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

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Kinetic solvent isotope effects (KSIEs) describe the effects of an isotopic solvent on rate constants of enzymatic reactions, including those catalyzed by RNA. Most commonly, deuterium is substituted for protium in the hydrogenic sites of water, leading to subsequent exchanges of deuterium into some positions of the dissolved enzyme and its substrate(s). The resulting KSIEs, which are usually expressed as ratios of the rate constants in H_2O and D_2O, are often useful in the characterization of the rate-limiting step(s) in an enzyme mechanism.[1] For example, recent studies have used KSIEs as evidence that a specific cytosine side chain with a perturbed pK_a of 6.1 is involved in proton transfer during self- (or cis-) cleavage of the genomic and antigenomic catalytic RNAs (ribozymes) from the hepatitis delta virus (HDV).[2−4] The genomic and antigenomic HDV ribozymes are closely related ∼85-nucleotide RNA motifs whose cis-cleavage is crucial to the rolling-circle replication of the circular HDV genome, an infectious human pathogen and satellite of the hepatitis B virus (HBV).[5,6]

We have previously used fluorescence resonance energy transfer (FRET) between a donor−acceptor fluorophore pair to measure the rate constants of global conformational changes upon binding, cleavage, and dissociation of an external substrate by a trans-acting form of the HDV ribozyme (Figure 1A).[7] In addition, we found that the pH dependence of substrate cleavage by this ribozyme is bell-shaped and thus similar in form to that of the cis-cleaving HDV ribozymes (Figure 1B). However, cleavage in trans is generally ∼10−100-fold slower than in cis, and the first pK_a derived from the pH dependence is lowered from 6.1 to 5.4 (Figure 1B).[6] A likely explanation is that the observed pK_a in trans is not directly related to titration of an ionizable group involved in reaction chemistry as assumed for cis-cleavage, but rather to the rate-limiting step changing from a chemistry-related ionization to a composite of ionization and conformational change.[7] Thus, KSIEs may vary in the two regimes (Figure 1B).

We therefore measured the KSIEs of the cleavage rate constants of our trans-acting HDV ribozyme under standard single-turnover reaction conditions over the accessible pL (pH or pD) range of 5−9 (Figure 1B). Briefly, for each pL the ribozyme concentration was varied from 50 to 1600 nM, and the resultant pseudo-first-order, single-exponential rate constants were plotted as a function of ribozyme concentration and fit to a binding equation to yield the limiting rate constant of cleavage (k_{cleav}) under standard conditions (25 mM MES for pL 5−6.5 and 25 mM HEPES for pL 7−9, 11 mM Mg^{2+}, at 25 °C).[8] Throughout the entire pL range, we observe significant KSIEs of 1.6−3.0-fold (Figure 1B), comparable to KSIEs of 3.5−4.6-fold and 2.3-fold in the cis-acting genomic and antigenomic HDV ribozymes, respectively.[2,4] The apparent pK_a's shift from 5.4 ± 0.2 in H_2O to 5.8 ± 0.2 in D_2O and from 8.8 ± 0.2 to 9.0 ± 0.2 (Figure 1B), similar to the first pK_a's of the cis-cleaving genomic and antigenomic ribozymes, which shift from 6.1 to 6.5 and 6.8, respectively (a second pK_a is not consistently observed).[2,4] Taken together, our findings raise two questions with relevance to RNA in general: (i) Do an apparent pK_a and a signif-
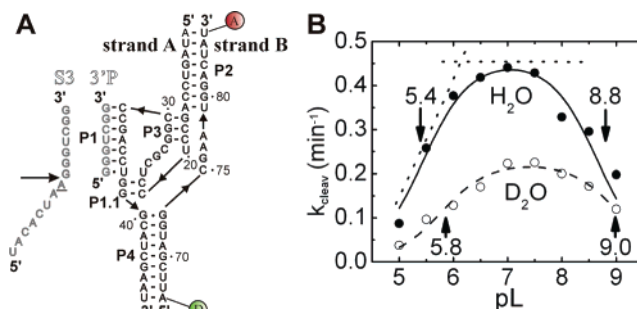

Figure 1. FRET-labeled trans-acting HDV ribozyme and its pL-dependent activity. (A) The ribozyme is shown in bold and consists of two separate RNA strands A and B. For FRET studies, donor (D, fluorescein) and acceptor (A, tetramethylrhodamine) fluorophores were coupled to strand B as indicated. 3′ Product (3′P) and substrate sequences (S3) are outlined; for structural studies the underlined nucleotide in S3 was 2′-O-methylated. (B) pH (●)- and pD (○)-dependent activity under standard single-turnover conditions. The data were fit, as described previously,[7] to yield apparent pK_a values as indicated. Dotted lines show two hypothetical processes, one pH-dependent, one pH-independent, that are rate-limiting in different regimes and thus may cause the first apparent pK_a.
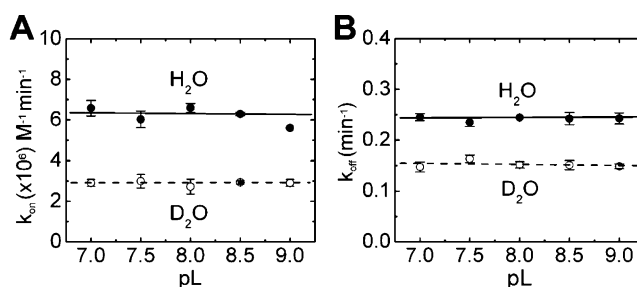

Figure 2. KSIEs of conformational changes of the HDV ribozyme. pH (●) and pD (○) dependence of the rate constants of conformational changes observed upon substrate binding (A) and substrate dissociation (B).

icant KSIE derived from the pL dependence of an RNA-catalyzed reaction necessarily relate to ionization of a functional group that acts as a general acid or base in rate-limiting reaction chemistry, as is commonly assumed? (ii) Can a pH independent conformational change have a significant KSIE?

To answer these questions, we determined the KSIEs of global conformational changes in our trans-acting HDV ribozyme upon substrate binding and dissociation by using FRET (Figure 2). Ribozyme strand B was labeled with a 5′ fluorescein and 3′ tetramethylrhodamine donor−acceptor pair. A chemically blocked, non-cleavable substrate analogue (ncS3) was obtained for these studies by modifying the cleavage site adenosine with a 2′ methoxy group (Figure 1A). In all fluorescence assays, a saturating 2-fold excess of ribozyme strand A and at least a 5-fold excess of ncS3 were used to ensure saturation of the labeled ribozyme strand B (Figure 1A) under standard conditions. Binding rate constants were obtained as previously described[7] by monitoring the FRET increase over time resulting from addition of varying concentrations of ncS3 to the assembled ribozyme, calculating pseudo-first-order rate con-
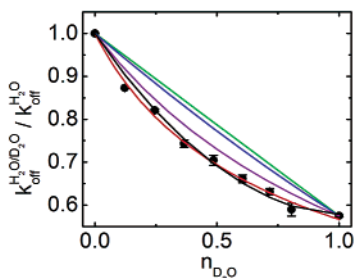
Figure 3. Proton inventory of the dissociation rate constant at pL 8.0. The overall isotope effect for the proton transfer is 1.75. Data were fit to the most common models of proton transfer: green and blue, one- and two-proton transfers in the transition state, respectively; purple, one-proton transfer in the reactant state. The best fits were obtained with eq 1 (red) and eq 2 (black).

stants from these time courses, and extracting a second-order substrate-binding rate constant (k_{on}) from their dependence on [ncS3] at each pL in the range of 7−9 (Figure 2A).[8] The binding rate constants are pL-independent, as expected, but show significant KSIEs of 2.0−2.5-fold throughout the entire pL range, comparable to those of cleavage.

Similarly, dissociation rate constants (k_{off}) were calculated at each pL from the rate constants of the FRET decrease resulting from the addition of a saturating concentration (at least 5-fold over [ncS3]) of 3′ Product (3′P) as a chase to the preformed ribozyme−substrate complex under standard reaction conditions (Figure 2B).[7,8] Again, dissociation rate constants are pL-independent, yet show quite significant KSIEs of 1.4−1.6-fold throughout the entire pL range. (Consequently, the equilibrium dissociation constants $K_D = k_{off}/k_{on}$ in D_2O are slightly increased compared to H_2O (by factors of ∼1.5) over the accessible pL range.) We observe similar (1.6-fold) KSIEs in a buffer containing no Mg^{2+}, but 300 mM Na^+ instead (data not shown), indicating that the observed KSIEs are not related to the shift in deprotonation equilibrium of the Mg^{2+}(aq) complex in D_2O.

Our results thus demonstrate that the pH-independent global conformational changes observed upon substrate binding and dissociation by the trans-acting HDV ribozyme have significant KSIEs. A change in rate-limiting step from a pH-dependent chemistry to a pH-independent conformational (or composite) step, both with significant KSIEs, may therefore explain the observed pH dependence and KSIEs of the overall cleavage rate constants of the HDV ribozyme and other catalytic RNAs (Figure 1B). These findings challenge the common assumption that the observed pL profiles and KSIEs of RNA-catalyzed reactions necessarily provide evidence for an ionization step to be rate-limiting.[2−4,9] This may come as no surprise considering that a large number of RNA protons involved in hydrogen bonding, including amino, imino, and sugar hydroxyl protons, rapidly exchange for deuterium from solvent.[10] If hydrogen and deuteron bonds are broken or formed during a kinetically slow conformational change, a resultant KSIE may be expected.

A common technique to further characterize KSIEs is to perform proton inventory experiments in H_2O/D_2O mixtures to determine the number of protons transferred in the rate-limiting step of the reaction.[1] Proton inventory experiments for cis-cleavage of the genomic HDV ribozyme have recently been reported to be consistent with a two-proton transfer mechanism,[3] while such experiments on the antigenomic HDV ribozyme suggest a one-proton transfer mechanism.[4] We have performed proton inventory experiments at pL 8.0 for substrate dissociation of our trans-acting HDV ribozyme (Figure 3). Dissociation rate constants were measured as described above in the presence of an increasing mole fraction of D_2O and expressed relative to the rate constant in H_2O. A plot of $k_{off}^{H_2O/D_2O}/k_{off}^{H_2O}$ over n_{D_2O} shows a concave or "bowl-shaped" curve (Figure 3).

Our proton inventory of substrate dissociation does not fit well to the standard proton-transfer models, but rather to more general models resulting in eq 1,[1] yielding a fractional proton transfer of $p = 0.46$ and a fractionation factor of $\phi = 0.575$, and eq 2,[1] yielding $Z = 0.23$ and $\phi = 0.42$ (Figure 3). Such models are consistent with the notion that there is an isotope-induced shift in the transition-state ensemble of substrate dissociation,[1] perhaps due to changes in the sequence of base pair melting in helix P1 (Figure 1A) when increasing n_{D_2O}. While a nonintegral p has no physical meaning, Z is a solvation term reflecting small fractionation factors at many protonic sites.[11] We propose that such an unusual proton inventory may generally indicate a purely conformational change involving breaking of multiple base pairs.

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

$$\frac{k_n}{k_0} = \frac{Z^n}{(1 - n + \phi n)} \tag{2}$$

In summary, our results demonstrate that KSIEs are observed in reactions involving pH-independent conformational changes of RNA. This suggests that KSIEs, observed throughout a broad pL range of an RNA-catalyzed reaction such as cleavage by the HDV ribozyme, are not necessarily proof that a measured pK_a reflects a real ionization event rather than a change in a rate-limiting step. Therefore, KSIEs have to be used with caution to characterize rate-limiting steps in RNA reactions and should be augmented by proton inventories.

Supporting Information Available: Examples of cleavage reactions, measurement of k_{cleav}, steady-state FRET, and substrate-binding and dissociation data (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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