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The Internal Equilibrium of the Hammerhead Ribozyme Reaction†

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ABSTRACT: The effects of temperature, pH, and magnesium ion concentration on the internal equilibrium of the hammerhead ribozyme reaction were determined in order to better understand why the ribozyme-bound substrate RNA is 99% cleaved at equilibrium. Cleavage of substrate is more efficient at higher temperatures because a large entropy gain upon cleavage outweighs an enthalpically unfavorable generation of a 2′,3′-cyclic phosphate product. The ΔH of the reaction is as expected from bond energies, and provides no indication of high-energy ribozyme/substrate interactions that are lost upon cleavage. The rate constants of both cleavage and ligation increase log-linearly with pH between 5.6 and 8.0, indicating that a deprotonation step is required for both cleavage and ligation. The magnesium ion dependence of the internal equilibrium suggests that either the number or the affinity of bound magnesium ions changes upon cleavage. Since the very slow rate of hydrolysis of the 2′,3′-cyclic terminus of product P1 was unaffected by the presence of the ribozyme, we conclude that hydrolysis is not a significant side reaction of the hammerhead cleavage reaction.

In a recent kinetic and thermodynamic analysis of the hammerhead RNA cleavage reaction, we have determined all elemental rate constants for a ribozyme (HH16, Figure 1) that recognizes its substrate by the formation of eight potential base pairs in helices I and III (Hertel et al., 1994). One interesting feature of this analysis was that the internal equilibrium constant, defined as the ratio of the chemical steps of the forward and reverse reactions ($K_{eq}^{int} = k_2/k_{-2}$), greatly favors product formation. The values of 1 min^{-1} for k_2 and 0.008 min^{-1} for k_{-2} give a K_{eq}^{int} of 130. When this value is compared with that for the overall solution reaction (Mohr & Thach, 1969), an effective concentration (EC) of 10^{-2} M for the two products bound to the ribozyme can be calulated (Hertel et al., 1994). This value is quite small compared to ECs measured to be 10^4-10^5 M for protein enzymes catalyzing phosphoryl transfer reactions (Burbaum & Knowles, 1989). Since the EC for the hairpin ribozyme which also generate 2′,3′-cyclic products was estimated to be approximately 100-fold higher (Feldstein & Bruening, 1993), it is of interest to determine why the hammerhead is different and to analyze the driving forces responsible for the preference of product formation on the hammerhead ribozyme.

In this paper, the effects of temperature, pH, magnesium concentration, identity of the supporting metal ion, and solvents on the magnitude of K_{eq}^{int} are determined. In contrast to protein enzymes which require very sophisticated experimental approaches to measure the internal equilibrium (Burbaum & Knowles, 1989), the slow rates of hammerhead cleavage and the tight binding of substrates and products make these measurements relatively straightforward. Since the internal equilibrium is directly related to the mechanism of cleavage, these experiments provide insight into how the hammerhead functions.

MATERIALS AND METHODS

RNA Synthesis. The oligonucleotides E,[1] P1, and P2 were synthesized as previously described (Hertel et al., 1994). All oligonucleotides were purified by gel electrophoresis, ethanol-precipitated, and stored at −20 °C in 50 mM Tris-HCl (pH 7.5). The concentrations of nonradiolabeled solutions were determined by UV absorption, assuming a residue extinction coefficient at 260 nm of 8.5×10^3 M^{-1} cm^{-1}. 5′-^{32}P-labeling of P1 was accomplished using [γ-^{32}P]ATP and the Pset1 mutant of polynucleotide kinase in order to maintain the terminal 2′,3′-cyclic phosphate (Soltis & Uhlenbeck, 1982). The concentrations of radiolabeled solutions were determined from specific activities.

The synthesis of P1 uniquely radiolabeled at the terminal 2′,3′-cyclic phosphate (P1>p*) was accomplished as follows. First, the oligonucleotide substrate containing a radiolabeled phosphate at the hammerhead cleavage site was generated by template-directed ligation of P1 and P2 according to Moore and Sharp (1992). A 50 μL solution of 2 μM P1 with a 3′-hydroxyl terminus, trace amounts of 5′-^{32}P-labeled P2, and 1 μM DNA template that had been used for transcription of S was incubated at room temperature for 14 h with 1 unit/μL of T4 DNA ligase in the presence of 1 mM ATP. The radiolabeled S was then purified by denaturing gel electrophoresis. In order to generate P1>p*, approximately 1 nM S* was incubated with 500 nM E at 25 °C in 50 mM Tris-HCl (pH 7.5) and 10 mM $MgCl_2$. After 20 min, when approximately 80% of the substrate had been converted to products, the solution was heated to 95 °C for 2 min to disrupt any ribozyme substrate or product complexes. Reequilibration at 25 °C allowed an additional round of substrate cleavage, resulting in less than 4% of uncleaved substrate. After an additional heat treatment at 95 °C and reequilibration to 25 °C, the reaction mixture contains

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[1] Abbreviations: E, ribozyme; S, substrate, GGGAACGUCGUCGUCGC; P1, 5′ product containing a 2′,3′-cyclic phosphate, GGGAACGUC>p; P1*, 5′-^{32}P-labeled P1; P1>p*, P1 uniquely labeled at the 2′,3′-cyclic phosphate; P2, 3′ product containing a 5′-hydroxyl terminus, GUCGUCGC; $K_{eq}^{int} = k_2/k_{-2}$ = [E·P1·P2]/[E·S].

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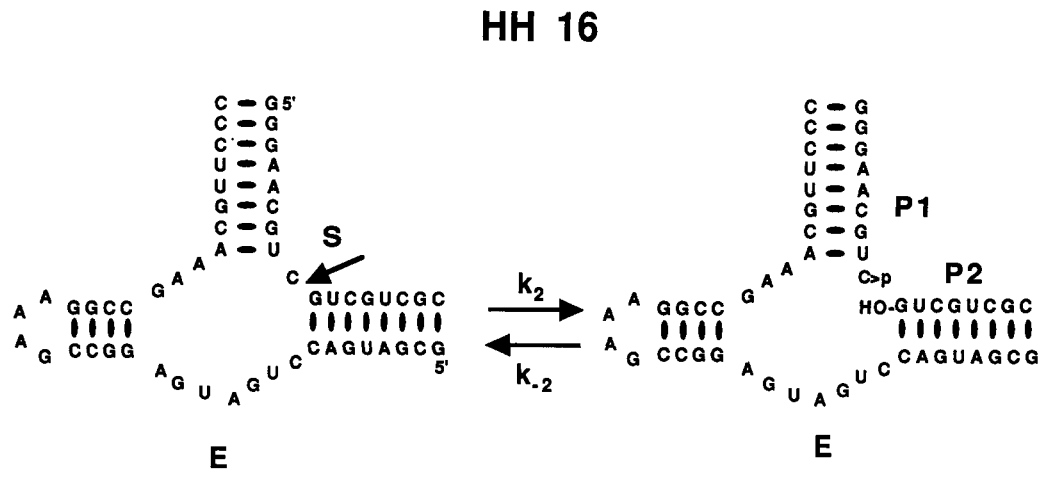

FIGURE 1: Secondary structure and reaction of hammerhead 16 (HH16). The arrow indicates the cleavage site. Cleavage of the 17 nucleotide substrate generates the 5′-product P1 and the 3′-product P2. The chemical step of cleavage is defined by the elemental cleavage rate constant k_2 and the chemical step of ligation by the rate constant k_{-2}. The internal equilibrium constand K_{eq}^{int} is defined as k_2/k_{-2}.

approximately 1 nM E·P1>p* and 1 nM E·P2, each fully bound to ribozyme since the ribozyme concentration (500 nM) is well above the K_d of the products (0.05−1 nM). However, since the ribozyme is well in excess of products, a given ribozyme will only have one bound product. This mixture was used directly in hydrolysis experiments.

Kinetics. All experiments were designed to ensure that the reaction rates reflected the chemical cleavage and ligation steps under each reaction condition. Since ribozyme was always in great excess of substrate, product release could never be rate-limiting in either direction. Since the binding of products and substrate to ribozyme closely follows the thermodynamics of RNA helix formation (Hertel et al., 1994), one can use the available data on RNA helix stability at different temperatures (Freier et al., 1986) to ensure that under all conditions used in this work, products and substrates were fully bound to ribozyme. In a few cases, this was tested experimentally by raising the concentration of substrate or product and showing no change in rate.

Ligation reactions were conducted at standard reaction conditions [50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, and 25 °C] unless stated otherwise. An 18 μL solution of 1.1 nM P1*, 550 nM E, and 1100 nM P2 in 55 mM Tris-HCl (pH 7.5) was heat treated at 95 °C for 1.5 min in order to disrupt potential RNA aggregates formed during storage. Under these conditions, all the P1* is bound in an E·P1·P2 complex. This solution was then allowed to equilibrate for 10 min at reaction temperature. The reaction was initiated by the addition of 2 μL of 100 mM $MgCl_2$. At times ranging from 15 s to 30 min, samples were removed and quenched in a 5× volume of denaturing quench buffer containing 8 M urea, 50 mM EDTA, pH 8, 0.02% bromophenol blue, and 0.02% xylene cyanol. Observed rate constants for the approach to the internal equilibrium were obtained from the slopes of semilogarithmic plots of the fraction P1* ligated, normalized to the final extent of the reaction, versus time. To determine the extent of the internal equilibrium, the ligation reaction was incubated for 4 h. To measure the dependence of pH on the rate of approach to the internal equilibrium and the magnitude of K_{eq}^{int}, standard ligation reactions were carried out except the pH was varied between 5.6 and 9.0. The sulfonate buffers used to maintain the desired pH were Mes (pH 5.6−6.5), Pipes (pH 6.2−7.0), Mops (pH 7.2−7.9), and Taps (pH 8.2−9.0) according to Dahm et al. (1993). The pK_a value for the Tris buffer used changes slightly with temperature. Therefore, the data for the temperature dependence of the reaction were corrected prior to analysis to the appropriate values using the pH profile reported herein. Each rate or equilibrium measurement was performed at least twice and was accurate to within 25%.

Hydrolysis of the 2′,3′-Cyclic Terminus of P1. The reaction mixture used to prepare P1>p* contained sufficiently high concentrations of ribozyme (500 nM) for the trace concentrations of products (1 nM) to be fully bound to the ribozyme. Because of the high ribozyme to product ratio and the fact that the solution had been heated and cooled, a given ribozyme molecule with a bound P1>p* will not also contain a P2. An aliquot of this reaction mixture was diluted directly into a buffer of appropriate pH to give a sample of trace P1>p* bound to E. To form E·P1·P2 complex, an aliquot of the reaction mixture was mixed with an excess of P2 (750 nM) to ensure that all the E·P1>p* complex also contained bound P2. An aliquot of the reaction mixture was also mixed with a large excess of P1 (10 μM) which was sufficient to compete with the radiolabeled P1>p* so that, after another heating and cooling step, all the P1>p* was free in solution. Three 20 μL reactions (E·P1, E·P1·P2, and free P1) were incubated at three different pH values (pH 6.5, 7.5, and 8.5), and aliquots were removed at intervals up to 48 h. The extent of hydrolysis was monitored by gel electrophoresis using 15% nondenaturing gel electrophoresis where P1>p* migrated faster than P1-3′p*. Alternatively, the oligonucleotide product was treated with 0.1 u/μL calf intestine phosphatase for 20 min at 37 °C to produce inorganic phosphate if P1>p* was hydrolyzed to P1-3′p*. Thus, by analysis of the digested products by poly(ethylenimine) thin-layer chromotography developed with 0.5 M ammonium acetate, the relative amount of ^{32}P and P1>p could be quantitated. Reaction rates were determined from semilogarithmic plots of the disappearance of the 2′,3′-cyclic phosphate versus time.

RESULTS

The Temperature Dependence of K_{eq}^{int}. The ligation efficiency was monitored as a function of temperature ranging from 0 to 37 °C. Ligation is more efficient at lower temperatures although product formation is favored at all measured temperatures. The van't Hoff plot (Figure 2A)

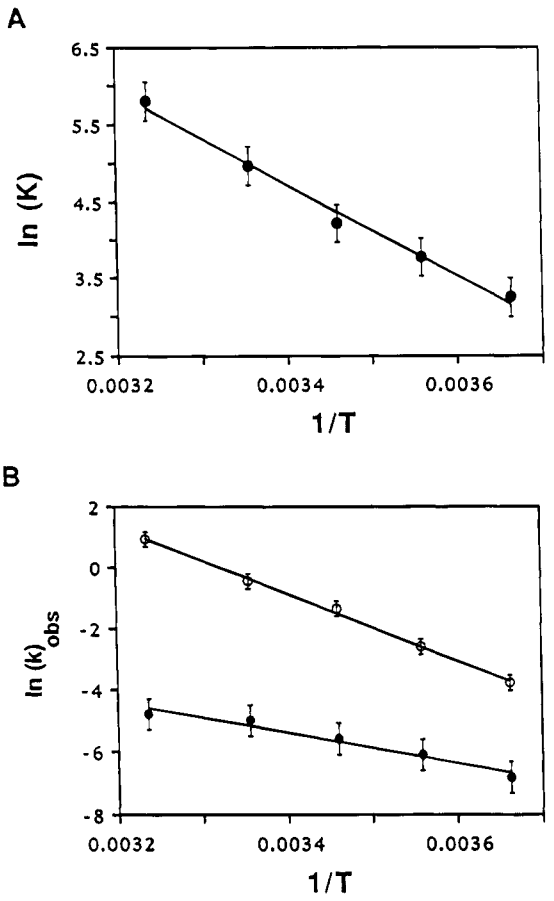

FIGURE 2: Temperature dependence of the internal equilibrium constant. (A) van't Hoff plot of the fraction of products ligated at equilibrium as a function of temperature, giving $\Delta H = 10 \pm 2$ kcal/mol and $\Delta S = 44 \pm 7$ eu. (B) Arrhenius plot of the temperature dependence of the rate constants k_2 (○) and k_{-2} (●), giving values for the activation energy of $E_a = 22 \pm 1$ for cleavage (○) and $E_a = 10 \pm 2.4$ for ligation (●). The error bars reflect 25% precision for equilibrium and forward rates and a 35% precision for the reverse rection.

gives values for $\Delta H = 10 \pm 2$ kcal/mol and $\Delta S = 44 \pm 7$ eu for the reaction of E·S to E·P1·P2. The data indicate that the cleavage reaction catalyzed by the hammerhead ribozyme is driven toward product formation by a large gain in entropy while the enthalpy of the reaction is unfavorable. The data in Figure 2A were fit to a line, although there appears to be some curvature. Most likely, the observed deviation arises from inaccurate data corrections associated with the temperature-dependent pK_a changes of the supporting Tris buffer. However, this does not rule out the possibility that hammerhead-specific factors, such as a temperature-dependent enthalpy of reaction, for example, are partly responsible for the minor curvature in Figure 2.

In addition to the extent of ligation, the rate constants for reaching K_{eq}^{int} were measured at various temperatures. Since k_2 is always much faster than k_{-2}, $k_{app} = k_2 + k_{-2}$ approximates k_2. With the values of k_2 and K_{eq}^{int}, the values of k_{-2} at all temperatures could be calculated. k_2 changed approximately 100-fold over the 36 °C range while k_{-2} changed only 10-fold. As shown in Figure 2B, the Arrhenius energies are $E_a = 22 \pm 1$ kcal/mol for the cleavage reaction and $E_a = 10 \pm 2.4$ kcal/mol for the ligation reaction. These permit calculation of the thermodynamic activation values, describing the difference in energy levels of reactants and the transition state at 25 °C (Table 1).

Table 1: Thermodynamic Parameters of Hammerhead Cleavage at 25 °C

	cleavage reaction, k_2	ligation reaction, k_{-2}
E_a (kcal/mol)	22 ± 1	10 ± 2.4
$\Delta G^{\ddagger}$ [a] (kcal/mol)	20 ± 1	23 ± 2
$\Delta H^{\ddagger}$ [b] (kcal/mol)	21 ± 1	9.5 ± 2.4
$\Delta S^{\ddagger}$ [c] (eu)	3 ± 0.2	−43 ● 10

[a] $\Delta G^{\ddagger}$ was calculated from the relationship: $\Delta G^{\ddagger} = -RT \ln(k_{obs}h/k_BT)$, where H is Planck's constant, k_B is Boltzmann's constant, and k_{obs} is the rate constant of cleavage at $T = 298$ K. [b] $\Delta H^{\ddagger}$ was calculated from the relationship: $\Delta H^{\ddagger} = E_a - RT$. [c] $\Delta G^{\ddagger}$ was calculated from the relationship: $\Delta G^{\ddagger} = \Delta H^{\ddagger} + T\Delta S^{\ddagger}$. Errors were estimated assuming a precision of 25% for k_2 measurements and 25% for internal equilibrium experiments.

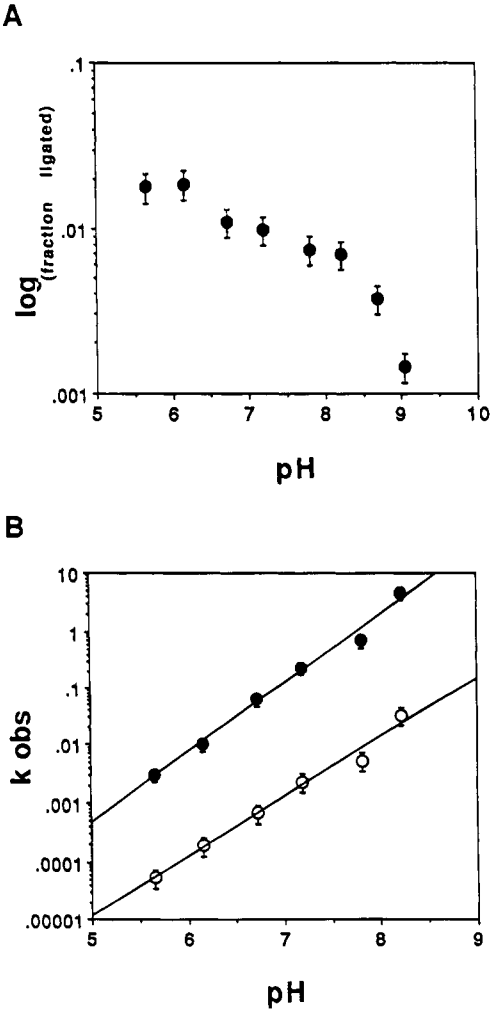

FIGURE 3: (A) pH dependence of the internal equilibrium constant. (B) pH dependence of k_2 (●) and k_{-2} (○). The slopes for the data in the logarithmic plot are 1.2 for k_2 (●) and 1.0 for k_{-2} (○). The error bars reflect 25% precision.

The pH Dependence of K_{eq}^{int}. The effect of hydroxide ion concentration on the internal equilibrium was determined over 3.4 pH units (pH 5.6–9.0). As shown in Figure 3A, the formation of product was greatly favored over ligation at all pH values. The extent of ligation at pH 5.6 was observed to be approximately 5-fold higher than observed at pH 8.7. This very modest change in K_{eq}^{int} over a 1000-fold change in hydroxide concentration suggests that the rate constants for the forward and the reverse reactions are similarly affected by the hydroxide concentration. This was confirmed by measuring $k_{obs} = k_2$ between pH 5.6 and 8.2

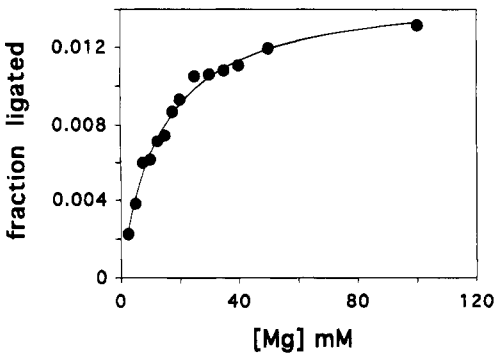

FIGURE 4: Internal equilibrium constant as a function of magnesium concentration. The line represents single ligand binding with K_{Mg} = 13 mM and the fraction of 0.015 as the maximal extent of ligation.

Table 2: Ionic Requirement for the Internal Equilibrium

	fraction ligated x 10^3 [a]	
metal	10 mM M^{2+}	2 mM M^{2+}, 0.5 mM spermine
Mg	13	16
Cd	6	16
Mn	21	24
Ca	<0.2	<0.2
Co	10	23
Zn[b]		1

[a] Detection limit 0.2 × 10^3; the measurements are precise within 25%. [b] 0.5 mM Zn^{2+} was used. All reactions were carried out at pH 6.5.

and using K_{eq}^{int} to calculate k_{-2}. The logarithm of the rate of both the cleavage and the ligation reactions increased linearly with pH (Figure 3B). Such a log-linear relationship had previously been shown for the cleavage step of another hammerhead (Dahm et al., 1993). The similar behavior observed here for the reverse reaction supports the view that the internal equilibrium reflects the chemical step of the reaction.

Ionic and Solvent Requirements of K_{eq}^{int}. The internal equilibrium was measured as a function of magnesium ion concentration ranging from 0 to 100 mM (Figure 4). The extent of ligation increased with magnesium concentration, and the data fit well to a simple binding equilibrium with an apparent binding dissociation constant for Mg^{2+} of 13 mM with the maximal fraction ligated of 0.015. A number of different divalent ions were tested at selected conditions to determine whether the internal equilibrium was significantly affected. To avoid metal precipitation, most experiments were conducted at pH 6.5 (50 mM Pipes) either in the presence of 0.5 mM spermine at 2 mM M^{2+} or in the absence of spermine at 10 mM M^{2+}. Equilibration was for 4 h, which is sufficent to reach an end point based on previously determined reaction rates (Dahm & Uhlenbeck, 1991; Dahm et al., 1993). Generally, the metal ion dependent formation of K_{eq}^{int} summarized in Table 2 correlates well with their ability to promote cleavage (Dahm & Uhlenbeck, 1991). Among the metal ions which support cleavage, Mg^{2+}, Mn^{2+}, Cd^{2+}, and Co^{2+}, the value of the internal equilibrium varied only up to 4-fold. The greater than 10-fold reduction of the fraction ligated observed for Zn^{2+} in the presence of spermine and the inability to detect a significant fraction ligated for Ca^{2+} could indicate a dramatic shift of the internal equilibrium toward product formation. When reactions containing 10 mM Mg^{2+} were supplemented with 0.2, 2, or 20 mM spermine, spermidine, or neomycin B, no significant change in K_{eq}^{int} was observed. Similarly, 7.5%, 15%, or 30% methanol or ethanol had no effect on the equilibrium.

Table 3: Rate Constants of Hydrolysis of P1>p Free in Solution, in the E·P1 Complex, and in the E·P1·P2 Complex

	rate constant × 10^{-5} [a]		
	pH 6.5	pH 7.5	pH 8.5
P1	0.2	3	7
E·P1	0.3	7	20
E·P1·P2	0.4	7	30

[a] The rate constants are precise within 2-fold.

The Rate Constant of Hydrolysis. The rate constant of hydrolysis of the uniquely radiolabeled 2′,3′-cyclic terminus of P1 was determined free in solution, as the E·P1 complex, and as the E·P1·P2 complex in 10 mM Mg^{2+} at pH 6.5, 7.5, and 8.5. As shown in Table 3, the reaction rates are very slow and base-catalyzed. At pH 7.5, the 2′,3′-cyclic phosphate at the end of P1 is converted to a terminal phosphate at 3 × 10^{-5} min^{-1}. The rate increases approximately 2-fold to 7.5 × 10^{-5} min^{-1} when P1 is bound to E, but does not increase further when P2 is present. The data indicate that the ribozyme is not able to significantly catalyze the attack of water (or hydroxide ion) on the 2′,3′-cyclic phosphate. For comparison, the rate of ligation is 8 × 10^{-3} min^{-1} or 270-fold faster.

DISCUSSION

Energetics of the Hammerhead Cleavage Reaction. The temperature dependence of the internal equilibrium constant and reaction rate constants provides a view of the energetics of the reversible cleavage step of the hammerhead ribozyme. The favorable free energy of the cleavage reaction [$\Delta G_{(298)}$ = −2.9 kcal/mol] is achieved by a very favorable entropic contribution (ΔS = 44 eu) overcoming the unfavorable enthalpy change (ΔH = 10 kcal/mol). It appears that the enthalpy change can be explained largely by the fact that the intrinsic bond energy of the 2′,3′-cyclic phosphate is greater than that of a 3′,5′-phosphodiester linkage because of strain within the pentacyclic phosphate ring fused to the ribose ring (Kumamoto et al., 1956; Gerlt et al., 1975). At 25 °C and pH 7.3, a value of ΔH = −8.1 kcal/mol was measured for the hydrolysis of cytidine 2′,3′-cyclic phosphate (Rudolph et al., 1971). The value for the enthalpy change of the reverse reaction is remarkably close to what is observed upon hammerhead cleavage.

If the ΔH of hammerhead cleavage primarily reflects the formation of cyclic phosphate, any additional factors that contribute to the internal equilibrium must have little or no net enthalpy change. This, in turn, places restrictions on proposed mechanisms for hammerhead cleavage. If, for example, one proposes that the bond energy of the 3′,5′-phosphodiester bond at the hammerhead cleavage site is greatly strained in the ground state (Mei et al., 1989), the associated enthalpy change upon cleavage must be offset by some other process. Similarly, any proposed change in the number of base pairs or tertiary interactions that occurs upon cleavage must in some way account for the absence of an associated enthalpy change, since such structural changes are generally assoicated with large values of ΔH (Freier et al., 1986).

The internal equilibrium favoring cleavage results from a large entropy gain which offsets the unfavorable change in enthalpy. There are many factors that could lead to the entropy change in the unimolecular reaction. The observation that the effective concentration of products bound to the ribozyme is much lower than is observed with other enzymes has led to the suggestion that the E·P1·P2 complex is relatively "floppy" (Hertel et al., 1994). It is possible that cleavage simply increases the degrees of freedom available to the hammerhead, making the E·P1·P2 complex thermodynamically more stable than the E·S complex. There is currently very little physical evidence that bears on this issue. The NMR spectra of cleaved and uncleaved hammerheads are quite similar (Odai et al., 1990; Pease & Wemmer, 1990; Heus & Pardi, 1991) and, if anything, show more structural detail in the cleaved state. In addition, cleavage of the hammerhead does not seem to greatly alter the relative configurations of helix I and helix II (Gast et al., 1994).

Another factor that may be responsible for the large entropy gain on hammerhead cleavage is a change in the associated water structure. Large solvation entropy changes can accompany relatively small changes in nucleic acid structure (Zieba et al., 1991). Similarly, a change in the metal ion binding properties upon cleavage could lead to a large entropy change. For example, the binding of Mg to 3′-AMP is accompanied by a ΔS of 21 eu, presumably due to the release of a water molecule (Izatt et al., 1971). Since 21 eu is about half of the change observed upon hammerhead cleavage, an alteration in the binding properties of a few of the many divalent ions bound to the hammerhead could account for the ΔS.

From the temperature dependence of the forward and reverse reaction rates, the enthalpy and entropy changes required to reach the transition state from the E·S and E·P1·P2 complexes were calculated. The activation enthalpy ($\Delta H^{\ddagger}$) for the reverse reaction (10 kcal/mol) is considerably more unfavorable than for the forward reaction (22 kcal/mol). This might reflect the fact that the strained 2′,3′-cyclic phosphate is energetically closer to the transition state than the 3′,5′-phosphodiester bond. Since the attack of the 2′-hydroxyl on the adjacent phosphodiester bond is a unimolecular reaction, the entropy change of activation ($\Delta S^{\ddagger}$) for cleavage of S is, as expected, near zero (Piszkiewicz, 1977). On the other hand, $\Delta S^{\ddagger}$ of the reverse reaction is quite negative, indicating that considerable ordering is required for the E·P1·P2 complex to react. Presumably the large value observed in the ligation reaction is because the ribozyme must place reactive groups properly in three-dimensional space to achieve an appropriate spatial proximity before catalysis by transition state stabilization can occur.

It would be of interest to compare the kinetic and thermodynamic data obtained here with those obtained with other ribozymes catalyzing the same reaction. However, such data are very limited. While there are several reports that cleavage rates for the hairpin and delta ribozymes are temperature-dependent (Hampel & Tritz, 1989; Wu et al., 1989), it is unclear whether the measured values reflect the chemical step of the reaction. However, a small intramolecular derivative of the hairpin ribozyme has been shown to undergo reversible circularization, suggesting that the extent of circularization is a direct measure of the internal equilibrium (Feldstein & Bruening, 1993). From the temperature dependence of that reaction, $\Delta H = 10$ kcal/mol and $\Delta S = 20$ eu can be estimated. The fact that the hammerhead and hairpin ribozymes show very similar values for ΔH supports the suggestion that the value simply reflects the higher bond energy of the 2′,3′-cyclic phosphate. The ΔS for the hairpin cleavage reaction is also favorable, but about half the value of the hammerhead, resulting in an overall internal equilibrium much closer to 1. Perhaps the larger size of the hairpin and its increased potential for tertiary folding result in less flexibility in the E·P1·P2 complex than is observed for the hammerhead. Alternatively, it is possible that the different ΔS for the hairpin cleavage reaction reflects the difference in its divalent ion requirements for cleavage (Chowrira et al., 1993).

pH Dependence of the Internal Equilibrium. Both the cleavage and ligation reactions are base-catalyzed. Cleavage requires abstraction of the 2′-proton to permit in-line attack of the 2′-oxygen on the phosphate. Similarly, the reverse reaction requires abstraction of the 5′-proton of P2 to permit attack of the 5′-oxygen on the cyclic phosphate. Since proton transfer rate constants between electronegative oxygens are extremely fast (Fersht, 1985), the rate-limiting step for hammerhead cleavage most likely reflects the formation or decomposition of the pentacoordinate transition state (or short-lived intermediate). The data further support the interpretation that the rate constants of cleavage and ligation reflect the chemical interconversion of substrate and products.

The internal equilibrium is only very slightly pH-dependent. Over 3 pH units, k_2 increases 5-fold more than k_{-2}. It is possible that this reflects a small pH-dependent change in structure upon cleavage. Alternatively, since magnesium ion was not saturating in the experiment, a pH-dependent change of affinity of magnesium ion to the sulfonic acid buffers (Good et al., 1974; Perrin & Dempsey, 1974) could be responsible for the effect. In any case, the pH−rate profiles of both the forward and reverse reactions are very similar.

The Ionic Requirements of the Internal Equilibrium. While the internal equilibrium is always strongly in favor of cleavage, the fraction cleaved decreases significantly as the magnesium concentration is increased. Although the magnesium concentration dependence of the forward and reverse rates was not measured in this study, the forward rate of HH16 and several other hammerheads has been shown to increase substantially with increasing magnesium concentration in the same range (Dahm & Uhlenbeck, 1991; Perreault et al., 1991; B. Clouet d'Orval, unpublished experiments). Thus, it is likely that both the cleavage and ligation reaction rate constants increase with magnesium concentration, but that the ligation rate constant increases more rapidly, resulting in more ligated RNA at high magnesium concentrations.

The magnesium dependence indicates that more ligation occurs at higher magnesium concentrations, although the reaction favors cleavage under all conditions. Surprisingly, the data fit quite well to a simple binding equilibrium with a K_d of 13 mM. Although the ionic strength was not maintained in our experiments, recent experiments at constant ionic strength with a similar hammerhead revealed a similar simple binding equilibrium (D. Long, unpublished experiments). This value of K_d is very similar to the affinity of magnesium for the 2′,(3′)- or 5′-phosphate of AMP (Izatt et al., 1971). A simple interpretation is therefore that the E·P1·P2 complex differs from the E·S complex in either the number or the affinity of bound magnesium ions. One

expects that, like most RNAs, both E·S and E·P1·P2 will bind a large number of magnesium ions at differing affinity. A fraction of these will be critical for the forward and reverse reactions and defining the internal equilibrium. While no cooperative binding of magnesium ions is observed, it is not yet possible to deduce how many magnesium ions are coupled to the internal equilibrium. As mentioned above, the large positive ΔS for the internal equilibrium may also partially reflect changes in metal ion binding on cleavage. A more detailed analysis of this point is in progress.

A survey of the effect a number of additional metal ions and solvent conditions had on the internal equilibrium did not reveal any conditions that favored ligation very much more than Mg^{2+}. However, Zn^{2+} and Ca^{2+} appear to favor substrate cleavage by 10−100-fold more than Mg^{2+}. This suggests that these metal ions coordinate to E·S and/or E·P1·P2 differently than Mg^{2+}. Solvent additions such as the antibiotic neomycin B, which has been shown to inhibit the hammerhead cleavage reaction (T. Stage, unpublished experiments), polyamines, which are known to stabilize RNA structure, or organic solvents, which generally destabilize RNA structure, had little effect on the internal equilibrium. This suggests that the structures of the E·P1·P2 and the E·S complex are similarly affected by the tested solvents since it was not possible to denature one selectively.

2′,3′-Cyclic Phosphate Hydrolysis. The attack of water or hydroxide ion on the 2′,3′-cyclic phosphate to give a 2′- or 3′-terminal phosphate resembles the reverse reaction, but due to the high concentration of water is effectively irreversible. Since this reaction is strongly catalyzed by several protein ribonucleases (Richards & Wycoff, 1971), it was of interest to see whether the hammerhead can also stimulate the reaction rate. Careful measurements of the very slow rate of conversion of P1>p to P1-2′(3′)-phosphate in the absence of ribozyme provided a measurement of the background, uncatalyzed rate constant of reaction of between 10^{-4} and 10^{-5} min^{-1}. This rate is only slightly higher than the background rate of phosphodiester bond cleavage of 10^{-6} min^{-1} (Hertel et al., unpublished experiments) and was virtually the same when the ribozyme was present, with or without the presence of the competing 5′-hydroxyl of P2. It is, of course, possible that the E·P1 complex does not contain enough of an intact active site to catalyze activation and the E·P1·P2 complex can effectively exclude water. However, these experiments indicated that, unlike protein ribonucleases, the hammerhead does not have a significant hydrolysis side reaction.

It is interesting to contrast the behavior of the hammerhead with a small Pb^{2+}-dependent cleavage motif that was recently isolated by *in vitro* selection strategy (Pan & Uhlenbeck, 1992; Pan et al., 1994) . Like the hammerhead, the Pb^{2+} motif cleaves RNA at a unique site to generate a 2′,3′-cyclic phosphate. However, unlike the hammerhead, the Pb^{2+} motif also carries out stereoselective hydrolysis of the 2′,3′-cyclic phosphate to a 3′-phosphate at a rate about 20-fold faster than the background rate. This hydrolysis reaction is favored over the reverse reaction. It is striking that the hammerhead ribozyme and other natural ribozymes generate 2′,3′-cyclic phosphates without the completing hydrolysis reaction. It is possible the self-cleaving RNA domains have evolved to preserve the high-energy 2′,3′-cyclic bond for further processing. Indeed, it has been suggested that the hammerhead, hairpin, or HDV motifs may also operate in the reverse direction as part of the replication cycle (Buzayan et al., 1986; Forster & Symons, 1987; Wu & Lai, 1989).

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