Significant Kinetic Solvent Isotope Effects in Folding of the Catalytic RNA from the Hepatitis Delta Virus

Rebecca A. Tinsley, Dinari A. Harris, and Nils G. Walter*

Department of Chemistry, University of Michigan, Ann Arbor, Michigan 48109-1055

RECEIVED DATE (automatically inserted by publisher); nwalter@umich.edu

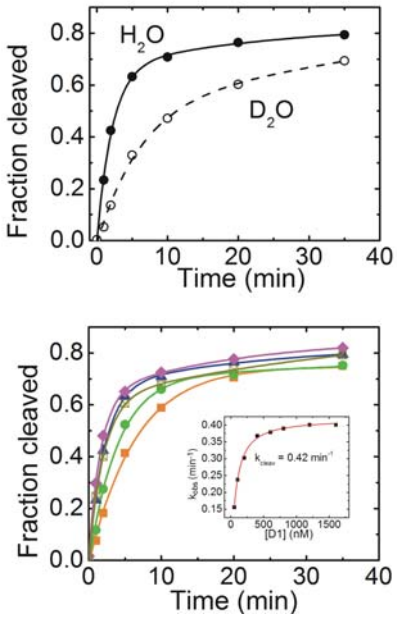
Supporting Information:

RNA Synthesis: All RNA oligonucleotides were obtained commercially from the HHMI Biopolymer/Keck Foundation Biotechnology Resource Laboratory RNA at the Yale University School of Medicine. RNA was deprotected by a two step protocol as previously described.[1] Deprotected RNA was purified by denaturing 20% polyacrylamide, 8 M urea, gel electrophoresis, diffusion elution into 0.5 M NH_4OAc, 0.1% SDS, and 0.1 mM EDTA overnight at 4 °C, chloroform extraction, ethanol precipitation, and C_8 reverse-phase HPLC with a linear acetonitrile gradient in triethylammonium acetate as described previously.[1] To obtain a chemically blocked, noncleavable substrate analogue for structural analyses, the substrate was modified with a 2' methoxy group at the cleavage site. The 3' product (3'P) had the sequence 5'GGGUCGG-3'. RNA concentrations were calculated from their absorption at 260 nm and corrected for the additional absorption of fluorescein and tetramethylrhodamine by using the relations $A_{260}/A_{492} = 0.3$ and $A_{260}/A_{554} = 0.49$, respectively.

Cleavage Reactions: The 5'-^{32}P-labeled substrate was prepared by phosphorylation with T4 polynucleotide kinase and [γ-^{32}P]ATP. All cleavage reactions were performed under single-turnover (pre-steady-state) conditions. Standard conditions used were 25 mM MES (for experiments at pH 5 to 6.5) and 25 mM HEPES (for experiments at pH 7 to 9) and 11 mM $MgCl_2$, at 25 °C, unless otherwise stated. All D_2O solutions and buffers were prepared in 99.9 atom-%-D deuterium oxide (Sigma). The final pD of each reaction was determined with a standard glass electrode by adding 0.4 to the reading.[2] Ribozyme was preannealed from strand A and twice the concentration of strand B in standard buffer, by heating to 70 °C for 2 min and cooling to room temperature. After preincubation for at least 15 min at 25 °C, a trace (<4 nM) amount of 5'-^{32}P-labeled substrate (also in standard buffer) was added to 50 nM to 1600 nM ribozyme (based on the strand A concentration). Aliquots (5 μL) were taken at appropriate time intervals and the reactions quenched with 10 μL of 80% formamide, 0.025% xylene cyanol, 0.025% bromophenol blue, and 50 mM EDTA. The 5' cleavage product was separated from the uncleaved substrate by denaturing 20% polyacrylamide, 8 M urea, gel electrophoresis and was quantified and normalized to the sum of the substrate and product bands using a Storm 840 PhosphorImager with ImageQuant software (Molecular Dynamics). Time traces of product formation were fit to the single-exponential first-order rate equation $y = y_0 + A(1 - e^{-t/\tau})$, employing Marquardt-Levenberg nonlinear least-squares regression (Microcal Origin), where A is the amplitude and τ^{-1} the pseudo-first-order rate constant k_{obs}. Ribozyme dependencies of this rate constant were fit to binding equation 1:

$$k_{obs} = k_{cleav}\frac{[Rz]}{[Rz]+K_M} \quad (1)$$

to yield k_{cleav} under standard conditions. Due to the fact that the dissociation rate constant of the substrate is comparable to the rate constant of the chemistry step, indicating a Briggs-Haldane mechanism for cleavage, this fit is a simplification for intermediate ribozyme concentrations.[3] However, we only use the plateau region of the ribozyme dependencies to extract k_{cleav}, which is independent of this simplification. k_{cleav} was then plotted as a function of pL and fit to the equation $k_{obs} = k_{max}/(1+10^{pK_{a1}-pH}+10^{pH-pk_{a2}})$, yielding two pK_a values with a precision of about ± 0.1.[1]



Supplemental Figure A (Top) Cleavage time course in 25 mM MES, pL 6.5 (pH in H_2O, pD in D_2O), and 11 mM $MgCl_2$ at 25 °C and at a ribozyme concentration of 400 nM. Data were fit to a single-exponential increase function to yield the pseudo-first order rate constant k_{obs}. Solid line with filled circle (●), cleavage time course in H_2O; dotted line with open circle (○), cleavage time course in D_2O. (Bottom) Cleavage time course in 25 mM MES, pH 6.5, and 11 mM $MgCl_2$ at 25 °C and at varying ribozyme concentrations [orange, 50; green, 100; blue, 400; pink, 800; dark yellow, 1600 nM]. Data were fit to single-exponential increase functions to yield the rate constants k_{obs} reported in the inset. (Inset) The Dependence of k_{obs} on the ribozyme concentration was fit to eq 1 yielding $k_{cleav} = 0.42$ min^{-1} and $K_M = 80$ nM. k_{cleav} is taken to be the saturation point of the ribozyme dependence or the maximum cleavage rate at saturating ribozyme concentrations.

Steady-state FRET Assays: Steady-state fluorescence spectra were recorded on an Aminco-Bowman Series 2 spectrofluorometer using our trans-acting HDV ribozyme D1, doubly-labeled with fluorescein and tetramethylrhodamine.[1] Annealed ribozyme [final concentration of 50 nM based on the strand B concentration; with an at least 2-fold (saturating) excess of unlabeled strand A] was incubated at 25 °C for at least 15 min in buffer supplemented with 25 mM dithiothreitol as a radical quencher, and was then transferred to a 150 μL cuvette. Noncleavable substrate analogue ncS3 was manually added to a (saturating) at least 5-fold excess. Fluorescein was excited at 490 nm (4 nm bandwidth) and fluorescence emission was recorded simultaneously at the fluorescein (520 nm, 8 nm bandwidth) and tetramethyrhodamine (585 nm, 8 nm bandwidth) wavelengths, by shifting the emission monochromator back and forth. A FRET ratio Q ($=F_{585}/F_{520}$) was calculated and normalized to the initial value. The resulting time traces were fit to single-exponential increase functions of the form $y = y_o + A(1-e^{-t/\tau})$ in MicroCal Origin 7.0 to extract the rate constants $k_{obs} = \tau^{-1}$. To obtain binding rate constants we varied the concentration of ncS3 from 250 nM to 1600 nM. Concentration dependences of the pseudo-first-order rate constants were fit to a linear regression, the slope of which reveals the bimolecular rate constant k_{off}. All D_2O solutions and buffers were treated as described above.

A recent study by Shih and Been reported that the 3' cleavage product (3'P) has a dissociation rate constant that is ~240 times slower than that of the substrate.[3] This makes 3'P a good chase for probing the dissociation rate constant of ncS3.[1,3] To perform dissociation assays, we first formed the ribozyme-ncS3 complex from 50 nM ribozyme (based on the strand B concentration) and 250 nM ncS3, then added 2.5 μM 3'P. Data were fit to a single-exponential decrease function of the form $y = y_o + Ae^{-t/\tau}$ as described above to directly yield the rate constant of substrate dissociation $k_{off} = \tau^{-1}$.[1] All D_2O solutions and buffers were treated as described above.

Proton inventory experiments were performed at pL 8.0 by varying n_{D_2O} and performing dissociation assays as described above. Equations used to fit the data and their corresponding line colors in Figure 3 are as follows:

$$\frac{k_n}{k_0} = (1 - n + 0.575n) \qquad \text{Green}$$

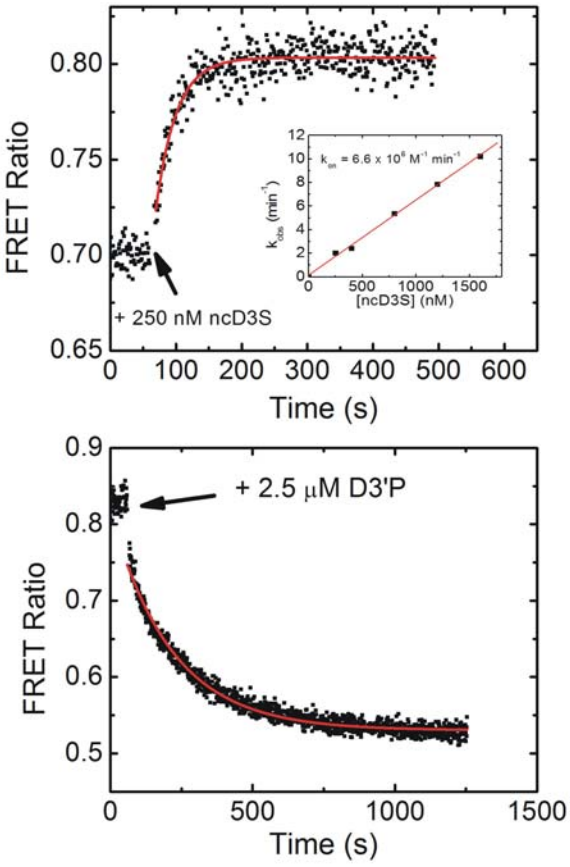
$$\frac{k_n}{k_0} = (1 - n + 0.758n)^2 \qquad \text{Blue}$$

$$\frac{k_n}{k_0} = \frac{1}{(1 - n + 1.739n)} \qquad \text{Purple}$$

$$\frac{k_n}{k_0} = \frac{1}{(1 - n + \phi n)^p} \qquad \text{Red}$$

$$\frac{k_n}{k_0} = \frac{Z^n}{(1 - n + \phi n)} \qquad \text{Black}$$

The equations for the green and blue lines assume one and two proton transfers, respectively, in the transition state, given our KSIE at 100% D_2O. The equation for the purple line represents a one-proton transfer in the reactant state. The equation for the black line represents a mechanism with multiple proton transfers in the transition state and a one-proton transfer in the reactant state. The equation for the red line is a general equation for multiple proton transfers during the reaction.



Supplemental Figure B (Top) Change over time in the FRET ratio of the doubly labeled ribozyme upon addition of 250 nM noncleavable substrate analogue (ncS3) at pH 8. The data were fit with a single-exponential increase function (solid line) to yield a rate constant of 2.1 min^{-1} (amplitude of 0.09). (Inset) Concentration dependence of the observed pseudo-first-order rate constant upon addition of excess ncS3 to the ribozyme. The slope of the linear regression line yields the bimolecular binding rate constant k_{on}. (Bottom) Dissociation of the noncleavable substrate analogue from its ribozyme complex at pH 8. The ribozyme-ncS3 complex (50 nM, with a 250 nM excess of ncS3) was chased with 2.5 μM 3'P, and the resulting decrease in the FRET ratio Q was fit to a single-exponential decrease function to yield a dissociation constant k_{off} of 0.24 min^{-1}. There is no significant dependence on the chase concentration, proving that a true substrate dissociation rate constant is derived.[1]

References

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