Kinetic and Thermodynamic Characterization of the Reaction Catalyzed by a Polynucleotide Kinase Ribozyme†

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ABSTRACT: We have previously isolated a series of ribozymes with polynucleotide kinase activity [Lorsch, J. R., & Szostak, J. W. (1994) *Nature 371,* 31−36]. In order to learn how such newly evolved RNAs effect catalysis, we have determined a number of the kinetic and thermodynamic parameters for the reaction catalyzed by one of these ribozymes. This ribozyme, a class I polynucleotide kinase, catalyzes the transfer of the γ-(thio)phosphate from ATP(-γS) to the 5′-hydroxyl of a 7-mer oligoribonucleotide. The k_{cat} for the reaction with ATP-γS is 0.17 min^{-1} with a K_m of ~3 mM. The K_m for the oligoribonucleotide substrate 5′-HO-GGAACCU-3′ is 2 μM, the same as the K_d for this substrate in the presence or absence of ATP-γS. Neither the binding of substrates nor the release of products is the rate-limiting step of the reaction. The binding of substrates and release of products appear to occur in a random fashion, with no synergy of binding between the ATP(−γS) and oligoribonucleotide substrates. The ribozyme binds the oligoribonucleotide substrate no more strongly than would be expected for the formation of a simple RNA−RNA duplex, suggesting that there are no tertiary contacts between the ribozyme and the RNA substrate. The oligoribonucleotide substrate binding site has been located, and the sequence specificity of the ribozyme could be altered by mutating this binding site. The ribozyme is specific for adenosine triphosphate substrates; GTP-γS reacts approximately 650-fold slower than ATP-γS. With ATP as the substrate, the K_ms remain unchanged, but k_{cat} decreases by a factor of 50, consistent with a rate-limiting chemical step occurring through a dissociative transition state. The pH independence (from pH 5.5 to 8.5) of k_{cat}/K_m and of the rate constant for the conversion of the ternary substrate complex into the ternary products complex is also consistent with a dissociative phosphoryl transfer mechanism. These results suggest that this newly evolved catalyst operates in a relatively simple manner, with independent substrate binding sites and without changing the mechanism of the underlying chemical reaction.

In previous work (Lorsch & Szostak, 1994), we isolated a large number of ribozymes with polynucleotide kinase activity from a pool of RNAs consisting of an ATP[1] binding domain (Sassanfar & Szostak, 1993) surrounded by regions of random sequence. One of these ribozymes (class I; Figure 1) was engineered (by deleting 40 bases at the 5′-end of the RNA) into a true enzyme, capable of transferring the γ-thiophosphate from ATP-γS to the 5′-hydroxyl of an exogenous oligoribonucleotide substrate with multiple turnovers, a reaction analogous to that catalyzed by T4 polynucleotide kinase. The full-length cis-acting ribozyme was shown to possess a saturable ATP-γS substrate binding site and to catalyze the transfer of the γ-thiophosphate from ATP-γS to the ribozyme's own 5′-hydroxyl. ATP was also found to be a substrate for the reaction, with a K_m identical to that for ATP-γS but a k_{cat} that is approximately 100-fold lower than for ATP-γS.

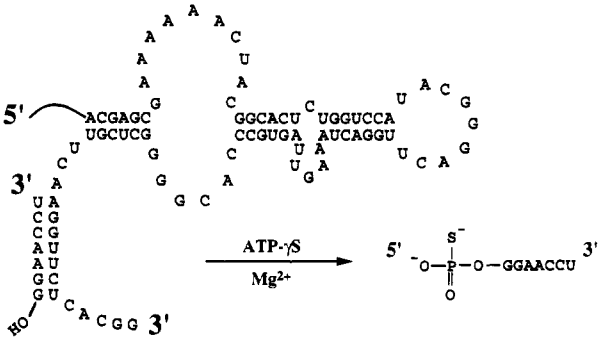

FIGURE 1: Secondary structure model for the trans-acting class I ribozyme, Kin.46 (Lorsch & Szostak, 1994). The ribozyme catalyzes the transfer of the γ-(thio)phosphate from ATP(-γS) to the 5′-end of the oligoribonucleotide 5′-HO-GGAACCU-3′. The black line on the 5′-end of the structure represents a 20-base sequence that is not required for catalysis. The ribozyme also has 18 bases 3′ of the sequence shown corresponding to the 3′-primer binding site of the original pool.

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[1] Abbreviations: ATP, adenosine triphosphate; ATP-γS, adenosine 5′-*O*-(3-thiotriphosphate); GTP-γS, guanosine 5′-*O*-(3-thiotriphosphate); E, polynucleotide kinase ribozyme; rS, 7-mer oligoribonucleotide substrate, 5′-GGAACCU-3′; rS*, ^{32}P body-labeled 7-mer substrate; rP, 5′-(thio)phosphorylated product oligoribonucleotide; K_d, equilibrium dissociation constant; K_m, Michaelis constant; K_m^{rS}, Michaelis constant for the oligoribonucleotide substrate; K_m^{A}, Michaelis constant for the adenosine (thio)triphosphate substrate; st, single-turnover parameter; PAGE, polyacrylamide gel electrophoresis; IGS, internal guide sequence, the oligoribonucleotide substrate binding site; IGS′, the 7-mer oligoribonucleotide with the same sequence as the IGS, 5′-AGGUUCU-3′; AMPSO, 3-[(1,1-dimethyl-2-hydroxyethyl)amino]-2-hydroxypropanesulfonic acid; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid); PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid).

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As the number of known ribozymes is small, a detailed study of the chemical mechanisms used by newly developed, non-natural ribozymes might be of value in attempts to understand the general mechanisms used by RNA in effecting catalysis. Although these polynucleotide kinase ribozymes have not been refined by several billion years of evolution, they are capable of remarkable rate enhancement and specificity. What mechanisms do they use to perform such catalysis?

In the last five years several studies have elucidated kinetic and thermodynamic frameworks for naturally occurring ribozymes (Herschlag & Cech, 1990a,b; Fedor & Uhlenbeck, 1992; Hertel et al., 1994; Michels & Pyle, 1995; Pyle & Green, 1994). These studies have greatly advanced our understanding of the chemical basis for the catalytic functions performed by each of the ribozymes. As a starting point for a mechanistic analysis of the class I polynucleotide kinase ribozyme we describe here the groundwork for a similar minimal kinetic and thermodynamic framework. The data suggest that the catalyzed reaction proceeds via a dissociative transition state, as does the uncatalyzed reaction.

MATERIALS AND METHODS

Reagents. Buffers and salts were from Sigma and were of the highest grade (>99%). Ultrapure ATP was purchased from Pharmacia. ATP-γS and GTP-γS were from Boehringer-Mannheim. The pH of ATP stock solutions was adjusted to 7.0 with KOH. In general, stocks of ATP contained 1 equiv of $MgCl_2$, and stocks of ATP-γS and GTP-γS contained 1 equiv of $MgCl_2$ and 0.05−0.1 M DTT (to give 10 mM final DTT in a reaction). Radioisotopes were purchased from DuPont-New England Nuclear. T7 RNA polymerase was either purchased from United States Biochemicals or purified from an overproducing strain. *Taq* DNA polymerase was from Promega.

RNA Synthesis and Purification. Ribozymes were synthesized by T7 RNA polymerase runoff transcription of PCR DNA templates. Products were purified by denaturing polyacrylamide gel electrophoresis (PAGE) and were eluted from the gel in 0.3 M NaCl overnight. The short oligoribonucleotide substrates were made either by automated solid-phase chemical synthesis using standard phosphoramidite chemistry (Expedite, Milligen/Biosearch) or by runoff transcription from synthetic DNA templates (Milligan et al., 1987). Synthetic oligoribonucleotides were deprotected in aqueous NH_4OH overnight at 50 °C, and the supernatant was removed, passed through a 0.2 μm filter to remove CPG particles, and dried under vacuum. The resulting solid was resuspended with sonication in 150−200 μL 1 M tetrabutylammonium fluoride (TBAF) in THF (Aldrich). The solution was stored in the dark for 1−2 days at room temperature, at which point 2 volumes of water were added, and the RNA was purified directly on a preparative anion exchange column (Dionex), using aqueous ammonium acetate as the eluent. Short oligoribonucleotides prepared via runoff transcription were purified by PAGE on 0.8 mm thick, 40 cm long 22.5% denaturing gels and then dephosphorylated using calf intestinal alkaline phosphatase (New England Biolabs). ^{32}P body-labeled 7-mer 5′-GGAACCU-3′ was synthesized by T7 RNA polymerase runoff transcription in the presence of α-^{32}P ATP.

General Kinetics. Assays were done using ^{32}P body-labeled RNA substrate unless otherwise specified. Standard reaction conditions were 50 mM $MgCl_2$, 5 mM $MnCl_2$, 400 mM KCl, 25 mM PIPES, pH 7.0. The ribozyme was annealed with an oligodeoxynucleotide complementary to the last 18 bases of its 3′-end (the 3′-constant region used for primer binding in reverse transcription and PCR in the selection; not shown in Figure 1) in the presence of KCl and the PIPES buffer at 80 °C for 2.5 min, and then cooled to room temperature. The oligodeoxynucleotide was included for consistency with the original selection (Lorsch & Szostak, 1994); the activity of the ribozyme was approximately 10-fold lower in the absence of the 18-mer. Presumably this effect is either due to the fact that when the 3′-end of the RNA is free it interferes with the proper folding of the ribozyme or because there are stabilizing contacts between the DNA−RNA duplex and the core of the ribozyme. Deleting the 3′-constant region also resulted in an enzyme with ~10 times lower activity. The 7-mer RNA substrate was added prior to the annealing step, although it was found that whether rS was added before or after annealing had no effect on the kinetic constants of the reaction (see Results). After the annealed ribozyme had cooled to room temperature, 1/10 volume of 10× $MgCl_2$/$MnCl_2$ (500 mM $MgCl_2$, 50 mM $MnCl_2$) was added, and the ribozyme solution equilibrated at the reaction temperature (30 °C, unless otherwise noted) for 15 min. The typical reaction volume was 20 μL. The reaction was initiated by the addition of ATP(-γS), and aliquots were withdrawn at various times and quenched in 2 volumes of 70 mM EDTA, 10 mM dithiothreitol, 76% formamide, 0.02% xylene cyanol, and 0.05% bromphenol blue. The time points were analyzed by PAGE on 22.5% denaturing gels (0.4 mm thick, 40 cm long). Unphosphorylated, phosphorylated, and thiophosphorylated 7-mer are well separated with this gel system. Initial rates were measured such that <15% of substrate was converted to product. Rate constants measured by following multiple reaction half-lives agree well with those from initial velocity experiments. Gels were quantitated using a phosphorimager (Molecular Dynamics), and the data were analyzed using the KaleidaGraph graphing and curve-fitting package (Abelbeck Software).

In some early experiments (e.g., the determination of the oligoribonucleotide binding site) labeled ATP-γ^{35}S or ATP-γ^{32}P were used instead of body-labeled rS* to follow the reaction. In these cases, a known quantity of the labeled substrate was spotted on the dried gel in order to determine the absolute amount of product in each band. This method is less accurate than observing a shift in mobility from rS* to rP*, as it requires accurate pipetting of aliquots from the reaction into the quench and when loading samples on the gel.

In some experiments (temperature curves, pH profiles) 55 mM $MgCl_2$ was used in place of the 50 mM $MgCl_2$ plus 5 mM $MnCl_2$, but this change was found to have no effect on the activity or on the kinetic parameters of the ribozyme with either the ATP or the ATP-γS substrates. The Mn^{2+} was included in most experiments for consistency with the conditions of the selection. The divalent metal ion concentration was not the optimum concentration but rather was that of the initial selection. The Mg^{2+} optimum for the full-length cis system appears to be considerably higher than 200 mM (the highest concentration tested). Such high concentrations of divalent metal ions can cause RNA aggregation and substrate insolubility problems.

ATP-γS is supplied as a tetralithium salt, and it was found that high concentrations of LiCl (>10 mM) have a slight inhibitory effect on the catalyzed reaction with ATP as a substrate. We estimate that our value of k_{cat} for the reaction with ATP-γS may be as much as 2-fold below the value in the absence of Li^+. This uncertainty does not affect any of the conclusions in this paper, however.

Single-Turnover, Saturating Ribozyme Experiments. In order to observe only events after substrate binding and before product release (i.e., the conversion of the ternary substrate complex to the ternary product complex), we performed experiments in which ribozyme was saturating over ^{32}P-labeled 7-mer oligoribonucleotide substrate. In most experiments, 10 μM ribozyme and 1 μM rS* were used. Using 0.1 μM rS* and 10 μM ribozyme did not affect the observed rate constant for the reaction. The reactions were initiated with excess (10−25 mM) ATP(-γS).

Pulse−Chase Experiments. Pulse−chase experiments were performed after the method described by Herschlag and Cech (1990a). Reactions were performed as described above for single-turnover measurements, except that the reaction was initiated by the addition of saturating ATP(-γS) and an excess (100−400 μM) of unlabeled rS. The time of incubation of E and rS* prior to the initiation of the reaction was varied from 15−30 min, with no effect on the results. Increasing the concentration of E also did not effect the outcome of the experiment. In the dilution−chase experiment, a 5 μL mix of 10 μM E and 1 μM rS* in reaction buffer was diluted into 1 mL of reaction buffer containing 20 mM ATP-γS·Mg^{2+}. Aliquots were removed at various times, quenched, and analyzed by PAGE as above.

Equilibrium Dialysis. Equilibrium dialysis measurements were performed using a Hoeffer equilibrium dialysis apparatus and 12−14 kD cutoff membranes. Subsaturating (0.1 μM) ^{32}P body-labeled rS* was placed in equal concentrations on each side of the membrane. The concentration of ribozyme (0.25−10.0 μM) on one side of the membrane was varied. After the system had equilibrated overnight, the fraction of rS* on each side of the membrane was quantitated directly using Cerenkov counting in a scintillation counter (except when the K_d for the [^{35}S]thiophosphorylated product, rP*, was measured, in which case aliquots were counted in scintillation fluid), and the fractional excess on the side containing ribozyme was calculated. The data were fitted to a saturation curve using KaleidaGraph. In every experiment one cell was set up without ribozyme and with rS* on only one side of the membrane. This cell was used to ensure that equilibrium had been reached at the end of the experiment. Prior to the experiment, the ribozyme was treated in the same way as for the kinetics experiments described above except that no ATP(-γS) was added. These experiments were done at 24 °C instead of 30 °C. Melting experiments have shown that $\Delta H°$ for the formation of the 5′-GGAACCU-3′/3′-UCUUGGA-5′ duplex is −50 kcal/mol. Using the integrated form of the van't Hoff equation,

$$K_{eq}(T) = (4/C_t)\exp[(\Delta H°/R)(1/T_m - 1/T)]$$

where $K_{eq}(T)$ is the equilibrium constant for the association of two complementary strands at the temperature T, C_t the total strand concentration, and T_m the measured melting temperature (312 K), the expected difference in K_ds between 24 and 30 °C is ~20%. Thus, while the binding of rS to the enzyme may not be exactly the same as for the simple duplex case (see Results and Discussion below), we feel that a conservative estimate of the difference between the K_d measured at 24 °C and the K_d at 30 °C is less than a factor of 2, about the error of the measurements.

Melting Temperature Experiments. Melting curves for the RNA oligos 5′-GGAACCU-3′ and 5′-AGGUUCU-3′ were measured on a Carey 1E UV−vis spectrophotometer equipped with a Carey temperature controller. The absorbance at 260 nm was measured from 10 to 85 °C, at a rate of 0.5 °C/min, with data collected every 0.5 °C. The temperature was monitored with a temperature probe inserted into a cuvette containing buffer. The buffer used was 400 mM KCl, 55 mM $MgCl_2$, 25 mM PIPES, pH 7.0 (at 24 °C). The oligos were preannealed in the KCl and PIPES (80°C) and then cooled to room temperature, at which time the $MgCl_2$ was added. The data were analyzed as described elsewhere (Roberts & Crothers, 1991) using a two state model.

Inhibition Experiments. Substrate inhibition experiments were done after the method of Herschlag and co-workers (Knitt et al., 1994; Narlikar et al., 1995; G. Narlikar, M. Khosla, and D. Herschlag, manuscript in preparation). In all cases, inhibition experiments were performed at subsaturating concentrations of the competing substrate and saturating concentrations of the other substrate. For the substrate inhibition and rP inhibition experiments the concentration of ribozyme used was 0.1 μM. For the ADP inhibition experiments the ribozyme concentration was 1.0 μM. The inhibition experiments were performed at two different concentrations of substrate (ADP inhibition, 100 and 250 μM ATP-γS; rP inhibition, 0.1 and 0.3 μM rS; substrate inhibition, 0.2 and 0.4 μM rS). The measured rate constants in each experiment were normalized to those for zero inhibitor concentration, and the data were fitted to a binding isotherm using KaleidaGraph (Abelbeck Software). For the product inhibition experiments, separate tests were done to ensure that the inhibition is competitive, that is, that K_m is altered by the presence of inhibitor while k_{cat} remains unchanged.

The thiophosphorylated product oligonucleotide, rP, was made by end-labeling 5′-HO-GGAACCU-3′ with ATP-γS and T4 polynucleotide kinase. The product was purified by PAGE on a 22.5% denaturing gel (0.8 mm thick, 40 cm long).

pH Profiles. Buffers were acetate (pH 4.9−5.3), PIPES (pH 6.1−7.6), HEPES (pH 7.6−8.2), and AMPSO (pH 8.2−9.0). The pH of each buffer was adjusted with KOH. The final concentration of buffer in all reactions was 50 mM. The pHs were corrected for temperature (30 °C) based on the reported ΔpH/ΔT values (Dawson et al., 1986); in all cases the changes were <0.1 pH units. At the end of each experiment the pH of the reaction solution was checked with pH paper. A 55 mM solution of $MgCl_2$ was used in these experiments instead of 50 mM $MgCl_2$, and 5 mM $MnCl_2$, because Mn^{2+} catalyzes RNA degradation at high pHs.

k_{cat}/K_m was measured at each pH by varying [ATP(-γS)] (10−50 μM). Since, under conditions of subsaturating substrate, $v = (k_{cat}/K_m)$[E][S], the slope of a plot of v vs [ATP(-γS)] divided by [E] yields a value for k_{cat}/K_m. In these experiments, the pH ranges of the buffers were overlapped to show that there are no significant buffer effects for the reaction. The rate constants for the single-turnover conversion of the ternary substrate complex to the ternary product

complex were measured as described above. In these single-turnover experiments, increasing the concentration of ribozyme did not affect the observed rate of the reaction at pH 4.9, 7.0, 7.6, or 9.0, indicating that saturating conditions were used at all pHs.

Error Values. Unless otherwise noted, reported error values are one standard deviation. When fewer than four independent experiments were performed, a range of values is reported instead, to give a sense of the reproducibility of the data. In general, experiments performed side by side were very reproducible (≤30% variation), but there was considerably more variability between experiments performed on different days (as much as 2-fold).

RESULTS AND DISCUSSION

Determination of Michaelis−Menten Parameters. The class I kinase was previously shown to act as a true enzyme: it exhibits saturation kinetics for both the 7-mer oligoribonucleotide substrate 5′-GGAACCU-3′ (rS) and for ATP-γS and can perform multiple turnovers. We have determined the apparent first-order rate constant, k_{cat}, for the reaction by measuring the velocity of the reaction at varying concentrations of ribozyme at concentrations of rS and ATP-γS previously determined to be saturating. Since $V_{max} = k_{cat}[E]$, the slope of the plot of velocity vs [E] is k_{cat}. The values for k_{cat} determined in this way were 0.16 and 0.18 min^{-1} in two independent experiments. These values are in good agreement with less accurate measurements from saturation curves (v vs [S]) which ranged from 0.10 to 0.17 min^{-1}.

The value of k_{cat}/K_m for ATP-γS was measured by varying the concentration of ATP-γS over a range from 0.01 to 0.2 of the previously estimated K_m. The concentration of rS was kept at saturation. The linearity of the plot of reaction velocity vs [ATP-γS] is further evidence that all concentrations of ATP-γS used were below K_m. Dividing the slope of the plot of velocity vs [S] by [E] yields the apparent second-order rate constant, k_{cat}/K_m, for the reaction, since $v = k_{cat}/K_m[E][S]$. The values obtained from such experiments, performed at two different concentrations of ribozyme, were 53 and 55 M^{-1} min^{-1}. Dividing the previously measured value of k_{cat} by the value for $k_{cat}/K_m^{ATP\text{-}\gamma S}$ yields $K_m^{ATP\text{-}\gamma S} =$ 3.1 mM. This agrees well with the value of 3.2 mM obtained from a saturation curve. These values are similar to those previously measured for the full-length cis-acting ribozyme (Lorsch & Szostak, 1994) for which k_{cat} is ~2 times higher and $K_m^{ATP\text{-}\gamma S}$ is ~6 times lower. Thus, the catalytic core of the truncated ribozyme must be largely intact.

k_{cat}/K_m^{st} and K_m^{st} for rS (under single-turnover conditions; st) were determined as follows. Reactions were carried out with a constant amount of labeled rS, and [E] was varied (0.05−0.4 μM) instead of varying [rS] in order to keep a constant amount of radioactivity in each reaction and to avoid a decrease in signal by adding unlabeled rS. Two such experiments, with [rS] at either 0.1 or 0.4 μM, gave values for $k_{cat}/K_m^{rS\text{-}st}$ of 7.5×10^4 and 8.0×10^4 M^{-1} min^{-1}. Again, dividing k_{cat} by the value for $k_{cat}/K_m^{rS\text{-}st}$ yields $K_m^{rS\text{-}st} = 2.2$ μM. A less accurate determination from a saturation curve (multiple-turnover conditions) gave a value of ~1 μM. The values for the Michaelis−Menten parameters are summarized in Table 1. In this system, the values determined under single-turnover conditions should be the same as those for multiple-turnover conditions (see data and discussion below).

Table 1: Kinetic Parameters of the Class I Polynucleotide Kinase Ribozyme[a]

	k_{cat} (min^{-1})	k_{cat}/K_m^A (M^{-1} min^{-1})	k_{cat}/K_m^{rS} (10^4 M^{-1} min^{-1})	K_m^A (mM)	K_m^{rS} (μM)
ATP-γS	0.17	57	8.5	3.1	2
ATP	0.003	0.9	0.14	3.4	2

[a] Kinetic constants for ATP and ATP-γS were determined under multiple-turnover conditions. Those for rS were determined under both single- and multiple-turnover conditions. See Materials and Methods for details.

In order to assess the role of the sulfur atom of ATP-γS in the reaction we also determined the Michaelis−Menten parameters when ATP is the substrate. As shown in Table 1, the K_m for ATP is the same as the K_m for ATP-γS, the K_m for rS remains unchanged, but k_{cat} with ATP is 50-fold lower than with ATP-γS ($k_{cat}^{ATP} = 0.003$ min^{-1}, $k_{cat}^{ATP\text{-}\gamma S} =$ 0.17 min^{-1}). The direction of this effect is consistent with the expected "thio effect" for the hydrolysis of a phosphomonoester (Breslow & Katz, 1968; Domanico et al., 1986; Herschlag et al., 1991): the sulfur atom of the thiophosphate should stabilize the metaphosphate-like transition state (Herschlag & Jencks, 1989) of the hydrolysis reaction by donating electron density to the partially positively charged phosphorus atom, relative to the reaction in which sulfur is replaced by oxygen. The magnitude of the effect is, however, 5−10 times greater than the reported values of 2−10-fold (Domanico et al., 1986; Herschlag et al., 1991; Hollfelder & Herschlag, 1995). There are several possible explanations for this observation. First, as the thio effect for the hydrolysis of a phosphoanhydride has never, to our knowledge, been measured, it may be that the effect is larger with this more activated bond than it is for a phosphomonoester. It is also possible that the ribozyme is specifically interacting with the sulfur atom of the thiophosphate and that this interaction is weaker when the sulfur is replaced by oxygen. However, the ribozyme does not require Mn^{2+} (the reaction with ATP-γS is always 50 times faster than the reaction with ATP whether it is performed in 50 mM Mg^{2+} plus 5 mM Mn^{2+}, the conditions of the initial selection, or in 55 mM Mg^{2+}; the rate constants themselves also remain unchanged), and thus a preferential interaction with the sulfur would have to be mediated by some group other than Mn^{2+}, which is the only component of the system known to be able to interact strongly with the sulfur atom of a thiophosphate (Eckstein, 1985; Pecoraro et al., 1984). Interestingly, the ribozymes in classes II−IV from the original selection all have an absolute requirement for the presence of Mn^{2+} (J. R. Lorsch and J. W. Szostak, unpublished results). There is, of course, the possibility that the class I ribozyme has an extremely tight binding site for Mn^{2+} and is able to use the very small amount of contaminating Mn^{2+} (~1 ppm) present in our $MgCl_2$ solutions. However, this seems unlikely because the binding site would have to have a K_d in the nanomolar range, several orders of magnitude lower than most Mg^{2+}/Mn^{2+} binding sites in RNA (Pan et al., 1993). Another possibility is that discrimination arises from the fact that Mg^{2+} is not able to interact with the sulfur atom of the thiophosphate (O/S coordination ratio for Mg^{2+} has been reported as 31 000; Pecoraro et al., 1984), and that a deleterious Mg^{2+}−O interaction, not present with the γ-thiophosphate, decreases the activity of the ribozyme.

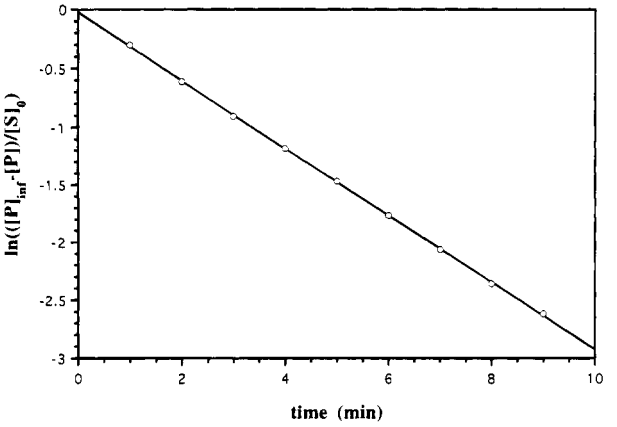

FIGURE 2: Plot of the conversion of the ternary complex E·rS·ATP-γS to E·rP·ADP (i.e., saturating ribozyme over rS). An endpoint of 98% substrate reacted was measured. The reaction was followed for over 3 half-lives and displays first-order kinetics during this period, demonstrating that no significant side reactions occur involving the oligonucleotide substrate. $[P]_{inf}$, concentration of product at infinite time (>10 half-lives); $[S]_0$, initial substrate concentration.

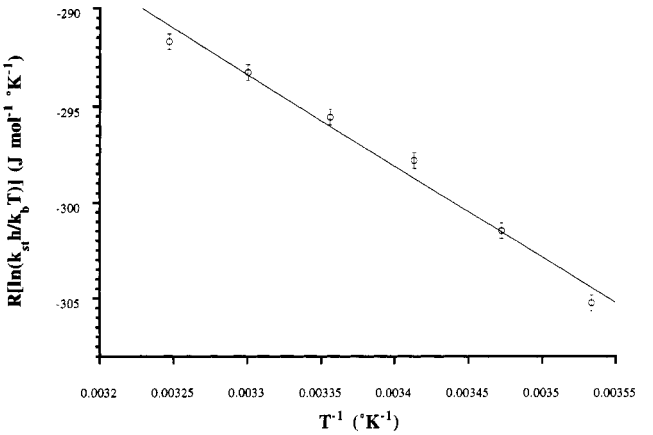

FIGURE 3: Eyring plot for the single-turnover conversion of the ternary substrates complex to the ternary products complex (E·rS·ATP-γS → E·rP·ADP). k_{st} is the single turnover rate constant (see text), k_b is the Boltzmann constant, h is the Planck constant, and T is the temperature. All units are standard SI units. The 10 and 20 °C values are the average of four separate experiments, the 30 °C value is the average of three experiments, and the 15, 25, and 35 °C values are the average of two experiments each. Error bars represent one standard deviation in the data for k_{st}. The (negative) slope of the plot gives a value of $\Delta H^{\ddagger} = 47$ kJ/mol (11 kcal/mol), and the Y-intercept gives a value of $\Delta S^{\ddagger} = -137.5$ J mol^{-1} K^{-1} (−33 eu). The curvature of the plot suggests that the system may have a significant heat capacity of activation (i.e., the enthalpy for attaining the transition state is not completely temperature independent). No correction was made for the change in pH of the buffer with temperature because (a) the buffer used (PIPES) has a very small change in pK_a with temperature and (b) the reaction is pH independent at 30 °C. Separate experiments showed that the [E] used (10 μM) is sufficient to saturate rS from 10 to 35 °C.

Rate-Limiting Step. The rate-limiting event for the catalyzed reaction could be any step or combination of steps along the reaction pathway, including substrate binding, chemistry, conformational change, or product release. If, as is the case in a number of engineered versions of naturally occurring ribozymes, release of the oligonucleotide product is rate-limiting, we would expect to see a pre-steady-state burst phase (Fedor & Uhlenbeck, 1992; Herschlag & Cech, 1990b; Hertel et al., 1994). However, no burst is seen when measuring steady-state rates with saturating substrates. Furthermore, the rate constant for the single-turnover reaction with saturating ribozyme (10 μM), ATP-γS (20 mM), and 1 μM rS is the same as k_{cat}, demonstrating that neither the rate of formation of the E·rS complex (assuming that rS can bind productively in the absence of ATP-γS; see below) nor the thiophosphorylated oligonucleotide product (rP) release is rate-limiting. This is because the single-turnover, saturating ribozyme reaction observes only the rate at which bound substrate reacts to give bound product (E·rS + ATP-γS → E·rP·ADP). We can therefore conclude that the dissociation of both products is faster than the rate-limiting step between substrate binding and product release. The order of addition of substrates has no effect on the measured rate constant for this reaction. Starting the reaction with either ATP-γS or rS (and preincubating with the other) yields the same rate constant. Furthermore, preannealing the ribozyme (80 °C denaturation followed by cooling to room temperature) with the oligonucleotide substrate has no effect on the measured rate. Thus, it appears that under these conditions all binding steps are fast.

The single-turnover reaction with saturating ribozyme (over rS) shows first-order kinetics for >3 half-lives (Figure 2), with a measured endpoint consistently at ~98% completion. These results indicate that there are no significant side reactions involving the oligonucleotide substrate.

The rate-limiting step is unlikely to be binding of ATP-γS, even at subsaturating concentrations. The apparent second-order rate constant for the reaction, $k_{cat}/K_m^{ATP\text{-}\gamma S}$ is 54 M^{-1} min^{-1}. This value is approximately 8 orders of magnitude lower than the rate of association for most small molecule−macromolecule interactions (Hammes & Schimmel, 1970). Although it seems unlikely that the active site of the ribozyme is this inaccessible to ATP-γS, it is harder to discount the possibility that the ATP(-γS) binding site is a rarely formed, metastable structure.

The fact that k_{cat} for the reaction with ATP is 50 times lower than with ATP-γS is consistent with a rate-limiting chemical step, since, as discussed above, ATP-γS is expected to be more reactive than ATP. However, the possibility of a rate-limiting conformational change that, for some reason, occurs more rapidly with ATP-γS than with ATP, cannot yet be completely ruled out (see below). It is also possible that ATP-γS is more than 50 times more reactive than ATP (either due to the thio effect or because of thiophosphate-specific contacts to the ribozyme in the transition state), and thus some event other than chemistry (i.e., conformational change) is rate-limiting with ATP-γS while the chemical step is rate-limiting with ATP.

Activation Parameters. In order to determine $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ for the conversion of the ternary substrate complex to the ternary product complex, the temperature dependence of the single-turnover rate constant when ribozyme is in saturating excess over rS was measured from 10 to 35 °C. Separate experiments (data not shown) demonstrated that the concentration of ribozyme used was saturating over rS at all temperatures at which the experiments were performed. Assuming that there is a single rate-determining step, Eyring analysis of the data gives a $\Delta H^{\ddagger} = 11 \pm 1$ kcal/mol and a $\Delta S^{\ddagger} = -33 \pm 3$ eu for the catalyzed reaction (Figure 3; Carey & Sunderberg, 1984; Jencks, 1987). The barrier to the transition state therefore has only a modest enthalpic

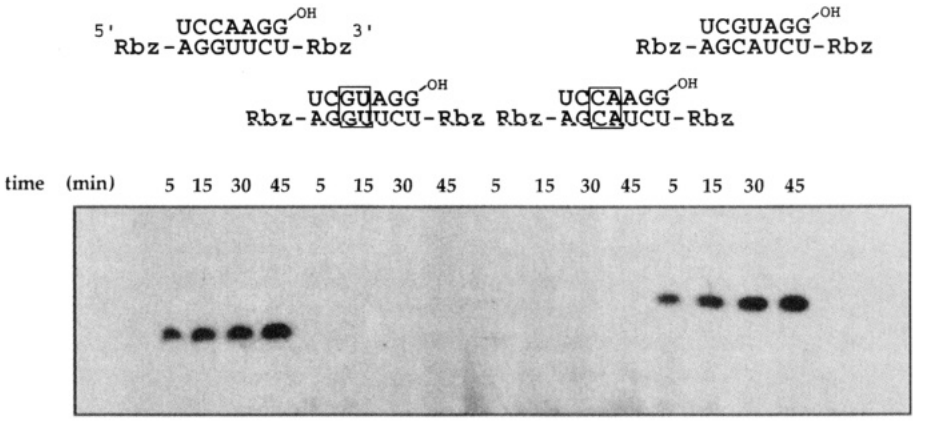

FIGURE 4: Defining the oligonucleotide substrate binding site (IGS) of the ribozyme. A mutant ribozyme was made that forms two internal mismatches with the "wild-type" substrate 5′-GGAACCU-3′. The mutation was introduced into the PCR DNA template for the RNA via a mismatched 3′-primer, and the RNA was synthesized by runoff transcription. This ribozyme shows no activity with the wild-type substrate. A mutant substrate that had two internal mismatches with the putative IGS shows no activity with the wild-type ribozyme. When the mutant substrate is used with the mutant ribozyme, full Watson−Crick complementarity is restored, and the ribozyme catalyzes the reaction with a k_{obs} identical to that for the wild-type ribozyme with wild-type substrate. Rbz: ribozyme. The reactions were performed using 10 mM ATP-γ^{35}S and 10 μM RNA substrate as described in Materials and Methods. Time points were analyzed by denaturing PAGE on a 22.5% gel.

component, but there appears to be a great deal of ordering in the transition state for the catalyzed reaction relative to the ground state. There may also be a slight curvature to the Eyring plot, suggesting that the system has some heat capacity of activation. These values are similar to those reported by Hertel and Uhlenbeck (1995) for the ligation (reverse) reaction catalyzed by the hammerhead ribozyme ($\Delta H^{\ddagger} = 9.5$ kcal/mol and $\Delta S^{\ddagger} = -43$ eu). One possible explanation for this similarity is that the ribozyme−substrate complexes for both enzymes are "floppy", and that many degrees of freedom within the ribozymes themselves must be frozen in order to attain the transition-state stabilizing structures. There is, in fact, some evidence that the hammerhead ribozyme−cleaved products complex (E·P1·P2) is floppy (Hertel et al., 1994). In any event, the entropy of activation for the class I polynucleotide kinase ribozyme is somewhat larger than would be expected for a phosphotransfer reaction in solution occurring with either a dissociative or an associative mechanism (Benkovic & Schray, 1971), suggesting that the transition state for the catalyzed reaction reflects changes in the enzyme as well as changes in the reactants.

Binding of the Oligonucleotide Substrate. Is the binding of the 7-mer substrate the formation of a simple Watson−Crick duplex with the internal guide sequence (IGS) of the ribozyme, or are there tertiary interactions, as is the case for the *Tetrahymena* ribozyme (Bevilacqua & Turner, 1991; Herschlag & Cech, 1990b; Pyle et al., 1990)? In order to answer this question we first had to demonstrate that the proposed IGS (Figure 1) is indeed the oligonucleotide substrate binding site. To do this we made a double-mutant version of both the ribozyme and the substrate. In the mutant ribozyme, the putative binding site was changed from 5′-ribozyme-AGGUUCU-ribozyme-3′ to 5′-ribozyme-AGCAU-CU-ribozyme-3′ (changes underlined) to give internal C·C and A·A mismatches with the original substrate (5′-HO-GGAACCU-3′). When this ribozyme was used with the wild-type substrate, no reaction was observed under conditions that were saturating (10 μM 7-mer) with the wild-type enzyme (Figure 4). In a reciprocal experiment a mutant substrate was made (5′-HO-GGAUGCU-3′) that created G·G and U·U mismatches with the putative IGS of the wild-type ribozyme. When this substrate was used with the wild-type ribozyme under the same conditions as above, no activity was detected. However, when the mutant ribozyme was used with the mutant substrate, activity was restored, with a k_{obs} for the reaction almost identical to that for the wild-type enzyme and substrate. Since the IGS of the mutant ribozyme can form an uninterrupted Watson−Crick duplex with the mutant substrate (Figure 4), this series of experiments provides good evidence that the putative IGS is the oligonucleotide substrate binding site for the ribozyme and that rS forms a Watson−Crick duplex with the IGS (at least at these two positions). Whether or not the terminal G·U wobble pair, which is conserved in all four members of the original class I ribozymes, is actually formed is not yet clear. It is interesting, however, that when the U of this proposed wobble base pair is mutated to a C, allowing a true Watson−Crick G·C pair to form, the ribozyme becomes inactive (>1000 times lower activity under conditions saturating with the wild-type ribozyme). This suggests either that the 3′-U of the IGS is required for catalysis or for structural reasons or that when the 5′-G of the substrate (the site of phosphorylation) forms a G·C base pair with the IGS it is no longer in the correct position or orientation to react with ATP-γS.

The fact that the internal guide sequence of the ribozyme can be changed at two internal positions to produce a fully active polynucleotide kinase with altered sequence specificity suggests the possibility that the ribozyme might be able to tolerate more drastic changes to its IGS. If so, this class of RNA enzymes could prove to be useful reagents for the site-specific phosphorylation of other RNA (and DNA) molecules.

In order to understand better how the binding of rS to the ribozyme compares with simple duplex formation, we measured the K_d of rS for the ribozyme and the K_d of a Watson−Crick duplex formed by rS and an oligonucleotide with the same sequence as the IGS. The Michaelis constant, K_m^{rS}, for the oligonucleotide substrate may or may not be equal to the K_d of rS for the ribozyme−ATP-γS complex, depending on the relative values of the rate constants for rS

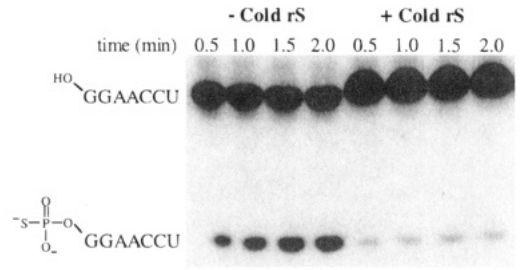

FIGURE 5: Pulse−chase experiment to determine the ratio k_{cat}/k_{-1} (where k_{-1} is the rate constant for rS dissociation from the ribozyme). The experiment was performed as described in Materials and Methods. The RNA substrate was ^{32}P body-labeled. −Cold rS, only ATP-γS in chase; +Cold rS, 200× excess of cold rS included in ATP-γS chase. No significant product formation is seen after the initial burst (0.5 min time point). The k_{cat}:k_{-1} ratio is 1:15. Thus, k_{-1} is ~2.5 min^{-1}. Experiments in which the amount of cold rS in the chase and the time of preincubation of labeled rS with E were varied gave similar results.

Table 2: Summary of Results of Pulse−Chase and Dilution−Chase Experiments[a]

[cold rS] in chase (mM)	extrapolated burst (% rS* converted to rP*)
50	6
100	8
200	6
dilution−chase	5

[a] Pulse−chase experiments were performed using saturating ribozyme over rS* (1 μM rS*, 10 μM E) and initiated with a chase of excess ATP-γS and varying amounts of cold rS. The dilution−chase experiment was set up similarly but was initiated by diluting the ribozyme + rS* mix 1:200 in reaction buffer containing excess ATP-gS. See Materials and Methods for details.

dissociating from the enzyme, k_{-1}, and for proceeding on to products, k_{cat}, and on whether or not intermediates can accumulate on the reaction pathway after the chemical step. We have already demonstrated that there is no accumulation of enzyme-bound intermediates or products (i.e., no burst phase seen, etc.; see above), and thus the one question that remains is the relative magnitudes of k_{-1} and k_{cat}. To measure this ratio we performed pulse−chase experiments (Figure 5; Table 2) in which the complex between saturating ribozyme and labeled oligonucleotide substrate was preformed, and then a chase of excess (50−200×) cold rS was added along with the other substrate, ATP-γS. In this experiment, once a labeled substrate molecule falls off the ribozyme it can no longer react because it is competed out by the excess cold oligonucleotide. A burst of 6%−8% labeled product (relative to labeled substrate) was seen, after which there was no significant product formation, indicating that the ratio of k_{-1}/k_{cat} is approximately 15. Since $k_{-1} \gg k_{cat}$, $K_m^{rS} = k_{-1}/k_1 = K_d^{rS}$.

A potential problem with this kind of experiment is that inhibition of the ribozyme by high concentrations of rS (i.e., due to binding to a low-affinity inhibitory site) or displacement of bound rS* by strand invasion could lead to artificially low values for the burst, and thus an overestimate of k_{-1}. However, the results of these experiments are independent of the concentration of the chase oligonucleotide over a 4-fold range, suggesting that neither of these potential artifacts occurs. As a further test for artifacts caused by the presence of the chase oligonucleotide, a dilution−chase experiment (Hertel et al., 1994) in which substrate rebinding

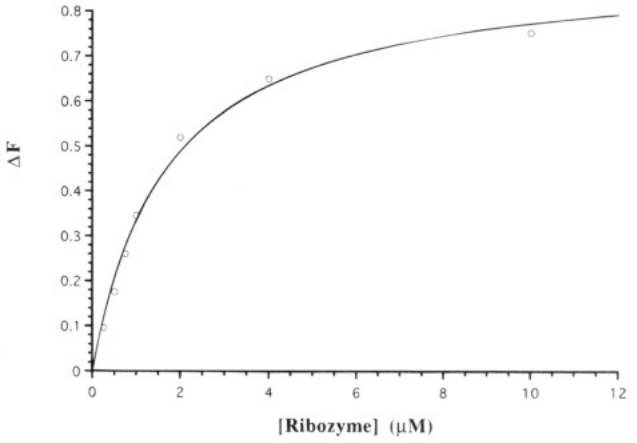

FIGURE 6: Equilibrium dialysis measurements of the binding of rS to the ribozyme in the absence of ATP(-γS). ΔF is the fractional excess of rS on the side of the membrane containing ribozyme. Each point is the average of three independent experiments, except the 10 μM ribozyme point, which was measured only once. The data were fitted to a theoretical saturation curve using KaleidaGraph. The curve fit gives a K_d of 1.7 μM, with a χ^2 of 0.003 and a correlation coefficient (R) of 0.995.

was prevented by dilution was performed. The ribozyme·rS* complex in reaction buffer was diluted 200 fold into reaction buffer containing 20 mM ATP-γS·Mg^{2+}. This experiment yielded the same result as the pulse−chase experiments, within experimental error. The fact that in these experiments some product formation is seen before the labeled rS falls off shows that rS can form a productive complex with the ribozyme in the absence of ATP-γS. As expected, in the pulse−chase experiments no product formation could be detected when only cold rS was added as a chase (i.e., in the absence of ATP-γS) or when ATP was used in place of ATP-γS, since ATP reacts 50 times more slowly than ATP-γS, and thus the ratio of k_{-1}/k_{cat} should be approximately 750. The value of 15 for k_{-1}/k_{cat} with ATP-γS as the substrate allows us to estimate the values of k_1 and k_{-1} (the second- and first-order rate constants for E + rS → E·rS and E·rS → E + rS, respectively), assuming the minimal kinetic mechanism shown in Figure 11: $k_{-1}/k_{cat} \approx 15$ and $k_{cat} = 0.17$ min^{-1}, therefore $k_{-1} \approx 2.5$ min^{-1}; since $K_m^{rS} = K_d^{rS} = k_{-1}/k_1 = 2$ μM, $k_1 \approx 1.2 \times 10^6$ M^{-1} min^{-1}. This calculated value of k_1 is over 1 order of magnitude lower than measured values for RNA−RNA helix formation (Craig et al., 1971; Nelson & Tinoco, 1982; Porschke & Eigen, 1971; Porschke et al., 1973). This may suggest that the oligoribonucleotide binding site is less accessible than an RNA strand in solution.

Using equilibrium dialysis we also measured the affinity of the ribozyme for the oligonucleotide substrate in the absence of ATP-γS (Figure 6). These experiments give a value of K_d for the free ribozyme and rS of ~1.7 μM (range: 1.25−2.7 μM), which is the same, within error, as the K_d of the E·ATP-γS complex and rS, indicating that the presence of ATP-γS does not significantly increase (or decrease) the affinity of the ribozyme for the oligonucleotide substrate. Taken together with the fact that the ribozyme can bind rS productively in the absence of the second substrate, ATP-γS, this result suggests that the full oligonucleotide substrate binding site is formed in the free ribozyme.

The dissociation constant of the model duplex formed by the oligonucleotides 5′-GGAACCU-3′ (the substrate) and 5′-

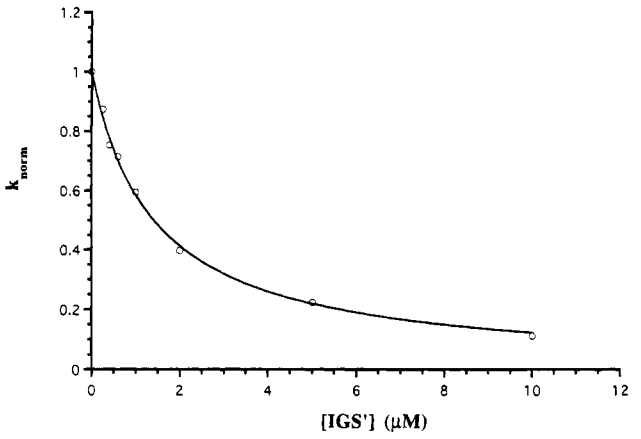

FIGURE 7: Substrate inhibition experiment to determine the K_d for the oligos 5′-GGAACCU-3′ and 5′-UCUUGGA-3′ (IGS′). k_{norm} is the observed rate constant normalized to that at [IGS′] = 0. Each point represents the average value from four separate experiments. The data were fitted to a binding isotherm using KaleidaGgraph, giving a curve with χ^2 of 0.002 and a correlation coefficient (R) of 0.998. The calculated K_i is 1.4 ± 0.4 μM. Since the experiment was done under conditions of subsaturating [E] and [rS], and thus most rS was unbound, the K_i represents a true dissociation constant.

AGGUUCU-3′ (the same sequence as the IGS; hereafter called IGS′) was determined in a variety of ways. To measure the K_d of the model duplex in our buffer conditions, melting experiments were performed using 5 μM of each strand. The data from these experiments were fitted to theoretical curves (Roberts & Crothers, 1991) to give values of $\Delta H^\circ \approx -50$ kcal/mol and $\Delta S^\circ \approx -135$ eu (data not shown). The estimated K_d calculated from these values is ~0.3 μM. The T_m was also measured at 2 and 10 μM concentrations of each strand, and a plot of $1/T_m$ vs $\ln(C_t/4)$ (where C_t is the total strand concentration; Borer et al., 1974; Petersheim & Turner, 1983) yielded a value for K_d of ~0.15 μM (data not shown).

Another method for determining the K_d of the IGS′ and the substrate is to use IGS′ as a competitive inhibitor of the reaction. Similar "substrate inhibition" experiments have been used to study the binding of substrate oligonucleotide to the *Tetrahymena* group I intron (Knitt et al., 1994; Narlikar et al., 1995; G. Narlikar., M. Khosla, and D. Herschlag, manuscript in preparation). The experiment was performed at subsaturating concentrations of ribozyme (0.1 μM) and ^{32}P body-labeled rS (0.2 and 0.4 μM), and the reactions were started by the addition of saturating (10 mM) ATP-γS. By varying the concentration of IGS′, K_i can be measured. Since the majority of rS is unbound, the K_i should be the true dissociation constant for the substrate−IGS′ duplex. Four such experiments yielded a value of 1.4 ± 0.4 μM (range: 0.95−2.0 μM) for the duplex K_d (Figure 7). The reason for the 5−10-fold discrepancy between the values calculated from T_m and kinetic measurements is not clear. The affinity of the ribozyme for the oligonucleotide appears to be no stronger than would be expected for a simple RNA−RNA duplex, and it may actually bind slightly more weakly than expected. Weaker binding could be a result of strained helix geometry between the oligonucleotide substrate and the internal guide sequence (due to the structure of the ribozyme) or of the possibility that the terminal G·U wobble pair contributes to the binding energy of the rS−IGS′ duplex in solution but not to the binding of rS to the ribozyme (the U may be involved in structural interactions within the ribozyme and thus not available to base pair with the oligonucleotide substrate; see above results on changing the U to a C). Our data suggest that the oligoribonucleotide substrate binds via the formation of a simple duplex, although we cannot rule out the possibility that there are favorable tertiary interactions to the ribozyme which are energetically offset by other, unfavorable interactions (either between the ribozyme and the substrate or between two or more groups on the ribozyme).

Binding of the Adenosine Triphosphate Substrate. The affinity of the ribozyme for the adenosine triphosphate substrate is too low to measure the K_d directly by equilibrium dialysis or other standard techniques. Several arguments, however, support the idea that the Michaelis constants for ATP and ATP-γS are equal to the dissociation constants of these substrates. We have already shown that no step after chemistry is rate-limiting. Thus, for K_m^A not to equal the dissociation constant for the adenosine triphosphate substrate would require that the ternary substrate complex not be in rapid equilibrium with ATP-γS and E·rS, that is, for k_{-1} be about equal to or smaller than k_{cat}. Since $K_m^{ATP\text{-}\gamma S} = (k_{-1} + k_{cat})/k_1 = 3$ mM, for k_{-1} to be approximately equal to k_{cat} would mean that k_1, the second-order rate constant for the association of ATP-γS and the ribozyme would have to be equal to approximately 100 M^{-1} min^{-1} [(0.17 min^{-1} + 0.17 min^{-1})/3 × 10^{-3} M = k_1], ~10^9-fold below the diffusion limit (Eigen & Hammes, 1963). The likelihood of this being the case is remote. For the K_m for ATP not to equal its K_d, would require k_1 to be equal to ~2 M^{-1} min^{-1}, nearly 11 orders of magnitude below the diffusion limit. The fact that the K_m for ATP is the same as the K_m for ATP-γS, but k_{cat} is 50 times lower with ATP than with ATP-γS, is further evidence for the equality of the Michaelis constants and dissociation constants, because for them to be different would require that the association rate constant for ATP with the ribozyme be 50 times lower than for ATP-γS with the ribozyme. It seems very unlikely that the substitution of a sulfur atom for an oxygen could produce this effect. Therefore, it seems likely that the Michaelis constants for both ATP and ATP-γS are equal to their respective dissociation constants.

The apparent K_m for ATP-γS at a concentration of E (0.1 μM) and rS (0.3 μM) below the K_m for rS (2 μM) was determined to be 3.6 mM, the same as the true K_m value with saturating rS. Similarly, the apparent K_m for rS with subsaturating ATP-γS was found to be the same, within error, as the true K_m^{rS}. These data support the conclusion that there is no significant synergy of binding between ATP-γS and the oligoribonucleotide substrates. These data also support a random substrate binding mechanism (Cleland, 1970).

Product Inhibition. Inhibition constants (K_i) were measured for ADP competing with ATP-γS for the E·rS complex and rP competing with rS for the E·ATP-γS complex, respectively (assuming random binding; Figures 8 and 9). In both cases the inhibition was shown to be competitive (K_m was altered by the presence of the inhibitor, k_{cat} was unchanged), and thus these K_is represent true dissociation constants. The K_i for ADP is 3.8 ± 1.5 mM and is the same, within error, as the K_m for ATP-γS and for ATP, which we believe to be the dissociation constants for these substrates. The simplest explanation of these data is that the γ-(thio)-phosphate of the adenosine triphosphate substrate makes no significant *net* favorable contacts with the ribozyme in the

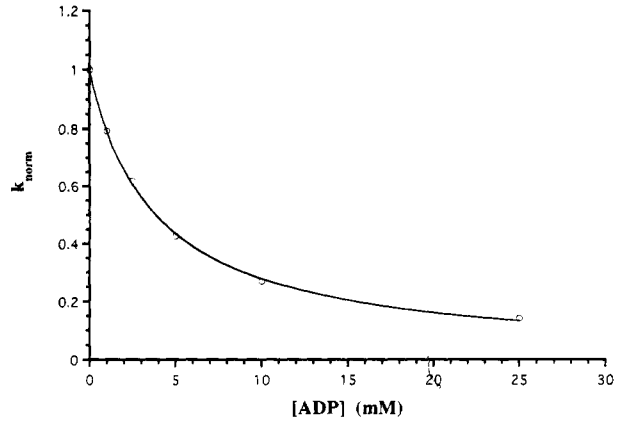

FIGURE 8: Inhibition curve for ADP. k_{norm} is the observed rate constant normalized to that at [ADP] = 0. Each point is the average of four independent experiments, performed at two different, subsaturating concentrations of ATP-γS (100 and 250 μM). The theoretical curve was fit with KaleidaGraph and has a χ^2 of 0.0004 and a correlation coefficient (R) of 0.9996. The measured K_i is 3.8 $\pm$ 1.5 mM. The inhibition was shown to be competitive in separate experiments (data not shown).

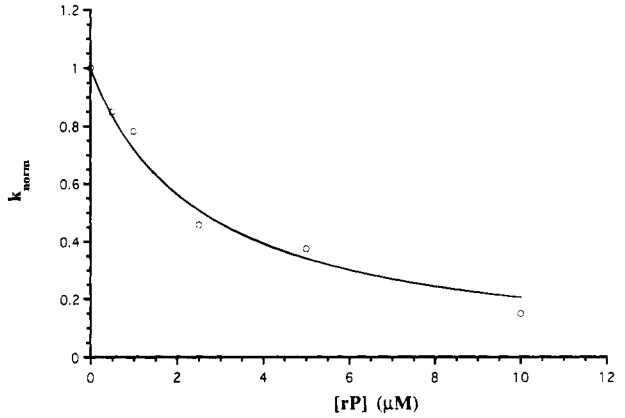

FIGURE 9: Inhibition curve for the thiophosphorylated product (rP), 5′-thiophosphate-GGAACCU-3′. k_{norm} is the observed rate constant normalized to the value at [rP] = 0. Each point is the average of four separate measurements, performed at two different, subsaturating concentrations of rS (0.1 and 0.3 μM; [E] = 0.1 μM). The data were fitted to a binding isotherm using KaleidaGraph. χ^2 for the fit is 0.01, and the correlation coefficient (R) is 0.990. K_i is 2.6 $\pm$ 0.5 μM. Separate experiments (data not shown) showed the inhibition to be competitive.

ground state. It also seems unlikely that any of the binding energy of the adenosine diphosphate portion of ATP(-γS) is used to destabilize the γ-(thio)phosphate (e.g., force it next to a negatively charged group, the destabilization being partially relieved upon attaining the metaphosphate-like transition state), since ADP and ATP(-γS) bind with similar affinities. Similarly, the K_i for the thiophosphorylated oligonucleotide product (2.6 $\pm$ 0.5 μM) is the same as the K_d for the RNA substrate (~2 μM). The K_d of rP and free ribozyme was directly measured by equilibrium dialysis and found to be approximately 1 μM, in reasonable agreement with the inhibition constant. From all of the above data it appears that the binding energies of the substrates and products to the ribozyme are largely balanced, with the reactive thiophosphate group playing no part in the energetics of the ground states. That the ribozyme does not stabilize this thiophosphate group in the ground state is reasonable, because no catalytic advantage can be gained by stabilizing the substrates of a reaction relative to the transition state (Fersht, 1985). The lack of ground-state destabilization also eliminates one possible explanation for the catalytic activity of the ribozyme.

Specificity. The ability of GTP-γS to act as a substrate for the reaction was studied under single-turnover conditions in which ribozyme was saturating over the oligonucleotide substrate (10 μM E, 1 μM rS*). Under these conditions, 20 mM GTP-γS reacts ~650 times more slowly than 20 mM ATP-γS. GTP-γS does, however, act as an inhibitor of the reaction with ATP-γS: under subsaturating conditions of ATP-γS (500 μM) the reaction was inhibited ~50% by 20 mM GTP-γS, giving a rough estimate for the K_i of GTP-γS as ~20 mM. While GTP-γS does appear to bind more weakly to the ribozyme than ATP-γS (~10-fold), this lower affinity cannot explain the large difference in reactivity. Most of the 650-fold lower reactivity with 20 mM GTP-γS must be due to catalytic effects rather than to weak binding. For example, GTP-γS may bind in a largely nonproductive manner, with its γ-thiophosphate not properly aligned for catalysis. Similar studies of other potential substrates and inhibitors for the reaction (e.g., other nucleotides and nucleotide analogs) could yield information about which parts of the adenosine triphosphate substrate are important for recognition by the ribozyme and whether the ribozyme recognizes ATP in the same way as the original ATP aptamer.

We have already shown that the ribozyme is readily able to distinguish between mismatched oligonucleotide substrates and the fully complementary substrate (see above). The ribozyme can also distinguish between complementary substrates of different lengths: K_m for the 6-mer 5′-HO-GGAACC-3′ is ~25 μM, over 10-fold higher than for the 7-mer 5′-HO-GGAACCU-3′. Interestingly, k_{cat} for the 6-mer is 0.05 min^{-1}, approximately 3-fold lower than with the 7-mer, suggesting that there may be minor catalytic or structural problems when the 3′-U of the substrate is removed. The ribozyme can also distinguish between RNA and DNA substrates. k_{obs} for the thiophosphorylation of 100 μM (50 $\times$ K_m for rS) 5′-HO-d(GGAACCT)-3′ is 0.003 min^{-1}, ~60-fold lower than k_{cat} with the all-RNA substrate. This effect may be due to differences in the conformation of the DNA−RNA helix vs the all RNA case, or one or more 2′-hydroxyls may be involved in orienting the substrate oligonucleotide relative to the ribozyme or directly in catalysis. The effect is not due to the replacement of the terminal U by a dT, because k_{cat} with the hybrid substrate molecule 5′-HO-GGAACC(dT)-3′ is the same as with the full RNA substrate.

pH Dependence of Catalysis. We have studied the pH dependence of both k_{cat}/K_m and of the conversion of the ternary complex, E·rS·ATP-γS, to products (i.e., saturating ribozyme over rS, saturating ATP-γS; we will call the observed rate constant for this process k_{st}). Neither of these parameters shows any pH dependence from pH 6.0−8.5 (Figure 10a,b). When ATP is used as a substrate the reaction is similarly pH independent (Figure 10c,d). As the pH is lowered below 6.0 and raised above 8.5, both k_{cat}/K_m and k_{st} begin to decrease, although still not in a log−linear fashion with pH, suggesting that groups on the bases in the ribozyme are beginning to titrate at their normal pK_as (~4.0−4.5 and ~9.5; Saenger, 1984). The fact that these parameters seem to be titrating at the normal pK_as for RNA nucleotides suggests that there are relatively few groups in the ribozyme

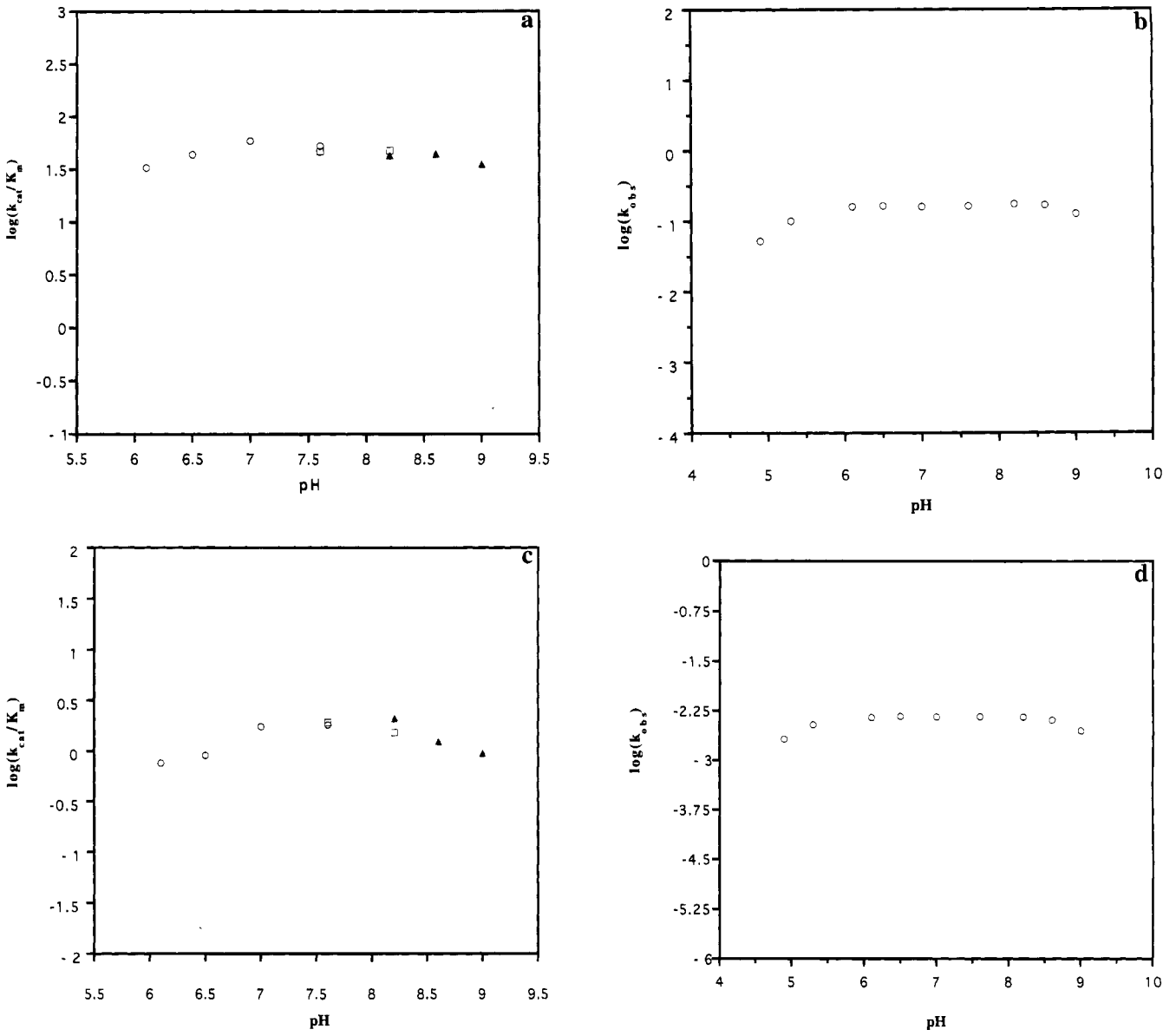

FIGURE 10: pH profiles of k_{cat}/K_m and the rate constant for the single-turnover conversion of the ternary substrate complex to the ternary product complex with ATP-γS and ATP as substrates. (a) k_{cat}/K_m for ATP-γS at pHs from 6.1 to 9.0. Different symbols represent different buffers (circles = PIPES, squares = HEPES, triangles = AMPSO). (b) pH profile for the single-turnover conversion of E·rS·ATP-γS → E·rP·ADP. See Materials and Methods for buffers used. (c) k_{cat}/K_m for ATP vs pH. Symbols are the same as in a. (d) pH profile for the single-turnover conversion of E·rS·ATP → E·rP·ADP. Separate experiments showed that 10 μM ribozyme is saturating over rS in the pH range studied.

whose protonation states affect the catalytic ability of the enzyme (Knitt & Herschlag, 1995). For example, if there were five groups each with a pK_a of 4.5, and protonation of each one abolished the activity of the ribozyme, at pH 4.5 the apparent activity (1 = full activity) of the ribozyme would be $0.5^5 = 0.03$. Thus the apparent pK_a would be considerably higher than the true pK_a of any individual group in the ribozyme.

The pH independence of both k_{cat}/K_m and k_{st} demonstrates that the rate-limiting step of the catalyzed reaction does not involve the net loss or gain of a proton. This is consistent with rate-limiting chemistry in which the transfer of (thio)-phosphate occurs via a dissociative transition state. The hydrolysis of Mg^{2+}·ATP is believed to occur via a dissociative mechanism and shows no dependence on [H^+] between pH 6 and pH 8.5 (Miller & Westheimer, 1966; Ramirez et al., 1980; S. Admiraal and D. Herschlag, manuscript in preparation). If the chemical step was rate-limiting and transfer of (thio)phosphate proceeded via an associative mechanism, then we would expect the rate of the reaction to increase with pH because deprotonation of the 5′-hydroxyl of the oligonucleotide substrate should be a requisite step for the reaction. Although we cannot rule out the possibility of a pH independent rate-limiting conformational change, arguments presented above make this seem unlikely.

Reaction Mechanism. Our data are most consistent with a mechanism in which substrates are bound and products released in a random fashion (random bi bi) by the class I polynucleotide kinase ribozyme (Figure 11). The fact that the K_d of the ribozyme for the oligonucleotide is the same in the presence and the absence of ATP(-γS) demonstrates that there is no synergy of binding between the adenosine triphosphate and oligonucleotide substrates. This conclusion is also supported by the fact that the apparent K_m values for ATP-γS with subsaturating rS and for rS with subsaturating ATP-γS are the same as the true K_m values (that is, the value

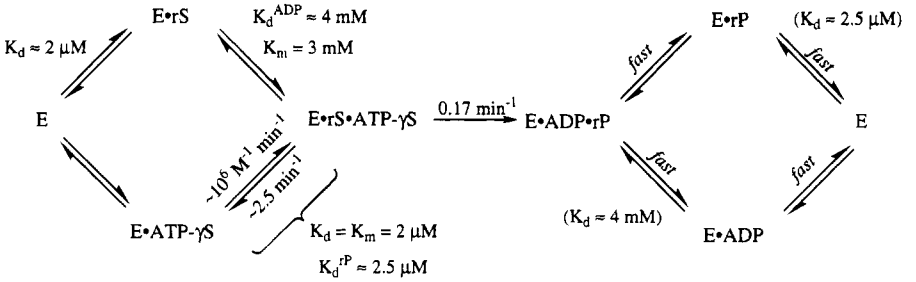

FIGURE 11: Model kinetic and thermodynamic scheme for the reaction catalyzed by the class I polynucleotide kinase. Values in parentheses are assumed, on the basis of a random binding mechanism and on the assumption that there is no synergy of binding between substrates and products.

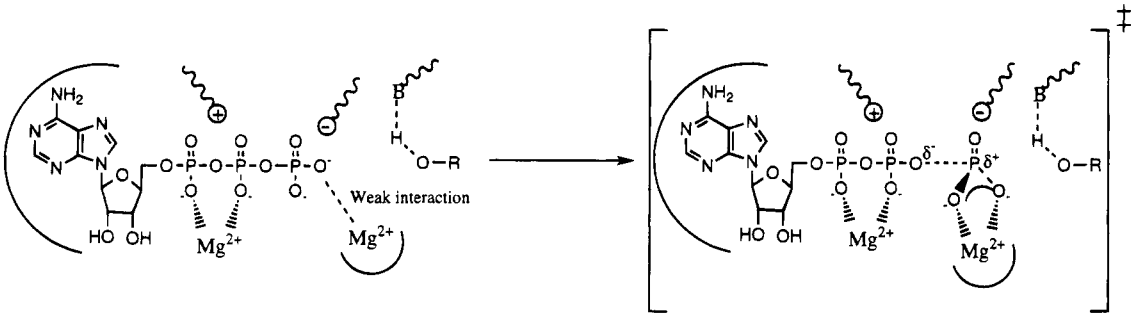

FIGURE 12: Several possible ways in which a biocatalyst could stabilize a dissociative transition state for a phosphotransfer reaction with ATP•Mg^{2+} (Herschlag & Jencks, 1990). The enzyme could stabilize the developing negative charge on the leaving group via an electrostatic interaction. Substrate destabilization could occur if a negative charge is placed near the γ-phosphate in the ground state. The charge repulsion would be partially relieved in the less negatively charged metaphosphate-like transition state. Since the bond length between the β- and γ-phosphates increases as the transition state is achieved, the enzyme could also position a positive charge such that it interacts more strongly with the negatively charged oxygens in the transition state than in the ground state ("strain"). Specific contacts that stabilize the trigonal bipyramidal transition state relative to the tetrahedral ground state would have a similar effect ("geometric stabilization"). Proximity and orientation effects will almost certainly play a major role in catalysis. These general mechanisms could also be employed in reactions involving phosphomonoesters. Any of these mechanisms could be utilized by the class I kinase ribozyme and are shown merely to illustrate possibilities for the enzymatic stabilization of a dissociative transition state.

with saturating second substrate). We have also shown that the oligonucleotide substrate can bind productively to the free ribozyme. In the absence of synergistic effects, the only way that substrate binding could be ordered is if ATP(-γS) must bind after rS for steric reasons (i.e., binding of ATP blocks part of the entrance to the rS binding site). The fact that no substrate inhibition by ATP or ATP-γS is seen suggests that this is not the case.

Our data are also most consistent with rate-limiting chemistry, in which the (thio)phosphate group is transferred via a dissociative, metaphosphate-like transition state (Figure 12). Both the pH independence of the reaction and the fact that ATP reacts more slowly than ATP-γS, with the entire effect in k_{cat}, suggest that chemistry is rate-limiting, although a slow conformational change whose rate is affected by the presence or absence of a terminal thiophosphate cannot be completely ruled out. It is also possible that the rate-limiting step changes from a slow conformational change to chemistry when the substrate is changed from ATP-γS to ATP. This possibility would require, however, that both of these steps be pH independent.

A Floppy Active Site? We have presented evidence that significant ordering of the ribozyme−substrate complex is required to achieve the transition state of the reaction. This is also the case for the ligation reaction catalyzed by the hammerhead ribozyme (Hertel & Uhlenbeck, 1995). The possibilities that both of these ribozymes are floppy in the ground state and that the transition-state-stabilizing structures of the ribozymes are only attained upon freezing of degrees of freedom in the RNA are somewhat daunting. Most structure-probing experiments look only at the ground-state structure of the ribozyme and substrates. Thus, if the ground-state structure(s) of the ribozyme is very dissimilar from that of the transition-state-stabilizing structure, and if the transition-state-stabilizing structure is attained only fleetingly during the course of normal thermal fluctuations in the ribozyme, it will be difficult to learn much about the structure of the ribozyme that specifically stabilizes the transition state of the reaction by direct physical studies. This problem occurs for at least one protein enzyme, tyrosyl-tRNA synthetase, in which one loop becomes rigidly structured only in the transition state (First & Fersht, 1993).

Both the hammerhead (~40 nucleotides) and class I kinase ribozymes (~80 nucleotides) are small, and this may be part of the reason that their ground states are floppy. Larger, more highly structured ribozymes can pay for the cost of attaining an ordered structure with the enthalpy from intramolecular contacts. For example, ribozymes such as the group I and group II introns are believed to use structures outside of the catalytic core as a scaffold that stabilizes the correct (i.e., most active) transition-state-stabilizing structure (Jaeger et al., 1991; Michel & Westhof, 1990). Eckland et al. (1995) have been able to evolve a ligase ribozyme to be 3 orders of magnitude more catalytically efficient than the original ribozyme. Many, if not most, of the mutations that produced increased activity appear to stabilize the catalytically competent structure of the ribozyme rather than to introduce new catalytic groups, suggesting that the original ribozyme may be either floppy or exists in a number of differently folded structures, only one of which is active.

The problem of stabilizing a particular folded structure may therefore represent an argument in favor of using long random pools for *in vitro* selection, in that more complex structures, capable of fixing a larger number of catalytically important groups in space, may be isolable. This is not to say, however, that the catalytic core of efficient ribozymes is necessarily large. Rather, with a long pool, once a catalytic core has been found, extraneous bits of sequence can be recruited for use in making the catalytically active core structure more rigid.

Associative vs Dissociative Phosphotransfer. Our results are consistent with a dissociative mechanism for phosphate transfer from ATP(-γS) to the 5′-hydroxyl of the substrate oligonucleotide. It has been argued that it is hard to imagine how an enzyme could specifically stabilize a dissociative transition state other than by destabilizing the charge on the γ-phosphate in the ground state (Hassett et al., 1982). However, a number of mechanisms have been proposed whereby an RNA or a protein could position catalytic groups to accelerate the rate of a dissociative phosphotransfer (Herschlag & Jencks, 1990; Figure 12). It is not yet clear which, if any, of these modes of transition state stabilization the class I kinases employ. However, our data appear to rule out substrate destabilization as a means of catalysis. Proximity and orientation are likely major factors in catalysis.

Associative and dissociative pathways for phosphotransfer reactions are really extremes of a continuum of possible mechanisms (Herschlag & Jencks, 1987; Westheimer, 1981, and references therein), and even mechanisms that are dissociative in nature probably go through a metaphosphate-like transition state rather than a true metaphosphate intermediate (Herschlag & Jencks, 1989). Thus the absence of racemization or positional isotope exchange in a phosphotransfer reaction, especially an enzyme-catalyzed reaction in which the substrates remain bound in the enzyme's active site during the course of the reaction, cannot be taken as evidence against a dissociative mechanism (Herschlag & Jencks, 1990; Westheimer, 1981, and references therein).

There is considerable evidence that the hydrolysis of ATP takes place in solution via a dissociative transition state (Miller & Westheimer, 1966; Ramirez et al., 1980; Admiraal & Herschlag, 1995). One might therefore expect a newly evolved enzyme to utilize this mechanism when accelerating the rate of ATP hydrolysis or other analogous reactions (e.g., transferring the γ-phosphate from ATP to the 5′-hydroxyl of an oligonucleotide). Assuming that the particular type of biopolymer in question (i.e., protein, RNA, etc.) is capable of stabilizing a dissociative transition state equally as well as an associative one, then it would take less energetic stabilization to produce a given rate enhancement for the dissociative pathway (the one that occurs in solution) than it would to change the mechanism by stabilizing this new higher energy transition state. Reactions catalyzed by most enzymes, especially primitive ones such as those isolated by *in vitro* selection and evolution, should by this reasoning have a transition state similar to that of the uncatalyzed reaction (Herschlag & Jencks, 1990). In recent years a number of groups have provided evidence that several protein enzyme-catalyzed phosphoryl transfer reactions proceed via a dissociative transition state (Halkides et al., 1991; Jones et al., 1991; Hollfelder & Herschlag, 1995; Weiss & Cleland, 1989; Westheimer, 1981, and references therein), although there is still considerable controversy on this topic.

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