Proc. Natl. Acad. Sci. USA
Vol. 95, pp. 6091–6096, May 1998
Biochemistry

Structural basis for heterogeneous kinetics: Reengineering the hairpin ribozyme

JOSE A. ESTEBAN*†, NILS G. WALTER*, GERD KOTZOREK‡, JOYCE E. HECKMAN*, AND JOHN M. BURKE*§

*Markey Center for Molecular Genetics, Department of Microbiology and Molecular Genetics, The University of Vermont, Burlington, VT 05405; and ‡Nucleic Acids Products Supply (NAPS), Göttingen GmbH, Rudolf-Wissell Str. 28, 37079 Göttingen, Germany

Edited by George Bruening, University of California, Davis, CA, and approved March 23, 1998 (received for review December 3, 1997)

ABSTRACT **The RNA cleavage reaction catalyzed by the hairpin ribozyme shows biphasic kinetics, and chase experiments show that the slow phase of the reaction results from reversible substrate binding to an inactive conformational isomer. To investigate the structural basis for the heterogeneous kinetics, we have developed an enzymatic RNA modification method that selectively traps substrate bound to the inactive conformer and allows the two forms of the ribozyme-substrate complex to be separated and analyzed by using both physical and kinetic strategies. The inactive form of the complex was trapped by the addition of T4 RNA ligase to a cleavage reaction, resulting in covalent linkage of the 5′ end of the substrate to the 3′ end of the ribozyme and in selective and quantitative ablation of the slow kinetic phase of the reaction. This result indicates that the inactive form of the ribozyme-substrate complex can adopt a conformation in which helices 2 and 3 are coaxially stacked, whereas the active form does not have access to this conformation, because of a sharp bend at the helical junction that presumably is stabilized by inter-domain tertiary contacts required for catalytic activity. These results were used to improve the activity of the hairpin ribozyme by designing new interfaces between the two domains, one containing a non-nucleotidic orthobenzene linkage and the other replacing the two-way junction with a three-way junction. Each of these modified ribozymes preferentially adopts the active conformation and displays improved catalytic efficiency.**

Studies of RNA structure and function usually must confront issues of alternative RNA folding and associated dynamics (1–5). Ribozymes serve as excellent models with which to address these problems, because their catalytic activity is strongly dependent on structure. There are two major difficulties in the analysis of the conformational heterogeneity of ribozymes. First, the existence of multiple conformers complicates the interpretation of kinetic data. Second, it is difficult to ascribe specific structures to species that can be defined kinetically. Nevertheless, structural heterogeneity is an intrinsic property of most RNA molecules *in vitro* and is also likely to be prevalent *in vivo* under many circumstances (6). Thus, we believe that analysis of conformationally heterogeneous ribozymes will provide important insights into the structural basis for RNA catalysis.

The hairpin ribozyme (7–11) is a very useful model for the analysis of relationships between RNA structure and catalytic function, because detailed kinetic descriptions of the reaction are available (12, 13), and its small size (50 nt) permits the use of a wide variety of methods, including solid-phase synthesis, to formulate and test structural hypotheses. However, its structural heterogeneity represents a significant challenge for high-resolution methods for structure determination. A model for the secondary structure of this ribozyme has been established through genetic and biochemical studies (14, 15). This model includes four helical elements (H1 to H4) and two internal loops (A and B), which contain most of the catalytically essential groups (see Fig. 1). These elements are organized into two independent folding domains (A and B, see Fig. 1 and ref. 16), which must interact for the ribozyme-substrate complex to be active. This conformation is expected to impose a sharp bend between H2 and H3 in what is termed the hinge region (see cartoon in Fig. 1 and refs. 15, 17, and 18).

Previous work in our laboratory has shown that all hairpin ribozyme constructs tested show biphasic kinetics. A chase experiment designed to measure substrate dissociation showed that the biphasic kinetics resulted from two distinct forms of the ribozyme (13). The fast phase of the biphasic reaction reflects the cleavage activity taking place on active ribozyme-substrate complexes, whereas the slow phase is the result of a slow substrate dissociation from inactive complexes followed by binding to and cleavage by active ribozymes. The amplitude of each phase reflects the relative amounts of active and inactive conformers. Moreover, mutagenesis experiments on the hinge region of the ribozyme suggest that the inactive conformation may correspond to an extended form of the ribozyme-substrate complex stabilized by stacking interactions between H2 and H3 (Fig. 1; J.E.H., E. K. O'Neill, and J.M.B., unpublished work). These results prompted us to investigate the molecular basis for the kinetic behavior of the hairpin ribozyme, by isolating and characterizing the putative conformational isomers.

MATERIALS AND METHODS

DNA and RNA Preparation. DNA templates for ribozyme transcription and RNA substrates were synthesized on an Applied Biosystems 392 DNA/RNA synthesizer by using standard DNA and RNA phosphoramidite chemistry. Ribozymes were synthesized by transcribing partially duplex synthetic DNA templates with T7 RNA polymerase, basically as described (19). All DNA and RNA molecules were purified by PAGE as described (20). In addition, RNA products of solid-phase synthesis were purified by reverse-phase HPLC liquid chromatography. RNA substrates were 5′-end-labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase. Internally labeled ribozymes were synthesized by *in vitro* transcription in the presence of [α-^{32}P]CTP.

An ortho-dihydroxymethyl-benzene-containing phosphoramidite was used for the chemical synthesis of the *o*-benzene-substituted ribozyme (see Fig. 4*B*). Synthesis of this phosphoramidite was as follows: 1,2-dihydroxymethyl-benzene (2.8 g, 20 mmol) was dissolved in 50 ml of dry pyridine; then

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

This paper was submitted directly (Track II) to the *Proceedings* office.
†Present address: Cold Spring Harbor Laboratory, Cold Spring Harbor, NY 11724.
§To whom reprint requests should be addressed. e-mail: jburke@zoo.uvm.edu.

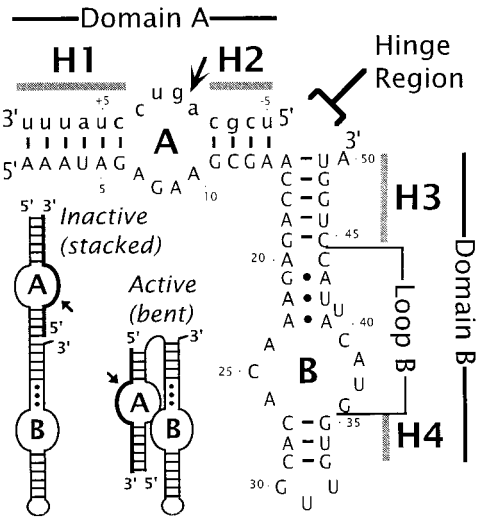

FIG. 1. Postulated secondary structure of the complex between the hairpin ribozyme and its cognate substrate. Ribozyme and substrate sequences are in uppercase and lowercase type, respectively. Ribozyme nucleotides are numbered from 1 to 50. Substrate nucleotides are numbered with negative numbers 5′ to the cleavage site and positive numbers 3′ to the cleavage site. A short arrow indicates the cleavage-ligation site. The four helical domains are designated as H1–H4, and the two internal loops as A and B. Proposed noncanonical base pairs at the top of loop B (22) are indicated with dots. Domain A is defined as the helical element comprising H1, loop A, and H2. Similarly, domain B stands for the H3-loop B-H4 element. Three base pairs (one in H1 and two in H2) of the naturally occurring sequences have been changed to minimize self-complementarity of the substrate (refs. 13 and 16; E. K. O'Neill, N.G.W., K. J. Hampel, J.E.H., and J.M.B., unpublished work). A rate-enhancing U39C mutation also was introduced. The putative hinge region is indicated. (*Inset*) Schematic cartoon showing the two proposed conformations of the hairpin ribozyme. See text for details.

dimethoxytrityl chloride (1.4 g, 4 mmol) was added. After stirring overnight at ambient temperature, the reaction was quenched by the addition of 2 ml of isopropanol and evaporated to near dryness. The residue was dissolved in ethyl acetate, washed with half-saturated sodium bicarbonate (2 × 50 ml) and brine (1 × 50 ml), and dried over anhydrous sodium sulfate. Removal of the solvent and coevaporation with toluene left the crude product that was purified on silica gel. Elution with *n*-hexane containing increasing amounts of CH_2Cl_2 gave 1,281 mg (2.9 mmol) of 1-*O*-(4,4′-dimethoxytrityl)-1,2-dihydroxymethyl-benzene (72.7%). A solution of this compound (517.5 mg, 1.2 mmol) in dry CH_2Cl_2 (30 ml) was treated with 2-cyanoethyl-chloro-*N*,*N*′-diisopropyl-phosphoramidite (320 μl, 1.4 mmol) in the presence of diisopropylethylamine (1.5 ml, 4.8 mmol) for 1 h at room temperature. The mixture was diluted with 90 ml of CH_2Cl_2, washed with 50 ml of half-saturated sodium bicarbonate and 50 ml of brine, and dried over anhydrous sodium sulfate. Removal of the solvent gave the crude product that was purified by silica gel chromatography. Elution with *N*-hexane/triethylamine (99.5/0.5, vol/vol) yielded 642 mg of 1-*O*-(4,4′-dimethoxytrityl)-2-*O*-(2-cyanoethyl-*N*,*N*′-diisopropyl-phosphoramidite)-1,2-dihydroxymethyl-benzene (83.5%). The chemical structure of this phosphoramidite was confirmed by ^{31}P-NMR spectroscopy. Standard solid-phase chemistry was used to synthesize the 5′-terminal strand of a two-piece hairpin ribozyme in which the *o*-benzene linker has been substituted for the original A_{14} residue. This construct was designed by eliminating the three-nucleotide closing loop of domain B and extending H4 with three extra bp (21). After synthesis, the presence of the linker in position 14 was confirmed by RNA sequencing methods. The RNA was labeled at its 5′ or 3′ end, and digested with sequence-specific RNases. In both cases, the sequence ladder was interrupted at the position corresponding to the ortho-benzene linker (not shown).

Ribozyme Cleavage Activity Assays. Reaction buffer was 50 mM Tris·HCl, pH 7.5/10 mM $MgCl_2$/3 mM DTT/10% dimethyl sulfoxide/10 μg/ml BSA/0.1 mM ATP. This buffer supports both ribozyme cleavage and T4 RNA ligase activity (see below). In the case of the ortho-benzene-containing ribozyme, this buffer reduced the stability of the ribozyme-substrate complex, probably because of the presence of dimethyl sulfoxide. Therefore, the standard hairpin ribozyme buffer (50 mM Tris·HCl, pH 7.5/12 mM $MgCl_2$) was used for reactions carried out with this construct. To allow T4 RNA ligase activity, 0.1 mM ATP was added. Although the activity of T4 RNA ligase was slightly lower in this buffer, the final extent of ligation was not affected (not shown). Cleavage assays were carried out at 25°C with saturating concentrations (empirically determined) of ribozyme (from 50 nM to 200 nM depending on the ribozyme) and a trace amount (<1 nM) of 5′-^{32}P substrate. When indicated, a saturating excess (1 μM) of a noncleavable 5′-phosphorylated substrate analog (deoxyriboadenosine at position −1) was incubated with 50 nM internally labeled ribozyme. Ribozyme and substrate RNAs were preincubated separately for 10 min at 37°C in reaction buffer; higher temperatures were avoided to prevent formation of ribozyme dimers (22). Solutions then were allowed to equilibrate for 10 min at 25°C, and reactions were initiated by mixing equal volumes of solutions containing ribozyme and substrate. Where indicated, 0.15 units/μl of T4 RNA ligase (Pharmacia) were added 30 s after initiation of the reaction. This incubation time is sufficient for a quantitative formation of the ribozyme-substrate complex (the binding half-time under these conditions is about 2 s). Aliquots of the reaction (10 μl) were removed and quenched with an equal volume of loading buffer (15 mM EDTA/97% formamide), and samples were analyzed by electrophoresis through 20% polyacrylamide-7 M urea gels. Radioactive bands were quantified by using a Bio-Rad GS-525 Molecular Imager system.

Cleavage Activity with Isolated Ribozyme Domains. A covalently linked ribozyme-substrate complex was obtained from a standard cleavage reaction carried out at 4°C for 15 h in the presence of T4 RNA ligase, as described above. These conditions are expected to favor enzymatic ligation rather than ribozyme cleavage. A trace amount (<1 nM) of the gel-purified ligated complex or isolated domain A (substrate hybridized to the substrate-binding strand; see ref. 16) was incubated for 20 min at 37°C in the presence of different concentrations (2–30 μM) of isolated domain B (16). The reaction buffer was 50 mM Tris·HCl, pH 7.5/12 mM $MgCl_2$. Samples were analyzed by gel electrophoresis and quantitated as described above.

Kinetic Analysis. Experimental data from cleavage activity time courses were fitted to single- and double-exponential equations. The single-exponential equation was

$$F = -A \cdot e^{-(r \cdot t)} + B,$$

where F is the fraction of substrate cleaved, A is the amplitude, and r is the rate of the exponential time course. The double-exponential equation was

$$F = -A_1 \cdot e^{(r_1 \cdot t)} - A_2 \cdot e^{-(r_2 \cdot t)} + B,$$

where A_1 and A_2 represent the amplitudes of the biphasic time course, and r_1 and r_2 stand for the corresponding rates. B represents the end point of the cleavage reaction, typically around 0.9. These parameters were estimated by nonlinear regression analysis using the Marquardt-Levenberg algorithm (Sigma Plot 5.0 and Origin/Microcal software). The standard error for fitted parameters was typically less than 10%. Ex-

perimental error from independent experiments was less than 50%. Experiments carried out side by side were highly reproducible (less than 5% error).

Gel Mobility-Shift Assays. Nondenaturing electrophoretic analyses were carried out by using a trace amount (<1 nM) of 5′-^{32}P noncleavable (dA_{-1}) substrate analog and saturating amounts (100 nM) of ribozyme. When indicated, the 5′ end of the substrate was covalently linked to the 3′ end of the standard two-piece ribozyme (21) by T4 RNA ligase reaction and gel-purification. Ribozyme-substrate complexes were formed on ice in the presence of 50 mM Tris·HCl, pH 7.5/12 mM $MgCl_2$/5% glycerol and were resolved on 10% polyacrylamide gels containing 25 mM magnesium acetate and 40 mM Tris-acetate, pH 7.5.

RESULTS AND DISCUSSION

Structural Identification of an Alternative RNA Conformation. It has been observed previously that forcing the coaxial stacking of H2 and H3 in the hairpin ribozyme yields an inactive molecule (17). On the other hand, we have detected kinetically an inactive conformation for the unmodified hairpin ribozyme (13). In this work, we postulate that helices 2 and 3 can stack spontaneously in the ribozyme-substrate complex, rendering an inactive molecule by inhibiting the interaction of the ribozyme's two folding domains. The presence of this hypothetical extended conformation of the ribozyme-substrate complex was tested by carrying out a standard single-turnover cleavage reaction in the presence of T4 RNA ligase (see *Materials and Methods*). The experimental rationale is as follows. Coaxial stacking of H2 and H3 is expected to place the 3′-OH end of the ribozyme in close proximity to the phosphorylated 5′ end of the substrate (see Fig. 1), so that ribozyme-substrate complexes adopting this extended conformation may become covalently joined by RNA ligase. T4 RNA ligase is most efficient in joining single-stranded RNA molecules. However, double-stranded RNA ends are expected to be ligated if they are in close proximity (23). As can be seen in the time course shown in Fig. 2 (*Inset*), most of the substrate underwent ribozyme-mediated cleavage. However, a significant amount of substrate was ligated into a slow-migrating molecule, corresponding to a covalent ribozyme-substrate complex. Only ligation of the 3′ end of the ribozyme and the 5′ end of the substrate can produce this species, because the 5′ end of the ribozyme is blocked by a transcriptionally generated triphosphate. The same complex was obtained with a noncleavable substrate analog (deoxyriboadenosine at position −1; not shown). Ligation was not observed with a noncognate substrate or in the absence of RNA ligase (data not shown)¶. Strikingly, the biphasic cleavage reaction of the hairpin ribozyme (13) became monophasic in the presence of RNA ligase (see Fig. 2). This monophasic reaction was essentially identical to the fast phase of the biphasic control, in both amplitude (fraction of substrate reacting in the fast phase) and rate (see legend of Fig. 2). Furthermore, the amount of substrate covalently linked to the ribozyme was equivalent to the extent cleaved in the slow phase of the control reaction in the absence of RNA ligase (Fig. 2). This equivalence was

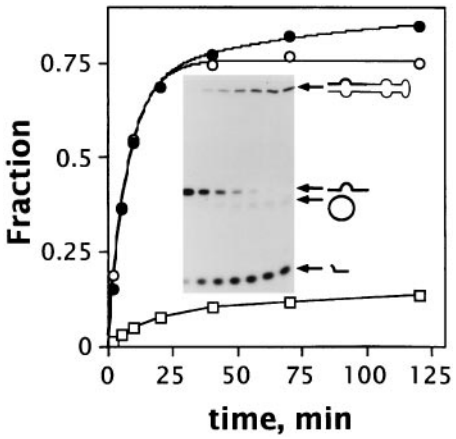

FIG. 2. Covalent trapping of the extended ribozyme-substrate complex with T4 RNA ligase. Substrate cleavage reactions were carried out either in the absence (●) or in the presence (○) of T4 RNA ligase (see *Materials and Methods*). Time courses were fitted to double- or single-exponential equations, respectively (see *Materials and Methods*). Amplitudes (A_1 and A_2) and rates (r_1 and r_2) for the biphasic reaction in the absence of RNA ligase were $A_1 = 0.71$, $r_1 = 0.13$ min^{-1}; $A_2 = 0.19$, $r_2 = 0.01$ min^{-1}. Parameters for the monophasic reaction in the presence of RNA ligase were $A = 0.73$, $r = 0.12$ min^{-1}. The fraction of substrate ligated to the ribozyme when T4 RNA ligase was present is also shown (□). Reactions with or without RNA ligase were carried out side by side with the same ribozyme and substrate solutions. (*Inset*) Electrophoretic analysis of a cleavage reaction carried out with 5′-end labeled substrate in the presence of T4 RNA ligase. Lanes from left to right correspond to the data points in the graph. The migration positions of the substrate-ribozyme covalent complex, uncleaved substrate, circularized substrate, and 5′ cleavage product are shown from top to bottom. Substrate strands are depicted with a thicker line. The residual amount of circularized substrate is the result of an intramolecular ligation reaction catalyzed by T4 RNA ligase on free substrate molecules (23). A similar electrophoretic analysis was carried out in the absence of RNA ligase (not shown).

observed with several ribozymes displaying different amplitudes for the fast and slow phases (not shown). Together, these facts strongly suggest that the inactive conformation responsible for the slow phase of the hairpin cleavage reaction is an extended conformation characterized by coaxial stacking between helices 2 and 3.

To evaluate the behavior of the entire population of ribozyme molecules, we carried out similar experiments with a saturating excess of a noncleavable substrate analog and a trace quantity of internally labeled ribozyme (see *Materials and Methods*). Under these reaction conditions the substrate excess is rapidly cyclized by the RNA ligase, but we have carried out a titration experiment to ensure that all of the ribozyme molecules are saturated with substrate. A noncleavable substrate was used to prevent active complexes from disassembly after substrate cleavage and product dissociation. It is important to bear in mind that in this experiment, unlike the one shown in Fig. 2, the proportion of ligated ribozyme is monitored, rather than that of the substrate. After incubation periods of up to 27 h, only about 10% of the total ribozyme molecules were covalently linked to the substrate by T4 RNA ligase, indicating that the majority of the ribozyme-substrate complexes remained in a nonstacked conformation over this time interval (not shown). These results are in agreement with our previous kinetic analysis of the hairpin ribozyme, where active and inactive conformations were shown to be essentially nonexchangeable (13).

Cleavage Activity on a Covalently Linked Ribozyme-Substrate Complex. To confirm that the ligated ribozyme-substrate complex represented the extended, inactive conformation of the ribozyme, it was gel-purified and used as a substrate in *trans*-cleavage reactions carried out with different

¶Under the reaction conditions described in *Materials and Methods*, ligation of the ribozyme-substrate complex catalyzed by T4 RNA ligase followed multiphasic kinetics, probably because of its complicated reaction mechanism (23). The weighted average rate for this reaction (sum of the exponential rate of each phase multiplied by the corresponding amplitude) was 0.03–0.04 min^{-1}. This ligation rate is significantly faster than the dissociation rate measured for inactive ribozyme-substrate complexes (0.01 min^{-1}; see ref. 13). Therefore, a quantitative ligation of the inactive conformation can be expected. T4 DNA ligase is also able to efficiently ligate RNA strands in duplex structures, but this reaction is strongly dependent on the presence of a complementary DNA strand acting as a bridge (24).

concentrations of the isolated domain B (see *Materials and Methods*). Parallel control reactions were carried out by using the isolated domain A (substrate hybridized to the substrate-binding strand; see ref. 16) as substrate. As shown in Fig. 3, the ligated substrate-ribozyme complex was cleaved as efficiently as was domain A alone. In addition, no cleavage was observed in the absence of domain B, confirming that the ligated complex lacks self-cleavage activity. These results clearly demonstrate that in the inactive, stacked, and ligated form of the ribozyme-substrate complex, domain A is assembled in such a way that substrate can be cleaved by domain B, and that domain B of the stacked complex does not interfere with association of domain B in *trans*. These conclusions are in agreement with previous observations obtained by forcing stacking of H2 and H3 (17).

Engineering the Hairpin Ribozyme to Favor the Bent Conformation. Having established the ligation assay as a diagnostic tool for the detection of the inactive extended conformation, we attempted to rationally reengineer the hairpin ribozyme so as to selectively favor the active, bent conformation. Two distinct strategies were followed. First, we designed a hairpin ribozyme in which a three-way junction replaces the H2-H3 interface in a manner that is expected to inhibit their coaxial stacking (Fig. 4*A Inset*). Three cytidine residues were kept unpaired in each strand at the connections with H2 and H3 to increase the flexibility of the junction. A second approach was explored by introducing a novel non-nucleotidic linker into the hinge region. We replaced A_{14} at the hinge with a rigid ortho-substituted benzene group, so that domains A and B of the ribozyme were connected to the phenyl group in an ortho-orientation through hydroxy-methyl residues (see *Materials and Methods* and Fig. 4*B Inset*). This linker is expected to impose a sharp dihedral angle between H2 and H3, and we anticipated that sufficient anisotropic flexibility still might be present to permit a functional interaction between the two domains.

To evaluate partitioning of these new ribozymes between active and inactive conformations, we carried out a cleavage reaction under single turnover conditions in the presence or absence of RNA ligase (see *Materials and Methods*). As shown in Fig. 4 *A* and *B*, the cleavage reaction carried out by these ribozymes in the absence of RNA ligase was nearly monophasic with only a very small fraction of the substrate cleaved during the slow phase. The residual slow phase was abolished in the presence of RNA ligase with the equivalent amount of substrate being ligated to the ribozyme. These results indicate that both modifications introduced at the hinge region (the three-way junction and the *o*-benzene linker) act to induce the ribozyme to fold into the active conformation, while destabilizing coaxial stacking between H2 and H3.

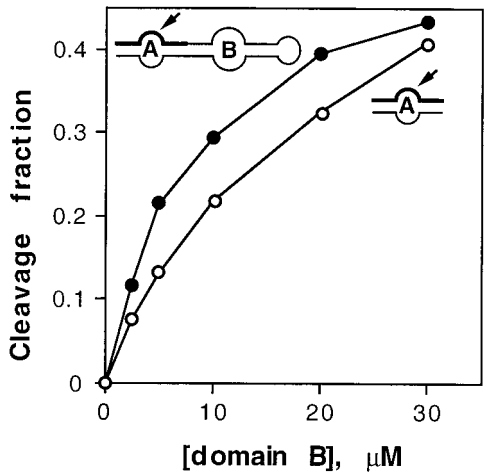

FIG. 3. Trans-cleavage activity on the covalently linked ribozyme-substrate complex and the isolated domain A of the hairpin ribozyme. Cleavage assay was carried out under single turnover conditions (see *Materials and Methods*), using different concentrations of domain B, and the isolated domain A (○) or the ribozyme-substrate covalent complex (●) as substrates.

This interpretation was further supported by gel mobility-shift assays using a noncleavable (dA_{-1}) substrate and different structural variants of the hairpin ribozyme (see *Materials and Methods*). For comparative purposes, the hairpin ribozyme used as control in this assay was a two-piece ribozyme obtained by extending H4 to 6 bp and eliminating the three-nucleotide closing loop of domain B (21). This ribozyme, containing the original A_{14} residue at the hinge region of the 5′-terminal strand, displayed the same kinetic behavior as the standard one-piece ribozyme (see Fig. 1) in the cleavage-ligation assay (not shown). The three-way junction hairpin ribozyme was not used in this comparative analysis because of its very different molecular weight. As shown in Fig. 4*C*, the ribozyme-substrate complex formed with the *o*-benzene linker migrated with a higher mobility and in a more homogeneous fashion than the complex formed with the analogous unmodified ribozyme (compare lanes 2 and 3). In addition, the *o*-benzene-substituted ribozyme was unable to adopt a perfect coaxially stacked conformation, as inferred from the heterogeneity of the ribozyme-substrate complexes formed when the 5′ end of the substrate was previously ligated to the 3′ end of the ribozyme (Fig. 4*C*, compare lanes 5 and 6). Electrophoretic mobility in nondenaturing gels shows a complex dependence on a number of factors, including static curvature, flexibility, and net charge (25). However, our results are consistent with the notion that the *o*-benzene linker increases the compactness of the ribozyme-substrate complex, suggesting that the active conformation of the hairpin ribozyme involves side-by-side packing of domains A and B.

What is the structural difference between the active and inactive species of the hairpin ribozyme? We know that the inactive species is a substrate for T4 RNA ligase, as concluded from the kinetic analysis, and therefore, it is probably adopting a structure in which helices 2 and 3 are stacked. The active species is not a substrate for RNA ligase, and so is unlikely to assume the stacked structure. Because ribozyme activity depends on functional interaction between the two domains (16), we infer that the active species is folded into a structure in which domains A and B can dock through an interaction that leads to catalytic activity. This structure must contain a sharp bend between helices 2 and 3.

Why is it that the stacked and bent species do not exchange with one another? The simplest model for the stability of the active species is that the two domains are stably docked, and remain so throughout the catalytic cycle. The two domains may simply fold into the active structure and then dock with one another. However, it is also possible that the initial interaction of the two domains is followed by an induced conformational change that leads to the active structure.

There are at least two plausible explanations for the stability of the inactive species. First, there could be a high activation energy for the conformational change from an inactive stacked structure to the active docked structure. If one focuses only on the modest energy of stacking at the helical interface, this hypothesis appears untenable. However, modeling studies (ref. 28 and this work) strongly suggest that bending at the helical interface requires unpairing of one or more base pairs, and docking of the two domains requires extension of the RNA chain at the hinge, resulting in a further loss of stacking energy, exposure of hydrophobic surfaces to solvent, and possibly torsional strain. Thus, the activation energy for the transition from stacked to docked forms of the ribozyme-substrate complex is likely to be significantly higher than that which would result from simple unstacking of the interface.

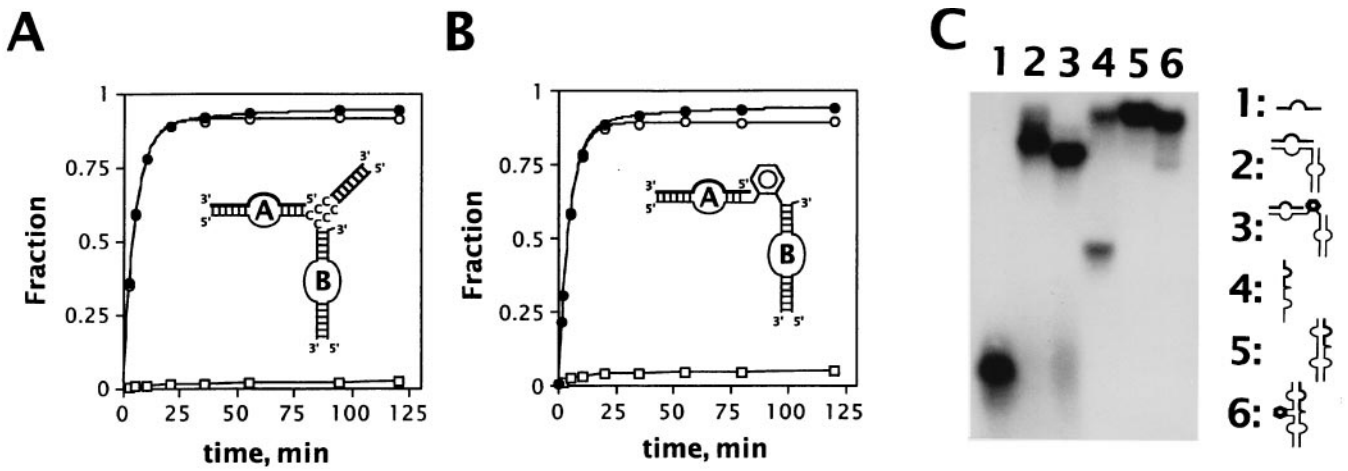

FIG. 4. Modifications of the hinge region of the hairpin ribozyme and analysis of the conformational partition with T4 RNA ligase. (*A*) Cleavage reactions were carried out and analyzed as described for Fig. 2. Parameters for the biphasic reaction in the absence of RNA ligase (●) were: $A_1 = 0.86$, $r_1 = 0.19\ min^{-1}$, $A_2 = 0.07$, $r_2 = 0.02\ min^{-1}$. Parameters for the monophasic reaction in the presence of RNA ligase (○) were: $A = 0.89$, $r = 0.18\ min^{-1}$. The fraction of substrate ligated to the ribozyme is indicated by □. (*Inset*) Schematic representation of the three-way junction hairpin ribozyme used in this study. An extra 7-bp helix with three unpaired cytidine residues at each strand has been substituted for the original hinge region. (*B*) A similar analysis was carried out with the hairpin ribozyme containing an ortho-dihydroxymethylbenzene linker at the hinge region. Parameters for the biphasic reaction in the absence of RNA ligase (●) were: $A_1 = 0.86$, $r_1 = 0.21\ min^{-1}$, $A_2 = 0.08$, $r_2 = 0.03\ min^{-1}$. Parameters for the monophasic reaction in the presence of RNA ligase (○) were: $A = 0.89$, $r = 0.21\ min^{-1}$. The fraction of substrate ligated to the ribozyme is indicated by □. (*Inset*) Schematic representation of the hairpin ribozyme containing the *o*-benzene linker. (*C*) Nondenaturing electrophoretic analysis of different ribozyme-substrate complexes (see *Materials and Methods*). Complexes were assembled with free noncleavable substrate (lanes 1–3) or with a noncleavable substrate covalently linked to the 3′-terminal strand of a two-piece ribozyme (lanes 4–6). In the former case, both 5′-terminal and 3′-terminal ribozyme strands were incubated with the substrate, whereas in the latter case, only the 5′-terminal strand was added. Lanes 1 and 4 represent controls with no addition. Complexes obtained with the standard ribozyme (lanes 2 and 5) or with the ribozyme containing the *o*-benzene linker (lanes 3 and 6) are shown.

The second hypothesis is that the inactive conformation represents a structure in which an incorrect folding event leads to a stably misfolded structure that precludes stable docking of the two domains. In this scenario, RNA ligase would covalently trap the substrate when helices 2 and 3 became stacked, even if stacking were a transient event. It is important to note that these two hypotheses for the inability of the inactive structure to convert to an active structure are not mutually exclusive.

CONCLUSION

We have shown that the hairpin ribozyme can adopt two alternative conformations. A catalytically inactive conformer that retains substrate binding activity can adopt an extended conformation in which helices 2 and 3 are coaxially stacked. Tertiary interactions between domains A and B stabilize the active conformation, which contains a sharp bend between helices 2 and 3. The hinge region appears to behave as a flexible linker, enabling specific tertiary contacts between the two domains to mediate the docking that is required for catalytic function. This configuration appears analogous to the interactions between the two segments of the P4-P6 domain of the *Tetrahymena* group I intron, where a sharply bent helical structure is clamped by specific contacts including adenosine platforms and ribose zippers (26, 27). In contrast, the helical junctions of the two new hairpin ribozyme constructs presented in this paper (three-way junction and *o*-benzene linker) are expected to play a more active role either in guiding the domains of the hairpin ribozyme into a side-by-side orientation, or alternatively, in destabilizing the stacking interactions between helices 2 and 3. In either way, the precise register of the two domains is expected to depend on specific tertiary contacts, possibly the proposed interactions between A_{10} and C_{25}, and between G_{11} and A_{24} (28).

The elucidation of the structural basis for heterogeneous ribozyme kinetics can be expected to help in the understanding of other cases of complex kinetic behavior and structural heterogeneity of RNA molecules. We believe that the resulting ability to engineer conformationally homogeneous ribozymes will be useful for simplifying *in vitro* analysis of structure and mechanism, and for increasing the activity of ribozymes in cellular RNA-targeting studies.

We thank Jillian Amaral for purification of T7 RNA polymerase, David Pecchia for the synthesis of DNA and RNA oligonucleotides, Fritz Benseler for help in designing the *o*-dihydroxymethylbenzene phosphoramidite, and Jeffrey Bond for molecular modeling studies. This work was supported by research grants from the National Institutes of Health. J.A.E. was the recipient of a postdoctoral fellowship from the Ministerio de Educación y Ciencia, Spain. N.G.W. was the recipient of a postdoctoral fellowship from the Alexander von Humboldt Foundation.

1. D'Orval, B. C., Carafa, Y. D., Sirand-Pugnet, P., Gallego, M., Brody, E. & Marie, J. (1991) *Science* **252,** 1823–1828.
2. Libri, D., Piseri, A. & Fiszman, M. Y. (1991) *Science* **252,** 1842–1845.
3. Eng, F. J. & Warner, J. R., (1991) *Cell* **65,** 797–804.
4. Dammel, C. S. & Noller, H. F. (1993) *Genes Dev.* **7,** 660–670.
5. Fortner, D. M., Troy, R. G. & Brow, D. A. (1994) *Genes Dev.* **8,** 221–233.
6. Herschlag, D. (1995) *J. Biol. Chem.* **270,** 20871–20874.
7. Prody, G. A., Bakos, J. T., Buzayan, J. M., Schneider, I. R. & Bruening, G. (1986) *Science* **231,** 1577–1580.
8. Buzayan, J. M., Gerlach, W. L. & Bruening, G. (1986) *Nature (London)* **323,** 349–353.
9. Haseloff, J. & Gerlach, W. L. (1989) *Gene* **82,** 43–52.
10. Feldstein, P. A., Buzayan, J. M. & Bruening, G. (1989) *Gene* **82,** 53–61.
11. Hampel, A. & Tritz, R. (1989) *Biochemistry* **28,** 4929–4933.
12. Hegg, L. A. & Fedor, M. J. (1995) *Biochemistry* **34,** 15813–15828.
13. Esteban, J. A., Banerjee, A. R. & Burke, J. M. (1997) *J. Biol. Chem.* **272,** 13629–13639.
14. Burke, J. M. (1994) *Nucleic Acids Mol. Biol.* **8,** 105–118.
15. Burke, J. M., Butcher, S. E. & Sargueil, B. (1996) *Nucleic Acids Mol. Biol.* **10,** 129–143.
16. Butcher, S. E., Heckman, J. E. & Burke, J. M. (1995) *J. Biol. Chem.* **270,** 29648–29651.

17. Feldstein, P. A. & Bruening, G. (1993) *Nucleic Acids Res.* **21,** 1991–1998.
18. Komatsu, Y., Koizumi, M., Nakamura, H. & Ohtsuka, E. (1994) *J. Am. Chem. Soc.* **116,** 3692–3696.
19. Milligan, J. F. & Uhlenbeck, O. C. (1989) *Methods Enzymol.* **180,** 51–62.
20. Sargueil, B., Pecchia, D. B. & Burke, J. M. (1995) *Biochemistry* **34,** 7739–7748.
21. Chowrira, B. M. & Burke, J. M. (1992) *Nucleic Acids Res.* **20,** 2835–2840.
22. Butcher, S. E. & Burke, J. M. (1994) *Biochemistry* **33,** 992–999.
23. Uhlenbeck, O. C. & Gumport, R. I. (1982) *The Enzymes* **XV,** 31–58.
24. Moore, J. M. & Sharp, P. A. (1992) *Science* **256,** 992–997.
25. Mills, J. B., Cooper, J. P. & Hagerman, P. J. (1994) *Biochemistry* **33,** 1797–1803.
26. Cate, J. H., Gooding, A. R., Podell, E., Zhou, K., Golden, B. L., Kundrot, C. E., Cech, T. R. & Doudna, J. A. (1996) *Science* **273,** 1678–1685.
27. Cate, J. H., Gooding, A. R., Podell, E., Zhou, K., Golden, B. L., Szewczak, A. A., Kundrot, C. E., Cech, T. R. & Doudna, J. A. (1996) *Science* **273,** 1696–1699.
28. Earnshaw, D. J., Masquida, B., Müller, S., Sigurdsson, S. T., Eckstein, F., Westhof, E. & Gait, M. J. (1997) *J. Mol. Biol.* **274,** 197–212.