# Ribozyme-Catalyzed and Nonenzymatic Reactions of Phosphate Diesters: Rate Effects upon Substitution of Sulfur for a Nonbridging Phosphoryl Oxygen Atom[†,‡]

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ABSTRACT: The L-21 *Sca*I ribozyme derived from the intervening sequence of *Tetrahymena thermophila* pre-rRNA catalyzes a guanosine-dependent endonuclease reaction that is analogous to the first step in self-splicing of this intervening sequence. We now describe pre-steady-state kinetic experiments, with sulfur substituting for the *pro*-$R_P$ (nonbridging) phosphoryl oxygen atom at the site of cleavage, that test aspects of a kinetic model proposed for the ribozyme reaction (Herschlag, D., & Cech, T. R. (1990) *Biochemistry 29*, 10159–10171). Thio substitution does not affect the reaction with subsaturating oligonucleotide substrate and saturating guanosine ($(k_{cat}/K_m)^S$), consistent with the previous finding that binding of the oligonucleotide substrate limits this rate constant. In contrast, there is a significant decrease in the rate of single-turnover reactions of ribozyme-bound (i.e., saturating) oligonucleotide substrate upon thio substitution, with decreases of 2.3-fold for the reaction with guanosine ($(k_{cat}/K_m)^G$) and 7-fold for hydrolysis [i.e., with solvent replacing guanosine; $k_c(-G)$]. These "thio effects" are consistent with rate-limiting chemistry, as shown by comparison with model reactions. Nonenzymatic nucleophilic substitution reactions of the phosphate diester, methyl 2,4-dinitrophenyl phosphate monoanion, are slowed 4–11-fold by thio substitution for reactions with hydroxide ion, formate ion, fluoride ion, pyridine, and nicotinamide. In addition, we have confirmed that thio substitution has no effect on the nonenzymatic alkaline cleavage of RNA (Burgers, P. M. J., & Eckstein, F. (1979) *Biochemistry 18*, 592–596). Considering the strong preference of $Mg^{2+}$ for binding to oxygen rather than sulfur, the modest thio effect on the chemical step of the ribozyme-catalyzed reaction and the absence of a thio effect on the equilibrium constant for binding of the oligonucleotide substrate suggest that the *pro*-$R_P$ oxygen atom is not coordinated to $Mg^{2+}$ in the E·S complex or in the transition state. General implications of thio effects in enzymatic reactions of phosphate diesters are discussed.

A sulfur atom has been substituted for a nonbridging oxygen atom of phosphate diester substrates in many enzymatic reactions, generally to determine the stereochemical course of these reactions (for review see Knowles (1980), Eckstein (1985), and Frey (1989)). In addition, "thio substitution" has been used to probe the rate-limiting step (Bryant et al., 1983; Mizrahi et al., 1985; McSwiggen & Cech, 1989; Griep et al., 1990; Patel et al., 1991; Wong et al., 1991). The latter approach relies on the expectation that substitution of sulfur will slow the nonenzymatic or "intrinsic" chemical reactivity of the phosphate diester; this expectation is derived from studies with phosphate triesters (Benkovic & Schray, 1973, 1978). The absence of a rate decrease in an enzymatic reaction could then be considered evidence for a rate-limiting step other than chemistry, such as substrate binding, product release, or a conformational change. The rate effect upon substitution of sulfur is commonly referred to as a "thio effect" (i.e., thio effect = $k_{phosphate}/k_{phosphorothioate}$).

A ribozyme, or RNA enzyme, derived from the intervening sequence of *Tetrahymena thermophila* pre-rRNA catalyzes the transesterification reaction shown in Figure 1 (Zaug et al., 1986). The substrate $G_2CCCUCUAGU$ forms a perfect "matched" duplex with the 5′ exon binding site. This reaction exhibits a small thio effect of $\lesssim$2-fold on the steady-state rate parameters when a sulfur atom is substituted for the *pro*-$R_P$ oxygen at the cleavage site (Figure 1B; McSwiggen & Cech, 1989). A detailed kinetic analysis has shown that, with subsaturating concentrations of the oligonucleotide substrate and saturating guanosine (i.e., $(k_{cat}/K_m)^S$), binding of the oligonucleotide substrate is rate-limiting; with saturating oligonucleotide substrate (i.e., $k_{cat}$), release of the product $G_2CCCUCU$ is rate-limiting (Herschlag & Cech, 1990a).[1] Furthermore, the chemical step[2] is calculated to be very fast ($\sim$350 $min^{-1}$) relative to substrate and product dissociation (0.2 and 0.1 $min^{-1}$, respectively), so that even if the chemical step were limited by the chemical reaction and not a conformational change, a large decrease in the rate of the chemical reaction upon substitution by sulfur would have been invisible to the steady-state analysis with saturating G.[3] Thus, no significant thio effect would be expected, consistent with the

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[1] The kinetic analysis was performed with a substrate with $A_5$ rather than AGU at its 3′ end. However, as shown herein, the endonuclease reactions of these substrates have the same kinetic parameters. This is reasonable because the L-21 *Sca*I ribozyme doesn't base pair with the 3′ of the cleavage site (see Figure 1) and doesn't appear to have any other net interaction with them as judged by equilibrium binding measurements (Herschlag & Cech, 1990a,b; Pyle et al., 1990).

[2] The term "chemical step" is used to refer to all steps that involve the ternary complex, i.e, the chemical reaction and any associated conformational steps.

[3] Abbreviations: G, guanosine; S*, 5′-$^{32}$P-end-labeled substrate (i.e., p*$G_2CCCUCUAGU$ and the analogous specifically thio-substituted oligonucleotide); MES, 2-(*N*-morpholino)ethanesulfonic acid; CHES, (cyclohexylamino)ethanesulfonic acid; PAGE, polyacrylamide gel electrophoresis; EDTA, (ethylenedinitrilo)tetraacetic acid; MDP, methyl 2,4-dinitrophenyl phosphate; MDPS, methyl 2,4-dinitrophenyl phosphorothioate; DNP, 2,4-dinitrophenol.

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results of McSwiggen and Cech (1989).

In contrast, the reaction of a "mismatched" substrate, which has G replacing U at position −3 from the cleavage site (Figure 1), is limited partially by binding of the oligonucleotide substrate and partially by the chemical step[2] when the oligonucleotide is subsaturating and guanosine is saturating (i.e., $(k_{cat}/K_m)^S$; Herschlag & Cech, 1990b). Thus, if thio substitution produced a large slowing of the chemical step, there should be a large thio effect on $(k_{cat}/K_m)^S$. However, the thio effect is $\lesssim 2$ (McSwiggen & Cech, 1989). The absence of a large effect might signify that the chemical step is limited by a conformational change. Alternatively, thio-substitution might have only a modest effect on the intrinsic reactivity of a phosphate diester.

In this paper we determine the thio effect on the chemical step for the ribozyme-catalyzed reaction and obtain for comparison the thio effect in some nonenzymatic reactions of phosphate diesters. The modest thio effects in both the ribozyme-catalyzed and nonenzymatic reactions are consistent with rate-limiting chemistry in the reaction of ribozyme-bound oligonucleotide substrate and avert the need to propose a rate-limiting conformational step. As thio effects for nonenzymatic intermolecular reactions of phosphate diesters have not, to our knowledge, been reported previously, general implications for the mechanism of enzymatic reactions of phosphate diesters are discussed.

## Materials and Methods

### *Procedures for Ribozyme Experiments*

*Materials.* The L-21 *Sca*I ribozyme was prepared as described previously (Zaug et al., 1988). The oligonucleotides $pppG_2CCCUCUAGU$ and $(R_P)$-$pppG_2CCCUCU_{(P-S)}AGU$[4] (i.e., with two nonbridging oxygen atoms at the UA phosphoryl group and with one sulfur atom and one oxygen atom, respectively) were transcribed from a synthetic DNA template by T7 RNA polymerase using ATP or $(S_P)$-ATP$\alpha$S by J. McSwiggen (McSwiggen & Cech, 1989). Only the $R_P$ substrate can be synthesized with T7 RNA polymerase, as $(R_P)$-ATP$\alpha$S is not a substrate (Griffiths et al., 1987). The oligonucleotide $pppG_2CCCUCUA_5$ was also transcribed by T7 RNA polymerase, as described by Zaug et al. (1988). RNA oligonucleotide substrates were 5′-end-labeled by treatment with calf alkaline phosphatase (New England Nuclear) followed by polynucleotide kinase (U.S. Biochemical) and [$\gamma$-$^{32}$P]ATP (New England Nuclear), essentially as described previously (Zaug et al., 1988) except that the labeled substrate was used directly following elution from a gel slice into 10 mM Tris/1 mM EDTA, pH 7.5, without ethanol precipitation. This alternative procedure had no effect on kinetic parameters.

*Kinetics.* Unless otherwise stated, reactions were carried out at 50 °C in 50 mM sodium MES, pH 7.0 (determined at 25 °C; pH 6.7 at 50 °C, calculated from Good et al. (1966))/10 mM $MgCl_2$, as described in Herschlag and Cech (1990a). Reactions were initiated by addition of the 5′-end-labeled oligonucleotide substrate after a 10-min preincubation of the ribozyme/$MgCl_2$/MES buffer/guanosine at 50 °C. For hydrolysis reactions, the guanosine was omitted. Typically about eight aliquots of 1–2 $\mu$L were removed from 20-$\mu$L reaction mixtures at specified times and quenched with ~2 volumes of 20 mM EDTA in 90% formamide with 0.005% xylene cyanol/0.01% bromophenol blue/1 mM Tris, pH 7.5. Product and substrate were separated by electrophoresis on 20% polyacrylamide 7 M urea gels, and their ratio at each time point was quantitated with use of an AMBIS radioanalytic scanner.

All reactions were single-turnover, performed with an excess of ribozyme and only radiolabeled oligonucleotide substrate ($\leq$1 nM). A small burst of $\leq$10% for reactions of the thio substrate in which the thio substitution caused a rate decrease is consistent with the expected contamination by a small amount of the "normal" substrate (McSwiggen & Cech, 1989). Aside from this, reactions were first-order for ~3 half-lives. Endpoints of ~95% for the oxygen substrate and ~90% for the thio substrate were obtained from the extent of reaction after 10 half-lives.

Pulse chase experiments were performed as described previously (Herschlag & Cech, 1990a).

### *Procedures for Nonenzymatic Experiments*

*Materials.* Methyl 2,4-dinitrophenyl phosphate (MDP), methyl 2,4-dinitrophenyl phosphorothioate (MDPS), [$\alpha$-$^{32}$P]pppGAGU and $(S_P)$-[$\alpha$-$^{32}$P]$pppG_{(P-S)}$AGU RNA were synthesized as described below. Dimethyl chlorothiophosphate, 2,4-dinitrophenol, triethylamine, lithium chloride, dimethyl phosphite and [$^{18}$O] water (97%) were from Aldrich; [$\alpha$-$^{32}$P]GTP was from New England Nuclear; acetone was from J. T. Baker Inc.; and silica gel was from Merck.

*NMR Spectral Characterization of MDP and MDPS.* $^1$H NMR spectra were recorded on a Varian VXR-300 spectrometer. $^{31}$P NMR spectra were recorded on a JOEL FX 90 Q spectrometer, and the chemical shifts (parts per million) are reported relative to an external capillary standard of 85% $H_3PO_4$.

*Synthesis of Substrates for Nonenzymatic Alkaline Hydrolysis of RNA with and without Phosphorothioate Substitution.* [$\alpha$-$^{32}$P]pppGAGU and $(S_P)$-[$\alpha$-$^{32}$P]$pppG_{(P-S)}$AGU RNA[4] were synthesized with use of 1 $\mu$M L-21 *Sca*I ribozyme to catalyze the reaction between 2.5 $\mu$M [$\alpha$-$^{32}$P]GTP and 1 $\mu$M $pppG_2CCCUCUAGU$ or 1 $\mu$M $(R_P)$-$pppG_2CCCUCU_{(P-S)}AGU$. After a 5–20-min reaction at 50 °C in 50 mM MES, pH 7.0/10 mM $MgCl_2$, the products were separated from the starting materials by denaturing PAGE, as described above for the ribozyme experiments, and the products, [$\alpha$-$^{32}$P]pppGAGU and $(S_P)$-[$\alpha$-$^{32}$P]$pppG_{(P-S)}$AGU (McSwiggen & Cech, 1989), were soaked from the gel. The thio-substituted RNA was resistant to digestion by RNase T1 under conditions that gave complete digestion of the oxygen-containing RNA, as observed previously (McSwiggen & Cech, 1989); the thio-substituted RNA was digested when incubated with higher concentrations of RNase T1 in the absence of competing tRNA.

*Lithium Methyl 2,4-Dinitrophenyl Phosphate (MDP).* MDP was prepared as described by Ba-Saif et al. (1989): mp 132–134 °C (lit. mp 133–137 °C; Kirby & Younas, 1970); $^1$H NMR ($D_2O$) $\delta$ 8.76 (m, 1 H, H-3, Ph), 8.40 (dd, 1 H, H-5, Ph), 7.56 (d, 1 H, H-6, Ph), 3.58 (d, 3 H, $J_{CH_3-P}$ = 13.2 Hz, $CH_3$); $^{31}$P NMR ($D_2O$) $\delta$ −4.41.

*Lithium Methyl 2,4-Dinitrophenyl Phosphorothioate (MDPS).* This procedure was analogous to that for the preparation of MDP except that dimethyl chlorothiophosphate replaced dimethyl chlorophosphate. To a solution of dimethyl chlorothiophosphate (2.8 g, 22 mmol) in dry acetone (25 mL) was added 2,4-dinitrophenol (3.2 g, 17 mmol) and triethylamine (1.95 g, 19.5 mmol). The mixture was stirred at room temperature. After about 5 min, a precipitate of triethylammonium chloride began to form. Stirring was continued for 90 min, and the resulting suspension was filtered. The filtrate was concentrated in vacuo to yield the triester as a

[4] (P–S) is used to represent replacement of a nonbridging phosphoryl oxygen atom by sulfur.

yellow oil. This residue was dissolved in a small amount of acetone, and lithium chloride (0.75 g) suspended in acetone (75 mL) was added. The mixture was refluxed for 60 min and then concentrated in vacuo. The residue was chromatographed on silica gel using triethylamine/acetone/methanol (10:85:5) as eluent. The fractions containing MDPS were pooled and concentrated to yield MDPS as a yellow oil: $^1$H NMR ($D_2O$) $\delta$ 8.74 (m, 1 H, H-3, Ph), 8.36 (d, 1 H, H-5, Ph), 7.61 (d, 1 H, H-6, Ph), 3.60 (d, 3 H, $CH_3$, $J_{CH_3-P}$ = 13.5 Hz); $^{31}$P NMR ($D_2O$) $\delta$ 54.28. This $^{31}$P chemical shift is consistent with that of other phosphorothioate diesters (e.g., Bartlett and Eckstein (1982) and Cosstick and Eckstein (1985)). No trace of MDP was observed (<5%).

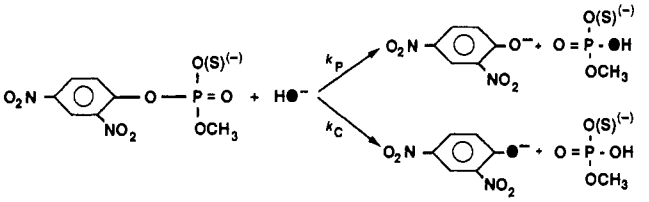
*Kinetics of Nonenzymatic Alkaline Hydrolysis of RNA.* Nonenzymatic alkaline hydrolysis of [$\alpha$-$^{32}$P]pppGAGU and ($S_P$)-[$\alpha$-$^{32}$P]ppp$G_{(P-S)}$AGU was carried out in NaOH at 37 °C. Aliquots containing ~1000 cpm of RNA were taken at varying times and were cooled to 0 °C to quench the reaction; quenching by neutralization with 0.67 equivalents of Tris-HCl gave the same observed extent of reaction, showing that the cold quench was effective. The reaction products were separated by denaturing PAGE, as described above. At early times in the reaction, four bands were observed, corresponding to the starting pppGAGU and, presumably, pppGAGp, pppGAp, and pppGp (see below); at late times only the fastest migrating band, pppGp, remained. The formation of pppGp was followed by comparing the amount of radioactivity corresponding to pppGp to the total radioactivity associated with all four bands. The appearance of the monomeric product, pppGp, from each substrate followed first-order kinetics for at least three half-lives. The addition of 1 mM EDTA and the doubling of the concentration of substrate had no effect on the observed rate constant.

The "p" on the right side, for example, of pppGp, represents a mixture of 2′,3′-cyclic phosphate and 2′- and 3′-phosphates, as the initial 2′,3′-cyclic phosphate product reacts with hydroxide ion to form the 2′- and 3′-phosphates (Bock, 1967). These species apparently comigrate in the PAGE separation system used. The presumed products pppGAGp and pppGp comigrated with the two products from a limited digestion of pppGAGU with RNase T1, which cleaves after guanosine residues (Uchida & Egami, 1971), and pppGAGp also comigrated with ppp$G_{(P-S)}$AGp, the single product from a limited digestion of ($S_P$)-ppp$G_{(P-S)}$AGU with RNase T1. RNase T1 initially gives 2′,3′-cyclic phosphate products that are more slowly hydrolyzed to 3′-phosphate esters (Uchida & Egami, 1971).

As stated above, the presence of sulfur in the ($S_P$)-[$\alpha$-$^{32}$P]ppp$G_{(P-S)}$AGU starting material was confirmed by resistance to digestion with RNase T1. When an alkaline reaction, in which ~75% of the non-thio-containing starting material had reacted, was treated with RNase T1, essentially all of the [$\alpha$-$^{32}$P]pppGAGU, [$\alpha$-$^{32}$P]pppGAGp, and [$\alpha$-$^{32}$P]-pppGAp was converted to [$\alpha$-$^{32}$P]pppGp; in contrast, analogous treatment of an alkaline reaction of the thio-containing compound caused cleavage of the starting material to [$\alpha$-$^{32}$P]ppp$G_{(P-S)}$AGp but gave no cleavage of [$\alpha$-$^{32}$P]-ppp$G_{(P-S)}$AGp or [$\alpha$-$^{32}$P]ppp$G_{(P-S)}$Ap. This is the expected result for cleavage at only the non-thio-containing phosphoryl linkage that is 3′ to a guanosine (McSwiggen & Cech, 1989) and suggests that the sulfur atom is not lost in the course of the alkaline hydrolysis reaction.

*Kinetics of Reaction of MDP and MDPS.* Reactions of MDP and MDPS at 39.0 °C and ionic strength 1.1 (KCl) were followed spectrophotometrically at 360 or 420 nm. Typically,

Scheme I




more than 10 time points were taken during each reaction. Most reactions were followed for 2–3 half-lives, and these reactions were first-order for this time. Initial rates were used to determine rate constants for the slower reactions: those with formate ion and most of the reactions with nicotinamide. Rate constants for reactions of nicotinamide determined by both methods were the same. Endpoints were determined after $\geq 10t_{1/2}$ following the addition of NaOH to a final concentration of 0.05–0.2 M. The rate constants obtained for reactions of MDP (Table I) are in reasonable agreement with those reported previously (Kirby & Younas, 1970). The same ratio of observed rate constants for reaction of hydroxide ion with MDP and MDPS was obtained spectrophotometrically and by $^{31}$P NMR analysis at a single time point of a reaction mixture containing both MDP and MDPS.

Phenyl phosphate esters can undergo attack at carbon, by nucleophilic aromatic substitution, as well as at phosphorus, as is of interest for this work (Kirby & Jencks, 1965a,b; Kirby & Younas, 1970). The nucleophiles studied herein were chosen because there is evidence that these nucleophiles, with the exception of hydroxide ion, have a large preference for attack at phosphorus (Kirby & Younas, 1970). In addition, the observation of the same thio effect for two pyridines of different p$K_a$ (pyridine and nicotinamide; Table I) shows that MDP and MDPS have the same sensitivity to nucleophiles; this provides evidence for attack at phosphorus with MDPS as has been shown with MDP (Kirby & Younas, 1970). We therefore assume that all the reactions studied, except those with hydroxide ion, proceed via attack at phosphorus rather than by nucleophilic aromatic substitution. The rate constant for attack by hydroxide ion at phosphorus was determined by product analysis with [$^{18}$O]hydroxide ion, as outlined below and in the supplementary material.

*Product Analysis from Reactions of MDP and MDPS with [$^{18}$O]Hydroxide Ion.* In order to obtain the thio effect for reaction at phosphorus, the relative amount of reaction at phosphorus and carbon ($k_P/k_C$) was determined in addition to the observed rate constant for reaction of hydroxide ion with MDP and MDPS (Scheme I and eq 1). The ratio $k_P$ to $k_C$

$$k_{obsd} = k_P + k_C \quad (1)$$

was determined by analysis of the products from reactions with [$^{18}$O]hydroxide ion. Equation 2 describes the relationship of

$$\frac{(\text{frac}^{18}\text{O})_{\text{methyl phosphate}}}{(\text{frac}^{16}\text{O})_{\text{methyl phosphate}}} = \frac{k_P(\text{frac}^{18}\text{O})_{\text{solvent}}}{k_C + k_P(\text{frac}^{16}\text{O})_{\text{solvent}}} \quad (2)$$

$k_P$ and $k_C$ to the fraction of methyl phosphate (or phosphorothioate) product that contains $^{18}$O and the fraction that contains only $^{16}$O, accounting for the fraction of $^{18}$O in the solvent. This product ratio from MDP and from MDPS was determined by $^{31}$P NMR spectroscopy, taking advantage of the ~0.03-ppm chemical shift difference between $^{18}$O and $^{16}$O-substituted methyl phosphate (Cohn & Hu, 1978; Lowe & Sproat, 1978; Lutz et al., 1978) as described in the supplementary material. Similarly, eq 3 describes the analogous relationship for the other reaction product, 2,4-dinitrophenoxide ion. The $^{18}$O content of this product from MDP and

$$\frac{(\text{frac}^{18}\text{O})_{\text{dinitrophenol}}}{(\text{frac}^{16}\text{O})_{\text{dinitrophenol}}} = \frac{k_C(\text{frac}^{18}\text{O})_{\text{solvent}}}{k_P + k_C(\text{frac}^{16}\text{O})_{\text{solvent}}} \quad (3)$$

MDPS was analyzed by mass spectroscopy, as described in the supplementary material. The product analysis and $k_{obsd}$ were used to obtain $k_P$ and $k_C$, with use of eqs 1 and 2 or 3. The product analyses of eqs 2 and 3 give redundant information, and the values of $k_P$ and $k_C$ calculated for MDP and for MDPS with use of each equation agree within 10%. The same values of $k_C$ are obtained for the two substrates, MDP and MDPS ($k_C$(MDP)/$k_C$(MDPS) = 1.1 ± 0.1). These values are expected to be the same for this nucleophilic aromatic substitution reaction with rate-limiting attack to form the tetrahedral intermediate (Bunnett et al., 1957; Kirby & Jencks, 1965b). We do not know why the ratio of $k_P$ to $k_C$ = 0.55 ± 0.05 obtained herein for MDP is slightly different from that of 1.0 ± 0.3 obtained previously (Kirby & Younas, 1970).

## Results and Kinetic Description[5]

Kinetic analysis of the ribozyme-catalyzed cleavage of the substrate $G_2CCCUCUA_5$ has led to a kinetic scheme that includes rate constants for individual steps of the reaction (Herschlag & Cech, 1990a). However, to study the effect of thio substitution solely at the cleavage site, the substrate $G_2CCCUCUAGU$ was designed; synthesis of this substrate by transcription in the presence of ($S_P$)-ATPαS gives stereospecific incorporation of a single $R_P$ sulfur at the UA phosphoryl group (Figure 1B; McSwiggen & Cech, 1989). Applicability of the kinetic scheme derived for the substrate ending in $A_5$ to the AGU substrate would facilitate comparison of the AGU substrate and its thio analogue.

*Comparison of the Substrates $G_2CCCUCUA_5$ and $G_2CCCUCUAGU$.* The following data show that it is indeed reasonable to adopt the same kinetic scheme for the substrates ending in $A_5$ and AGU. Side-by-side comparisons of the two substrates in reactions with ribozyme in excess gave values of $(k_{cat}/K_m)^S$ and $(k_{cat}/K_m)^G$ that are the same, within experimental uncertainty (10–20%; data not shown). $(k_{cat}/K_m)^S$ is the second-order rate constant for reaction of E·G with S, previously shown to represent binding for the substrate ending in $A_5$ (Herschlag & Cech, 1990a). Thus, the data suggest that $(k_{cat}/K_m)^S$ for the substrate ending in AGU is similarly limited by substrate binding and that the two substrates bind at the same rate (this is shown directly below). $(k_{cat}/K_m)^G$ is the second-order rate constant for reaction of E·S with G, representing binding of G and the chemical step[2] for the substrate ending in $A_5$. There is evidence that binding of G is independent of binding of the oligonucleotide substrate (Herschlag & Cech, 1990a), so that the equivalence of $(k_{cat}/K_m)^G$ for the two substrates suggests that the chemical step occurs at the same rate. The equilibrium binding constants for the two substrates are also the same (see below). Turnover with saturating S and G occurs at the same rate for the two oligonucleotide substrates (Zaug et al., 1988; McSwiggen & Cech, 1989), as expected since $k_{cat}$ is limited by dissociation of $G_2CCCUCU$, which is formed from both substrates (Herschlag & Cech, 1990a). The only difference between the substrates in their interactions with the ribozyme appears in the rate of the hydrolysis reaction that occurs in the absence of G: the rate of hydrolysis of S bound to the ribozyme is 3-fold slower for the AGU substrate (data not shown). The reason for this lone difference is not known.

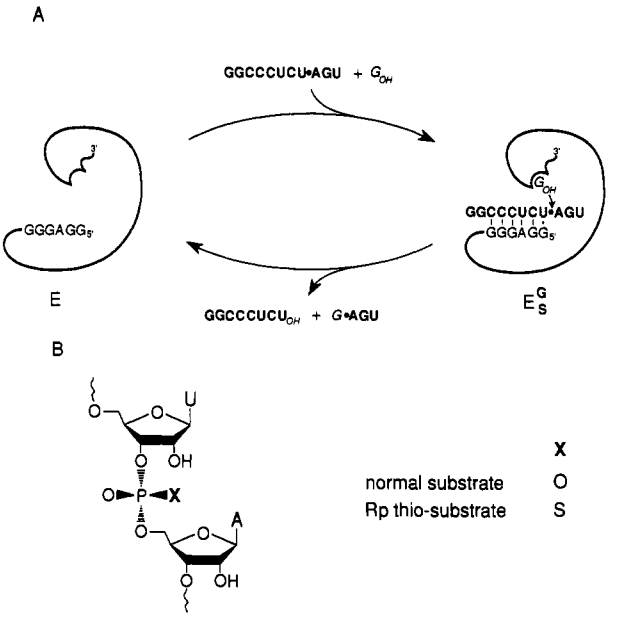



FIGURE 1: Endonuclease reaction catalyzed by the L-21 *ScaI* ribozyme derived from *Tetrahymena* pre-rRNA (Zaug et al., 1988). (A) The reaction of the oligonucleotide substrate $G_2CCCUCUAGU$ (S) with guanosine (G) is shown. S is shown base paired to the 5′ exon binding site of the ribozyme; the G·U directly preceding the cleavage site is conserved in group I intervening sequences (Cech, 1988). Tertiary interactions involving the backbone also contribute to binding (Herschlag & Cech, 1990a; Pyle et al., 1990). (B) Structures of the substrates used in this work are shown. The "normal" substrate has all phosphates in its backbone, and the "thio substrate" has the $R_P$ phosphorothioate at the cleavage site (i.e., at UA) and phosphates at all other positions. It should be noted that the *pro*-$R_P$ oxygen of the normal substrate is replaced by sulfur to give the $R_P$ thio isomer.

The similar behavior of the substrates ending in $A_5$ and AGU might have been expected, because there are no base pairing interactions of these residues with the 5′ exon binding site of the L-21 *ScaI* ribozyme (Figure 1) and the $pA_5$ portion of $G_2CCCUCUA_5$ provides no net contribution to binding (i.e., $G_2CCCUCUA_5$ and $G_2CCCUCU$ have essentially the same binding constants; Herschlag & Cech, 1990a; Pyle et al., 1990).

*Thio Effect on the Ribozyme Reaction with Subsaturating S.* McSwiggen and Cech (1989) compared steady-state rate parameters for reactions of $G_2CCCUCUAGU$ and ($R_P$)-$G_2CCCUCU_{(P-S)}AGU$ with saturating G (i.e., $(k_{cat}/K_m)^S$). Their data indicated that the thio effect is ≲ 2-fold. In order to determine if there is *any* thio effect, we measured $(k_{cat}/K_m)^S$ using single-turnover conditions. This provides two advantages: (1) data can be accumulated over several half-lives of the reaction, providing a larger "signal" than that from initial rate measurements under multiple-turnover conditions; and (2) the variable of substrate concentration is removed, since the first-order rate constant for disappearance of substrate is determined by the concentration of ribozyme and not by the concentration of substrate, as in multiple-turnover experiments.

Figure 2 shows the disappearance of normal and thio substrates with saturating G and a subsaturating concentration of ribozyme. The thio effect (i.e., the ratio of values of $(k_{cat}/K_m)^S$) obtained from Figure 2 and other side-by-side comparisons is 1.06 ± 0.1; $(k_{cat}/K_m)^S$ equals $9 \times 10^7$ $M^{-1}$ $min^{-1}$ for both substrates. (The ratio is more accurate than the rate constant.) A pulse chase experiment with the normal and thio

[5] The following nomenclature is used throughout: E or ribozyme represents the L-21 *ScaI* ribozyme; S represents the oligonucleotide substrate, with or without sulfur (Figure 1); G represents guanosine; and P represents the product $G_2CCCUCU$. The normal substrate refers to that without thio substitution, $G_2CCCUCUAGU$, and the thio substrate refers to ($R_P$)-$G_2CCCUCU_{(P-S)}AGU$ (Figure 1B).

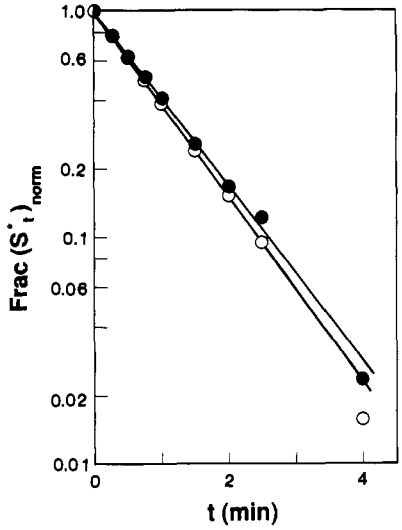



FIGURE 2: Thio effect on $(k_{cat}/K_m)^S$. Reaction of $p^*G_2CCCUCUAGU$ (○) or $p^*G_2CCCUCU_{(P-S)}AGU$ (●), ~1 nM, and 800 μM guanosine catalyzed by 10 nM L-21 *Sca*I ribozyme (50 mM MES, pH 7, 10 mM $MgCl_2$, 50 °C). The fraction of S* remaining at each time was normalized by subtracting the fraction of S* that did not react at long times (0.05 for the normal substrate and 0.10 for the thio substrate) and then dividing by the fraction of S* that was reactive: i.e., $frac(S^*_t)_{norm} = (fracS^*_t - fracS^*_{t\to\infty})/(1 - fracS^*_{t\to\infty})$. Each time point is the average of three separate determinations.

substrates was performed and analyzed as described previously (Herschlag & Cech, 1990a). Ribozyme and S* were incubated to form E·S*, followed by the simultaneous addition of G (to give the E·S*·G ternary complex) and unlabeled P (to trap free ribozyme and E·G and thereby prevent rebinding of any S* that dissociates). With both the thio and normal substrates, essentially all of the S* was converted to P* (>95% of the reactive S*; data not shown). This means that the free energy barrier for dissociation of the E·S* complex is higher than that for the chemical reaction. Thus, binding, rather than the chemical step, limits $(k_{cat}/K_m)^S$ for both the normal and thio substrate $[(k_{cat}/K_m)^S = k_{on}^S]$. The absence of a thio effect on the rate constant for binding is consistent with the proposal that the rate of binding is limited by the formation of a base-paired duplex between the oligonucleotide substrate and the 5′ exon binding site (Herschlag & Cech, 1990a).

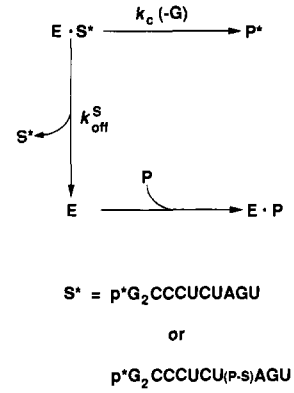
*Thio Effect on $K_d(E·S)$.* A pulse chase experiment was performed in order to determine if thio substitution affects the rate constant for dissociation $(k_{off}^S)$, and thus the equilibrium binding constant. The partitioning of bound substrate between the ribozyme-catalyzed hydrolysis reaction $(k_c(-G))$ and dissociation $(k_{off}^S)$ was determined for the normal and thio substrates (Scheme II). The formation of the product $p^*G_2CCCUCU$ during this partitioning is shown in Figure 3. These data, along with the values of $k_c(-G)$ that were measured independently, give $k_{off}^S$ in two ways. (1) The final amount of P* formed is determined by $k_c(-G)$ and $k_{off}^S$ according to eq 4, which was derived from Scheme II. The values

$$frac(P^*)_{t\to\infty} = k_c(-G)/\{k_c(-G) + k_{off}^S\} \quad (4)$$

of $frac(P^*)_{t\to\infty} = 0.49$ and 0.17 (Figure 3; these values have been corrected for the extent of reaction during the preincubation and the fraction of unreactive S*) and $k_c(-G) = 0.24$ and 0.035 $min^{-1}$ (see below) give $k_{off}^S = 0.24$ and 0.14 $min^{-1}$ for the normal and thio substrate, respectively. (2) The rate constant for formation of P*, $k_{obsd}$, is also determined by $k_c(-G)$ and $k_{off}^S$, according to eq 5. The values of $k_{obsd}$ =

$$k_{obsd} = k_c(-G) + k_{off}^S \quad (5)$$

Scheme II



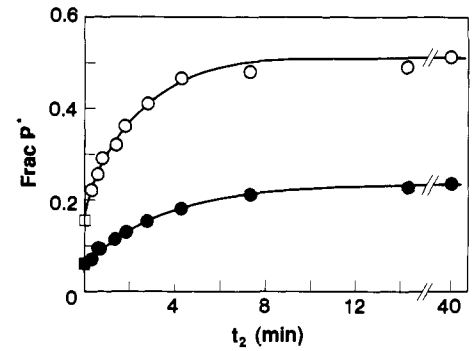




FIGURE 3: Thio effect on the rate constant for dissociation of substrate bound to the ribozyme $(k_{off}^S)$. Labeled substrate, $p^*G_2CCCUCUAGU$ (○) or $p^*G_2CCCUCU_{(P-S)}AGU$ (●), ~5 nM, was incubated with 100 nM L-21 *Sca*I ribozyme for $t_1$ = 0.67 min (50 mM MES, pH 7, 10 mM $MgCl_2$, 50 °C) to allow formation of the E·S* complex. The mixture was then diluted 10-fold with buffer containing 3 μM of unlabeled product, $G_2CCCUCU$. The formation of labeled product (P*) during $t_2$ following the dilution is shown. The large excess of unlabeled product that binds strongly to the ribozyme ($K_d$ = 1 nM; Herschlag & Cech, 1990a) prevents rebinding and reaction of labeled substrate that has dissociated during $t_2$. Note that *no* guanosine was added in these experiments so that P* was formed via the site-specific hydrolysis reaction (Herschlag & Cech, 1990a; and see text); P* present at $t_2 = 0$ was from hydrolysis during $t_1$. Control experiments in which the rate of P* formation was increased by the addition of guanosine at $t_2 = 0$ along with the unlabeled product gave complete conversion of S* to P* (>95%), showing that all of the S* is bound productively during $t_1$. The amount of P* present at $t_2 = 0$ (squares), due to the hydrolysis reaction during $t_1$, was determined in a separate time course. The lines are best fits to semilogarithmic plots of the data (not shown) that give $k_{obsd}$ = 0.42 and 0.27 $min^{-1}$ for the normal substrate (○, □) and the thio substrate (●, ■), respectively. (See Figure 3B of Herschlag and Cech (1990a) for an analogous plot.)

0.42 and 0.27 $min^{-1}$ obtained from first-order plots of the data of Figure 3 (not shown) and the values of $k_c(-G)$ give $k_{off}^S$ = 0.18 and 0.24 $min^{-1}$ for the normal and thio substrate, respectively. The values of $k_{off}^S$ = 0.18–0.24 $min^{-1}$ for the normal substrate and $k_{off}^S$ = 0.14–0.24 $min^{-1}$ for the thio substrate are the same, within experimental uncertainty. The uncertainty presumably arises because the results from independent experiments are combined to give the values of $k_{off}^S$. (A value of $k_{off}^S$ = 0.2 $min^{-1}$ was also obtained for $G_2CCCUCUA_5$; Herschlag & Cech, 1990a.)

The absence of a thio effect for both $k_{off}^S$ and $k_{on}^S$ (see above) indicates that there is no significant thio effect on the equilibrium binding of substrate to the ribozyme ($K_d(E·S) = k_{off}^S/k_{on}^S = 0.2\ min^{-1}/9 \times 10^7\ M^{-1}\ min^{-1} = 2$ nM).

*Thio Effect on the Ribozyme Reaction with Saturating S.* With saturating S and subsaturating G, the ribozyme reaction is between E·S and G; $(k_{cat}/K_m)^G$ is the second-order rate constant for this reaction (eq 6). We have previously argued

$$E{\cdot}S + G \xrightarrow{(k_{cat}/K_m)^G} \text{products} \quad (6)$$

that $(k_{cat}/K_m)^G$ is limited by conversion of the E·S·G ternary complex to products, including chemistry and any associated conformational steps, and not binding of G because $(k_{cat}/K_m)^G \cong 10^6\ M^{-1}\ min^{-1}$ is several orders of magnitude smaller than the rate constant for diffusional encounter. However, the possibility of very slow binding of G, because of occlusion of the binding site or a requirement for a conformational change to form the binding site, was not eliminated (Herschlag & Cech, 1990a). In this section the thio effect on $(k_{cat}/K_m)^G$ is determined. The modest, but significant, effect provides strong evidence that binding of G is *not* rate-limiting. The subsequent sections show that this modest thio effect is similar to the thio effects in nonenzymatic reactions of phosphate diesters, consistent with conversion of the ternary complex being limited by the actual chemical reaction.

The inset in Figure 4 shows that thio substitution slows the reaction of eq 6. Quantitation of these and analogous data gave a thio effect of 2.3 ± 0.2 for $(k_{cat}/K_m)^G$ (Figure 4).[6] Experiments in the absence of G gave a thio effect of 7 ± 2 for $k_c(-G)$, the ribozyme-catalyzed hydrolysis reaction (Figure 4, intercepts). The occurrence of a significant thio effect on $(k_{cat}/K_m)^G$ strongly suggests that the binding of G is not fully rate-limiting. There is evidence that the binding of the oligonucleotide and the binding of G are independent so thio substitution in the oligonucleotide substrate is not expected to affect the binding of G (Herschlag & Cech, 1990a; Pyle et al., 1990; D.H. and P. Legault, unpublished results). A lower limit for the rate constant for binding of G of $k_{on}^G > 10^6\ M^{-1}\ min^{-1}$ is obtained, as the binding of G must be faster than the overall rate constant of $(k_{cat}/K_m)^G \cong 10^6\ M^{-1}\ min^{-1}$ in order for another step to be rate-limiting.

*Why Is the Thio Effect in the Ribozyme-Catalyzed Reaction So Small? Nonenzymatic Model Reactions.* The thio effect was investigated for reactions of two phosphate diester: RNA, which is cleaved in an intramolecular reaction with the ribose 2′-$O^-$ serving as the nucleophile, and methyl 2,4-dinitrophenyl phosphate, which is cleaved in intermolecular reactions with nucleophiles.

The alkaline cleavage of [$\alpha$-$^{32}$P]pppGAGU and ($S_P$)-[$\alpha$-$^{32}$P]ppp$G_{(P-S)}$AGU to form the monomeric products [$\alpha$-$^{32}$P]pppGp and [$\alpha$-$^{32}$P]ppp$Gp_{(P-S)}$, respectively, occurred with $k = 2.5 \times 10^{-4}\ s^{-1}$ and $2.2 \times 10^{-4}\ s^{-1}$ (±10%), respectively (0.3 N NaOH, 37 °C; data not shown). Thus, there is no significant thio effect. This conclusion also follows from the observation that all three products, pppGAGp, pppGAp, and pppGp, had similar initial rates of formation for the substrate that contained the phosphorothioate as well as for the substrate with no thio substitution; there was no selection for or against cleavage of the phosphorothioate bond. Analogous results were obtained in reactions with 0.2 and 0.5 N NaOH. The rate constants for cleavage of the oxygen- and sulfur-containing substrates in 0.5 N NaOH are the same, within error, as those previously reported for UpA and its phosphorothioate (Burgers & Eckstein, 1979).

Although there was no significant thio effect in the alkaline cleavage of RNA, intermolecular reactions of phosphate diesters may provide a better model for the enzymatic reactions. We investigated intermolecular reactions by obtaining the rate constants for nucleophilic substitution reactions of MDP and

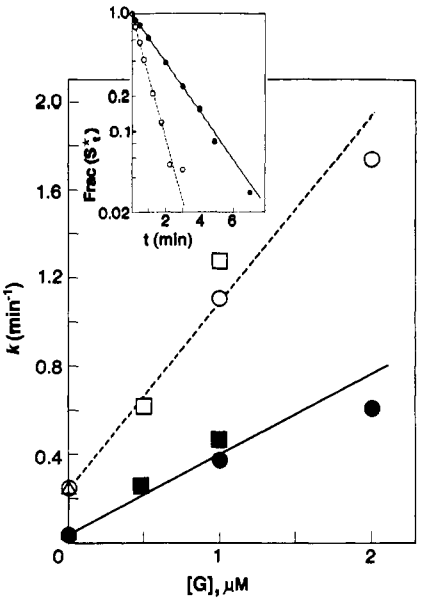



FIGURE 4: Thio effect on $(k_{cat}/K_m)^G$: determination of the second-order rate constant $(k_{cat}/K_m)^G$ for the reaction of E·S* and guanosine (eq 6), where S* is the normal substrate (open symbols and dashed lines) or the thio substrate (closed symbols and solid line), from reactions with ~1 nM S* and 80 (circles), 100 (triangles), or 400 (squares) nM L-21 *Sca*I ribozyme. The slopes of the lines give $(k_{cat}/K_m)^G = 8.5 \times 10^5\ M^{-1}\ min^{-1}$ and $3.7 \times 10^5\ M^{-1}\ min^{-1}$ for the normal and thio substrate, respectively, a thio effect of 2.3 ± 0.2. The intercepts are the rate constants for the ribozyme-catalyzed hydrolysis of S*: $k_c(-G) = 0.24$ and $0.035\ min^{-1}$ for the normal and thio substrate, respectively, a thio effect of 7 ± 2. Pseudo-first-order rate constants, $k_{obsd}$, were obtained from semilogarithmic plots as shown in the inset for the reaction of ~1 nM labeled substrate, p*$G_2$CCCUCUAGU (dashed line) or p*$G_2$CCCUC$U_{(P-S)}$AGU (solid line), with 1 μM guanosine catalyzed by 400 nM L-21 *Sca*I ribozyme (frac($S^*_t$) was normalized as described in the legend to Figure 2). A small correction of $k_{obsd}$ was performed to obtain $k = (k_{cat}/K_m)^G[G]$. The correction used a steady state *approximation* to account for the contribution of binding of S* to $k_{obsd}$; i.e., $1/k_{obsd} = 1/(k_{on}^S[E]) + 1/k$, from the reaction shown below. ($k_{on}^S = (k_{cat}/K_m)^S = 9 \times 10^7\ M^{-1}\ min^{-1}$; see text.) This correction is <20% in all cases and does not affect the conclusions.

$$S^* \xrightarrow{k_{on}^S[E]} E{\cdot}S^* \xrightarrow{(k_{cat}/K_m)^G[G]} \text{products}$$

MDPS listed in Table I. The thio effect with these nucleophiles is modest; it ranges from 4 to 11. These results are similar to the thio effect "on the order of 10" reported for the attack by an amine on the diester analogue methyl *N*-cyclohexylphosphoramidate (Hamer, 1965).

*What Is the Rate-Limiting Step for Reaction of the Ternary Complex E·S·G and for Hydrolysis of Ribozyme-Bound Substrate?* The thio effects of 2.3 and 7 for the reactions of E·S with G and solvent, respectively (i.e., $(k_{cat}/K_m)^G$ and $k_c(-G)$), are similar to the thio effects of 4–11 for several nonenzymatic intermolecular reactions and 1 for intramolecular alkaline cleavage of RNA. Thus, the modest thio effects in the ribozyme-catalyzed reactions are consistent with rate-limiting chemistry. However, because a thio effect on a conformational change is possible, a rate-limiting conformational change cannot be eliminated (see Discussion). It is interesting to note that had the previous assumption of a large intrinsic thio effect on the reactions of phosphate diesters been adopted, the data for G attack would have been interpreted to suggest that the chemistry is *not* rate-limiting.

*Why Is the Thio Effect for the Guanosine-Dependent Endonuclease Reaction Smaller than That for the Hydrolysis Reaction?* Three possible explanations for the smaller thio effect on $(k_{cat}/K_m)^G$ than on the $k_c(-G)$ (2.3 vs 7) have been

[6] The value and its error limits were determined by ratios from side-by-side comparisons of the normal and thio substrates; this provides greater precision than a simple comparison of the slopes.

Table I: Thio Effect for Nonenzymatic Reactions of Methyl 2,4-Dinitrophenyl Phosphate and Phosphorothioate[a]

| nucleophile | $10^4 k_2$ ($M^{-1}$ $min^{-1}$) phosphate | $10^4 k_2$ ($M^{-1}$ $min^{-1}$) phosphorothioate | thio effect $k$(phosphate)/$k$(phosphorothioate) |
|---|---|---|---|
| hydroxide ion[b] | 170 | 41 | 4.1 ± 0.4 |
| formate ion[c] | 1.1 | 0.12 | 9 ± 2 |
| fluoride ion[d] | 19 | 4.3 | 4.5 ± 0.5 |
| pyridine[e,f] | 62 | 5.6 | 11 ± 2 |
| nicotinamide[f,g] | 8.9 | 1.1 | 8.3 ± 1.5 |

[a] Reactions at 39 °C and ionic strength 1.1 (KCl) (see Methods). Intercepts in the absence of added nucleophile were determined in the presence of the specified buffer. [b] The rate constants and thio effect are for reaction at phosphorus only (Scheme I, $k_P$). The values were obtained from the observed rate constants of $k_{obsd} = 470 \times 10^{-4}$ $M^{-1}$ $min^{-1}$ and $310 \times 10^{-4}$ $M^{-1}$ $min^{-1}$ for MDP and MDPS, respectively (determined from 5 reactions with 0.05–0.2 M NaOH and 0.1–1 mM MDP or MDPS), and the fraction of the reaction proceeding via attack of phosphorus. This fraction was determined by product analysis of reactions with [$^{18}$O]hydroxide ion, as described in Methods. For MDP, values of $k_P$ = 160, 180, and $170 \times 10^{-4}$ $M^{-1}$ $min^{-1}$ were obtained in two product analyses by mass spectroscopy and one by $^{31}$P NMR; for MDPS, values of $k_P$ = 41 and $40 \times 10^{-4}$ $M^{-1}$ $min^{-1}$ were obtained by mass spectroscopy and by $^{31}$P NMR. [c] Determined from 10 reactions with 0.1–1.0 M potassium formate and 1–3 mM MDP or MDPS in 30 mM potassium phosphate buffer, pH 7. The same thio effect was obtained from reactions with 2.0 M potassium formate (ionic strength 2.1). [d] Determined from 10 reactions with 0.2–1.0 M potassium fluoride and 0.05–1 mM MDP or MDPS in 50 mM CHES buffer, pH 9. The same thio effect was obtained with 1.8 M potassium fluoride (ionic strength 1.9). [e] Determined from 16 reactions with 0.1–1.0 M pyridine and 0.1–1 mM MDP or MDPS in 50 mM CHES buffer, pH 9, 30 mM potassium bicarbonate buffer, pH 10, or 30 mM potassium phosphate buffer, pH 7. The identity of the buffer had no effect on the rate constant. [f] The amount of free pyridine and free nicotinamide were calculated with use of $K_{aa}$ = 0.5 and 0.35 $M^{-1}$, the self-association constants for pyridine and nicotinamide, respectively (39 °C, $I$ = 1 (KCl); Kirby & Jencks, 1965a); plots of $k_{obsd}$ vs nucleophile concentration were linear only after making this correction. This correction does not affect the value of the thio effect. [g] Determined from 7 reactions with 0.1–1.6 M nicotinamide and 0.1–1 mM MDP or MDPS with 30 mM potassium phosphate buffer, pH 7.

Table II: Thio Effects on Rate and Equilibrium Constants in the Ribozyme-Catalyzed Endonuclease Reaction

| | thio effect ($k_{phosphate}/k_{phosphorothioate}$ or $K_{phosphate}/K_{phosphorothioate}$) |
|---|---|
| $(k_{cat}/K_m)^S$[a] | 1.06 ± 0.1 |
| $k_{on}^S$[b] | 1.06 ± 0.1 |
| $k_{off}^S$ | 1[c] |
| $K_d$(E·S) | 1[c] |
| $(k_{cat}/K_m)^G$[d] | 2.3 ± 0.2 |
| $k_c$(–G)[d] | 7 ± 2 |

[a] From Figure 2, as described in the text. [b] $k_{on}^S = (k_{cat}/K_m)^S$, as shown by the pulse chase experiment described in the text. [c] Error limits can be estimated from the range of values given in the text. [d] From Figure 4, as described in the figure legend and text.

considered: (1) there are small differences in the intrinsic thio effect with G and water (or their respective anions) as nucleophiles or small differences in the active site in the presence of G and water that cause differences in interactions with the phosphoryl group; (2) partially rate-limiting binding of G lessens the thio effect on $(k_{cat}/K_m)^G$; and (3) a conformational change partially or fully limits $(k_{cat}/K_m)^G$. The following experiments and analysis of these three possibilities suggest that explanation 1 is most likely to hold.

(1) The nonenzymatic reactions of MDP and MDPS give different thio effects for attack by hydroxide ion and formate ion of 4 and 9, respectively (Table I). Analogous small nucleophile-specific differences could account for the difference in thio effects for the ribozyme-catalyzed reactions with G and water. It is also reasonable that the presence of G or water in the active site could lead to small differences in the interaction with the phosphoryl group in the transition state resulting in the observed 3-fold difference in the thio effects (see also Discussion). The large difference of $\sim 10^3$-fold in the rate constants for the hydrolysis and guanosine-dependent reactions, with $k_c$(–G) = 0.24 $min^{-1}$ (Figure 4) and $k_c$(guanosine-dependent) $\cong$ 350 $min^{-1}$ (Herschlag & Cech, 1990a), shows that there are significant energetic differences between the two transition states.

(2) The following data suggest that partially rate-limiting binding of G for $(k_{cat}/K_m)^G$ is not responsible for the smaller thio effect on $(k_{cat}/K_m)^G$ compared with $k_c$(–G). Decreasing the pH to 5.2 slows $(k_{cat}/K_m)^G$ and $k_c$(–G) each by $\sim$50-fold but leaves the thio effect on $k_c$(–G) larger than that on $(k_{cat}/K_m)^G$ (thio effects of 8 (±2) and 3 (±1), respectively; data not shown). This is consistent with a thio effect of 3 (±1) on $(k_{cat}/K_m)^G$ that represents the thio effect for the chemical step, undiminished by binding that is partially rate-limiting, and a mechanism in which the sole effect of lowering the pH is to slow the chemical step. Alternatively, if decreasing the pH disrupted the G binding site, such that G bound to E·S and not to E·H$^+$·S, then binding would remain partially rate-limiting at the lower pH (if it were partially rate-limiting at the higher pH).[7] However, the following data suggest that the binding of G is unaffected by the decrease in pH and thus provide evidence against this alternative.[8] The value of $K_m^G$ at pH 5.2 is no larger than that at pH 6.1, despite the $\sim$8-fold smaller value of $(k_{cat}/K_m)^G$ at pH 5.2. (Only a lower limit for $K_m^G$ at pH 6.1 could be obtained because the reaction at high concentrations of G became too fast to measure accurately; data not shown.) Furthermore, the occurrence of the same value of $K_m^G$ in the DNA endonuclease reaction (Herschlag & Cech, 1990c) at pH 7 as in the faster RNA endonuclease reaction at pH 5.2 suggests an absence of kinetic complexity so that $K_m^G$ is expected to equal $K_d^G$ in these reactions (D.H. and P. Legault, unpublished results). Two possibilities remain: (i) the rate of binding and dissociation of G could both be slowed with decreasing pH so that there is a rate effect in the absence of an equilibrium effect on G binding or (ii) G could have the same affinity for E·S and E·H$^+$·S, but the interconversion of E·S·G and E·H$^+$·S·G could be slow so that only G that binds to E·S reacts at both the low and high pH. Either model would allow the binding of G to remain partially rate-limiting at the lower pH despite the decrease in $(k_{cat}/K_m)^G$ and lack of effect on $K_d$(E·G). However, both of these models seem unlikely. Furthermore, pulse chase experiments in which the pH is changed upon addition of G and unlabeled P to the E·S* complex provide no indication of the slow proton exchange suggested in (ii) above; e.g., E·S* preincubated at pH 7.0 prior to a change in pH to 5.2 concomitant with G and P addition behaves identically with E·S* that was preincubated at pH 5.2 prior to reaction at pH 5.2.

(3) The thio effect on $(k_{cat}/K_m)^G$ could arise from a fully rate-limiting conformational change that coincidentally had a thio effect similar to that seen in the nonenzymatic reactions. Thus, the different thio effects could arise from different rate-limiting steps: a conformational change in the G-dependent reaction (thio effect = 2.3) and chemistry in the hydrolysis reaction (thio effect = 7). We again emphasize that

[7] This can be understood as follows: the only reactive species would be the unprotonated ribozyme at both pH values so that the rate-limiting step would not change with pH.

[8] These experiments to follow the binding of G as a function of pH were conducted at 30 °C, rather than 50 °C, because the slower reaction and stronger binding of G (suggested by kinetic studies; D.H. and P. Legault, unpublished results) facilitated these experiments.

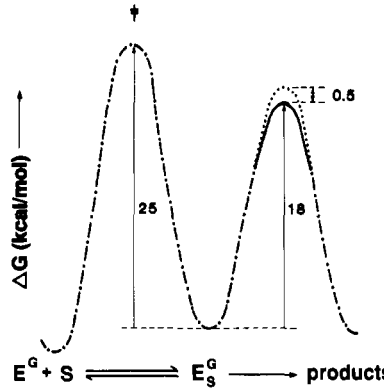



FIGURE 5: Free energy reaction profile for RNA cleavage by the ribozyme. Reaction with saturating guanosine and subsaturating oligonucleotide substrate ($G_2CCCUCUAGU$, solid line; $G_2CCCUCU_{(P\text{-}S)}AGU$, dotted line); i.e., "$(k_{cat}/K_m)^S$ conditions". The free energy profile is drawn for [oligonucleotide substrate] = 0.1 nM, [ribozyme] $\ll$ 0.1 nM, and [G] $\gg K_d$(E·G). The profile is drawn to scale, except that the increase in free energy for the chemical step upon thio substitution is exaggerated by 2-fold for clarity. The numbers refer to the actual free energy differences in kilocalories per mole.

there are no data to warrant proposal of a kinetically significant conformational step.

## Discussion

*Kinetic Implications of the Thio Effects.* Thio effects in the ribozyme-catalyzed cleavage of RNA (Figure 1) are summarized in Table II. The different thio effect on $(k_{cat}/K_m)^S$ and $(k_{cat}/K_m)^G$ strongly suggests that different steps limit these rate constants. The free energy reaction profile of Figure 5 describes the reaction with subsaturating oligonucleotide substrate and saturating G ("$(k_{cat}/K_m)^S$ conditions"). Under these conditions there is no thio effect, despite the thio effect on the chemical step, because binding of the oligonucleotide substrate is rate-limiting (Figure 5, ‡) and is unaffected by thio substitution. The thio effect of 2.3 with saturating oligonucleotide substrate and subsaturating G ($(k_{cat}/K_m)^G$) provides evidence that the chemical step,[2] rather than the binding of G, is rate-limiting. The thio effect is consistent with the actual chemical reaction limiting this rate constant, since the thio effect is similar to those for nonenzymatic reactions of phosphate diesters measured herein. The thio effect of 7 on the hydrolysis of ribozyme-bound substrate ($k_c$(–G)) is likewise consistent with rate-limiting chemistry. However, a mechanism with $(k_{cat}/K_m)^G$ and/or $k_c$(–G) limited by a conformational change, which coincidentally has a sensitivity to thio substitution similar to that observed in the nonenzymatic reactions, cannot be ruled out. This underscores the significant limits to general conclusions that can be reached from the determination of a thio effect.

The thio effect can be useful in diagnosing whether or not there is a change in the rate-limiting step, either with a change in reaction conditions, as described above, or with a change to a mutant ribozyme (B. Young, D.H., and T.R.C., manuscript in preparation). In general however, more information is needed to determine what step limits the rate (see below).

*Mechanistic Implications of the Ribozyme Thio Effects.* $Mg^{2+}$ (or $Mn^{2+}$) is required for ribozyme activity (Grosshans & Cech, 1988). It is tempting a priori to favor a mechanism in which a $Mg^{2+}$ ion directly coordinates one or both of the phosphoryl oxygen atoms in the ground state and transition state (Haydock & Allen, 1985; Cech, 1987; Rajagopal et al., 1989). Substitution of sulfur for the *pro-*$R_P$ oxygen atom at the cleavage site of the substrate has no affect on its equilibrium binding constant (Table II). If this oxygen coordinated $Mg^{2+}$, a decrease in binding upon thio substitution would be expected, as $Mg^{2+}$ is coordinated more strongly by oxygen than sulfur by an estimated 31 000-fold for the β-phosphoryl group of ATPβS (Jaffe & Cohn, 1978, 1979; Pecoraro et al., 1984). Furthermore, the thio effect on $(k_{cat}/K_m)^G$ is no larger than expected simply from the small effect on reactivity seen in nonenzymatic reactions, so that there is no indication of an interaction of the *pro-*$R_P$ oxygen atom with $Mg^{2+}$ in the transition state.

$Mg^{2+}$ or a hydrogen-bond donor from the ribozyme could, of course, interact with the other oxygen atom; i.e., the *pro-*$S_P$ oxygen atom. Indeed, substitution of this oxygen atom by sulfur in a multipartite ribozyme system derived from the *Tetrahymena* IVS gives a thio effect of $\sim 10^3$-fold, much larger than the thio effect of 2.3 obtained herein from substitution of the *pro-*$R_P$ oxygen atom (Rajagopal et al., 1989; their reaction is analogous to the reverse of that in Figure 1, so the $R_p$ thio isomer in their reaction is equivalent to the $S_P$ thio isomer in the reaction studied herein). The systems are different, and assembly or conformational steps may be important in the more complex system of Rajagopal et al. (1989). However, it is likely that the active sites contain the same catalytic groups, and conformational problems with the more complex system might be expected to diminish, not enhance, the thio effect. Thus, the different thio effects with the two isomers suggest that there is direct contact of the ribozyme (or a ribozyme-bound $Mg^{2+}$ ion) with the *pro-*$S_P$ oxygen atom of the oligonucleotide substrate of Figure 1 (i.e., the oxygen atom that is *not* replaced in forming the $R_P$ thio isomer studied herein). This is currently being tested by studying reactions of both isomers with the same ribozyme. Analogous isomer-specific thio effects have been observed with protein enzymes (e.g., Burgers and Eckstein (1979) and Bryant and Benkovic (1979) and Sheu et al. (1979); see below). Thio substitution at the cleavage site of a hammerhead ribozyme substrate results in a large thio effect that is relieved by replacement of $Mg^{2+}$ by $Mn^{2+}$, which has a higher affinity for sulfur than $Mg^{2+}$, or by increased concentrations of $Mg^{2+}$. This provides the best evidence of which we are aware for direct participation of a metal ion at the active site of a ribozyme (S. C. Dahm and O. C. Uhlenbeck, manuscript in preparation).

*Thio Effects in Nonenzymatic Reactions: Correlation with Transition-State Structure.* Thio substitution slows reactions of phosphate triesters ~10–160-fold (Ketelaar et al., 1952; Heath, 1956a,b; Cox & Ramsay, 1964; Fanni et al., 1986) but speeds reactions of a phosphate monoester dianion 3–10-fold (Breslow & Katz, 1968; Domanico et al., 1986). We now see that phosphate diesters give an intermediate thio effect ($k_{phosphate}/k_{phosphorothioate}$) of 4–11 for MDP and 1 for the intramolecular alkaline cleavage of RNA.

As shown in Figure 6, the trend in the thio effects, phosphate triester > phosphate diester > phosphate monoester, correlates with the transition state changing from associative to dissociative, as determined by linear free energy relationships and isotope effects (compiled in Herschlag and Jencks (1989b); see also Hengge and Cleland (1990)). Furthermore, thio substitution stabilizes the metaphosphate ion ($PO_3^-$) so that thiometaphosphate ion ($PO_2S^-$) is stable enough to exist in ethanol, a nucleophilic solvent, whereas metaphosphate ion apparently is not (Cullis & Iagrossi, 1986; Herschlag & Jencks, 1989a). The formation of the thiometaphosphate intermediate is consistent with thio substitution stabilizing the

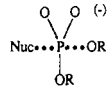
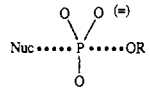
| Phosphate | $k_{ox}/k_s$ | Transition state | |
|---|---|---|---|
| triester | 10-160 | associative |  |
| diester | 4-11 | intermediate |  |
| monoester | 0.1-0.3 | dissociative |  |

FIGURE 6: Correlation between thio effects and transition state structures for reactions of phosphate esters. The thio effect for substitution of a nonbridging phosphoryl oxygen atom by sulfur in phosphate diester reactions is from Table I, and those for the triesters and monoesters are from references given in the text. The term "associative" is used for transition states in which there is a larger amount of bonding to both the incoming nucleophile and outgoing leaving group, while "dissociative" indicates little bonding to either the incoming nucleophile or the outgoing leaving group. Evidence for these characterizations of the transition states is summarized in Herschlag and Jencks (1989b), and further support comes from Hengge and Cleland (1990). The bond order to the nonbridging phosphoryl oxygen (sulfur) atom(s) presumably increases as the dissociative character of the transition state increases; this is not shown explicitly here. There is evidence for a pentavalent intermediate in an intramolecular reaction of a phosphate triester (Kluger et al., 1969) and evidence against such an intermediate in an intermolecular phosphate triester reaction (Ba-Saif et al., 1989); this issue is not addressed in the qualitative transition-state pictures for intermolecular phosphoryl-transfer reactions shown here.

dissociative, metaphosphate-like transition state (relative to the ground state) for reactions of phosphate monoesters to give the observed rate increase.

The thio effects can be rationalized by the greater electronegativity of oxygen than sulfur. Thio substitution slows reactions of phosphate triesters, for which the electron-withdrawing oxygen atom can stabilize the increased electron density in the associative transition state. In contrast, thio substitution speeds reactions of phosphate monoesters, which require charge donation from the phosphoryl substituents. The effect with phosphate diesters is intermediate, consistent with an intermediate reaction pathway.

It has been suggested that phosphorothioate monoester and diester anions contain a single bond to the sulfur atom so that more charge is localized on the sulfur atom than on oxygen, despite the lower electronegativity of sulfur (Frey & Sammons, 1985). If such a preference for single-bonded sulfur relative to single-bonded oxygen held in the ground state and transition state for all phosphate esters, thio substitution might be expected to *increase* the reactivity of phosphate triesters and *decrease* the reactivity of phosphate monoesters, opposite to the observed effects (Figure 6). The phosphate triester reaction presumably involves a change of a P=O(S) double bond to a P–O(S) single bond. Thus, a general preference for a P–S single bond over a P–O single bond, relative to the P=S and P=O double bonds, respectively, would be expected to favor the phosphorothioate triester reaction. Similarly, formation of a P=O double bond in the metaphosphate-like transition state of phosphate monoester reactions would be expected to be easier than the formation of a P=S double bond in the phosphorothioate monoester reactions. The direction of the observed rate effects from thio substitution suggests that these naive arguments may be too simplistic and that electronegativity effects may be dominant in determining the stability of the transition state.

Although there is a gross trend, with phosphate monoester reactions accelerated, diester reactions slowed, and triester reactions slowed still more, the thio effect varies within each group as the nucleophile substituent, and solvent are changed (Figure 6). Thus, there are no unique numbers that provide hallmarks for mono-, di-, and triester reactions. In the case of the phosphate diester MDP, thio substitution slows the reaction by a factor of 4–11 for the limited set of nucleophiles in Table I. These values are all greater than the thio effect of 1 for alkaline cleavage of RNA, perhaps because of differences in the leaving group or perhaps because of differences between the inter- and intramolecular reactions. The opening of the uridine 2′,3′-cyclic phosphate in base, to give uridine 2′- and 3′-phosphate, exhibits a thio effect of 6 (Eckstein, 1968). Since this reaction is analogous to the reverse of the RNA cleavage reaction, the thio effect of 6 on the opening of the cyclic phosphate and the thio effect of 1 on the formation of the cyclic product by RNA cleavage (Results; Burgers & Eckstein, 1979) suggest that thio substitution stabilizes the 2′,3′-cyclic phosphate relative to the 2′- and 3′-phosphates by ~1 kcal/mol.

*General Implications of Thio Effects for Mechanisms of Enzymes.* As described above, thio substitution has an imperceptible effect on alkaline cleavage of RNA and only a modest effect on nucleophilic substitution reactions of MDP. Considering these results, what can be concluded from thio effects in enzymatic reactions of phosphate diesters? We consider three situations.

(1) If there is no rate effect upon thio substitution, chemistry or any other step could be rate-limiting. $(k_{cat}/K_m)^S$ for the *Tetrahymena* ribozyme reaction described above has a thio effect of 1, because the rate-limiting step is binding of the substrate, not chemistry. However, since there is a thio effect of 1 for the alkaline cleavage of RNA, there could be enzymes that have a thio effect of 1 with chemistry rate-limiting. The smallest thio effect for a RNase of which we are aware is ~2-fold on $k_{cat}$ for RNase T2 (Burgers & Eckstein, 1979).

(2) If there is a rate decrease upon thio substitution, then chemistry or any other step in which the contact of the enzyme to the phosphoryl group changes in the transition state could be rate-limiting. cAMP-dependent protein kinase is activated only by the $S_P$ isomer of cAMPαS; the $R_P$ isomer binds but does not activate the kinase, showing that thio substitution can affect a conformational change (deWit et al., 1982; O'Brian et al., 1982; Kerlavage & Taylor, 1982; Rothermel et al., 1983). Further limiting interpretation, the step that is rate-limiting with the thio-substituted substrate need not be rate-limiting with the normal substrate. If chemistry is rate-limiting, there are several factors that could contribute to the observed thio effects. (i) The thio effect could represent the amount that the reaction rate is decreased by thio substitution in the analogous nonenzymatic reaction. This may be the case for the opening of uridine 2′,3′-cyclic phosphate, which has a thio effect on $k_{cat}$ of 5 with RNase A for both thio isomers and a thio effect of 6 for the nonenzymatic reaction with hydroxide ion (Eckstein, 1968, 1970). (ii) A larger thio effect than that observed in nonenzymatic reactions could arise from unfavorable interactions of the enzyme with the phosphorothioate group in the transition state due to differences in bond lengths and charge distribution. (See also (3) below). (iii) The thio effect could be larger or smaller than that observed in nonenzymatic reactions because an alternative mechanism is used by the enzyme (e.g., protonation of a phosphoryl oxygen atom). (iv) A larger thio effect than that observed in model reactions could in principle arise from a change to a more

associative transition state on the enzyme, with the enzyme reducing the entropic barrier to the more associative reaction (Haake & Allen, 1980; see also Herschlag and Jencks (1990) and Dietze and Jencks (1989)). However, the observed thio effect cannot be used to determine if the enzymatic transition state is monoester-, diester-, or triester-like, because the intrinsic effect of thio substitution on chemical reactivity can be obscured by differential interactions of the enzyme with oxygen and sulfur (i.e., (ii) above; see also (3) below and Knowles, (1980) and Herschlag and Jencks (1990)). (v) A thio effect on an internal equilibrium for a chemical, binding, or conformational step could contribute to a thio effect on the observed rate. DNA polymerase exhibits a different thio effect for the forward and reverse reactions of the Michaelis complexes, suggesting that the thio substitution perturbs the equilibrium for the reaction (Mizrahi et al., 1985). The thio effect of 70 ($S_P$ thio isomer) on $k_{cat}$ for cleavage of 5′-*O*-adenosyl 3′-*O*-uridyl phosphate by RNase A, compared to the thio effect of 5 on $k_{cat}$ for the opening of the cyclic phosphate product, suggests that the enzyme favors the RNA substrate relative to the cyclic phosphorothioate, if the opening by water can be considered essentially the reverse of the cleavage reaction in which a 5′-hydroxyl of a nucleoside is the leaving group (Eckstein, 1970; Burgers & Eckstein, 1979). Finally, there are examples of thio effects on equilibrium binding constants (e.g., deWit et al. (1982) and Rothermel et al. (1983) and Garcia et al., (1990)).

(3) If there is a different rate or binding effect for the two thio isomers, then a strong conclusion can be drawn: there is contact of the enzyme (or a cofactor such as a metal ion) with the phosphoryl group. Different rates for different diastereomers will in general arise from specific geometrical or charge effects in the active site, since in solution the isomers react at essentially the same rate (Eckstein, 1968; Burgers & Eckstein, 1979). The rate decrease for reactions of DNA restriction endonucleases upon thio substitution *adjacent to* the cleavage site also demonstrates that thio substitution can affect reactions because of differences in size and charge and not just because of differences in chemical reactivity (e.g., Vosberg and Eckstein (1982) and Stec et al. (1984) and Taylor et al. (1985) and Koziotkiewicz et al. (1986)). The metal-ion-dependent selectivity of enzymes for diastereomers of thio-ATP has been used to determine the coordination and screw sense of enzyme-bound metal·ATP complexes (e.g., Jaffe and Cohn (1979) and Eckstein (1985)).

Thus in general, experiments with thio-substituted substrates provide limited information about the rate-limiting step. Despite these limitations, thio substitution can, in conjunction with other approaches, be valuable in understanding a mechanism.

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## Supplementary Material Available

Descriptions of the $^{31}P$ NMR and gas chromatograph/mass spectroscopy experiments that were used to obtain the ratio of [$^{18}O$]hydroxide ion attack at phosphorus and carbon in the reactions of MDP and MDPS (eqs 2 and 3 in text) (3 pages). Ordering information is given on any current masthead page.

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