Single-molecule mechanical unfolding and folding of a pseudoknot in human telomerase RNA

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ABSTRACT

RNA unfolding and folding reactions in physiological conditions can be facilitated by mechanical force one molecule at a time. By using force-measuring optical tweezers, we studied the mechanical unfolding and folding of a hairpin-type pseudoknot in human telomerase RNA in a near-physiological solution, and at room temperature. Discrete two-state folding transitions of the pseudoknot are seen at ~ 10 and ~ 5 piconewtons (pN), with ensemble rate constants of $\sim 0.1 \text{ sec}^{-1}$, by stepwise force-drop experiments. Folding studies of the isolated 5'-hairpin construct suggested that the 5'-hairpin within the pseudoknot forms first, followed by formation of the 3'-stem. Stepwise formation of the pseudoknot structure at low forces are in contrast with the one-step unfolding at high forces of ~ 46 pN, at an average rate of $\sim 0.05 \text{ sec}^{-1}$. In the constant-force folding trajectories at ~ 10 pN and ~ 5 pN, transient formation of nonnative structures were observed, which is direct experimental evidence that folding of both the hairpin and pseudoknot takes complex pathways. Possible nonnative structures and folding pathways are discussed.

Keywords: single-molecule; optical tweezers; telomerase RNA pseudoknot; folding pathways; unfolding/folding kinetics; force-jump/drop

INTRODUCTION

RNA must adopt various secondary and tertiary structures for its diverse biological functions (Tinoco and Bustamante 1999; Gesteland et al. 2006). The building blocks for secondary structure include canonical stems, hairpins, internal loops, bulges, and multibranches; tertiary structure involves interactions between the secondary structure building blocks, for example, loop-stem, loop-loop, and stem-stem interactions (Tinoco 1996; Tinoco and Bustamante 1999; Leontis et al. 2006). A hairpin (H)-type pseudoknot (Fig. 1B) involves base-pairing between nucleotides in a hairpin loop with nucleotides in a single-stranded region outside the hairpin (Pleij et al. 1985; Puglisi et al. 1988; Tinoco 1996; Su et al. 1999; Staple and Butcher 2005). The H-type pseudoknot is stabilized by base-pairing interactions in the two stems (5' stem1 and 3' stem2), which in turn facilitate the tertiary interactions involving loop-stem and loop-loop contacts. The thermodynamics of the H-type pseudoknot formation has been

studied by thermal unfolding (Puglisi et al. 1988; Wyatt et al. 1990; Theimer and Giedroc 2000; Theimer et al. 2003, 2005; Soto et al. 2007), but unfolding/folding dynamics and kinetics information are scarce (Wyatt et al. 1990; Green et al. 2007).

Recent developments in single-molecule techniques have facilitated RNA unfolding and folding reactions in near-physiological solutions and temperatures by applying mechanical force and following the reaction trajectories of individual molecules (Liphardt et al. 2001; Onoa et al. 2003). Mechanical unfolding/folding dynamics, kinetics, and thermodynamics information can provide insight into mechanical processes in biology (Bustamante et al. 2004; Tinoco et al. 2006). Here, we use optical tweezers (Smith et al. 1996; Tinoco et al. 2006) to study the mechanical unfolding and folding of a modified pseudoknot in human telomerase RNA (Fig. 1B; Cech 2004; Chen and Greider 2004; Autexier and Lue 2006; Blackburn 2006; Collins 2006; Legassie and Jarstfer 2006; Theimer and Feigon 2006), one molecule at a time, at 200 mM NaCl, 10 mM Tris-HCl (pH 7.3), 0.1 mM EDTA, at room temperature. This H-type pseudoknot was chosen as a model system because there are three-dimensional NMR structures available for a truncated pseudoknot and a constituent 5'-hairpin (Fig. 1E; Theimer et al. 2003,

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FIGURE 1. RNA constructs for single-molecule study. The constructs are derived from human telomerase RNA. The directions of stretching force by optical tweezers are shown by arrows. (A) The isolated stem1 hairpin construct (HPi). The noncanonical base pairs revealed by NMR are indicated with dots. The NMR construct of the stem1 hairpin (E, left) consists of residues 7–35, with an additional 3' dangling C. (B) The pseudoknot construct. Tertiary contacts revealed by NMR are indicated with dots. The NMR construct of the pseudoknot (E, right) consists of the residues 7–35 and 63–79 (with residues 35 and 63 covalently linked), and an additional 3' dangling A. The conserved single U-bulge (deleted in this construct) is located between residues 73 and 74. (C) The intermediate state during pseudoknot folding (HPp), in which the stem1 hairpin forms but stem2 does not. (D) The optical tweezers setup for single-molecule experiments. The RNA and DNA are shown in color and gray, respectively. The RNA molecule shown is from the NMR structure only for representation; only the structures shown in panels A, B, and C were studied here. The drawing is not to scale. (E) The three-dimensional NMR structures of the stem1 hairpin (Theimer et al. 2003) and pseudoknot derived from human telomerase RNA (Theimer et al. 2005).

2005), and because it is functionally essential to the catalytic activity of the telomerase ribonucleoprotein enzyme (Comolli et al. 2002; Chen and Greider 2005; Theimer et al. 2005).

To facilitate single-molecule mechanical unfolding and folding reactions, the pseudoknot was modified from the NMR construct (Theimer et al. 2005) and flanked by two DNA/RNA handles (with ~500 and ~600 base pairs [bp]), which are attached to two micron-sized polystyrene beads (Liphardt et al. 2001). One bead is held in a dual-beam force-measuring optical trap, and the other on a micropipette by suction (Fig. 1D). Force-jump and force-drop methods (Li et al. 2006b) were employed to directly measure the unfolding and refolding kinetics. Stepwise force-drop experiments mapped the sequential folding of the pseudoknot structure and allowed direct measurement of the kinetics. Folding studies of the isolated 5'-hairpin construct (Fig. 1A) suggested that the 5'-hairpin within the pseudoknot (Fig. 1C) forms first, followed by formation of the 3'-stem. Transient formation of nonnative structures was observed with similar extension changes as native structures;

these transient folding events reveal complex folding pathways for RNA.

RESULTS

Constructs for single-molecule study

The NMR structure of a truncated pseudoknot from human telomerase RNA (Fig. 1E, right panel) reveals extensive tertiary interactions (schematically shown in Fig. 1B) around the junction region involving sequences that are conserved among vertebrates (Theimer et al. 2005). The 5' (Fig. 1B, stem1, shown in red) and 3' (Fig. 1B, stem2, shown in blue) stems of the pseudoknot are almost collinear. The NMR structure of an isolated stem1 hairpin (Fig. 1E, left panel; Theimer et al. 2003) reveals that to form the phylogenetically conserved 9-bp A•U-rich stem2 and tertiary contacts of the pseudoknot, six base pairs (two canonical and four noncanonical) within the loop of stem1 hairpin (schematically shown in Fig. 1C) have to be broken (Theimer and Feigon 2006).

The modified human telomerase RNA pseudoknot construct (Fig. 1B) for single-molecule study is based on the NMR construct (Fig. 1E; Theimer et al. 2005), the wild-type pseudoknot,

and functional analysis (Antal et al. 2002; Comolli et al. 2002; Chen and Greider 2004, 2005; Theimer et al. 2005). To follow the single-molecule reactions with distinct molecular extension changes (>5 nm) for individual reaction steps, stem1 and loop2 (Fig. 1B) were lengthened from the NMR construct. Stem1 was lengthened from 6 bp in the NMR construct to 12 bp, and the 29 nucleotides (nt) in loop2 were modified from the wild type. The loop1, which lies in the major groove of stem2, was maintained the same as in the NMR construct and the wild type. All the functionally important residues surrounding the junction (Comolli et al. 2002; Chen and Greider 2004, 2005; Theimer et al. 2005) were maintained except for the conserved single U-bulge in stem2 between residues 73 and 74 (see Fig. 1B), which is also absent in the NMR study (Theimer et al. 2005). An isolated stem1 hairpin construct (HPi) (Fig. 1A) was made by extending the DNA/RNA handle (see Materials and Methods) to cover the 3'-side of the stem1 hairpin, thus preventing the formation of loop2 and stem2. The details of the NMR and single-molecule constructs are described in the Figure 1 caption.

Mechanical force range of pseudoknot unfolding/folding

The force-ramp (with an approximately constant-force loading/unloading rate) method (Li et al. 2006b) was first used to find the force ranges of individual unfolding/folding reactions. In a force-ramp experiment (Fig. 2), the force on the RNA molecule is gradually increased or decreased; during the process, an unfolding/folding transition is indicated by a sudden increase/decrease in extension and decrease/increase in force. The abrupt unfolding and folding transitions are termed rips and zips, respectively. Representative force-ramp trajectories for the HPi construct are shown in Figure 2A. The HPi unfolding (Fig. 2A, gray traces) is indicated by a rip at ~ 24 piconewtons (pN) with an extension increase of ~ 18 nm, consistent with the worm-like-chain model (Bustamante et al. 1994) for 41 nt

of the hairpin unfolded, with a persistence length of 1 nm and contour length of 0.59 nm/nt for single-stranded RNA (Table 1; Smith et al. 1996; Liphardt et al. 2001).

In contrast to unfolding, the folding of HPi occurred in a wide range of force. At least three types of folding processes (Fig. 2A, black traces) are observed for five molecules of HPi, with a total of 193 traces: (1) Most (81%) of the traces fold without a clear zip transition until below 10 pN, as shown in curves 1 and 2; (2) $\sim 16\%$ of the traces show multiple zipping/unzipping processes at or below 10 pN as shown in curves 3 and 5; and (3) only 3% of the traces fold above 10 pN with a clear one-step zip as shown in curve 4. Types (1) and (2), but not the rare events of type (3), were observed in all the molecules. The folding zip (Fig. 2A, indicated with gray arrows) at low forces (≤ 10 pN) for the first two types have shorter extension change than that calculated by the worm-like-chain model for folding 41 nt (Table 1); the difference is probably caused by the transient formation of small structures before the formation of HPi (see below).

In contrast to the hairpin structure, the pseudoknot structure unfolds in one step at relatively high force (~ 50 pN), with rip size of ~ 36 nm (Fig. 2B, curves 2,3), consistent with the number of nucleotides expected for the unfolding of the pseudoknot (Table 1). The cooperative unfolding at high force of the pseudoknot is presumably due to the tertiary interactions, and to the fact that in pseudoknots the applied force is parallel to the helix axis of the stems (Fig. 1B, indicated with gray arrows). This applies a shear force to the stem, instead of an unzipping force that breaks base pairs one at a time, as in a hairpin (Fig. 1A,C, indicated with gray arrows). Unfolding a kissing hairpin complex is similar and also leads to unfolding forces higher than those for the component hairpins (Li et al. 2006a).

Distinct folding zip transitions are not consistently observed for the pseudoknot construct in its force-ramp relaxing trajectories (Fig. 2B). Some zips at ~ 10 pN (for example, Fig. 2B, curve 2) might be due to formation of the stem1 hairpin within the pseudoknot construct (HPp) (Fig. 1C), as observed in the HPi construct (Fig. 2A). It is likely that the HPp folds into a pseudoknot below 10 pN, which is barely resolved in the force-ramp trajectory. The folding transition below 10 pN is due to relatively slow folding rates compared to the force relaxation rate. Although the folding zip transitions are not resolved, the pseudoknot structure sometimes forms, as indicated by the subsequent pulling trajectories that show an unfolding force of ~ 50 pN and an extension increase of ~ 36 nm (see Fig. 2B, curves 2,3). The pseudoknot construct sometimes shows similar unfolding force and extension changes as those of HPi, indicating that only the HPp structure forms even when the force is decreased as low as 3 pN (see Fig. 2B, curve 1). Thus, these results support the conclusion that HPp folds first at ~ 10 pN during the formation of the pseudoknot. Slow folding of HPp at or below 10 pN masks the further folding zip transition to form the pseudoknot.

FIGURE 2. Representative force-extension curves from force-ramp experiments. The single RNA molecule is first pulled with a loading rate of 100 nm/sec (shown in gray) and then relaxed with an unloading rate of 100 nm/sec (shown in black). Stem1 hairpin or pseudoknot unfolding transitions are indicated by the rips at ~ 24 and ~ 50 pN, respectively. Folding zip transitions typically occur below 10 pN and mostly are not resolved. (A) Five traces of force-ramp trajectories of the HPi construct, which can be characterized as three distinct types by the folding trajectories (see Results). Folding at or below 10 pN with one folding zip: type (1), curves 1 and 2; and with multiple zipping/unzipping: type (2), curves 3 and 5. Curve 4 rarely occurs and belongs to type (3) with a folding zip above 10 pN. The folding zip transitions (indicated with gray arrows) shown in types (1) and (2) have a shorter extension change than that calculated by the worm-like-chain model for 41-nt single-stranded RNA. The multiple zipping/unzipping before the final folding zip transitions shown in type (2) might be due to formation/disruption of nonnative collapsed structures. The curves are ordered by occurrence from the highest to lowest. (B) Three traces of force-ramp trajectories of the pseudoknot construct. In curve 1, only HPp forms as indicated by the unfolding force of ~ 24 pN. In curves 2 and 3, the pseudoknot forms as indicated by unfolding forces of ~ 50 pN.

TABLE 1. Parameters obtained from force-jump/drop experiments

Construct	Reaction ^a	Force (pN)	ΔX measured (nm)	ΔX predicted (nm)	$\Delta X^\ddagger$ (nm)	Number of nucleotides folded or disrupted at transition state
HPi	S-H	10.0	9.6 $\pm$ 1.0	13.6	-4.1 $\pm$ 1.6	16 $\pm$ 4 ^b
		10.5	10.9 $\pm$ 1.0	14.0		
		11.0	11.5 $\pm$ 1.1	14.0		
	$\ln k_{S-H} = (-1.0 \pm 0.4)F + (8.4 \pm 4.2)^c$					
	H-S	22.0	17.5 $\pm$ 0.7	16.9	7.0 $\pm$ 0.4	15 $\pm$ 1
		22.5	17.5 $\pm$ 0.6	16.9		
		23.0	17.4 $\pm$ 0.6	16.9		
		23.5	17.7 $\pm$ 0.7	16.9		
$\ln k_{H-S} = (1.7 \pm 0.1)F - (39.6 \pm 1.2)^c$						
Pseudoknot ^d	S-H	10.0	10.0 $\pm$ 0.9	13.6	-4.5 $\pm$ 1.6	17 $\pm$ 4 ^b
		10.5	10.3 $\pm$ 0.9	14.0		
		11.0	11.7 $\pm$ 0.8	14.0		
	$\ln k_{S-H} = (-1.1 \pm 0.4)F + (9.4 \pm 4.2)^c$					
	H-S	22.5	18.3 $\pm$ 0.6	16.9	10.7 $\pm$ 0.4	23 $\pm$ 1
		23.0	18.1 $\pm$ 0.6	16.9		
		23.5	18.1 $\pm$ 0.7	16.9		
	$\ln k_{H-S} = (2.6 \pm 0.1)F - (60.2 \pm 0.1)^c$					
	H-P	5.0	7.2 $\pm$ 0.7	7.0	-4.1 $\pm$ 1.6	
		5.5	7.3 $\pm$ 0.8	7.4		
		6.0	7.5 $\pm$ 0.6	8.2		
	$\ln k_{H-P} = (-1.0 \pm 0.4)F + (3.0 \pm 2.5)^c$					
	P-S	45.0	36.4 $\pm$ 1.0	34.0	0.8 $\pm$ 0.4	
		46.0	36.1 $\pm$ 0.9	34.0		
		47.0	36.4 $\pm$ 0.9	34.0		
		$\ln k_{P-S} = (0.2 \pm 0.1)F - (11.6 \pm 0.9)^c$				

The worm-like-chain model is used to calculate the extension changes (ΔX , predicted), and conversion of $\Delta X^\ddagger$ to the number of nucleotides folded or disrupted at transition state.

^a(S) Single strand; (H) stem1 hairpin; (P) pseudoknot structure. S at 10.0–11.0 pN might contain nonnative small hairpins.

^bThe helix diameter of 2 nm is taken into account for calculation of the number of nucleotides folded at the transition state. Owing to formation of nonnative small hairpins at ~ 10 pN before the folding transition, the transition state might be farther away from the apical loop.

^cThe values are from the fits to Equation 1 as shown in Figure 5. k is the rate constant in sec^{-1} , and F is force in piconewtons. Extrapolation of rate constants to zero force is not reliable because $\Delta X^\ddagger$ varies over the large force range.

^dThe end-to-end extension of the folded pseudoknot is approximated to be $21 \times 0.26 = 5.5$ nm.

Stem1 hairpin unfolding/folding kinetics

As shown by force-ramp experiments (Fig. 2), formation of HPP and of pseudoknot structure is indicated by unfolding forces of ~ 24 and ~ 50 pN, respectively. Both of the HPi and pseudoknot constructs fold at relatively low force (typically, <10 pN), with folding zip transitions largely not resolved. To resolve the folding transitions, we employed force-jump and force-drop methods (Li et al. 2006b). In a force-jump/drop experiment (Fig. 3), force is quickly (<0.1 sec) increased/decreased to a preset force, and the preset force is kept constant by electronic force feedback. The unfolding/folding transition is indicated by a sudden increase/decrease of the molecular extension at constant force. The lifetimes of the molecule at each state (folded or unfolded) can be obtained for many cycles at different forces. By keeping the force above 9 pN, only HPP is observed to form; the pseudoknot structure does not form, thus allowing repetitive unfolding and folding of the

HPP within the pseudoknot construct. The HPP unfolds (H-S, transition from hairpin to single strand) at ~ 23 pN upon force-jump, with an abrupt molecular extension increase of ~ 18 nm, and folds (S-H, transition from single strand to hairpin) at ~ 10 pN upon force-drop, with an abrupt extension decrease of ~ 10 nm (Fig. 3; Table 1).

The folding transitions are ~ 2 – 4 nm shorter in extension than those predicted by the worm-like-chain model for single-stranded RNA (Table 1), which might be due to formation of small hairpins before the folding transition at ~ 10 pN. According to the nearest-neighbor model and a two-state mechanism for hairpin opening/closing (Tinoco et al. 1971; Serra et al. 1994; Xia et al. 1998; Mathews et al. 2004; Lu et al. 2006), the native 2-bp pentaloop between U17 and A25 (see Fig. 1C) has a predicted unfavorable free energy of 0.1 kcal/mol at zero force at 22°C in 1 M NaCl. The melting temperature is predicted to be 21°C in 1 M NaCl. Thus, the 2–4-nm deviation in extension is unlikely due to formation only of the native 2-bp pentaloop hairpin.

FIGURE 3. Representative force-jump/drop traces of the pseudoknot construct. Time courses of extension and force are shown on the *top* and *bottom* of each panel, respectively. Extension changes due to sudden force-jump/drop and unfolding/folding transition at constant force are indicated by vertical dashed lines. (S) Single strand; (H) native stem1 hairpin; (P) native pseudoknot; (C) nonnative stem1 hairpin; (C') collapsed nonnative conformation of the pseudoknot. S at ~ 10 pN might contain nonnative small hairpins. (A) A trace without transient folding events. The stepwise force-drop (from 15.0 to 10.0 pN and from 10.0 to 5.0 pN) and force-jump (from 40.0 to 45.0 pN) are indicated with black arrows. The lifetimes (τ) before unfolding/folding transitions are also shown. (B) A trace with two transient folding events (indicated with gray arrows) to nonnative HPP (S-C rapidly followed by C-S) before the formation of native stem1 hairpin (S-H) at 10.0 pN and one transient folding event (indicated with gray arrows) to nonnative conformation of the pseudoknot construct (H-C' rapidly followed by C'-H) before the formation of the native pseudoknot (H-P) at 5.0 pN. (C) A close-up view of a trace with two transient folding events (indicated with gray arrows) to nonnative HPP (S-C followed by C-S). The measured lifetimes for single-stranded (S) and collapsed states (C) are shown.

It is probably due to formation of nonnative small hairpins. The nonnative small hairpins probably form during the force-drop process (during the dead time of the instrument, <0.1 sec). Deviations from the expected extensions are also observed in the folding zip transitions below 10 pN (Fig. 2A, indicated with gray arrows) in force-ramp trajectories. Presumably, the lifetime of the single-stranded RNA without any nonnative small hairpin at ~ 10 pN is too

short to resolve, thus resulting in the apparent one-step native hairpin folding (S-H) transition with the ensemble kinetics listed in Table 2.

Repeated unfolding and folding of HPP within the pseudoknot by force-jump and force-drop, respectively, give single exponential distributions of lifetimes of the folded and unfolded molecules (Fig. 4); the respective ensemble kinetics parameters are $k_{H-S} = 0.08\text{--}1.00\text{ sec}^{-1}$ between 22.5 and 23.5 pN and $k_{S-H} = 0.07\text{--}0.21\text{ sec}^{-1}$ between 10.0 and 11.0 pN (Table 2). Thus, by force-drop experiments, the intermediate state with only HPP folded is available, and its folding kinetics can be directly measured. The unfolding and folding forces of ~ 23 and ~ 10 pN were chosen to provide experimentally accessible lifetimes.

The distances to transition states ($\Delta X^\ddagger$) along the reaction coordinate can be obtained from force-dependent unfolding/folding kinetics, $k(F)$ (Fig. 5; Tables 1, 2; Tinoco et al. 2006):

$$k(F) = k_0 e^{F\Delta X^\ddagger/k_B T}, \quad (1)$$

where k_0 is the apparent unfolding/folding rate constant at zero force, k_B is the Boltzmann constant, and T is the temperature in kelvins. The value of $\Delta X_{S-H}^\ddagger$ (from single strand to HPP) is -4.5 ± 1.6 nm (Table 1), equivalent to the formation of a stem-loop containing ~ 17 nt (for example, a pentaloop with ~ 6 bp, i.e., from G12 to C30 in Fig. 1C). Due to likely formation of nonnative small hairpins, the single-stranded RNA deviates (with 2–4 nm shorter extension; see above) from the worm-like-chain-model at ~ 10 pN. Thus, the HPP folding transition state might be 2–4 nm (3–5 bp) further away from the apical loop. Possible nonnative local structures include the 5-bp triloop formed between G21 and C33 (Fig. 6; see Discussion). Note that the helix diameter of 2 nm has been taken into account ($-\Delta X^\ddagger + 2 = 4.5 + 2 = 6.5$ nm) for calculation of the number of nucleotides folded at the transition state (Table 1). The NMR structure of an isolated stem1 hairpin (Fig. 1E, left panel; Theimer et al. 2003) reveals a single dominant conformation with four noncanonical base pairs (schematically shown in Fig. 1A). Presumably, the consecutive noncanonical U•C and U•U pairs observed in the NMR structure are not independently stable without closing Watson–Crick pairs on both sides (Chen and Turner 2006) at ~ 10 pN. Thus, closing of the U-rich internal loop at 10.0–11.0 pN is the rate-limiting step for the native stem1 hairpin folding. Slow folding caused by the internal loop at the transition state measured here is consistent with slow folding caused by a 3-nt bulge observed previously in HIV TAR RNA (Li et al. 2006b).

The HPP unfolding transition state has $\Delta X_{H-S}^\ddagger = 10.7 \pm 0.4$ nm, corresponding to disruption of ~ 23 nt from the terminal ends, which is close to the U-rich internal loop (Fig. 1A; Table 1). It indicates that (nearly) all the

TABLE 2. Ensemble reaction rate constants derived by single exponential fit (k), and the reciprocal of average ($1/\langle\tau\rangle$) of the measured lifetimes from force-jump/drop experiments

Reaction ^a	Force (pN)	Pseudoknot construct			HPi construct		
		Number of traces	k (sec ⁻¹)	$1/\langle\tau\rangle$ (sec ⁻¹)	Number of traces	k (sec ⁻¹)	$1/\langle\tau\rangle$ (sec ⁻¹)
S-H	10.0	132	0.21 ± 0.01	0.16	186	0.22 ± 0.01	0.17
	10.5	121	0.17 ± 0.01	0.13	149	0.09 ± 0.01	0.08
	11.0	125	0.07 ± 0.01	0.07	180	0.08 ± 0.01	0.07
S-C	10.0	35	0.25 ± 0.01	0.25	53	0.36 ± 0.01	0.31
	10.5	40	0.23 ± 0.01	0.21	11	0.15 ± 0.02	0.15
	11.0	17	0.11 ± 0.01	0.12	9	0.09 ± 0.01	0.08
C-S	10.0	35	21 ± 1	18	53	22 ± 1	17
	10.5	40	35 ± 2	28	11	42 ± 5	19
	11.0	17	30 ± 4	22	9	34 ± 5	30
H-S	22.0	—	—	—	136	0.06 ± 0.01	0.05
	22.5	119	0.08 ± 0.01	0.08	154	0.12 ± 0.01	0.09
	23.0	131	0.28 ± 0.01	0.27	165	0.28 ± 0.01	0.25
	23.5	129	1.00 ± 0.01	0.87	77	0.67 ± 0.01	0.64
H-P	5.0	64	0.15 ± 0.01^b	0.12 ^b			
	5.5	91	0.13 ± 0.01	0.11			
	6.0	89	0.06 ± 0.01	0.05			
H-C'	5.0	17	0.27 ± 0.02	0.24			
	5.5	24	0.14 ± 0.01	0.13			
	6.0	26	0.10 ± 0.01	0.08			
C'-H	5.0	17	21 ± 2	17			
	5.5	24	2.3 ± 0.1	2.2			
	6.0	26	12 ± 1^b	9 ^b			
P-S	45.0	75	0.0359 ± 0.0007	0.0343			
	46.0	92	0.0417 ± 0.0005	0.0445			
	47.0	111	0.0519 ± 0.0009	0.0510			

Similar rate constants obtained by the two methods indicate apparent two-state reactions. Uncollapsing (C-S or C'-S) rate constants are less accurate because of fewer traces obtained and short lifetimes of the collapsed states. Pseudoknot unfolding (P-S) rate constants are given extra significant digits for comparison.

^a(S) Single strand; (H) native stem1 hairpin; (P) native pseudoknot; (C) nonnative stem1 hairpin; (C') collapsed nonnative conformation of the pseudoknot construct upon the second force-drop. S at ~10 pN might contain nonnative small hairpins.

^bOne or two points are deleted with the longest lifetimes.

Watson–Crick pairs are disrupted at the unfolding transition state of HPP. The large block of G•C-rich base pairs results in an unfolding force higher than 20 pN, a movement of the unfolding transition state closer to the apical hairpin loop, and hysteresis between folding and unfolding trajectories. These observations are consistent with previous modeling and experimental results (Liphardt et al. 2001; Viereggs and Tinoco 2006; Woodside et al. 2006a,b). Similar unfolding/folding kinetics data were obtained for the HPi construct (Fig. 5B; Tables 1, 2), as expected. In addition, folding of the 9-bp stem2 hairpin (with 41 nt in the loop) before stem1 is unlikely and would result in an end-to-end extension decrease of ~20 nm at ~10 pN, larger than we observed (~10 nm). Therefore, we conclude that the stem1 hairpin folds (Fig. 1C) first and independently on the way to the formation of the pseudoknot structure (Fig. 1B). This folding pathway is consistent with that of thermal folding (Theimer et al. 2005).

Pseudoknot unfolding (P-S) kinetics

In contrast to the stepwise folding of the pseudoknot, one-step pseudoknot unfolding transitions (P-S, transition from pseudoknot to single strand) are observed with force-jump experiments by rapidly increasing the force from 40 to ~46 pN (Fig. 3A). A sudden molecular extension increase of ~36 nm with an ensemble rate constant of 0.05 sec⁻¹ is seen (Table 1,2), although our pseudoknot can sometimes unfold at forces lower than 40 pN (with ~5% frequency). The pseudoknot unfolding transition state at ~46 pN has $\Delta X_{P-S}^\ddagger = 0.8$ nm (Table 1), which indicates that the unfolding transition state is close to the fully “pseudoknoted” native state at 46 pN, consistent with the brittle characteristics of tertiary interactions with unfolding kinetics relatively less force-dependent (Liphardt et al. 2001; Onoa et al. 2003). A small force range (45–47 pN) was used because higher forces result in breaking of the digoxigenin/anti-digoxigenin antibody interactions (see Materials and

FIGURE 4. Representative plots of the counts of unreacted molecules as a function of time. Single exponential fits give the ensemble unfolding/folding rate constants at specific forces shown on top of each panel. (A) Pseudoknot folding from H-P (H-P) at 5.0 pN. (B) H-P folding from single strand (S-H) at 10.0 pN. (C) H-P unfolding to single strand (H-S) at 23.0 pN. (D) Pseudoknot unfolding to single strand (P-S) at 46.0 pN.

Methods), and lower forces result in an even slower unfolding rate.

Pseudoknot folding (H-P) kinetics

Once the HPp forms at ~ 10 pN after the first force-drop, the following pseudoknot folding transitions (H-P, transition from hairpin to pseudoknot) are observed after a second force-drop by lowering the force rapidly from ~ 10 to ~ 5 pN. A molecular extension decrease of ~ 7 nm at ~ 5 pN occurs with an ensemble rate constant of 0.1 sec^{-1} (Fig. 3; Table 2). The pseudoknot folding transition state has $\Delta X_{\text{H-P}}^\ddagger = -4.1 \text{ nm}$ (Table 1). To form the pseudoknot from HPp, the UCGCU pentaloop and six base pairs adjacent to the pentaloop formed between G12 and C30 (Fig. 1C), which are not significantly affected by the local force of ~ 5 pN exerted on terminal ends, have to be reorganized and (partially) opened to initiate successful base-pairing with the 3'-single-strand to form the 9-bp stem2 of the pseudoknot (Fig. 1B). The 3'-single-strand is adenine-rich and designed to have no appreciable structures (Mathews et al. 2004). The value of $\Delta X_{\text{H-P}}^\ddagger = -4.1 \text{ nm}$ is consistent with the fact that shortening of the extension of the 3'-single-strand is required to base pair with the loop of HPp.

Transient folding to nonnative structures of stem1 hairpin

After force is quickly dropped to ~ 10 pN, a stable native HPp structure forms with a sudden extension decrease, which is stable within our experimental time of up to 5

min. However, before HPp forms and after the force is stably maintained at the preset value, transient folding/unfolding events are observed with folded lifetimes typically shorter than 0.1 sec, which are significantly shorter than that of the native stem1 hairpin (Fig. 3B,C; Table 2). The corresponding extension changes (~ 10 nm) are similar to those of native HPp folding transitions from single strand (S-H). We term this relatively short-lived state as a “collapsed state” (C), the transient folding transition as “collapsing” (S-C), and the unfolding transition from the collapsed state as “uncollapsing” (C-S). Transient folding to a collapsed state is also observed for the HPi construct (Table 2). There can be a mixture of collapsed structures with similar end-to-end extension changes as the native state. The native HPp unfolds with an ensemble rate constant of $0.08\text{--}1.00 \text{ sec}^{-1}$ between 22.5 and 23.5 pN (Table 2). By extrapolation of the force-dependent kinetic equation: $\ln k_{\text{H-S}} = (2.6 \pm 0.1)F - (60.2 \pm 0.1)$ (Fig. 5; Table 1), the native HPp would unfold with a calculated ensemble rate constant of near $10^{-14} \text{ sec}^{-1}$ at 10.0–11.0 pN, many orders of magnitude slower than the “uncollapsing” rate constant of $20\text{--}40 \text{ sec}^{-1}$ for the collapsed species at the same force (Table 2). Thus, the

FIGURE 5. Plots of the natural logarithm of the ensemble rate constants (sec^{-1}) versus force. The data are fit to Equation 1 with the fitting results shown in Table 1. (A) The pseudoknot construct. Pseudoknot folding (H-P) at 5.0–6.0 pN, HPp formation (S-H) at 10.0–11.0 pN, and pseudoknot unfolding (P-S) at 45.0–47.0 pN. (B) Stem1 hairpin unfolding (H-S) at 22.0–23.5 pN and folding (S-H) at 10.0–11.0 pN for (○) HPi construct and (●) HPp in the pseudoknot construct. Force-dependent ensemble reaction rate constants were measured covering a minimum force range of 1 pN with 0.5-pN intervals. Each data point on this figure was normally obtained from fitting of >100 transitions (see Fig. 4; Table 2).

FIGURE 6. Native (left panel) and possible nonnative collapsed structures (middle and right panels) of the stem1 hairpin. The collapsed structures are predicted by the RNAstructure program. The free energies (in kilocalories per mole, at 37°C in 1 M NaCl) and corresponding values in piconewtons-nanometers (pN·nm) are shown at the bottom of each structure.

observed “collapsing/uncollapsing” transitions do not correspond to folding/unfolding of native HPP.

We observe one transient folding to nonnative HPP for approximately every seven constant-force trajectories at 11.0 pN, and the frequency increases with lower force. The average collapsing rate constants (k_{S-C}) differ by a factor of 2 or less between 10.0 and 11.0 pN, which is similar to the ratio of native folding rate constants (k_{S-H}) (Table 2). Note that, for those trajectories with transient folding events, the lifetime (τ_{S-H} , from the single-stranded conformation to native HPP or HPI structure) is calculated after the last uncollapsing step, which is also done for the pseudoknot folding transition from native HPP (τ_{H-P} , see below) (Fig. 3C).

To confirm that the transient folding is not due to the finite-speed electronic force feedback, we designed a different force-ramp experiment for the HPI construct. The molecule was pulled repeatedly between 10 and 30 pN, with 10 sec of incubation time at 10 pN, in a passive mode, that is, without force feedback and thus without adjusting the pipette bead position (Fig. 7; Wen et al. 2007). As expected, the native HPI structure folds (S-H) at ~ 10 pN during the 10-sec incubation period, as indicated by an abrupt force increase of ~ 1 pN, corresponding to a molecular extension decrease (~ 10 nm) upon folding. The formation of the native HPI conformation was confirmed by the subsequent force-ramp pulling trajectory with an unfolding force of ~ 24 pN (H-S) (see Fig. 7, top panel). Interestingly, transient force increase (~ 1 pN) events (S-C) were also observed in the passive mode trajectories before the formation of the native HPI structure, consistent with transient folding to nonnative collapsed structures (Fig. 7). Thus, occurrence of nonnative collapsed structures is not an artifact of the force-feedback mechanism.

Transient folding to nonnative structures of the pseudoknot construct

Upon the second force-drop by rapidly lowering force from ~ 10 to ~ 5 pN, the pseudoknot folds from native HPP at ~ 5 pN with an ensemble rate constant of 0.1 sec^{-1} . Transient folding events are also observed in the trajectories of constant force with lifetimes typically shorter than 0.5 sec (Fig. 3B; Table 2). Analogous to the observation that transient folding events (collapsed states, C) occur before the formation of the native stem1 hairpin (see above), these short-lived species are termed collapsed nonnative states of the pseudoknot construct (C'). The native pseudoknot structure is stable once formed, as indicated by the absence of transient folding events.

Since the 3'-single-strand of stem1 (Fig. 1B,C) was designed to have no appreciable structure (Mathews et al. 2004), it is unlikely that the transient folding is due to local collapsing or folding from the 3'-single-strand. Thus, the collapsed nonnative conformations C' are probably due to nonproductive intramolecular collisions, without enough stable interactions, between the loop of HPP and the 3'-single-strand. The frequency of collapsing events seems to be relatively independent of force from 5.0 to 6.0 pN, with similar collapsing rate constants as those of folding to native

FIGURE 7. Transient folding (S-C rapidly followed by C-S, indicated with gray arrows) of the HPI construct during a passive mode experiment (without force feedback). The single strand (S) might contain nonnative small hairpins. Time courses of both force and extension are shown. Note that only the trap bead moves upon a transition during a passive mode and thus force increases and extension decreases for collapsing transition (S-C), but force decreases and extension increases rapidly for the following uncollapsing transition (C-S). Force increases and extension decreases when the native hairpin forms (S-H). Unfolding of the native stem1 hairpin (H-S) is indicated by a rip at ~ 24 pN during the force-ramp. The bottom panel shows a close-up view of the top panel as indicated. For the force-ramp regions of this trace, the unloading rate is 200 nm/sec, and the loading rate is 100 nm/sec.

pseudoknot (k_{H-P} versus $k_{H-C'}$) (Table 2). More folding trajectories with transient folding events are needed for a rigorous force-dependent collapsing and uncollapsing analysis.

DISCUSSION

Mechanical stretching forces perturb the RNA folding landscape by favoring the more extended conformations and facilitate direct mapping of unfolding/folding pathways and measurement of kinetics. Figure 8 shows the measured unfolding/folding kinetics of the dominant pathway of the modified human telomerase RNA pseudoknot in this study. The stochastic transient folding events suggest that RNA folding can take complex pathways and show multiple conformations (Figs. 3B,C, 7). Force can be used to distinguish the native and nonnative conformations with similar molecular extensions but different structural stability.

Low-force folding and high-force unfolding

The stem1 hairpin and pseudoknot have different unfolding and folding forces and extension changes, which facilitate distinct observables for individual reactions (Fig. 8; Tables 1, 2). The force hysteresis larger than 10 pN for stem1 hairpin unfolding/folding (Fig. 2A) is mainly due to slow folding rates of the HPi and HPp, because of the rate-limiting step of closing the U-rich internal loop at the transition state (Fig. 1A,C). The folding forces of 10–11 pN result in formation of nonnative small hairpins with extension decreases of 2–4 nm. The lower limit for the disruption rate of one of the possible nonnative 5-bp

triloops (formed between G21 and C33) (see Fig. 6) before the native folding transition is approximately measured to be 20–40 sec^{-1} at zero force (see below). Thus, disruption of the nonnative small hairpins at 10.0–11.0 pN is not the rate-limiting step for the native folding of the stem1 hairpin ($k_{S-H} = 0.07\text{--}0.21 \text{ sec}^{-1}$).

Pseudoknot formation from HPp is even slower involving closing of the 29-nt loop2 (Fig. 1B,C), and it requires even lower force (Figs. 4, 5A). The mechanical stretching force is orthogonal to the stem1 helix axis before the loop2 closing step (Fig. 1C, indicated with gray arrows), but the force becomes parallel to the helix axis after the pseudoknot forms (Fig. 1B, indicated with gray arrows). Requirement of low forces for folding was also observed for other structures, especially those involving tertiary interactions, such as a kissing hairpin complex (Li et al. 2006a) and the *Tetrahymena* group I ribozyme (Onoa et al. 2003).

In contrast to low-force folding, significantly higher force (~ 50 pN) is needed to unfold the pseudoknot. Relatively high force is required mainly because the force is applied parallel to the helix axis of the pseudoknot (a shearing mechanism) (see Fig. 1B). High-force unfolding by a shearing mechanism was observed previously for an RNA-kissing hairpin complex and for frameshift-inducing pseudoknots (Li et al. 2006a; Green et al. 2007; Hansen et al. 2007). In contrast, relatively low force is needed to unfold a helix with force applied orthogonal to the helix axis (an unzipping mechanism) (see Fig. 1A,C). An unzipping mechanism breaks the secondary structure interactions one base pair at a time, and, thus, should have much lower energy barriers than a shearing mechanism.

Tertiary interactions, for example, compact consecutive base triples around the junction region add mechanical stability to the pseudoknot (Fig. 1B). These tertiary interactions form in the NMR construct (Fig. 1E; see Fig. 1B, base triples) in a buffer containing 200 mM KCl (Theimer et al. 2005). Thus, the tertiary interactions probably form in the pseudoknot construct used for the single-molecule study in 200 mM NaCl (Fig. 1B). Deletion of the single U-bulge (between residues 73 and 74) in stem2 (see the residue numbering in Fig. 1B) stabilizes the pseudoknot by 4 kcal/mol as measured for the NMR construct (Fig. 1E, right panel; Theimer et al. 2003, 2005), which affects unfolding pathways and adds mechanical stability. Magnesium ions stabilize the secondary and tertiary interactions of the pseudoknot further (Wyatt et al. 1990; Theimer and Giedroc 2000; Soto et al. 2007), which would require even higher unfolding forces (>50 pN). The effect of magnesium ions was not studied here due to the mechanical stability limit of DNA/RNA handles, which can be overstretched above 60 pN (Smith et al. 1996).

By ensemble thermal unfolding experiments in equilibrium, tertiary contacts were found to melt first followed by melting of stem2 and finally melting of stem1 for the NMR constructs (Fig. 1E; see Fig. 1B, structural scheme;

FIGURE 8. Schematic drawing of the folding landscapes involving the major conformational states of single-strand (S), stem1 hairpin (H), and pseudoknot (P) at different forces at 200 mM NaCl, 10 mM Tris-HCl (pH 7.3), 0.1 mM EDTA, at room temperature (22°C). Measured kinetic rate constants and distances to the transition states are also shown.

Comolli et al. 2002; Theimer et al. 2003, 2005). With a shearing mechanism as studied here for the modified pseudoknot (Fig. 1B), mechanical unfolding disrupts tertiary and secondary structures cooperatively. Obviously, mechanical unfolding and thermal unfolding take different pathways. Force, as a vector, can unfold an RNA structure via different directions along the energy landscape and via nonequilibrium and equilibrium pathways with different kinetics. However, mechanical folding and thermal folding follow roughly the same pathway for the pseudoknot structure: first folding of stem1 followed by stem2.

Transient folding and possible collapsed structures

Transient formations of nonnative collapsed structures (S-C and H-C') (Fig. 3B,C) are observed in both steps (S-H and H-P) of the stepwise folding of the pseudoknot construct. The single strand (S) at ~ 10 pN likely contains nonnative small hairpins (see below; Fig. 6). The collapsed nonnative states can be on-pathway or off-pathway to the native states. On-pathway collapsed states are directly converted to native state by thermal fluctuations with similar molecular extensions, and so they are not directly detectable in our experiments. The collapsed states we can detect are off-pathway.

Two possible nonnative structures of stem1 hairpin predicted by the RNAstructure program (Mathews et al. 2004) are shown in Figure 6 (both containing the same nonnative 5-bp CUU triloop). Based on kinetic modeling of thermal folding (Cao and Chen 2007), an intermediate nonnative structure consisting of the 5-bp CUU triloop was also proposed for the 6-bp NMR construct (Fig. 1E; see Fig. 1A, sequence; Theimer et al. 2003). Perhaps the nonnative 5-bp CUU triloop structure (formed with the wild-type sequence between G21 and C33) has a faster folding rate than the native structure (formed between G12 and C30) (Fig. 6). Subsequent closing of the bulge or multibranch followed by formation of six to eight native G•C pairs adjacent to terminal ends (Fig. 6, middle and right panels) results in an apparent two-state transient folding with a similar molecular extension decrease as that of native stem1 hairpin folding. As shown in Table 2, the force-dependent collapsing (S-C) kinetics is similar to that of folding to the native hairpin (S-H). This proposed transient folding pathway follows the nucleation-zipping mechanism, that is, the nucleation of several closing base pairs is the rate-limiting step, which is followed by fast zipping of the remaining stem. It is less likely that the collapsing pathway is initiated by formation of several native G•C pairs adjacent to the terminal ends, which would have more force-dependent kinetics than we see here. It cannot be ruled out that multiple pathways are involved in the transient folding. More folding trajectories with transient folding events are needed for a rigorous force-dependent collapsing and uncollapsing analysis.

The postulated nonnative structures are significantly less stable than the native stem1 hairpin (Fig. 6). The work required to unfold the native stem1 hairpin to form a single strand at 23 pN is 414 pN•nm ($W = F\Delta X$, including the stretching of single-stranded RNA of ~ 100 pN•nm) (Tinoco 2004) with an extension increase of 18 nm (Table 1). In contrast, the work required to unfold the collapsed structure to nonnative small hairpins at 10 pN is 100 pN•nm (including the stretching of single-stranded RNA of ~ 35 pN•nm) (Tinoco 2004) with an extension increase of 10 nm (Fig. 3B,C), which is comparable to the predicted values at 37°C and 1 M NaCl at zero force (Fig. 6). Thus, the six or eight native G•C pairs in the proposed collapsed structures might be bistable at ~ 10 pN (considering the force, temperature, and salt effects), consistent with our observations.

When the nonnative collapsed structures of HPI or HPP form, on-pathway and off-pathway mechanisms may compete for the following steps. The six to eight native G•C pairs (Fig. 6, middle and right panels) under tension tend to unfold and result in an uncollapsing transition to single-stranded RNA (an off-pathway mechanism). However, the bottom part of the collapsed structures, including the nonnative 5-bp triloop favors the rearrangement to the more stable native hairpin conformation (see Fig. 6), resulting in an on-pathway reaction. Thus, the probability to follow the on- or off-pathway route to the hairpin formation is basically determined by the kinetics and stability of different parts of the collapsed structures. The force exerted on the terminal ends does not significantly affect the stability of the nonnative 5-bp triloop. Disruption of the nonnative 5-bp triloop is the rate-limiting step to reform the native stem1 hairpin from the nonnative collapsed structures. Thus, the average uncollapsing rate ($20\text{--}40\text{ sec}^{-1}$) (Table 2) provides a rough lower limit for the opening rate of the nonnative 5-bp triloop at zero force.

The disruption rate of the nonnative 5-bp triloop can also be predicted from equilibrium free energies assuming a hairpin formation rate ($k_{\text{formation}}$) between 10^4 and 10^5 sec^{-1} (Turner 2000). The free energy for formation of the nonnative 5-bp triloop without tension is predicted (Serra et al. 1994; Xia et al. 1998; Mathews et al. 2004; Lu et al. 2006) to be -6.2 kcal/mol [$K_{\text{eq}} = \exp(-\Delta G_i/RT) = 4 \times 10^4$] at 22°C at 1 M NaCl. From the hairpin folding free energy and formation rate range at zero force, the hairpin disruption rate is calculated ($k_{\text{disruption}} = k_{\text{formation}}/K_{\text{eq}}$) to be $0.3\text{--}3\text{ sec}^{-1}$, $\sim 1\text{--}2$ orders of magnitude slower than the uncollapsing rate observed ($20\text{--}40\text{ sec}^{-1}$). Of course, fluctuation of the adjacent bulge or multibranch and lower salt concentration (200 mM NaCl) facilitate a faster opening rate than that predicted by only considering an isolated hairpin of the nonnative 5-bp triloop at 1 M NaCl. Thus, the proposed collapsed structures are reasonable. It is also reasonable that native folding of the stem1 hairpin can occur via rapid disruption of the nonnative 5-bp triloop at ~ 10 pN.

Transient folding during the second step of pseudoknot folding probably follows a “capture” mechanism, which was proposed for ribonucleoprotein assembly and RNA tertiary structure folding (Weeks and Cech 1996; Batey and Williamson 1998; Leulliot and Varani 2001; Chadalavada et al. 2002; Casiano-Negroni et al. 2007). It is probable that one of the pseudoknot folding transition pathways is initiated by reorganizing the UCGCU pentaloop formed between G12 and C30 (Fig. 1 C) and formation of several base pairs at the end of stem2 (Fig. 1B). Subsequent opening of the 6 bp (two Watson–Crick and four non-Watson–Crick) adjacent to the pentaloop, which is “captured” by the 3′-single-strand, leads to a successful pseudoknot folding transition (H-P). Accordingly, uncollapsing (C′-H) occurs if the adjacent 6 bp are not opened for base-pairing with the 3′-single-strand. Such a pathway explains the observation of similar molecular extension decreases for native and transient pseudoknot folding. Other native and transient pseudoknot folding pathways are also possible (Cao and Chen 2007).

The force exerted on the terminal ends of stem1 (Fig. 1C) does not significantly affect the opening rate of the pentaloop formed between G12 and C30 to initiate formation of stem2 with the 3′-single-strand. Thus, the apparent force-dependent pseudoknot folding kinetics (Fig. 5A; Tables 1, 2) reflects the rate-limiting step of bringing the loop1 in proximity to the 3′-single-strand and closing the 29-nt loop2 (Fig. 1B). The transition state positions for native and transient pseudoknot folding are less defined than that of hairpin folding. Our results provide a benchmark for testing theoretical simulations of the pseudoknot folding transition under tension in a more detailed molecular level, such as closing of loop2 (Fig. 1B,C) by the $\sim 90^\circ$ flipping of stem1 in HPp, by the reorganization of stem1 hairpin loop residues (Theimer et al. 2003; Yingling and Shapiro 2005; Reipa et al. 2007), and by relaxation of the stretched 3′-single-strand (Hyeon and Thirumalai 2006; Manosas et al. 2006).

Transient folding characterized by force and molecular extension changes

In the constant-force mode, the abrupt folding/unfolding transition with extension decrease/increase is accompanied by a small abrupt force increase/decrease (Wen et al. 2007). The force burst is due to the finite speed of force feedback, which is not fast enough to follow the trap bead movement when the RNA folds or unfolds, resulting in a time delay (~ 0.1 sec in that system) before force is gradually restored to the preset value (Wen et al. 2007). Thus, the force burst amplitude is determined by the extent of extension changes upon folding/unfolding, and the effective stiffness of the optical trap and DNA/RNA handles. Such a force-feedback delay can cause a bistable RNA hairpin to fold/unfold at forces below/above preset values.

As expected, a force burst of up to 1 pN is observed for both transient and native folding of the stem1 hairpin at

~ 10 pN and is less significant for pseudoknot folding transition at ~ 5 pN (Fig. 3B,C). The restoring of the force to the preset values is significantly less than 0.1 sec in the current system. Thus, lifetimes shorter than 0.1 sec can still be accurately measured at constant force (see Fig. 3C; Manosas et al. 2007). Force bursts of up to 1 pN from a preset value of ~ 10 pN or ~ 5 pN do not cause the native stem1 hairpin or pseudoknot to unfold, because it is still far below the unfolding forces of the native stem1 hairpin (~ 23 pN) and pseudoknot (~ 46 pN). In addition, transient folding is observed for the HPi construct in the passive mode at ~ 10 pN (without force feedback) (Fig. 7). Thus, the transient folding events we observed are not due to the finite time response of the force feedback, which, however, can affect the kinetics of uncollapsing (Table 2).

Transient sampling of other nonnative species (with extension changes smaller than 5 nm) are also observed for HPi at ~ 10 pN and for the pseudoknot construct at ~ 10 and ~ 5 pN (data not shown). This is consistent with the fluctuations observed on the force-ramp relaxing trajectories below 10 pN, probably due to formation/disruption of local structures (Fig. 2; Li et al. 2007). These conformations, including the nonnative 5-bp triloop (Fig. 6), may result in smaller extension decreases on folding than that predicted by the worm-like-chain model at 10 pN or below (Table 1; Smith et al. 1996; Liphardt et al. 2001). The loop2 and stem1 of the pseudoknot studied here were mutated to simplify the folding pathways, and thus we expect that more complex folding trajectories might be observed for the structure of the wild-type sequence (Gavory et al. 2006).

Transient folding in other single-molecule and ensemble experiments

Transient folding events of the pseudoknot construct are seen in both constant-force trajectories at ~ 10 and ~ 5 pN (Fig. 3B,C). The multiple zipping/unzipping events observed in force-ramp relaxing trajectories at ~ 10 pN or lower force might also be due to the transient formation of nonnative collapsed structures (see Fig. 2A, curves 3,5). Nonnative structures have been observed in both single-molecule and ensemble experiments previously (Fang et al. 2002; Buchmueller and Weeks 2003; Tan et al. 2003; Perez-Salas et al. 2004; Russell et al. 2006; Li et al. 2007). Misfolded structures were observed in HIV-1 TAR RNA folding by force-ramp and constant-force experiments by optical tweezers (Li et al. 2007). An intermediate state located at a single mismatch position was observed (lifetime ~ 0.1 sec) for a DNA hairpin, with the fully folded state sampled transiently (lifetime ~ 0.1 – 0.3 msec) (Woodside et al. 2006a). Multiple folding pathways for DNA and RNA hairpins were also suggested by ensemble temperature-jump studies (Ansari et al. 2001; Ma et al. 2006, 2007). Collapsed structures in protein mechanical folding have been observed by atomic force microscopy (Walther et al. 2007).

FRET is a useful observable for detecting molecular extension changes for RNA tertiary structure unfolding/folding (Ha et al. 1999; Stone et al. 2007). Based on two-state analysis of single-molecule FRET trajectories, a collapsed state has been inferred as the transition state for intramolecular docking transitions between loops A and B of two-helix hairpin ribozymes (Bokinsky et al. 2003) and for GAAA tetraloop–receptor interactions (Hodak et al. 2005). Heterogeneity of the undocking kinetics of the hairpin ribozyme was attributed to formation of various near-native states with different stabilities (Bokinsky et al. 2003). A collapsed intermediate state was directly observed by single-molecule FRET in a four-way multibranch hairpin ribozyme with mutation of an essential G•C pair between loop A and loop B (Tan et al. 2003). A near-native collapsed state was also observed for a cleaved form of the four-way multibranch hairpin ribozyme, presumably because of loss of native contact between “cleaved loop A” and loop B (Nahas et al. 2004).

Collapsed nonnative states have been observed by ensemble measurement for tertiary folding of the group I ribozyme and RNase P RNA (Fang et al. 2002; Buchmueller and Weeks 2003; Perez-Salas et al. 2004; Russell et al. 2006). Thus, near-native collapsed states can form in both secondary and tertiary structures. Studies from many functional RNA molecules indicate that misfolded or collapsed species can fold into native structures, probably facilitated by thermal fluctuation and/or mechanical force, in some ways similar to the action of chaperones.

By employing force–ramp and force–jump/drop methods, the short-lived nonnative collapsed states can be directly revealed, by following the complex folding pathways in real time. Utilization of both molecular extension and mechanical force provides powerful probes for perturbing and mapping the folding energy landscapes of both secondary and tertiary structures one molecule at a time. Compared with traditional methods to study RNA folding by varying solvent and temperature, the advantage of mechanical folding is that the experiments can be done under physiologically relevant conditions. Better understanding of RNA folding and unfolding may provide insight into therapeutics targeting telomerase.

MATERIALS AND METHODS

Preparation of RNA and single-molecule constructs

Single-molecule constructs were prepared as described previously (Liphardt et al. 2001). In brief, the chemically synthesized DNA oligonucleotide (Operon) corresponding to modified human telomerase RNA pseudoknot was cloned into the pBR322 vector (NEB) between the EcoRI and HindIII sites. The ~1.2-kb RNA with the pseudoknot sequence, flanked by ~500 and ~600 nt, on the 5' and 3' halves, respectively, was *in vitro* transcribed. The corresponding ~500-bp (handle A) and ~600-bp (handle B) DNA sequences were generated by PCR. RNA was annealed with

complementary strands of handle A and B by slowly cooling from 85°C. A constituent hairpin construct (HPi) was made by extending the handle B to cover the 3'-side of the pseudoknot sequence. A biotin group was added to the 3'-end of handle A by biotin-linked dUTP (Roche) with T4 DNA polymerase (NEB); and a digoxigenin group was added at the 5'-end of the handle B via the primer (Operon) during PCR. The biotin and digoxigenin at the opposite ends of DNA/RNA handles provide affinity binding with streptavidin and anti-digoxigenin-coated polystyrene beads (Spherotech), respectively. During a single-molecule manipulation experiment, ~2 μ m (diameter) streptavidin and ~3 μ m anti-digoxigenin beads were held by micropipette and optical trap, respectively.

Optical tweezers

The force-measuring optical tweezers instrument described previously (830-nm laser) (Smith et al. 1996; Liphardt et al. 2001) was modified with a nanodrive piezoelectric stage (MCL), which can be moved and recorded with nanometer precision. Force was measured from the displacement of the trap bead. The extension change was measured from movement of the pipette bead, which was linked to the piezoelectric stage and the reaction chamber, and the trap bead.

Force–ramp and force–jump/drop experiments

The single-molecule mechanical unfolding/folding reactions were done at 200 mM NaCl, 10 mM Tris-HCl (pH 7.3), 0.1 mM EDTA, at room temperature ($22^{\circ}\text{C} \pm 2^{\circ}\text{C}$). In a force–ramp experiment, the piezoelectric stage, which is linked to the micropipette bead, was moved back and forth continuously at a constant velocity (e.g., 100 nm/sec) (Smith et al. 1996; Liphardt et al. 2001; Li et al. 2006b). In a force–jump/drop experiment, an electronic feedback control was used to rapidly change and maintain the force (Li et al. 2006b). A typical cycle of the force protocol is shown as follows:

- (1) Unfolding: Force–ramp from 5 to 40 pN, followed by a fast force–jump to 46 pN, which is maintained constant until the pseudoknot unfolds as indicated by an abrupt extension increase.
- (2) First force–drop: Force–ramp up to 50 pN (to confirm complete structural unfolding) and then force–ramp down to 15 pN, followed by a force–drop to 10 pN until stem1 folds as indicated by abrupt extension decrease.
- (3) Second force–drop: Force–drop to 5 pN until pseudoknot folds as indicated by abrupt extension decrease.

The data were acquired at 100 Hz by a LabView program and analyzed by a MatLab program. The lifetimes obtained from the force–jump/drop experiment were sorted from shortest to longest. The *i*th lifetime (τ_i) was given a count of ($N - i$), where *N* is the total number of lifetimes obtained. The single exponential fits of ($N - i$) versus τ_i give the ensemble rate constants for individual two-state reactions. The rate constants can also be obtained from the reciprocal of the average of all the lifetimes of individual reactions ($1/\langle\tau\rangle$). Similar rate constants obtained by the two methods indicate apparent two-state reactions. Uncollapsing rate constants are less accurate due to fewer data obtained and short lifetimes of the collapsed states.

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