A Positive Entropy Change for Guanosine Binding and for the Chemical Step in the *Tetrahymena* Ribozyme Reaction†

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ABSTRACT: The ribozyme derived from the group I intron of *Tetrahymena thermophila* binds an exogenous guanosine nucleotide, which acts as the nucleophile in the sequence-specific cleavage of oligonucleotides. By examining the temperature dependence of the reaction under conditions where $K_m = K_d$, we conclude the following: (1) Guanosine 5′-monophosphate (pG) binds to the closed ribozyme−oligonucleotide substrate complex with a positive entropy change ($\Delta S°' = +23$ eu) and an enthalpy change ($\Delta H°'$) close to zero. This is contrary to the expectation that binding would cause increased order (negative $\Delta S°$) and be driven by a negative $\Delta H°$. (2) Inosine and 2-aminopurine riboside, each lacking two hydrogen-bonding moieties relative to guanosine, also bind with a positive entropy value and an unfavorable (positive) $\Delta H°'$. From this result, we suggest that the hydrogen-bonding moieties make an enthalpic contribution to guanosine binding overcoming an intrinsic unfavorable ΔH. (3) At 0 °C, there is equally tight binding of pG in the presence and absence of oligonucleotide substrate bound to the ribozyme. Thus, energetic interactions responsible for the thermodynamic coupling between pG and oligonucleotide substrate binding seen at higher temperatures are indirect. (4) The activation barrier of the chemical step is stabilized by a positive $\Delta S^{\ddagger}$ (+31 to 39 eu). This stabilization is seen in four reactions using substrates with two different leaving groups in the presence and absence of pG, suggesting that the entropic contribution is inherent to the active site. The positive ΔS values for the chemical step and for the binding of pG can be explained by a conformational change or release of water. Thus, although hydrogen bonding contributes to binding of nucleotides to this RNA enzyme as previously thought, it is these other events which produce a positive ΔS that provide the energetic driving force for binding.

Similar values for the dissociation constant of guanosine from the *Tetrahymena* L-21 *Sca*I ribozyme (E)[1] have been reported at significantly different temperatures (McConnell *et al.*, 1993; Moran *et al.*, 1993). Since temperature-independent binding indicates a negligible enthalpy change ($\Delta H° \sim 0$), it would seem that a positive entropy change ($\Delta S°$) drives the binding of the guanosine nucleotide. Because the binding of a small molecule causes a system to lose translational and rotational degrees of freedom (negative $\Delta S°$), other events that increase disorder in the system would therefore be implicated. By measuring the binding of guanosine 5′-monophosphate (pG) and other guanosine analogs over a 60-deg temperature range, we give support for this conjecture and test its implications. Further, this study provides additional evidence that the cooperativity between oligonucleotide substrate and guanosine binding is due to an indirect interaction. In the process of measuring the temperature dependence of K_m(pG), the thermodynamic parameters of activation of the guanosine-dependent and independent reactions are also determined.

The L-21 *Sca*I ribozyme, a truncated form of the group I intron from *Tetrahymena thermophila*, catalyzes an endonuclease reaction analogous to the first step of self-splicing (eq 1). Previous studies of the L-21 *Sca*I ribozyme have

$$\underset{(S)}{\text{CCCUCUpA}} + \text{G} \rightleftharpoons \underset{(P)}{\text{CCCUCU}} + \text{GpA} \qquad (1)$$

produced a kinetic and thermodynamic framework for studying the reaction pathway (Cech *et al.*, 1992, 1993). By manipulating the concentration and chemical structure of individual components, detailed questions can be asked about the binding of guanosine and the chemical step.

We previously established conditions where the Michaelis constant for guanosine, K_m(G), in the ribozyme reaction is equal to the equilibrium dissociation constant, K_d(G) (McConnell *et al.*, 1993). In addition, the observed rate constant at saturating guanosine (E·S·G) is the rate constant of the chemical step, k_c(G) (eq 2). Thus, in the present study we

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[1] Abbreviations: E, *Tetrahymena* L-21 *Sca*I ribozyme; S, oligonucleotide substrate whose identity depends on the experiment; $(E·S)_c$, closed complex in which substrate is bound and docked in the ribozyme active site; I, inosine; pG, guanosine 5′-monophosphate; 2AP, 2-aminopurine riboside; $k_c(-G)$, the rate constant of the chemical step in the absence of added guanosine or pG; k_c(pG), the rate constant of the chemical step at saturating [pG]; IGS, internal guide sequence; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Nuc, a G analog nucleophile. $\Delta H°'$ and $\Delta S°'$ represent values measured under the reaction conditions by the methods herein, and the symbols $\Delta H°$ and $\Delta S°$ represent the true state functions; $\Delta G^{\ddagger}$, $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ represent values for reaching the transition state of the reaction.

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$$E{\cdot}S + G \overset{K_d(G)}{\rightleftharpoons} E{\cdot}S{\cdot}G \xrightarrow{k_c(G)} E{\cdot}P{\cdot}GA \qquad (2)$$

are able to determine both $K_d(G)$ and $k_c(G)$ values in the same experiment and evaluate how each is affected by temperature.

The guanosine binding site is located predominantly in paired region P7 of the ribozyme [Figure 1 (Michel *et al.*, 1989; Been & Perrotta, 1991; Yarus *et al.*, 1991; Legault *et al.*, 1992)]. In the model of Figure 1B, the exogenous G forms a base triple with the G264•C311 base pair and makes additional interactions such that the G is tilted relative to the plane of the base pair. Stacking interactions (not shown) have also been proposed to be important for binding in the G-site (Yarus & Majerfeld, 1992). By looking at the K_m for inosine and 2-aminopurine, this study will distinguish how changing the hydrogen-bonding interactions in the guanosine binding site affects nucleotide binding versus the chemical step. Most important for this study, the temperature dependence of binding these guanosine analogs will provide the thermodynamic contribution of hydrogen bonding in the G-site.

Previously, the $\Delta S^{\ddagger}$ for reaching the transition state of the chemical step in the guanosine-independent reaction was determined to be positive (Legault *et al.*, 1992). However, it was unclear whether this observation was specific to the guanosine-independent reaction that was studied or was a general characteristic of the ribozyme's active site. Here, the temperature dependence of cleavage of two different substrates in the guanosine-dependent and independent reactions expands our understanding of this initial observation.

The establishment of a positive ΔS for the binding of pG and the chemical step contribute to a better understanding of molecular recognition and catalysis by RNA.

MATERIALS AND METHODS

L-21 ScaI Ribozyme. Ribozyme (E) was prepared by transcription of *Sca*I-cut pT7L-21 DNA template using T7 RNA polymerase with purification by polyacrylamide gel electrophoresis and size exclusion chromatography (Zaug *et al.*, 1988). The ribozyme concentration was determined spectrophotometrically (Zaug *et al.*, 1988).

Oligonucleotide Substrates and Products. Oligonucleotides were synthesized chemically on an Applied Biosystems (ABI) Model 380B DNA synthesizer as described (Wu *et al.*, 1989; Scaringe *et al.*, 1990) using phosphoramidites (ABI, American Bionetics, Milligen Biosystems, or Glen Research). Phosphorothioate-containing oligonucleotides were either used as a mixture of the R_P and S_P isomers or used as the isolated R_P isomer after purification by HPLC, essentially as described (Slim & Gait, 1991). Oligonucleotides were 5′ end-labeled by treatment with T4 polynucleotide kinase (New England Biolabs) and [γ-^{32}P]ATP (New England Nuclear) and purified by electrophoresis on a 20% polyacrylamide/7 M urea gel followed by a 24% polyacrylamide gel without urea. Oligonucleotides were 3′ end-labeled by treatment with poly(A) polymerase (U.S. Biochemicals) and cordycepin [α-^{32}P]triphosphate (New England Nuclear) and purified as above. Concentrations of labeled oligonucleotides were estimated on the basis of specific activity.

G Analogs. Guanosine 5′-monophosphate (pG) and inosine (I) were purchased from Chemical Dynamics. Because of the insolubility of the guanosine nucleoside at low temperatures (McConnell *et al.*, 1993), the more soluble nucleotide, pG, was used to obtain the binding data. 2-Aminopurine riboside (2AP) was synthesized in one step from 6-mercaptoguanosine (Sigma) as described (Fox *et al.*, 1957) and purified by preparative thin-layer chromatography (90:10 1-propanol/NH_4OH; 2AP $R_f = 0.26$, 6-mercaptoguanosine $R_f = 0.11$, G $R_f = 0.07$). 2AP gave a single 3′-product after ribozyme reaction that migrated faster than GA^*A_H in 20% polyacrylamide/7 M urea gel electrophoresis. Characterization by UV and ^{1}H NMR spectroscopy and fast atom bombardment mass spectroscopy (M + 1 = 268) confirmed the purified compound was 2AP (Nair *et al.*, 1986).

Kinetics. Single-turnover reactions were performed with less than 5 nM ^{32}P-radiolabeled oligonucleotide and excess ($\geq$10-fold) ribozyme. Reactions were carried out over a temperature range from 0 to 60 °C in 50 mM Mes, sodium salt, at pH 7.0 or 5.5. The pH values were determined at 25 °C. Due to the temperature dependence of the pK_a for Mes, the actual pH of the pH 7.0 buffer ranged from 6.6 (60 °C) to 7.3 (0 °C) and the actual pH of the pH 5.5 buffer ranged from 5.1 (60 °C) to 5.8 (0 °C) [0.011 ΔpK_a/°C for Mes (Good *et al.*, 1966)]. No correction for the change of pH has been made in the rate constants presented; however, thermodynamic values derived from these rate constants were subsequently corrected for the pH dependence of the buffer. Ribozyme was preincubated at 50 °C in the presence of buffer and 10 mM Mg^{2+} for 20 min to allow formation of a single active species ($\geq$90%) of ribozyme, as previously described (Herschlag & Cech, 1990; McConnell *et al.*, 1993). For reactions at temperatures other than 50 °C, the samples were subsequently incubated at the desired temperature for 5 min. Reactions were initiated by simultaneous addition of labeled oligonucleotide and pG. Typically, six aliquots (~2 μL each) were removed from a reaction mixture (20 or 40 μL) and quenched with 2−3 volumes of stop buffer containing 80% formamide, 50 mM EDTA, 0.01% bromophenol blue, 0.01% xylene cyanol, 0.05% orange G, and 2 mM Tris−boric acid, pH 7.5. Reaction products were separated on 20% polyacrylamide/7 M urea gels. When reactions were followed for more than 2 h, tubes were kept submerged and/or centrifuged periodically to prevent concentration of the sample by evaporation. The fraction of substrate remaining relative to the sum of the substrate and the product at each time point was quantitated using a PhosphorImager (Molecular Dynamics).

Determining K_m(Nuc) Values. Typically, for each nucleophile (Nuc) first-order kinetics were observed for approximately 3 half-lives with end points of 95% reacted at 50 and 40 °C, 90% at 30 and 20 °C, and 85% at 10 and 0 °C, indicating a single active species ($\geq$85%) of oligonucleotide substrate. Values for the Michaelis constant for each nucleophile, K_m(Nuc), were obtained from a plot of the dependence of the observed rate constant on the Nuc concentration and fit to the equation of $k_{obs} = k_c(\text{Nuc})[\text{Nuc}]/\{K_m(\text{Nuc}) + [\text{Nuc}]\}$, as previously described (McConnell *et al.*, 1993).

Pulse−Chase Experiments. Ribozyme was preincubated in 10 mM Mg^{2+} and 50 mM Mes, pH 7.0, at 50 °C for 20 min and 0 °C for 5 min as described above. To compare the dissociation rate constant of an oligonucleotide and the rate of the chemical step, 5 and 3000 nM ribozyme was

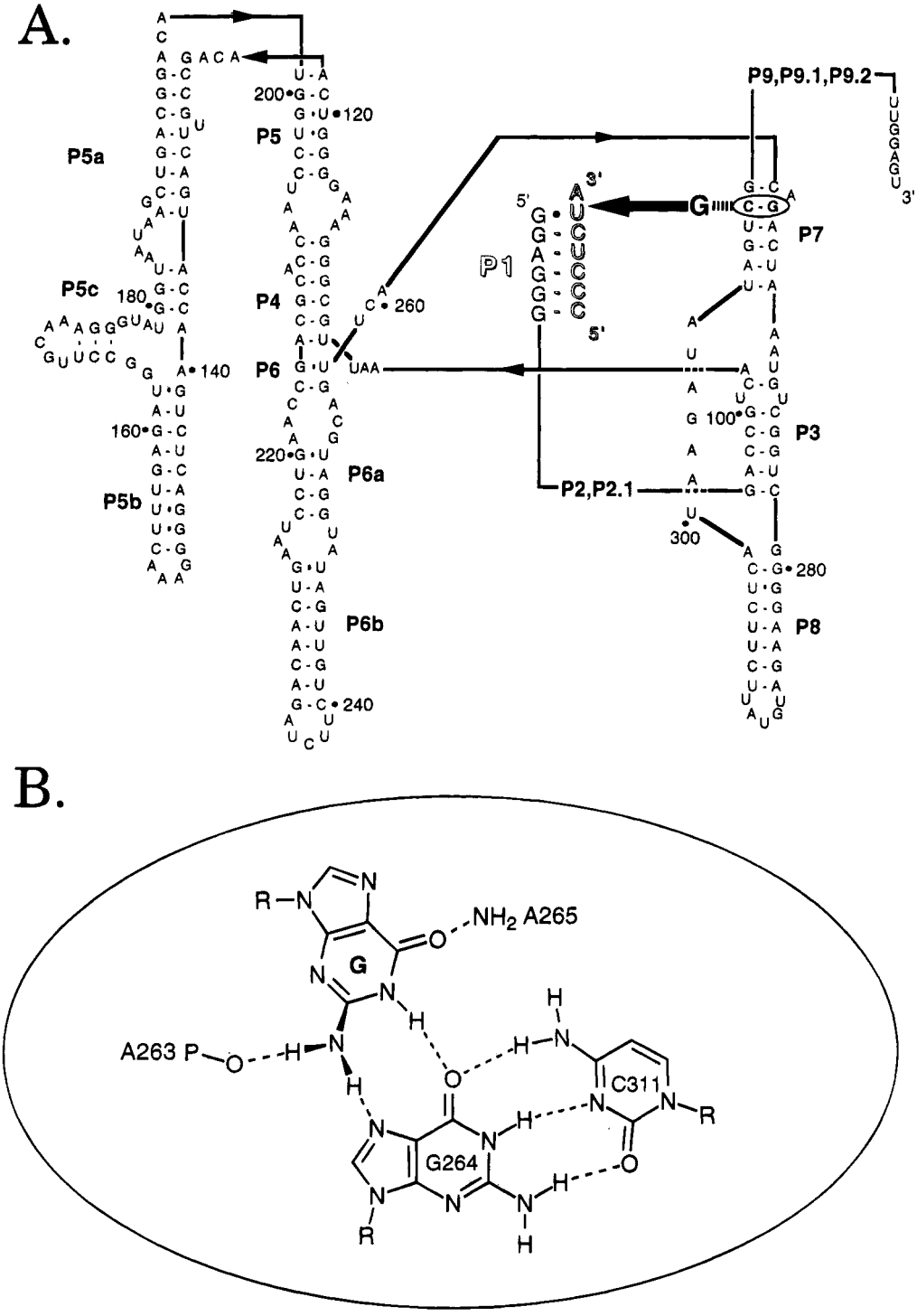

FIGURE 1: Ribozyme secondary structure and G binding site. (A) The secondary structure of the L-21 *Sca*I ribozyme in the new format (Cech *et al.*, 1994; Salvo & Belfort, 1992) is shown with the G264•C311 base pair of the G-site in P7 (ellipse). The bound G (bold) is positioned for nucleophilic attack on the oligonucleotide substrate CCCUCUA (open letters) at the phosphodiester bond between UA. The substrate is bound by the IGS forming the P1 helix via Watson–Crick base pairs except for the phylogenetically conserved G•U wobble pair at the cleavage site. Omitted for simplicity are the secondary structures of the paired regions P2, P2.1, P9, P9.1, and P9.2. (B) Model for G binding showing the N1 imino and N2 exocyclic amino groups of the exogenous G (bold) hydrogen bonding with the O6 and N7 positions, respectively, of the guanine base of G264 in P7 (Michel *et al.*, 1989). Also shown are the O6 and the N2 positions of the exogenous guanosine interacting with the 6-amino group of A265 and the phosphate of A263, respectively. In this axial III model (Yarus *et al.*, 1991), exogenous guanosine is not parallel to the plane of the paper. The 6-amino group of A265 and the phosphate of A263 should be envisioned below and above the plane of the paper, respectively.

allowed 10 min to completely bind labeled CCCUCUA and C(dU)CUA, respectively. The sample was chased by diluting 100-fold in chase solution containing 10 mM Mg^{2+}, 50 mM Mes, pH 7.0, 2 mM pG, and 10 nM G264A•C311U mutant L-21 ribozyme (AU mutant ribozyme) (Legault *et al.*, 1992). The AU mutant ribozyme binds oligonucleotide substrates tightly but reacts slowly (Pyle *et al.*, 1990; Legault *et al.*, 1992). In the reaction with C(dU)CUA, although the AU mutant ribozyme is not in excess of wild-type ribozyme (10 nM AU mutant L-21:30 nM wild-type L-21), the 100-

Table 1: Kinetic Parameters for Ribozyme Reactions at Different Temperatures[a]

temp (°C)	K_m(pG), E (mM)	K_m(pG), $(E{\cdot}S)_c$ (mM)	k_c(pG), CCCUCUA (min^{-1})	k_c(−G), CCCUCUA (min^{-1})	k_c(pG), CCCUC(dU)A (min^{-1})	k_c(-G), CCCUC(dU)A (min^{-1})	k_{ox}/k_{Rp}[c]
0	0.04	0.049	0.036	2.9×10^{-5}	1.2×10^{-5}		7
10	0.11	0.060	0.74	2.3×10^{-4}			5
20	0.15[d]	0.047[d]	3.4	5.1×10^{-3}	2.2×10^{-3}	5.8×10^{-5}	4
30	0.30	0.035	10[b]	0.011	0.012	4.4×10^{-4}	4
40	0.34	0.050	25[b]	0.05	0.035	2.8×10^{-3}	4
50	0.25	0.059	80[b]	0.2	0.11	9.8×10^{-3}	4
60	0.12	0.030	240[b]	1.1	0.35	4.9×10^{-2}	4

[a] Except where noted, K_m and k_c(pG) values were determined by a least squares fit to the data. The values for k_c(−G) were determined directly from exponential fits to the loss of substrate or linear fits for the initial rates of product formation. Error was determined from the variation of multiple measurements of each value. Error was ≤20% for each kinetic constant measured. To obtain values for K_m(pG) for reaction with $(E{\cdot}S)_c$, saturating ribozyme (100 nM at 0−50 °C, 3000 nM at 60 °C) was used with respect to two substrates, CCCUCUA for temperatures 0−20 °C and CCCUC(dU)A for temperatures 30−60 °C. To obtain values for K_m(pG) for reaction with free E, subsaturating concentrations of ribozyme (10, 20, and 50 nM at 0 °C, 100 and 200 nM at 10 and 20 °C, 20 nM at 30 and 40 °C, 200 nM at 50 °C, 300 nM at 60 °C) were used with respect to two different substrates, C(dU)CUA for temperatures from 0−20 °C and d(CCCUC)Ud(AAAAA) for temperatures 30−60 °C. All data in this table were collected at pH 7.0 and 10 mM Mg^{2+}. [b] These values were determined as the product of K_m(pG) for reaction with E·CCCUC(dU)A and $(k_{cat}/K_m)^{pG}$ for reaction with E·CCCUCUA. [c] These values represent the ratio of the observed rate constant of the all phosphate substrates [d(CCCUC)Ud(AAAAA), C(dU)CUA, CCCUCUA, and CCCUC(dU)A] over the single R_P isomer phosphorothioate substrates [d(CCCUC)U_{Ps}-d(AAAAA), C(dU)CU_{Ps}A, CCCUCU_{Ps}A, and CCCUC(dU)$_{Ps}$A]. The values represent the average value over the pG concentration range extending from subsaturating to saturating [pG] with a variation in values at each temperature of ±2. [d] Not shown are K_m(pG) values for reaction with E + d(CCCUC)Ud(AAAAA) [K_m(pG) = 0.2 ± 0.04 mM, 20 nM E, 20 °C] and for reaction with E·CCCUC(dU)A [K_m(pG) = 0.04 ± 0.01 mM, 100 nM E, 20 °C].

fold dilution is a sufficient chase. The chase is sufficient because after the oligonucleotide dissociates the second-order rate constant for the reaction of C(dU)CUA is slow (5×10^4 M^{-1} min^{-1}). However, the AU mutant ribozyme (~200 × [wild-type L-21]) was needed to effectively bind all dissociated CCCUCUA and prevent it from reacting. At varying times, aliquots were removed and quenched in stop buffer. Lack of dilution and/or absence of competitor mutant ribozyme or unlabeled oligonucleotide resulted in the normal rate of chemistry.

To measure the association rate constant of CCCUCUA, 5 nM L-21 was incubated with labeled oligonucleotide. At varying times after this initiation, aliquots were diluted 3-fold into a chase solution of excess unlabeled CCCUCU (200 nM, ≥100[E]), 2 mM pG, 10 mM Mg^{2+}, and 50 mM Mes, pH 7.0, to prevent further binding of CCCUCUA while allowing the bound portion to react. After 2 min the chase reaction was accelerated by incubation at 50 °C for 2 min before the reaction was quenched. In any of the pulse−chase experiments, reversing the order of addition of chase solution and the labeled oligonucleotide resulted in negligible reaction (<5% over 2 h).

Determining $\Delta H°'$ and $\Delta S°'$ Contributions to G Analog Binding. By measuring the binding constant for pG, or any other guanosine analog, over a range of temperature (T), one can determine the $\Delta H°'$ and $\Delta S°'$ for binding using the van't Hoff equation (eq 3). This determination can be understood

$$\ln K_d(\text{pG}) = \Delta H°'/RT - \Delta S°'/R \quad (3)$$

by combining eqs 4 and 5 and solving for ln K_d(pG). The state equation (eq 4) relates a change in free energy ($\Delta G°$) to changes in enthalpy and entropy, and eq 5 relates free energy with the binding constant of pG.

$$\Delta G° = \Delta H°' - T\Delta S°' \quad (4)$$

$$\Delta G° = -RT \ln K_a(\text{pG}) = RT \ln K_d(\text{pG}) \quad (5)$$

RESULTS

Determining the Temperature Dependence of K_m(pG). To obtain thermodynamic values for the binding of the guanosine cofactor to the L-21 *Sca*I ribozyme, K_m(pG) was determined at 10-deg increments from 0 to 60 °C (Table 1). The 60-deg temperature range was chosen so the data would be sensitive to any nonlinearity in ΔG as a function of temperature. To measure each K_m value, the observed rate is plotted as a function of the concentration of the G analog (Figure 2). At high concentrations of the G analog the observed rate constant becomes independent of concentration, and a K_m value can be determined.

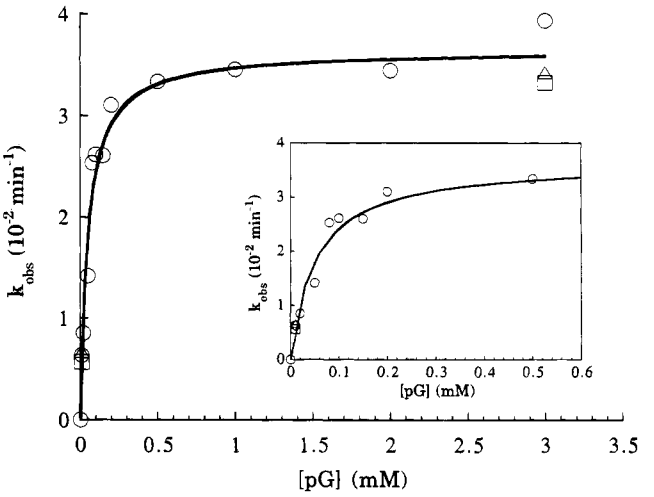

FIGURE 2: Example of determination of K_m(pG): reaction with E·S at 0 °C. The rate of cleavage of the oligonucleotide substrate CCCUCUA by saturating ribozyme (○) (100 nM) at 0 °C, pH 7.0, and 10 mM Mg^{2+} is shown. The ribozyme concentration was varied from (△) 50 to (□) 200 nM L-21 at 0.01 and 3 mM pG. The values of the fit shown (solid line) are K_m(pG) = 0.049 ± 0.009 mM and k_c = 0.036 ± 0.003 min^{-1}. The inset shows the lower [pG] data in detail.

For each K_m value at least two tests were performed to confirm that K_m(pG) is equal to K_d(pG). First, the ribozyme concentration was varied at low and high pG concentrations, demonstrating the predicted ribozyme concentration independence of reactions involving E·S and the linear dependence of those involving free E. Therefore, there was no change in the molecularity of ribozyme over the pG

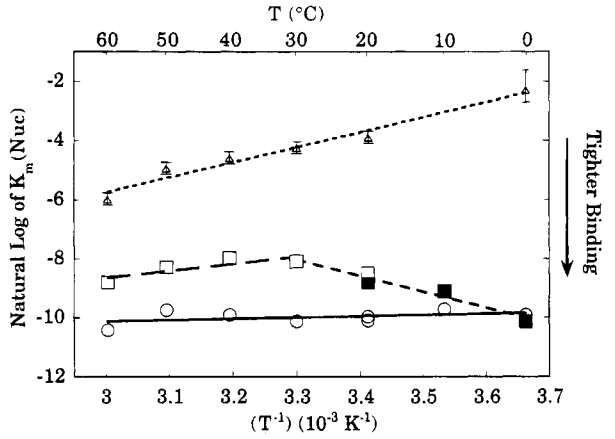

FIGURE 3: van't Hoff plot: zero slope for pG binding to E·S, nonlinear function for pG binding to free E, and positive slope for I binding to E·S. To obtain values for K_m(pG) (○) and K_m(I) (△) for reaction with E·S, saturating ribozyme (see Table 1; concentrations for I were the same as for pG) was used with respect to two substrates, CCCUCUA for temperatures 0−20 °C and CCCUC(dU)A for temperatures 20−60 °C. To obtain values for K_m(pG) for reaction with free E, concentrations of ribozyme (see Table 1) were subsaturating with respect to the substrate, C(dU)CUA (■) for temperatures 0−20 °C or d(CCCUC)Ud(AAAAA) (□) for temperatures 20−60 °C. Two different substrates were used for studies of binding pG to E because binding of d(CCCUC)Ud(AAAAA) becomes tighter at lower temperatures. The identity of the substrate does not affect K_m(pG) for reaction with free E since the dissociation rate constants of the substrates are fast relative to the chemical step and [E] is below the K_d for each substrate: E + S + pG ⇌ E·G + S → E + GA_n + P (n = 1 or 5). All data on this graph were collected at pH 7.0 and 10 mM Mg^{2+}. Error bars represent the maximum range of K_m values determined in independent experiments. For the I data shown, this uncertainty was ±20% in the K_m(I) values, except for the data point at 0 °C where the error was 50%. For all other data shown, the error bars are within the confines of the symbols.

concentration range. Second, to indicate that the chemical step was rate limiting, oligonucleotide substrates with a phosphorothioate (Ps) at the cleavage site [CCCUCU$_{Ps}$A and CCCUC(dU)$_{Ps}$A] were shown to react with an appropriately reduced rate constant throughout the pG concentration range (Table 1). Model studies have shown that cleavage of a phosphorothioate diester is 2−8-fold slower than that of the analogous phosphate ester (Herschlag *et al.*, 1991). If the dissociation rate constant of pG were slower than the rate constant for the chemical step, then the phosphorothioate effect would not be seen and K_m(pG) would be greater than K_d(pG). Also, the presence of a phosphorothioate effect implies that there is no change in the rate-limiting step, which would make K_m(pG) less than K_d(pG). Thus, the presence of a phosphorothioate effect tests criteria we previously set for K_m(pG) to be equal to K_d(pG) (McConnell *et al.*, 1993).

van't Hoff Plots of K_m(pG) and K_m(I) at pH 7.0 and 10 mM Mg^{2+}. The natural log of each K_m value (Table 1) is plotted as a function of the inverse temperature (Figure 3). The values for K_m(pG) and K_m(I) in reactions with E·S show a good linear dependence with temperature. The values for K_m(pG) show a slope close to 0, whereas the values for K_m(I) display a positive slope. These slopes are directly proportional to $\Delta H^{\circ\prime}$ (eq 3) and are shown in Table 2. Although slopes may be influenced by the 0 °C point, exclusion of any single point, including 0 °C, did not change the conclusions (see error limits, Table 2). The average change in entropy is positive for both pG and I binding to the E·S complex.

Table 2: Thermodynamic Parameters of the Binding of pG and I to E·S at pH 7.0 and 10 mM Mg^{2+} [a]

nucleophile	K_m(Nuc) (mM)	$\Delta G^{\circ\prime}$ (kcal/mol)	$\Delta H^{\circ\prime}$ (kcal/mol)	$\Delta S^{\circ\prime}$ (eu)
pG	0.049	−6.2 ± 0.3	0.9 ± 1	23 ± 4
I	7	−3.2 ± 0.2	10 ± 1	42 ± 4

[a] $\Delta G^{\circ\prime}$ represents the value at 50 °C with error determined from the variation in K_m(pG) at 50 °C. The values $\Delta H^{\circ\prime}$ and $\Delta S^{\circ\prime}$ are determined using eq 3 and the linear fit, $y = mx + b$, of the data in Figure 3; $\Delta H^{\circ\prime} = mR$ and $\Delta S^{\circ\prime} = -bR$, where R is the gas constant. Errors in $\Delta H^{\circ\prime}$ and $\Delta S^{\circ\prime}$ are based on variation of fits to the data excluding any single point.

The binding of pG to the free ribozyme shows biphasic behavior over the 60-deg temperature range (Figure 3). From 30 to 60 °C, pG binds to the free ribozyme (E) 4 to 9 times more weakly than to the E·S complex, as documented at 30 °C previously (McConnell *et al.*, 1993). In this temperature range $\Delta H^{\circ\prime}$ and $\Delta S^{\circ\prime}$ are +4.8 kcal/mol and +31 eu, respectively. Below 30 °C, K_m(pG) for reaction with the free ribozyme becomes lower with decreasing temperature. Only at 0 °C does pG bind to the free ribozyme as tightly as it binds to the E·S complex. Although two different substrates were utilized in these experiments in order to follow the reaction over such a wide temperature range, note that the identity of the substrate does not affect K_m(G) for reaction with free E (see Figure 3 legend). The nonlinear behavior of pG binding to the free ribozyme suggests there is a temperature-dependent conformational change of the free ribozyme. This conformational change converts a form of the ribozyme that binds pG weakly to a form that binds pG tightly. This latter form likely resembles the (E·S)$_c$ complex, except that the substrate is not bound.

pG and Oligonucleotide Substrate Binding Are Thermodynamically Uncoupled in the Tight-Binding Form of the Free Ribozyme. For the free ribozyme at 0 °C, K_m(pG) is unaffected whether C(dU)CUA is bound or unbound (Figure 4). These K_m(pG) values are equivalent to K_m(pG) for reaction with E·CCCUCUA (Table 1). The reverse is also true; the K_m value of C(dU)CUA is unaffected by the presence of pG. Thus, unlike binding at 30 °C (McConnell *et al.*, 1993), at 0 °C there is no cooperativity between pG binding and oligonucleotide substrate binding.

To confirm that the K_m(pG) for reaction with free ribozyme and C(dU)CUA is equal to K_d(pG), K_m(pG) was measured when the chemical step was slowed by lowering the pH. The value for K_m(pG) changed only 2-fold, while the reaction rate decreased 37-fold over a 1.5 unit pH change [K_m(pG) = 0.075 mM and 0.040 mM (Table 1), and $(k_{cat}/K_m)^S$ = 1.2 × 10^3 M^{-1} min^{-1} and 4.4 × 10^4 M^{-1} min^{-1} at pH 5.5 and 7.0, respectively, for reaction of subsaturating E (30 nM) and C(dU)CUA at 0 °C, 10 mM Mg^{2+}]. At 30 °C a similar slight pH dependence for pG binding to E·CCCUC(dU)A has been observed [K_m(pG) = 0.047 mM at pH 7.0 (Table 1) and K_m(pG) = 0.090 mM at pH 5.5 (McConnell *et al.*, 1993)]. Previously, we showed that guanosine binding is pH independent from pH 5.5 to pH 7.0 (McConnell *et al.*, 1993). Thus, it appears that addition of a 5′-phosphate to guanosine (pG) produces a slight pH dependence in binding. It is noteworthy that it is at the higher pH where the 5′-phosphate is more negatively charged that binding is tighter to the polyanionic ribozyme.

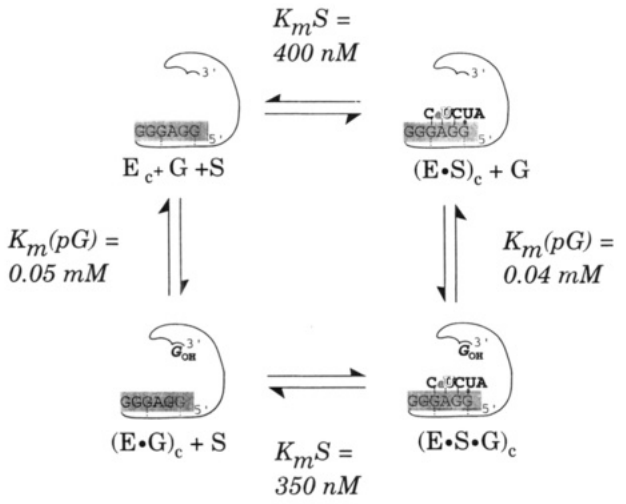

FIGURE 4: Equally tight binding of pG in the presence and the absence of C(dU)CUA at 0 °C. To measure K_m(pG) for reaction with E·S [where S = C(dU)CUA], saturating ribozyme concentrations (2000 and 3000 nM) were used at 0 °C, pH 7.0, and 10 mM Mg^{2+}. For the value of K_m(pG) for reaction with E, subsaturating ribozyme concentrations (10, 20, and 50 nM) were used. To measure K_m(S) [where S = C(dU)CUA] for reaction with E·pG, saturating concentrations of pG (1 and 3 mM) were used. For the value of K_m(S) for reaction with E, subsaturating concentrations of pG (0.010 and 0.020 mM) were used. Dotted lines between the IGS and the ribozyme indicate tertiary interactions (Strobel & Cech, 1993); the "closed" or tight-binding state of the free ribozyme (E_c) and of the ribozyme−G complex [$(E•G)_c$] is shown having made tertiary interactions characteristic of the $(E•S)_c$, but this particular structural model has not been directly tested.

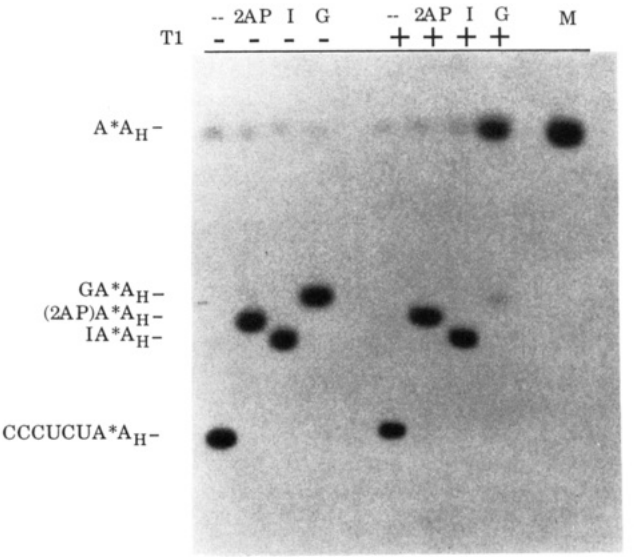

FIGURE 5: Product analysis demonstrates purity of G, I, and 2AP nucleophiles. CCCUCUA*A_H (where *A_H represents cordycepin label) was reacted in the presence of 100 nM ribozyme with 1 mM G, 5 mM I, and 10 mM 2AP at 50 °C, pH 7.0, and 100 mM Mg^{2+} for 5 min. The initial substrate and the reaction products were then each digested with 0.1 unit of T1 nuclease for 15 min at 50 °C. The A*A_H marker (M) was created by digesting CCCUCUA*A_H with RNase A [1 Kunitz unit (Sigma), 15 min at 50 °C in water]. Note that reacting E·CCCUCUA*A_H with pG (not shown) resulted in a product (pGA*A_H) that ran with faster mobility than CCCUCUA*A_H; no other bands were observed on the gel.

As a second test for the equivalence of K_m(pG) and K_d(pG) at 0 °C, pulse−chase experiments were performed to compare the rate constants for the chemical step with the dissociation rate constants of CCCUCUA and of C(dU)CUA. The value of K_m(pG) for reaction of the free ribozyme and C(dU)CUA would not equal K_d(pG) if the dissociation rate constant of C(dU)CUA were slow. In these pulse−chase experiments, the ^{32}P-labeled substrate bound to the ribozyme is challenged with saturating pG, a 10 nM quantity of the poorly reactive AU mutant ribozyme, and a 100-fold dilution (see Materials and Methods). The substrate either reacts with pG forming product [CCCUCU or C(dU)CU] or the substrate dissociates. If CCCUCUA dissociates, it is bound by (~200-fold) excess mutant ribozyme. If C(dU)CUA dissociates, it then reacts at its slow second-order rate constant [$(5 \pm 2) \times 10^4$ M^{-1} min^{-1}]). The pulse−chase experiments (data not shown) showed that at 0 °C, as at 50 °C (Herschlag & Cech, 1990), CCCUCUA reacts much faster than it dissociates. In contrast, C(dU)CUA dissociates much faster than it reacts, as predicted from its weak K_m value. The fast dissociation of C(dU)CUA suggests that the chemical step is rate limiting at all ribozyme concentrations and further supports the argument that K_m values for pG and C(dU)CUA shown in Figure 4 are equilibrium dissociation constants.

Additionally, the association rate constant of CCCUCUA with the ribozyme at 0 °C is $(1.3 \pm 0.3) \times 10^8$ M^{-1} min^{-1} with 85% of the substrate reacting in a single exponential as measured by a pulse−chase experiment. This value is the same as has been measured for matched RNA substrates at 30 and 50 °C (Herschlag & Cech, 1990; McConnell *et al.*, 1993). Also, multiple turnover experiments at 0 °C show a burst of product formation equal to 0.9[E]. The significance of these results is two part: (1) duplex formation is still rate limiting for CCCUCUA at 0 °C, and (2) at least 85% of the ribozyme is in an active conformation at 0 °C.

Reaction of Guanosine Analogs, I and 2AP. Although reactions of I and 2AP have been studied previously (Bass & Cech, 1984; Tanner & Cech, 1987; Michel *et al.*, 1989; Yarus *et al.*, 1991; Legault *et al.*, 1992), it was necessary to confirm the purity of these nucleophiles because a small contaminant of G (<1%) would skew the K_m values obtained. Although the 5′-product of the reaction, CCCUCU, is the same for all reactions, the 3′-product is dependent on the G analog used. In Figure 5, 3′-cordycepin-labeled substrate (CCCUCUA*A_H, where the asterisk represents the position of the ^{32}P-radiolabeled phosphate) is reacted with ribozyme in the presence of 2AP, I, and G. Reactions with 2AP, I, and (not shown) pG resulted in single products with no product comigrating with GA*A_H, as would result from a guanosine contamination. Ribonuclease T1 reactivity is sensitive to the carbonyl (O6), amino (N2), and imino (N1) substituents of G (Takashashi & Moore, 1982). As shown (Figure 5), the GA*A_H product is completely cleaved by ribonuclease T1, whereas (2AP)A*A_H and IA*A_H are insensitive to the same treatment. Thus, the observed K_m values for ribozyme reactions with each G analog are a result of reacting with that nucleophile and no other.

Removal of Hydrogen-Bonding Moieties from Guanosine Results in Unfavorable $\Delta H^{o\prime}$ for Binding. To firmly establish that hydrogen bonding provides an enthalpic contribution to the binding of guanosine, the temperature dependences of the binding of 2AP and I were compared to that of pG at pH 5.5 and 100 mM Mg^{2+} (Figure 6). The reaction conditions were changed from those used in Figure 3, because 2AP inhibited the reaction at high concentrations. Higher [Mg^{2+}] prevented inhibition by 2AP and lower pH provided conditions likely to result in K_m(Nuc) equaling

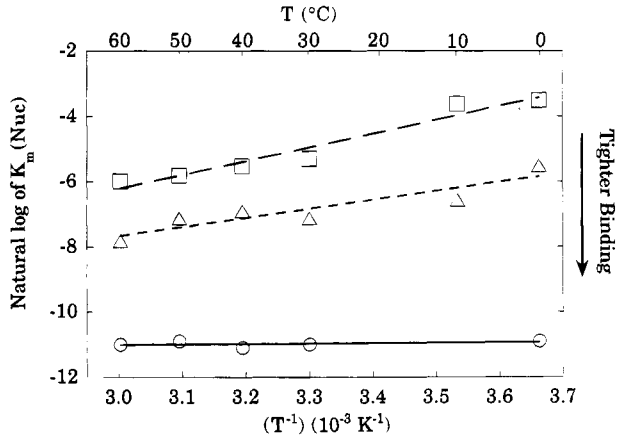

FIGURE 6: Removal of hydrogen-bonding groups from guanosine results in weaker binding and a positive $\Delta H°$. K_m values were measured for reaction of pG (○), 2AP (△), and I (□) with E·S, using saturating ribozyme (100−10000 nM) with respect to two substrates, CCCUCUA for temperatures 0−10 °C and C(dCC)-UC(dU)A for temperatures 30−60 °C. All data on this graph were collected at pH 5.5 and 100 mM Mg^{2+}. The error bars are within the confines of the symbols.

K_d(Nuc). Under these conditions, the phosphorothioate rate effect was present over the concentrations of G analogs used at all temperatures ($k_{ox}/k_{Rp} = 1.8-4$). This change in reaction conditions also provided the opportunity to test the generality of the thermodynamic measurements.

The substrate used for the higher temperatures for the experiments in Figure 6 was C(dCC)UC(dU)A instead of CCCUC(dU)A. This change was necessary because miscleavages at −3 U and −2 C were enhanced ~50 fold in the presence of 2AP. With the deoxyribonucleotide at the cleavage site, these miscleavage products were significant enough to complicate analysis of the results. Removal of the 2′-hydroxyl groups at the −4 and −5 positions of the oligonucleotide substrate weakens the docking of the P1 helix (see Figure 1A) into alternative conformations and reduces the rate of miscleavage (Herschlag, 1992). In binding in the correct conformation the 2′-hydroxyl groups at the −4 and −5 positions contribute <1 kcal/mol in ground-state binding and do not contribute anything additional to stabilization of the transition state of the chemical step (Bevilacqua & Turner, 1991; Pyle *et al.*, 1992; Herschlag *et al.*, 1993a).

At pH 5.5 and 100 mM Mg^{2+} the temperature dependences of the binding of pG, 2AP, and I to the E·S complex show good linear behavior (Figure 6). On the basis of van't Hoff plots, the slopes for I and 2AP binding are positive while the slope is 0 for pG binding (as was true for I and pG binding at pH 7.0 and 10 mM Mg^{2+}; Figure 3). Both I and 2AP bind more tightly at higher temperatures. Improved binding with higher temperatures indicates that the loss of the guanosine hydrogen-bonding moieties results in a loss in enthalpic binding even though there is no net enthalpic contribution to pG binding ($\Delta H° \approx 0$). Quantitation of the thermodynamic values for binding of each G analog is shown in Table 3. 2AP and I bind ~2 and 3 kcal/mol weaker than pG, respectively. The values for $\Delta H°'$ and $\Delta S°'$ are positive for 2AP and I binding.

While comparison of Tables 2 and 3 suggests that Mg^{2+} and pH do not greatly affect pG binding, there is some influence. The binding of pG tightens somewhat at higher Mg^{2+} ion concentration [K_m(pG) is 0.060 ± 0.01 and 0.020 ± 0.004 mM at Mg^{2+} concentrations of 10 and 100 mM, respectively, for reaction with E·CCUC(dU)A at pH 7.0, 50 °C]. Moreover, unlike G binding, pG binding is slightly pH dependent [K_m(pG) = 0.09 ± 0.01 and 0.04 ± 0.01 mM for reaction with E·CCUC(dU)A at pH 5.5 and 7.0, respectively, 10 mM Mg^{2+}, 30 °C; Table 1 and McConnell *et al.* (1993)]. This pH dependence may be minimized at high Mg^{2+} [K_m-(pG) = 0.017 and 0.014 mM at pH 5.5 and 7.0, respectively, 100 mM Mg^{2+}, 50 °C, for reaction with E·C(dCC)UC(dU)A]. Inosine binding shows almost no Mg^{2+} dependence at higher temperatures and perhaps a modest effect at lower temperatures [9 ± 3 and 8 ± 2 mM at 10 and 100 mM Mg^{2+}, respectively, for reaction with E·CCUC(dU)A at 50 °C, pH 7.0; 100 ± 50 and 40 ± 10 mM at 10 and 100 mM Mg^{2+}, respectively, for reaction with E·CCUCUA at 10 °C, pH 7.0]. The modest effect of the 5′-phosphate on pG binding may reflect the binding of a Mg^{2+} ion. Such a model is consistent with the weaker binding of pG at lower pH and the suppression of this pH effect at high Mg^{2+} concentration. The small difference in the thermodynamic values for pG binding between the high and low Mg^{2+} conditions suggests that it is unlikely that a Mg^{2+} interacting with the 5′-phosphate is responsible for the large positive $\Delta S°'$. Also, the thermodynamic values for I, which contains no 5′-phosphate, do not change between 10 and 100 mM Mg^{2+}.

Table 3: Thermodynamic Parameters of the Binding of pG, I, and 2AP to E·S at pH 5.5 and 100 mM Mg^{2+} [a]

nucleophile	K_m(Nuc) (mM)	$\Delta G°'$ (kcal/mol)	$\Delta H°'$ (kcal/mol)	$\Delta S°'$ (eu)
pG	0.018	−7.0 ± 0.2	0.2 ± 0.2	23 ± 3
I	3	−3.7 ± 0.3	8.4 ± 0.6	37 ● 3
2AP	0.8	−4.6 ± 0.3	5.5 ± 1.8	32 ± 6

[a] $\Delta G°'$ represents the value at 50 °C. Fits and error analysis were performed as described in Table 2 using values determined from Figure 6.

Thermodynamics of Reaching the Transition State of the Chemical Step. The Arrhenius plot in Figure 7 shows the temperature dependence of the rate constants for the chemical step in four ribozyme reactions, cleavage of bound CCCUCUA and CCCUC(dU)A in the presence or absence of pG. All sets of data are linear with a rate increase of (5 ± 2)-fold every 10 deg. Although 3′-product analysis shows that the G-independent reaction has multiple products including the expected hydrolysis product pA*A_H, they appear to behave as a single reaction if one observes the reaction with a 5′-labeled substrate.

The activation energies and thermodynamic values for reaching the transition state of the chemical step for these four reactions are shown in Table 4. In each case a positive $\Delta S^{\ddagger}$ stabilizes the transition state ($\Delta H^{\ddagger} > \Delta G^{\ddagger}$). At 50 °C, $T\Delta S^{\ddagger}$ is worth 10 ± 2 kcal/mol in free energy toward transition-state stabilization!

Changing the conditions from pH 7.0 and 10 mM Mg^{2+} to pH 5.5 and 100 mM Mg^{2+} affects the thermodynamics of the chemical step. At pH 5.5 and 100 mM Mg^{2+}, $\Delta S^{\ddagger}$ values for k_c(pG), k_c(I), and k_c(2AP) ($\Delta S^{\ddagger}$ = 14 ± 8, 15 ± 9, and 14 ± 8 eu, respectively) are smaller than those values shown in Table 4 for k_c(pG) of E·CCCUC(dU)A·pG. However, $\Delta G^{\ddagger}$ and $\Delta H^{\ddagger}$ are not greatly changed [$\Delta G^{\ddagger}$(50 °C) = 24.0, 24.3, and 23.9 kcal/mol and $\Delta H^{\ddagger}$ = 28 ± 3, 29 ± 3, and 29 ± 3 kcal/mol for k_c(pG), k_c(I), and k_c(2AP), respectively]. This difference in $\Delta S^{\ddagger}$ is not likely due to the difference in

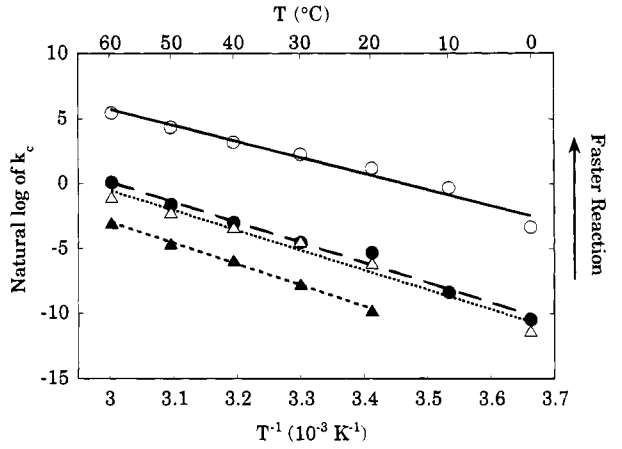

FIGURE 7: Temperature dependence of the chemical step. The rate constant at saturating pG, k_c(pG), for reaction of E·CCCUCUA·pG (○) and E·CCCUC(dU)A·pG (△) is plotted as a function of inverse temperature. The rate constant for reaction without added nucleophile, $k_c(-G)$, for reaction of E·CCCUCUA (●) and E·CCCUC(dU)A (▲) is also plotted. These data were obtained simultaneously with the K_m data. Note that, for the higher temperatures (°C), k_c(pG) for reaction of E·CCCUCUA·pG was too fast to measure directly, so it was calculated using the product of K_m(pG) for reaction with E·CCCUC(dU)A and $(k_{cat}/K_m)^{pG}$ for reaction with E·CCCUCUA (see Table 1). All data on this graph were collected at pH 7.0 and 10 mM Mg^{2+}. The error bars are within the confines of the symbols.

Table 4: Thermodynamic Parameters of Activation for the G-Dependent [k_c(pG)] and for the G-Independent Reactions [$k_c(-G)$][a]

rate constant	E_a (kcal/mol)	$\Delta G^{\ddagger}$ (kcal/mol)	$\Delta H^{\ddagger}$ (kcal/mol)	$\Delta S^{\ddagger}$ (eu)
k_c(pG) [CCCUCUA]	29	18.4 ± 0.3	29 ± 4	31 ± 10
k_c(pG) [CCCUC(dU)A]	35	22.6 ± 0.4	34 ± 6	36 ± 20
$k_c(-G)$ [CCCUCUA]	35	22.2 ± 0.3	35 ± 2	39 ± 4
$k_c(-G)$ [CCCUC(dU)A]	37	24.1 ± 0.3	36 ± 2	38 ± 4

[a] $\Delta G^{\ddagger} = -RT \ln[hk_c/k_BT]$, calculated using the standard state of the activity of water equal to 1; T = 323 K (50 °C), R = gas constant, h = Planck's constant, k_B = Boltzman's constant, and k_c = rate constant of the chemical step. $\Delta S^{\ddagger} = (\Delta H^{\ddagger} - \Delta G^{\ddagger})/T$; 1 eu = 1 cal mol^{-1} K^{-1}. $\Delta H^{\ddagger} = E_a - RT$, with E_a determined from data shown in Figure 7 that was subsequently corrected for the change in pH due to the temperature dependence of the pK_a of the buffer (see Materials and Methods). This correction resulted in increases of 4 kcal/mol for E_a, 4 kcal/mol for $\Delta H^{\ddagger}$, and 15 eu for $\Delta S^{\ddagger}$, and a decrease of 0.3 kcal/mol for $\Delta G^{\ddagger}$ at 50 °C.

substrates [CCCUC(dU)A vs C(dCC)CU(dU)A] because deoxyribonucleotide substitutions along the substrate at sites other than −1 have ground-state effects (Herschlag *et al.*, 1993a,b). Since at lower pH the probability that nucleophile molecules are in the reactive (unprotonated) form is decreased, it is expected that $\Delta S^{\ddagger}$ for the reaction would be less favorable. Thus, it is likely that the lower pH and possibly the higher Mg^{2+} concentration are responsible for the smaller $\Delta S^{\ddagger}$.

DISCUSSION

We have shown that pG binding to $(E{\cdot}S)_c$ is temperature independent over a 60-deg range, suggesting that binding is entropically driven ($\Delta S^{\circ\prime}$ is positive). $\Delta H^{\circ\prime}$ is almost 0 for pG binding. Removal of hydrogen-bonding moieties from the guanine base (I and 2AP) results in the loss of binding energy; $\Delta H^{\circ\prime}$ becomes unfavorable, while $\Delta S^{\circ\prime}$ is not as affected and remains the thermodynamic driving force. The positive $\Delta H^{\circ\prime}$ for binding I and 2AP indicates that there is an enthalpic barrier counterbalanced by hydrogen bonding to the guanine base.

In addition, we have shown evidence suggesting that a temperature-dependent conformational change affects the thermodynamic coupling between the binding of pG and oligonucleotide substrate. Because at 0 °C pG binding is independent of oligonucleotide binding, the interactions responsible for the thermodynamic coupling seen at higher temperatures must be indirect. Evaluation of this temperature-dependent conformational change as it relates to the well-studied step of P1 docking into the ribozyme active site is important for understanding the involvement of ribozyme structural dynamics in catalysis.

The increase in disorder continues after guanosine is bound. There is a positive $\Delta S^{\ddagger}$ for reaching the transition state of the chemical step. Under conditions where K_m does not equal K_d, the values of K_m and k_{cat} may not be independent of each other (Fersht, 1977). Since we have determined conditions where K_m(pG) equals K_d(pG), the values of K_m(pG) and k_c(pG) are independent. Because the positive $\Delta S^{\circ\prime}$ and $\Delta S^{\ddagger}$ values for binding pG and for chemistry are independent, they are likely to result from unique physical events.

Thermodynamics of Binding a Small Molecule. Binding of a small molecule results *a priori* in the loss of translational and rotational degrees of freedom as two molecules become one (Jencks, 1975; Page, 1977). However, the effects of water and ions in solution cannot be ignored. It is typically thought that hydrophobic and charged molecules are surrounded by ordered water molecules. Some of these are released upon interaction with a cognate hydrophobic and charged molecule, respectively (Jencks, 1969). The energetic value for the release of ordered water molecules seems to vary; this variability is likely due to the degree of order in the neighboring waters and the number of molecules released (Jencks, 1969; Connelly *et al.*, 1994). Hydrogen bonding is classically thought to provide enthalpy-driven binding (negative ΔH°). However, there are exceptions. For example, FK506 binding protein (FKBP-12) loses a hydrogen bond to FK506 when tyrosine-82 is changed to phenylalanine (Connelly *et al.*, 1994). Surprisingly, loss of this hydrogen bond results in a more favorable enthalpy of binding. This anomaly is understood from the crystal structure of FKBP-12. There are two ordered water molecules released when the ligand binds. The event of water release results in a positive ΔS° (7 eu/H_2O molecule), since it is the dissociation of a small molecule (increasing rotational and translational degrees of freedom), as well as in a positive ΔH°. In general, an unfavorable ΔH° can result from the ordered water molecules breaking their hydrogen-bonding interactions, either to a polar group as in FKBP-12, to the neighboring water molecules in hydrophobic interactions, or to the ion that is ordering them (Jencks, 1969; Connelly *et al.*, 1994).

Guanosine Binding and the Concomitant Release of Water. We propose that there is a release of one or more water molecules upon binding of guanosine. An increase in the degrees of freedom due to water dissociation that exceeds the loss of degrees of freedom involved in binding guanosine would account for the overall positive ΔS° for the event. There is also a loss of favorable hydrogen-bonding energy upon water dissociation due to interactions the bound water molecules had with neighboring hydrogen-bonding constitu-

ents. The hydrogen bonding of the guanine base moiety compensates for that enthalpic deficit.

Removal of the hydrogen-bonding moieties from the guanine base has the expected effect of increasing $\Delta H^{\circ\prime}$. The $\Delta\Delta G^{\circ}$ values of +2.4 and +3.3 kcal/mol and $\Delta\Delta H^{\circ\prime}$ values of 5.3 and 8.2 kcal/mol for comparing pG to 2AP and I binding, respectively, are reasonable values for the loss of two hydrogen bonds in nucleic acid−nucleic acid interactions (Freier *et al.*, 1986; Turner *et al.*, 1987; SantaLucia *et al.*, 1992). The $\Delta\Delta H^{\circ\prime}$ values suggest that these hydrogen bonds provide the expected enthalpic contribution even though ΔH° is ~0. It possible that the larger difference in $\Delta\Delta G^{\circ}$ for the pG to I change reflects the loss of a 2-amino interaction with the A263 phosphate, as proposed by Yarus *et al.* (1991) (Figure 1B). The polar−charge hydrogen bond is generally worth more than a polar−polar one (Weiner *et al.*, 1984; Fersht *et al.*, 1985). The more positive $\Delta S^{\circ\prime}$ values for I and 2AP binding suggest that one or more water molecules released upon binding are not fixed to the hydrogen-bonding moieties of the guanine base. The water molecules could be interacting with the charged or polar groups of the G-site on the ribozyme (Figure 1B). It is also possible that the released water molecules are ordered against the π face of an exposed base in the G-site or that they are bound by charged phosphates or Mg^{2+} ions in the G-site. Finally, a conformational tightening of the ribozyme secondary or tertiary structure could accompany nucleoside binding, with water molecules being released from sites not directly part of the binding site.

The difference in pG versus 2AP or I binding ($\Delta\Delta G^{\circ}$ = +2.4 and +3.3 kcal/mol, respectively) is strikingly similar to the difference in K_m values of G versus that of 2AP or I measured for the self-splicing reaction [+2.8 and +2.9 kcal/mol (Bass & Cech, 1984)]. The values for $\Delta\Delta G^{\circ}$ for the comparison of pG to 2AP and I binding here are somewhat larger than those measured when G, 2AP, and I were bound in the context of 3′-exon analogs [+1.8 and +~2 kcal/mol, respectively (Moran *et al.*, 1993)]. As suggested by the authors (Moran *et al.*, 1993), other interactions of the 3′-exon upstream of G may perturb guanosine binding. These perturbations may be important for the second step of splicing.

The maximal rates of the I and 2AP reactions do not differ greatly from that of the pG reaction [k_c(I) = 0.5k_c(pG) and k_c(2AP) = 1.1k_c(pG) for reaction with C(dCC)UC(dU)A at pH 5.5 and 100 mM Mg^{2+} from 30 to 60 °C]. This suggests that all three nucleophiles are binding in the same manner and are capable of catalyzing the reaction at the same rate once bound.

The phosphate of pG is not responsible for the positive $\Delta S^{\circ\prime}$ upon binding. The binding constant for G has the same value at 30 and 50 °C (McConnell *et al.*, 1993), suggesting a similar thermodynamic behavior for G as for pG (positive ΔS° and $\Delta H^{\circ} \approx 0$). By comparison of the values of pG binding with those of pG and G previously obtained (McConnell *et al.*, 1993), the phosphate of pG is seen to be worth as much as 0.5 kcal/mol of binding energy. This free energy change is too small to account for the large positive $\Delta S^{\circ\prime}$ in pG binding without also a large change in ΔH° (−6 to −7 kcal/mol) for G binding (at 50 °C a ΔG° of 0.5 kcal/mol is equivalent to 1.5 eu). If a large favorable ΔH were to occur for G binding, G would bind 2-fold tighter at 30 °C than at 50 °C. A 2-fold difference in G binding would have been detected in the previous study. The positive $\Delta H^{\circ\prime}$ and $\Delta S^{\circ\prime}$ values of the *unphosphorylated* I and 2AP provide additional evidence that the 5′-phosphate is not responsible for its entropy-driven binding.

It is also possible to explain the positive $\Delta S^{\circ\prime}$ for the binding pG and its analogs by a conformational change that relaxes a restricted part of the ribozyme or releases bound water molecules at some distance from the G-site. Electron micrograph and cross-linking data suggest that ribozyme is dynamic, not maintaining a single rigid structure in the presence of Mg^{2+} (T. Nakamura, Y.-H. Wang, J. Griffith, and T. R. Cech, unpublished data; Wang *et al.* 1993). There are examples of proteins, such as hexokinase, which undergo large conformation changes releasing a large number of water molecules upon binding small molecules (Rand *et al.*, 1993). Structural evidence indicates that no global conformational change is induced by guanosine binding (Latham & Cech, 1989; Downs & Cech, 1990). However, a conformational change sufficient to release a few water molecules might be too subtle to be detected by chemical probing experiments. Studies of the pH dependence of the second-order rate constant $(k_{cat}/K_m)^G$ in the single turnover reaction with E•CCCUCUAAAAA showed an apparent pK_a of 6.9 that is argued to result from a conformational change (Herschlag & Khosla, 1994). However, the apparent pK_a is unlikely a result of pG binding becoming rate limiting (Herschlag & Khosla, 1994) and would seem not to be responsible for the positive $\Delta S^{\circ\prime}$ measured here. At this point there no evidence for a conformational change for pG binding. Since a concomitant water dissociation model does not require an additional step in the mechanism, it currently provides a good working model.

Enthalpy−Entropy Compensation Model: Proteins Binding Small Molecules with Large Changes in Heat Capacity. Calorimetric studies of proteins have led to a model in which large changes in heat capacity (ΔC_p) affect ΔS° in an opposing direction to the temperature dependence of ΔH°, resulting in temperature-independent binding (Sturtevant, 1977). For glyceraldehyde-3-phosphate dehydrogenase binding NAD^+, the hydrophobic and vibrational contributions to ΔC_p are directly related to their contribution to ΔS°. The conclusion is that the favorable hydrophobic contribution to ΔS° decreases at higher temperatures, while the unfavorable vibrational contribution to ΔS° increases. This compounding effect leads to an increasingly unfavorable change in ΔS° with temperature that is counterbalanced by an increasingly favorable change in ΔH° leaving ΔG° unchanged. Since our approach of measuring ΔG° for pG binding over a temperature range is insensitive to the temperature-dependent changes in ΔS° or ΔH°, we cannot rule out this model. This explanation of our thermodynamic data would not rule out either physical model, a conformational change or water release. However, it does present a question about the thermodynamic nature of the ribozyme.

In terms of binding guanosine, does the ribozyme behave like a protein or a nucleic acid? The values of ΔS° and ΔH° for duplex, triplex, and tetraplex formation are negative, so a positive entropy change for nucleic acid−nucleic acid interactions is not expected (Jin *et al.*, 1990; Plum *et al.*, 1990; Turner *et al.*, 1990; Xodo *et al.*, 1990; Lu *et al.*, 1993; Shindo *et al.*, 1993). Also, for most nucleic acid−nucleic acid interactions ΔC_p is negligible. In one exception, however, it is argued that, at pH ~5, a triple helix may form

with a negative ΔC_p, resulting in temperature-independent triplex formation (Shindo *et al.*, 1993). In measurements of the thermodynamics of some nucleic acid binding proteins, positive as well as negative values of $\Delta S°$ have been seen for binding of nucleotides (Merino *et al.*, 1979; Burz & Allewell, 1982; Mateo *et al.*, 1986; Hu & Sturtevant, 1987). If the ribozyme interacts with guanosine as glyceraldehyde-3-phosphate dehydrogenase does with NAD^+ described above, perhaps the ribozyme is behaving more like a globular protein than an RNA triplex.

A Temperature-Dependent Conformational Change That Affects the Binding of pG to the Free Ribozyme. The thermodynamics of pG binding to the free ribozyme are biphasic, suggesting that more than one event is occurring (squares in Figure 3). With the information available, the most reasonable explanation for this biphasic behavior is that a conformational change is responsible for the thermodynamic coupling between the binding of oligonucleotide substrate and pG and that this conformational change is temperature dependent. As the temperature is decreased from 30 to 0 °C the free ribozyme changes from the weak pG binding state to a tight pG binding state. At 0 °C guanosine can bind tightly in the absence of bound oligonucleotide substrate. Although this change in ribozyme states occurs in the absence of oligonucleotide and therefore is distinct from the docking of P1, it remains possible that the conformational change could involve positioning of the IGS strand of P1 (Wang *et al.*, 1993). Perhaps at 0 °C the IGS without substrate bound is correctly docked into the ribozyme promoting a closed form (Figure 4). A prediction from this model is that at 0 °C the binding and docking of oligonucleotide substrate would occur in a single step and would not have the energetic contributions of tertiary interactions with the IGS, as has been seen at low temperatures by Bevilacqua *et al.* (1994).

A Positive Entropy Change To Reach the Transition State of the Chemical Step. The $\Delta S^‡$ for reaching the transition state is positive in all the observed ribozyme reactions. Others have measured the thermodynamics for similar ribozyme reactions including E·CCCUCUAAAAA reacting in the absence of G (Legault *et al.*, 1992) and the circle form of the intron reacting in the presence or absence of CU (Sugimoto *et al.*, 1988, 1989). In these cases $\Delta S^‡$ was positive (+30 to 50 eu). We suggest that the ribozyme uses a mechanism of catalysis that produces a positive $\Delta S^‡$.

As mentioned above, $T\Delta S^‡$ provides 10 kcal/mol in free energy at 50 °C. This value approaches the estimated 11 orders of magnitude (16 kcal/mol) rate enhancement over the uncatalyzed reaction rate (Herschlag & Cech, 1990). The interactions or events responsible for the positive $\Delta S^‡$ appear to account for much of the catalysis by the ribozyme.

This positive $\Delta S^‡$ for reaching the transition state of a transphosphoesterification reaction is unexpected from model studies. For example, the hydrolysis of bis-(2,4-dinitrophenyl)phosphate (Kirby & Younas, 1970) and other phosphate diesters (Osterheld, 1972) occurs with negative $\Delta S^‡$ values [−25 eu for bis(2,4-dinitrophenyl)phosphate]. Even for the intramolecular imidizole-catalyzed cyclization/cleavage of the 2-(*p*-nitrophenyl) phosphate ester of propylene glycol (an analog for a ribose diester), $\Delta S^‡$ is negative (−44 eu) (Breslow *et al.*, 1989).

As with the models for pG binding, two models to explain a positive $\Delta S^‡$ for reaching the transition state of the chemical step are water release or a conformational change. A model for water release upon binding is shown in Figure 8. Release of one or more water molecules from one or more bound, but still partially hydrated, Mg^{2+} ions could be the cause of a positive $\Delta S^‡$. Two candidates for such an event are at the 3′-oxygen of U-1 and at the *pro*-S_P oxygen of the reactive phosphate. A metal ion interaction with the 3′-oxygen of U-1 has already been shown to be important for catalysis (Piccirilli *et al.*, 1993). A metal ion specificity switch has not been demonstrated for the *pro*-S_P oxygen (Herschlag *et al.*, 1991) . The S_P isomer is ~5000-fold less reactive than the phosphate diester and is only rescued 20-fold by Mn^{2+} (T. S. McConnell and T. R. Cech, unpublished data). The failure of Mn^{2+} to restore the reaction rate of the S_P isomer to a value approaching that of the phosphate diester may be because this metal site has a high specificity for Mg^{2+}, and Mn^{2+} may bind poorly in the ground state.

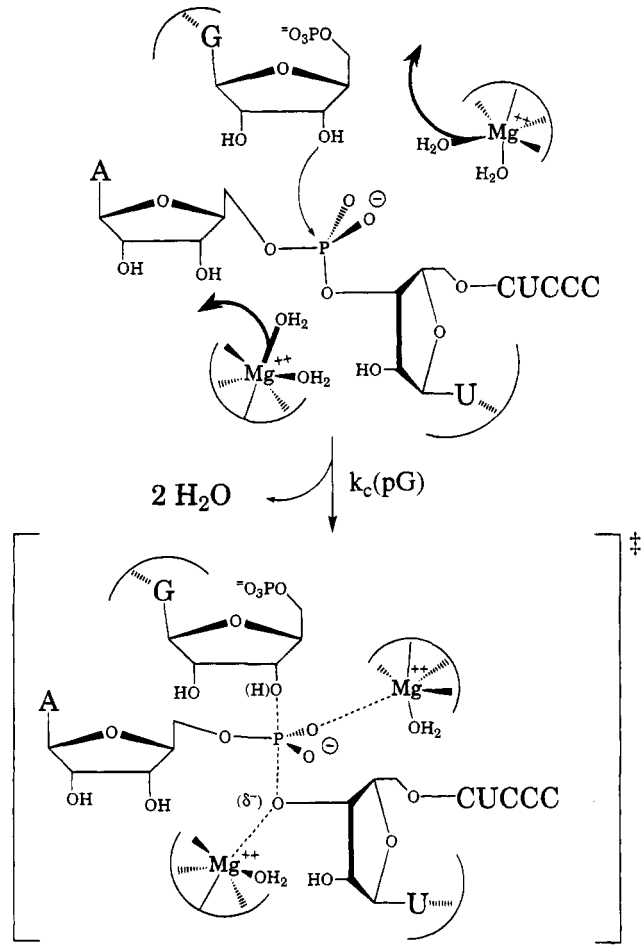

FIGURE 8: Model of release of water from catalytic Mg^{2+} ions to reach the transition state of the chemical step. The Mg^{2+} interaction with the 3′-hydroxyl leaving group of U-1 has been established (Piccirilli *et al.*, 1993), while that with the *pro*-S_P oxygen is more speculative (Herschlag *et al.*, 1991; Steitz & Steitz, 1993). Binding of either metal ion in the transition state would release bound water. The *pro*-S_P interaction would stabilize the pentacoordinate conformation around the phosphorus atom in the transition state, and the 3′-hydroxyl interaction would stabilize the developing negative charge on the leaving group.

A recent study has provided evidence that the ribozyme utilizes some of its excess binding energy of oligonucleotide substrate to force unfavorable interactions in ground-state binding (Narlikar *et al.*, 1995). These unfavorable interactions are not found in oligonucleotide product binding,

implying that they are alleviated in the transition state. It is possible that part of the positive $\Delta S^{\ddagger}$ for the chemical step results from abatement of these unfavorable interactions by mechanisms of solvent release or from relaxation of induced strain upon reaching the transition state. A predicted 40-fold weaker binding of oligonucleotide substrate at 50 °C could contribute as much as 11 eu of entropic stabilization.

The pH profile studies of the ribozyme reaction suggest that there is deprotonation of the 3′-hydroxyl of guanosine preceding the chemical step (Herschlag *et al.*, 1993a,b; Herschlag & Khosla, 1994). Deprotonation of the 3′-hydroxyl of guanosine would have a predicted negative $\Delta S^{\ddagger}$ (Izatt *et al.*, 1971) which would be unfavorable to reactivity. From the evidence presented, we suggest that the events responsible for the positive $\Delta S^{\ddagger}$ in the ribozyme reaction must overcome the entropic effect of deprotonation. It is also possible that a conformational change like the one proposed in a recent pH study is responsible for the positive $\Delta S^{\ddagger}$ seen here for the chemical step (Herschlag & Khosla, 1994). Current information cannot eliminate this model. A positive ΔS° is also seen for the equilibrium of the chemical step of the hammerhead ribozyme, and similar models are being considered in that system (K. Hertel, D. Long, and O. Uhlenbeck, personal communication).

Conclusions. We have demonstrated that the binding of the nucleotide pG to $(E{\cdot}S)_c$ is temperature independent over a 60-deg range, indicating a positive $\Delta S^{\circ\prime}$ for binding. The increase in the disorder of the system upon pG binding could be due to a conformational change or release of bound water. There are still enthalpic contributions to binding from the hydrogen-bonding moieties of the guanine base, which, although insufficient to drive binding alone, account for G binding more tightly than I or 2AP. We have also provided evidence that there is a temperature-dependent conformational change responsible for the thermodynamic coupling between pG and oligonucleotide substrate. Finally, the unusual positive $\Delta S^{\ddagger}$ for reaching the transition state of the chemical step appears to be a general characteristic of the ribozyme's active site and is perhaps due to the release of bound water from a catalytic Mg^{2+} ion or ions.

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