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# Motions of the substrate recognition duplex in a group I intron assessed by site-directed spin-labeling

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## Abstract

The *Tetrahymena* group I intron recognizes its oligonucleotide substrate in a two-step process. First, a substrate recognition duplex, called the P1 duplex, is formed. The P1 duplex then docks into the pre-folded ribozyme core by forming tertiary contacts. P1 docking controls both the rate and the fidelity of substrate cleavage, and has been extensively studied as a model for the formation of RNA tertiary structure. However, previous work has been limited to study millisecond or slower motions. Here we investigated nanosecond P1 motions in the context of the ribozyme using site-directed spin labeling (SDSL) and electron paramagnetic resonance (EPR) spectroscopy. A nitroxide spin label (R5a) was covalently attached to a specific site of the substrate oligonucleotide, the labeled substrate was bound to a pre-folded ribozyme to form the P1 duplex, and X-band EPR spectroscopy was used to monitor nitroxide motions in the 0.1 – 50 nanosecond regime. Using substrates that favor the docked or the undocked states, it was established that R5a was capable of reporting P1 duplex motions. Using R5a-labeled substrates it was found that the J1/2 junction connecting P1 to the ribozyme core controls nanosecond P1 mobility in the undocked state. This may account for previous observations that J1/2 mutations weaken substrate binding and give rise to cryptic cleavage. This study establishes the use of SDSL to probe nanosecond dynamic behaviors of individual helices within large RNA and RNA/protein complexes. This approach may help in understanding the relationship between RNA structure, dynamics, and function.

RNA can adopt versatile three-dimensional structures and act as catalysts and regulators in gene expression and maintenance. Knowledge of RNA structure has advanced dramatically[1], but information regarding RNA dynamics is scarce despite the central importance of dynamic transitions in nearly all RNA-mediated processes[2]. A number of experimental and computational approaches have been used to investigate RNA dynamics[2]. In particular, studies using spectroscopy methods, such as NMR[3] and fluorescence[4], have revealed complex RNA motions ranging from picoseconds to milliseconds. Nevertheless, NMR dynamic studies have been limited to RNA systems <100 nucleotides. Fluorescence spectroscopy, combined with single molecule methodologies, has been used to study larger RNA and RNA/protein systems[4-6], but such approaches are typically limited to probing millisecond or slower conformational transitions.

Here, we report work characterizing nanosecond motions of a specific RNA element within an approximately 400 nucleotide ribozyme, the group I intron of *Tetrahymena thermophila*. The *Tetrahymena* intron was the first catalytic RNA discovered and has served as a model system to study RNA structure, folding, and catalysis[7,8]. This ribozyme recognizes its exon junction substrate by forming a duplex, designated as P1, between the substrate and an internal

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guide sequence (IGS) within the intron. Extensive studies have revealed that the P1 duplex first forms as a secondary structure element referred to as the "open complex", then docks into the pre-folded ribozyme core via tertiary interactions to form the catalytically competent "closed complex" (Fig. 1A)[8-10].

P1 docking controls both the rate and the fidelity of substrate cleavage and provides a model for the formation of RNA tertiary structure[8]. Pre-steady state kinetic and single molecule fluorescence studies have provided extensive information about the rate constants for transition between the open and closed complexes[9,11,12]. Formation of the closed complex is surprisingly slow, given the apparent simplicity of docking a duplex, which is connected by a short tether to the ribozyme, to a preformed tertiary structure. Understanding the origin of this slow process will require more in-depth understanding of the properties of the open, undocked state.

For this reason, we investigated P1 duplex motions in the open and closed states by using site-directed spin labeling (SDSL) and electron paramagnetic resonance (EPR) spectroscopy[13,14]. A stable nitroxide radical (R5a) was attached to the $S_P$-phosphorothioate diastereomer substituted at the +3 position of the oligonucleotide substrate[15] (i.e., three residues 3' of the cleavage site; Fig. 1A), and this substrate was hybridized with the ribozyme to form the P1 duplex (supplemental data S.I). X-band continuous-wave EPR spectroscopy was used to monitor R5a rotational motions in the 0.1 – 50 nanosecond time regime. We were thus able to isolate nanosecond motions of the P1 duplex in the context of the rest of the *Tetrahymena* ribozyme helices and junctions.

Our study used two R5a-labeled oligonucleotides with identical base sequences that pair with IGS to form the P1 duplex (Fig. 1A). The "$S_C^{SL}$" substrate contains all RNA residues (except a deoxyribose residue at the cleavage site to slow substrate cleavage) and is predominantly docked to the wild type ribozyme[11,12]. The "$S_O^{SL}$" substrate has one additional modification relative to the dockable $S_C^{SL}$ substrate, a 2'-methoxy at a position distal from R5a (−3 from the cleavage site, indicated by "mU" in Fig. 1A). This modification destabilizes the closed complex and favors the open complex[12]. Biochemical studies showed that R5a labeling did not perturb P1 docking, although the P1 helix secondary structure was destabilized (supplemental data S.II.A).

$S_C^{SL}$ and $S_O^{SL}$ were bound to a pre-folded ribozyme, and EPR spectra were measured in aqueous solutions at 25 °C (Fig. 1B, wild type ribozyme). Both spectra were distinct from those for the individual oligonucleotides (supplemental data S.II.B), indicating behavior specific to the ribozyme complex. The $S_C^{SL}$ spectrum showed broader lines compared to that of $S_O^{SL}$ (Fig. 1B), revealing a lower R5a mobility in the $S_C^{SL}$ complex that favors the closed state. Control experiments established that these spectral differences arise from a reduction in P1 mobility in the closed complex (supplemental data S.II.C). Although R5a was not rigidly fused to the P1 duplex, it remains sufficiently coupled to the RNA to report on P1 duplex motions.

Mutant ribozymes were studied to assess how P1 motions are affected by structural elements in the ribozyme. The J1/2 junction, which consists of three A residues and physically connects the P1 duplex to the rest of the ribozyme, was either extended by five A residues (+5A ribozyme) or deleted (-3A ribozyme) (Figs. 1A & 2). For either mutant, $S_C^{SL}$ and $S_O^{SL}$ substrates gave identical spectra, and these spectra were similar to that obtained for the wild type ribozyme in the open complex (Fig. 1B and supplemental data S.II.D). These similarities suggest that the mutations destabilize the docked complex, as previously demonstrated by biochemical experiments[10,16].

Despite the overall similarities and indication of enhanced motions, the open states for the +5A, -3A, and wild type ribozymes show distinct spectral features, with the differences most clearly observed in the low field peak (arrows in Fig. 2; supplemental data S.II.E). The -3A ribozyme

spectrum has a split low-field peak, a feature that arises from incomplete averaging of the anisotropic magnetic tensors as the nitroxide undergoes restricted motions[17,18]. The split low-field peak is partially merged in the wild type ribozyme and becomes one peak in the +5A ribozyme. These spectral variations qualitatively indicate that R5a motions become less restricted as J1/2 is extended. Furthermore, the open complex spectra were simulated using an order parameter (S) and an effective correlation time ($\tau$) to describe the nitroxide diffusive motions under the restraint of an ordering potential[17-20] (supplemental data S.I). The simulated spectra matched very well with those measured experimentally and revealed a decrease in S as J1/2 is extended (Fig. 2 & supplemental data S.II.F). This modeling supports the conclusion that a longer J1/2 reduces R5a motional restriction. Because J1/2 mutations are remote from the nitroxide attachment site, it is unlikely that they alter the local environment around R5a (i.e., R5a/RNA contacts and dynamics of the nucleotide at the labeling site). Instead, extending J1/2 likely increases P1 duplex motions, consequently lessening the restrictive ordering potential experienced by the nitroxide (Fig. 2).

Variations in P1 nanosecond motions reported above likely reflect changes in P1 flexibility upon mutating J1/2. Such changes may contribute to the phenotypes of the J1/2 mutants, namely weaker substrate binding and cryptic cleavage[10,16,21]. For the +5A mutant, an extended J1/2 gives higher P1 mobility and increases flexibility of the open complex, presumably raising the entropic cost of docking and lowering substrate binding strength[16,21]. For the -3A mutant, which lacks a J1/2, P1 mobility is more restricted. Docking presumably occurs at the energetic expense of some distortion of the ribozyme[16], leading to a more severe reduction in the strength of substrate binding[16]. Alterations in P1 flexibility may also differentially affect the registers in which the P1 duplex docks, giving rise to the specific cryptic cleavage patterns observed for J1/2 mutant ribozymes[10,16].

In summary, SDSL was used to monitor nanosecond motions of a single RNA helix, the P1 duplex, within the complex group I ribozyme. We found that in the open complex of the ribozyme, an RNA element, the J1/2 junction, can modulate P1 duplex motions. In the closed complex, the P1 duplex is considerably less dynamic, and this limited motion presumably contributes to positioning the bound substrate for chemical catalysis. The methodology reported here may be applied to obtain basic dynamic information on a variety of states of large RNAs with complex tertiary structures. Such studies may help unravel the relationship between RNA structure, dynamics, and function.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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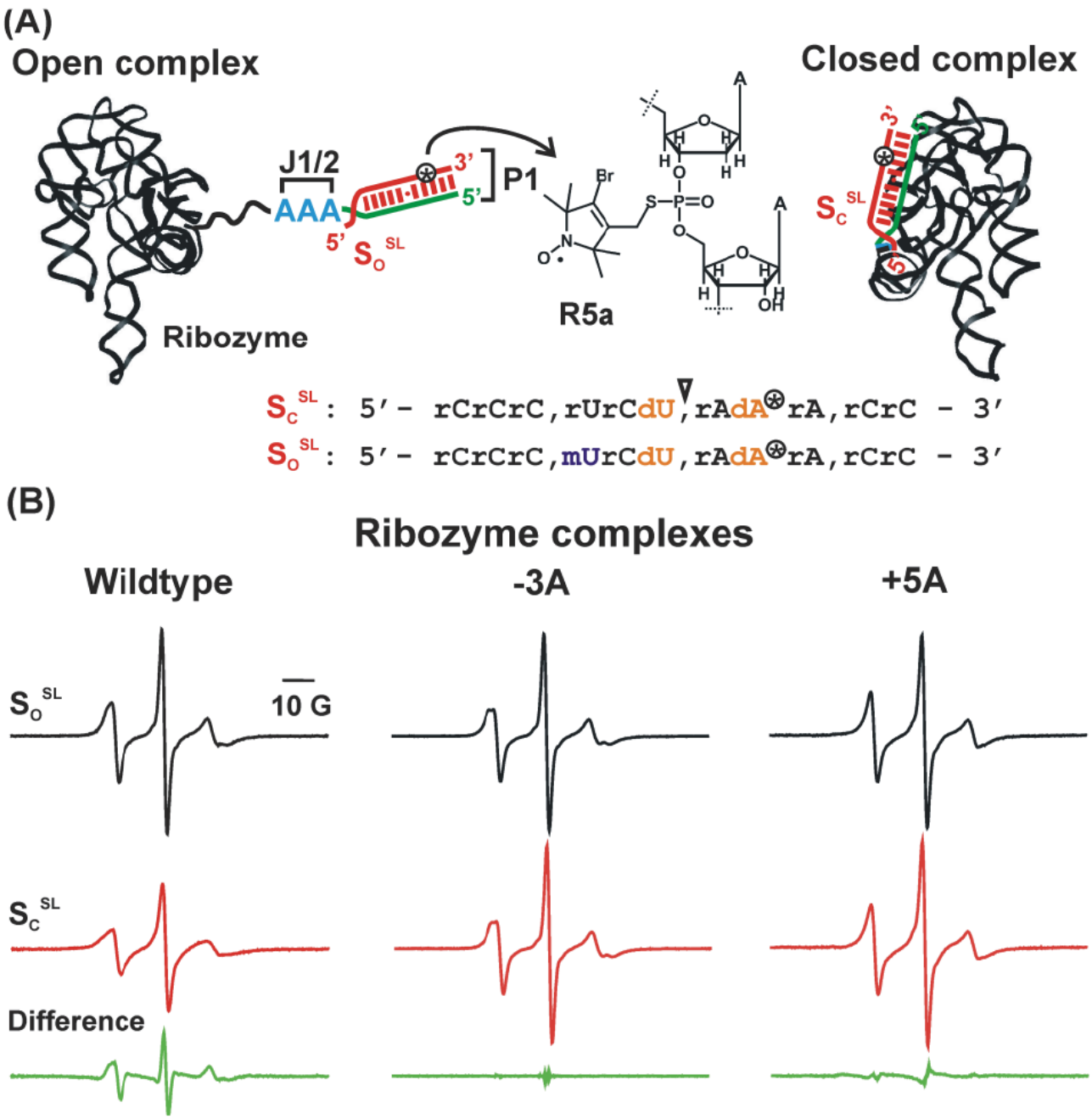


**Figure 1.**

SDSL probing of P1 helix docking. (A) Schematic renditions of the open and closed ribozyme complex. The substrate sequences, the R5a spin label (*), the cleavage site (∇), and the J1/2 junction are marked. (B) EPR spectra of various substrate/ribozyme complexes.

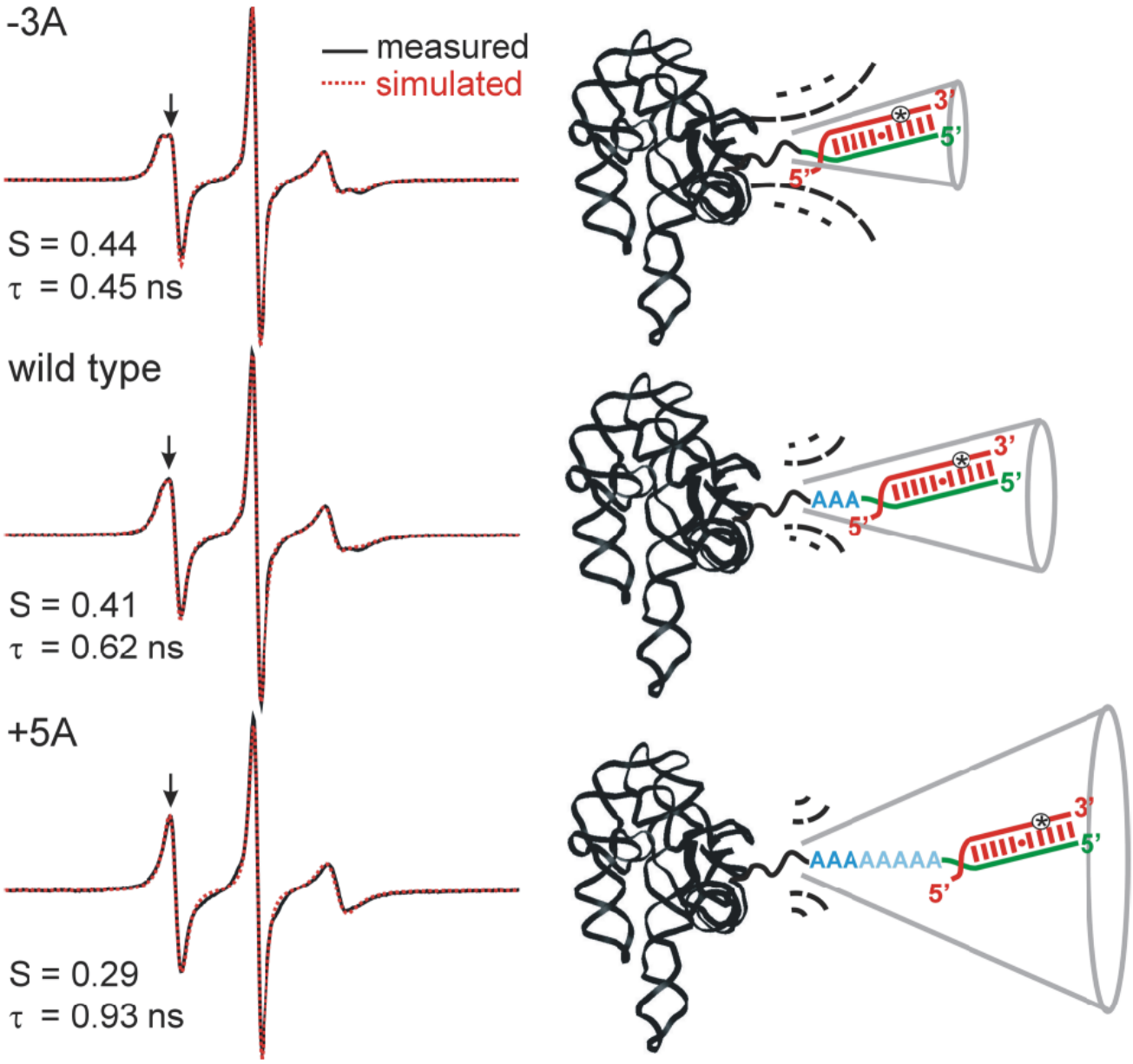


**Figure 2.**

J1/2 mutations affect P1 dynamics in the open state. Shown on the left are measured (black) and simulated (dotted red) spectra for the $S_O^{SL}$ substrate bound to the -3A, wild type, and +5A ribozymes. The data revealed a decrease of motional restriction as J1/2 is extended. This is depicted by the schematic renditions shown on the right.