaturation. Therefore, the two kinetically distinct populations of ribozymes were not the result of 5′ end sequence heterogeneity.

Effects of Renaturation Conditions on the Ribozyme Multiple-Turnover Rate. In a previous study, electrophoresis of 5′ end-labeled ribozyme preparations on polyacrylamide gels under native conditions revealed three different ribozyme species: a monomer, a putative dimer, and a trimer (Bevilacqua, 1993). Likewise, after electrophoresis of wild-type ribozyme renatured in 10 mM $MgCl_2$ at 50 °C for 10 min on a nondenaturing 4% polyacrylamide gel, we also observed bands consistent with monomer (89% of the total) and multimers (11% of the total). Multimers were not resolved with incubation at 50 °C for up to 60 min (11% of the total) but were partially resolved by incubation at 65 °C for 30 min (3% of the total). However, preincubation of wild-type ribozyme at 65 °C for 30 min resulted in substantial ribozyme degradation visible on denaturing gel electrophoresis and a 57% reduction in the stoichiometric burst under multiple-turnover conditions. Thus, it proved impractical to test whether different aggregated states of the ribozyme were responsible for the biphasic kinetics of product dissociation.

DISCUSSION

The P1 Sequence Does Not Affect Tertiary Interactions between the P1 Helix and the Ribozyme Core. Tertiary interactions between the ribozyme core and the P1 helix are critical for docking of the P1 helix into the active site. The ribozyme core recognizes the 2′-hydroxyls of position −3 of the product strand, and of G22 and G25 of the ribozyme IGS (Bevilacqua & Turner, 1991; Pyle et al., 1992; Strobel & Cech, 1993) and also recognizes the exocyclic amine of G22 as positioned by the G-U wobble pair (Strobel & Cech, 1995, 1996). Furthermore, formation of tertiary interactions requires the correct secondary structure of the P1 helix (Doudna et al., 1989) and proper positioning of P1 within the core by the ribozyme J1/2 region (Young et al., 1991). We have studied the effects of a number of compensatory IGS and oligonucleotide product mutations, in catalytically active ribozymes, on the binding of product by equilibrium and kinetic methods.

The mutant ribozymes used in our studies were not systematically designed. Rather, three ribozymes were identified from a library of IGS mutants by virtue of their efficient cleavage of a long (818-nucleotide) substrate RNA (Campbell & Cech, 1995). Two additional catalytically active ribozymes present within the ribozyme library, but not identified by the library screening, were studied for comparison. These five ribozymes were chosen for this study because the IGS mutations had little effect on the rate of catalysis under $(k_{cat}/K_m)^S$ conditions but produced up to a 10-fold change in the multiple-turnover rate.

In the present study, the conserved G-U pair was maintained at the first base pair in all P1 helixes. Even though the P1 base changes were not systematically designed, and only a limited number of P1 sequences were tested (Figure 1A), in aggregate, they involve changes at every base pair in positions 2−6 of P1 and they represent both transitions (e.g., A-U → G-C) and transversions (e.g., G-C → C-G). Given the linear relationship between the free energy change of ribozyme binding and the free energy of duplex formation (Figure 2), we conclude that these changes affected duplex stability but had no discernable effect on tertiary interaction energy. Thus, the results of our equilibrium binding studies are in agreement with the model in which docking of the P1 helix results from 2′-hydroxyl interactions and the conserved G-U pair, and the strength of these interactions is not influenced by changes in base pairs 2−6 of P1.

Mutations in the Ribozyme IGS Change K_d^P by Increasing or Decreasing k_{off}^P. The IGS mutations in the ribozymes in our study resulted in an up to 140-fold difference in K_d^P relative to that of the wild-type ribozyme. The rate of association of product, as judged by measuring $(k_{cat}/K_m)^S$, was not affected much. Instead, the changes in K_d^P for the IGS mutant ribozymes were caused by increases or decreases in the rate of product dissociation. Similarly, modification

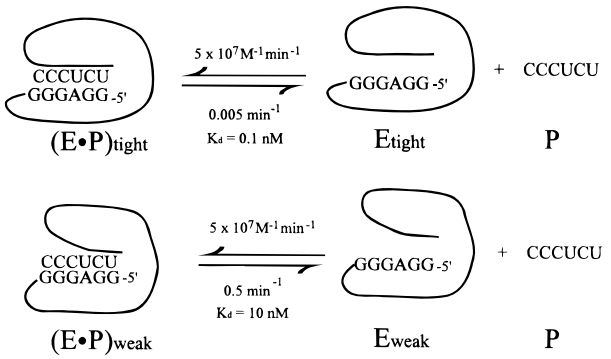

FIGURE 7: Schematic representation of the two-conformation model for docking of the P1 helix. Formation of the P1 helix is indicated by binding of P (5′-CCCUCU) to the ribozyme IGS (5′-GGAGGG). In the case of the wild-type ribozyme, 80% of the ribozyme preparation exists as the E_{tight} conformation which docks P1 tightly. Approximately 20% of the ribozyme preparation exists as E_{weak} which docks P1 weakly. The value of K_d for E_{weak} was calculated assuming that $(k_{on}^P)_{weak} = (k_{on}^P)_{tight} = 5 \times 10^7\ M^{-1}\ min^{-1}$.

of 2′-hydroxyls within the P1 helix and small insertions or deletions in J1/2 do not affect the substrate association rate, and changes in K_d^P result from changes in k_{off}^P (Strobel & Cech, 1993; Young et al., 1991).

The Multiple-Turnover Rate [k_{cat}(mt)] Equals the Average Rate of Product Dissociation from Two Different Ribozyme Populations. The values of k_{off}^P determined by gel-shift analysis are in good agreement with the values of K_d^P also determined by gel-shift analysis and the values of $k_{on} = (k_{cat}/K_m)^S$ determined by single-turnover kinetics. In other words, $k_{off}^P = k_{on}^P K_d^P$ as expected. Despite these consistencies, the values of k_{off}^P for the wild-type and mutant ribozymes are significantly less than the corresponding values for k_{cat}(mt). Initially, this result was troubling, because it seemed to indicate that multiple turnover was occurring much faster than one of the steps in the catalytic cycle. This discrepancy can be explained by a simple model in which each ribozyme preparation consists of two unique conformations (or aggregation states) of ribozymes: one with a fast k_{off}^P (E_{weak}) and one with a slower k_{off}^P (E_{tight}; Figure 7). Under conditions where $[E] > (K_d^P)_{weak} >> (K_d^P)_{tight} >> [P]$ (Figures 1 and 3), there is competition between E_{weak} and E_{tight} for binding of product and only the ribozyme conformation that binds product tightly is measured. In contrast, under conditions where $[P] >> [E] > (K_d^P)_{weak} >> (K_d^P)_{tight}$ (Figures 4 and 5), the binding sites of both E_{tight} and E_{weak} are saturated and both populations are measured. We cannot exclude the existence of an isomerization step between E_{weak} and E_{tight}. However, the product dissociation curves for $[P] >> [E]$ fit the equation that describes exponential decay by two independent unimolecular processes (Figure 5) and $k_2 = (K_d^P)_{tight}(k_{cat}/K_m)^S = (k_{off}^P)_{tight}$. These findings suggest that, if an isomerization step exists, the rate for the isomerization step is equal to, or slower than, the rate of product release from E_{tight}.

Our data suggest that the increased value of k_{off}^P for E_{weak} is due to the loss of about half of the energy of tertiary interactions between the P1 helix and the ribozyme core. Approximately 80% of the wild-type ribozyme preparation exists as E_{tight} which is capable of tight docking of P1 with $\Delta G°_{binding} = -14.3$ kcal/mol. Approximately 20% of the ribozyme preparation exists as E_{weak} which provides only weak docking of P1 ($\Delta G°_{binding} = -11.7$ kcal/mol; this estimate assumes a similar k_{on}^P for both ribozyme populations, which seems reasonable since the loss of tertiary interactions does not affect k_{on}^S). Thus, assuming that the free energy of duplex formation is unchanged, there is a loss of −2.6 kcal/mol in tertiary interactions when E_{tight} and E_{weak} are compared (−14.3 kcal/mol − −11.7 kcal/mol = −2.6 kcal/mol). Given a total $\Delta G°_{tertiary}$ of −4.9 kcal/mol for E·P (Table 1), about half of the tertiary binding energy has been lost. The 2.6 kcal/mol difference is similar to the energetic contributions measured for the 2′-hydroxyls of ribozyme IGS positions G22 and G25 ($\Delta\Delta G°_{tertiary} = -2.6$ and −2.1 kcal/mol, respectively; Strobel & Cech, 1993). The loss of the G22 tertiary interaction does not affect the rate of the chemical step of catalysis, and the loss of the G25 tertiary interaction results in only a 10% reduction in the rate of the chemical step (Strobel & Cech, 1993). Since $k_c \approx 200\ min^{-1}$ (Herschlag & Khosla, 1994), if the E_{weak} conformation is the result of a loss of either the G22 or G25 interaction, the rate of multiple turnover for both E_{tight} and E_{weak} would still be limited by the rate of product dissociation [$(k_{off}^P)_{tight} = 0.005-0.02\ min^{-1}$ and $(k_{off}^P)_{weak} = 0.2-0.5\ min^{-1}$; Table 3].

While we have not measured the proportion of E_{tight} and E_{weak} for the various mutant ribozymes, the discrepancy between k_{off}^P and k_{cat}(mt) for mutant ribozymes (Table 2) provides clear evidence that they also consist of two populations. Given that the ratio $k_{cat}(mt)/k_{off}^P$ varies in the mutant ribozymes, it appears that the proportion of E_{tight} and E_{weak} is affected by IGS base substitutions. Because we have not measured the proportion of E_{tight} and E_{weak} in preparations of mutant ribozymes, the difference in docking energy between the two forms cannot be calculated. The wild-type ribozyme can bind and cleave substrate in different registers to give products CCCUC and CCCU (Herschlag, 1992). An alternative model in which the two distinct rates of product dissociation are due to alternative binding registers for product is unlikely since miscleavage of substrate was not observed during ribozyme multiple turnover and since significant cleavage of product was not observed during binding of ribozyme and product. We also considered the possibility that our results reflected two ribozyme populations produced by damage or mutation of a portion of the ribozyme during synthesis. This "damaged ribozyme" model is unlikely since three different methods of ribozyme synthesis gave similar results and 5′ end heterogeneity was not detected by nuclease digestion.

Summary. Changes in the base composition of P1 in the *Tetrahymena* ribozyme do not affect tertiary interactions between the P1 helix and the ribozyme core. Several different preparations of the wild-type ribozyme were heterogeneous and contained two ribozyme populations with distinctly different rates of product dissociation. The weighted average of these two rates of product dissociation is equal to the rate of multiple-turnover catalysis. Our findings suggest that IGS mutant ribozymes have a E_{tight} and E_{weak}, too. Given that the difference between k_{cat}(mt) and k_{off}^P (measured with ribozyme excess) varies with the nucleotide composition of the ribozyme IGS, it is likely that the proportion of ribozyme that gives weak docking of the P1 helix can be changed by IGS mutations.

ACKNOWLEDGMENT

We are grateful to Phil Bevilacqua for suggestions on performance of gel-shift analyses and mathematical analysis of the data. We also thank Phil Hammond, Scott Strobel, and Art Zaug for helpful discussions.

REFERENCES

Been, M. D., & Cech, T. R. (1986) *Cell 47*, 207−216.
Bevilacqua, P. C. (1993) Ph.D. Thesis, University of Rochester, Rochester, NY.
Bevilacqua, P. C., & Turner, D. H. (1991) *Biochemistry 30*, 10632−10640.
Bevilacqua, P. C., Kierzek, R., Johnson, K. A., & Turner, D. H. (1992) *Science 258*, 1355−1358.
Bevilacqua, P. C., Johnson, K. A., & Turner, D. H. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 8357−8361.
Campbell, T. B., & Cech, T. R. (1995) *RNA 1*, 598−609.
Davies, R. W., Waring, R. B., Ray, J. A., Brown, T. A., & Scazzocchio, C. (1982) *Nature 300*, 719−724.
Doudna, J. A., Cormack, B., & Szostak, J. W. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 7402−7406.
Downs, W. D., & Cech, T. R. (1994) *Genes Dev. 8*, 1198−1211.
Fersht, A. (1985) *Enzyme structure and mechanism*, 2nd ed., pp 129−132, W. H. Freeman and Company, New York.
He, L., Kierzek, R., Santa Lucia, J., Walter, A. E., & Turner, D. H. (1991) *Biochemistry 30*, 11124−11132.
Herschlag, D. (1992) *Biochemistry 31*, 1386−1399.
Herschlag, D., & Cech, T. R. (1990a) *Biochemistry 29*, 10172−10180.
Herschlag, D., & Cech, T. R. (1990b) *Biochemistry 29*, 10159−10171.
Herschlag, D., & Khosla, M. (1994) *Biochemistry 33*, 5291−5297.
Knitt, D. S., Narlikar, G. J., & Herschlag, D. (1994) *Biochemistry 33*, 13864−13879.
Murphy, F. L., & Cech, T. R. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 9218−9222.
Narlikar, G. J., Gopalakrishan, V., McConnell, T. S., Usman, N., & Herschlag, D. (1995) *Proc. Natl. Acad. Sci. U.S.A. 92*, 3668−3672.
Pyle, A. M., McSwiggen, J. A., & Cech, T. R. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 8187−8191.
Pyle, A. M., Murphy, F. L., & Cech, T. R. (1992) *Nature 358*, 123−128.
Pyle, A. M., Moran, S., Strobel, S. A., Chapman, T., Turner, D. H., & Cech, T. R. (1994) *Biochemistry 33*, 13856−13863.
Strobel, S. A., & Cech, T. R. (1993) *Biochemistry 32*, 13593−13604.
Strobel, S. A., & Cech, T. R. (1995) *Science 267*, 675−679.
Strobel, S. A., & Cech, T. R. (1996) *Biochemistry 35*, 1201−1211.
Turner, D. H., Sugimoto, N., & Freier, S. M. (1990) in *Nucleic Acids* (Saenger, W., Ed.) pp 201−227, Springer-Verlag, Berlin.
Wang, J. F., Downs, W. D., & Cech, T. R. (1993) *Science 260*, 504−508.
Waring, R. B., Towner, P., Minter, S. J., & Davies, R. W. (1986) *Nature 321*, 133−139.
Young, B., Herschlag, D., & Cech, T. R. (1991) *Cell 67*, 1007−1019.
Zaug, A. J., Been, M. D., & Cech, T. R. (1986) *Nature 324*, 429−433.
Zaug, A. J., Grosshans, C. A., & Cech, T. R. (1988) *Biochemistry 27*, 8924−8931.

BI960510Z