Comparison of pH Dependencies of the Tetrahymena Ribozyme Reactions with RNA 2'-Substituted and Phosphorothioate Substrates Reveals a Rate-Limiting Conformational Step†

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ABSTRACT: The L-21 ScaI ribozyme (E) derived from the self-splicing group I intron of Tetrahymena pre-rRNA catalyzes an RNA endonuclease reaction analogous to the first step in self-splicing: CCCUCUAAAAA (S) + G → CCCUCU + GAAAAA (P)

(S)

(1)

This result was surprising because the titratable groups of RNA have pKₐ values of ≈4 or ≈9. Thus, two models were considered: (i) the ribozyme structure perturbs a pKₐ such that the pKₐ of 6.9 corresponds to an actual titration or (ii) the pKₐ of 6.9 is a kinetic pKₐ, reflecting a change in the rate-limiting step rather than an actual titration. Oligonucleotide substrates with -H (deoxyribose), -F (2'-fluoro-2'-deoxyribose), and -OH (ribose) substitutions at the 2' position of the U residue at the cleavage site [U(-1)] vary considerably in their intrinsic reactivities. In the ribozyme reaction these substrates reacted at very different rates at low pH, but approached the same limiting reaction rate at high pH. Similarly, substitution of the pro-Rp nonbridging oxygen atom of the reactive phosphoryl group by sulfur lowers the intrinsic reactivity of the oligonucleotide substrate. In the ribozyme reaction, this "thio effect" was 2.3 below pH 6.9, whereas the thio substitution had no effect on the rate above pH 6.9. The variations in the rate caused by these substitutions below pH 6.9 suggest that the chemical cleavage step is rate-limiting at low pH whereas the similar rates above pH 6.9 suggest that a conformational step is rate-limiting at higher pH (model ii above). Further, the pH dependence suggests that a proton is lost from the E-S-G ternary complex prior to the chemical cleavage step. The approaches described herein should be useful in deconvoluting individual steps in more complex reactions such as self-splicing of group I introns and intron excision from pre-mRNA by the spliceosome.

In 1982 Cech and co-workers discovered that the intron from pre-rRNA of Tetrahymena thermophila could self-splice, excising itself in the absence of proteins (Kruger et al., 1982). Subsequently, the intron was transformed into an RNA enzyme or ribozyme that catalyzes a reaction analogous to the first step in the self-splicing reaction, but utilizing a 5' exon analog supplied in trans so that multiple rounds of turnover can be performed (eq 1; Zaug & Cech, 1986; Zaug et al., 1988). The ribozyme system has allowed manipulation of the concentration of individual reaction components, which has been instrumental in constructing a kinetic and thermodynamic framework for the reaction of this RNA catalyst (Figure 1). This framework has in turn allowed detailed interpretation of the effects of ribozyme mutations, changes at the level of single atoms in the reaction substrates, and changes at the atomic level in the ribozyme itself (Cech et al., 1992; Herschlag et al., 1993a,b; Piccirilli et al., 1993; Strobel & Cech, 1993).

In the experiments herein, variations in the oligonucleotide substrate (Chart 1)† have been used to uncover a new conformational step, and it is shown that a proton is lost prior to the chemical step. These results further define the kinetic and thermodynamic framework for the ribozyme reaction. In addition, the ability to distinguish between conformational and chemical steps should help distinguish individual steps in the more complex self-splicing reaction of group I introns.

MATERIALS AND METHODS

Materials. Ribozyme was prepared by in vitro transcription with T7 RNA polymerase and purified as described previously (Zaug et al., 1988). Oligonucleotides were made by solid-phase synthesis, 5'-end-labeled with [γ-32P]phosphate using [γ-32P]ATP and T4 polynucleotide kinase, and purified by electrophoresis on a non-denaturing 24% polyacrylamide gel, as described previously (Herschlag et al., 1993a, and references therein). The phosphorothioate substrates were kindly supplied by Nassim Usman of Ribozyme Pharmaceuticals, Inc.

Kinetics. All reactions were single-turnover, with ribozyme (E) in excess of 5'-labeled oligonucleotide substrate (S*) as

† All residues are ribose unless otherwise demarked. -1d refers to a 2'-deoxyribose residue at position -1; similarly -1F refers to a 2'-fluoro-2'-deoxyribose residue at position -1. -1(F-S) refers to a phosphorothioate at the UA junction; although the oligonucleotide is a racemic mixture of the Rp and Sp thio isomers, only the reaction of the Rp isomer was followed (see Materials and Methods). The following additional abbreviations are used: E, L-21 ScaI ribozyme; MES, 2-(N-morpholino)-ethanesulfonic acid; EDTA, (ethylenedinitri10)tetraacetic acid; EPPS, N-(2-hydroxyethyl)piperazine-N'-(2-hydroxyethyl)piperazine-2'-ethanesulfonic acid.

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described previously, using the substrates in Chart 1 (Herschlag & Cech, 1990; Herschlag et al., 1991; Herschlag et al., 1993a,b). Briefly, reactions were carried out at 50 °C in 10 mM MgCl₂ with the following buffers (50 mM): sodium acetate, pH 4.6; NaMES, pH 5.0–6.8; NaEPPS, pH 6.8–8.9; NaHEPES, pH 7.6. [pH values were determined at 25 °C and corrected to 50 °C using the enthalpy of deprotonation from Good et al. (1966) or determined directly at 50 °C.] Ribozyme and G were incubated with 10 mM MgCl₂ and buffer for 15 min followed by initiation of the reaction by addition of S* to a final concentration of ~5 nM. At specified times, six aliquots of ~1–2 μL were taken from 20-μL reactions and quenched with ~2 volumes of 20 mM EDTA in 90% formamide with 0.005% xylene cyanol, 0.01% bromophenol blue, and 1 mM Tris, pH 7.5. The 5′-labeled cleavage product P* was separated from S* by electrophoresis on 20% polyacrylamide/7 M urea gels, and their ratio quantitated with use of a Phosphorimager (Molecular Dynamics). Unless otherwise noted, P* was the only significant reaction product.

Reactions were followed for ≥ 3 t₁/₂, and the first-order rate constant for the disappearance of S* was determined by a nonlinear least-squares fit to the data (Kaleidagraph, Synergy Software, Reading, PA). End points of 95% were used to account for ~5% unreactive starting material, except for reactions of the phosphorothioate substrates −1(P-S),rS and −1d,−1(P-S),rS (Chart 1). These substrates contained a mixture of the RP and SP thio isomers, but only the RP thio isomer is cleaved by the ribozyme on the time scale of these reactions (Rajagopalan et al., 1989; Herschlag et al., 1991; J. A. Piccirilli and T. R. Cech, personal communication). An end point of 55% reacted substrate was determined from reactions of the phosphorothioate substrates taken to > 5 t₁/₂ and was used to obtain the first-order rate constant for reaction of these substrates. The 45% unreacted substrate presumably corresponds to the substrate present at the SP thio isomer. For reactions of −1d,−1(P-S),rS, there was significant cleavage of this “unreactive” S* in a second slower phase to form shorter products, CCCUC and CCCU. These alternative products presumably arise because reaction at the normal cleavage position is inhibited by the SP phosphorothioate substitution, so that slow cleavage at alternative phosphodiester bonds is favored over cleavage at the normal position, as has been previously observed (Herschlag et al., 1991; Herschlag et al., 1993b). This second phase of cleavage was not cleanly separated from the first phase in reactions of −1d,−1(P-S),rS; the contribution from this second phase was therefore separated by determining the ratio of the normal product (CCCUCU) to all other species (i.e., S* remaining and the shorter products). This gave end points of 55% for the formation of the normal product after > 5 t₁/₂, consistent with 55% of the substrate present as the RP thio isomer and reacting to give the normal product. Although this procedure was necessary to obtain the most accurate rate constants, none of the conclusions herein rely upon these corrections. This procedure was not necessary for reactions of −1(P-S),rS as the second phase of shorter product formation does not interfere; this is because the 2′-hydroxyl at position −1 of −1(P-S),rS greatly speeds cleavage at the normal position relative to normal cleavage of −1d,−1(P-S),rS.

**Determination of (kₐᵤᵤ / Kₛ)⁺.** Second-order rate constants for the reaction of E-S* and G, (kₐᵤᵤ / Kₛ)⁺, were obtained by determining the observed first-order rate constants for disappearance of S* as a function of G concentration. Typically 200 nM E and six concentrations of G were used, with the concentrations chosen to remain at least 5-fold below the dissociation constant of G from the E-S-G complex (Figure 1; McConnell et al., 1993) to ensure that G was not partially saturating. The G concentrations were also chosen to give observed first-order rate constants of <2 min⁻¹ to allow accurate determination of the rate constants by manual pipetting. The observed first-order rate constants were independent of [E] over the range 80–200 or 200–400 nM for each substrate over the entire pH range, indicating that the ribozyme was saturating with respect to S*. Thus, reaction of the E-S* complex was followed. Plots of the observed constants vs [G] were linear, and their slopes gave the values of (kₐᵤᵤ / Kₛ)⁺ for each oligonucleotide substrate at each pH (see Figure 2A for examples).
pH Dependence of the Tetrahymena Ribozyme

RESULTS AND DISCUSSION

pH Dependence of \((k_{\text{cat}}/K_m)^0\): An Unexpected Apparent pKₐ

Figure 2A shows the G concentration dependence of the rate of cleavage of the all-RNA oligonucleotide substrate, rS, at several different pH values. The slopes of these lines give \((k_{\text{cat}}/K_m)^0\) (eq 2), which is shown as a function of pH in Figure 2B. Surprisingly, there is a break at pH 6.9, even though the titratable groups of the RNA bases, sugar, and phosphate have pKₐ values of \(<4\) and \(>9\) (Saenger, 1983). Control reactions have indicated that there are no significant buffer- or salt-specific effects or ionic strength effects that can account for the pH dependence (D. Knitt and D. Herschlag, unpublished results). The ribozyme has a 5'-triphosphate that could titrate near pH 7 and could in principle affect ribozyme activity; however, control experiments with ribozyme lacking this 5'-triphosphate indicate that a titration of the triphosphate also does not account for this pH dependence (data not shown). Thus, we considered two explanations for the apparent pKₐ of 6.9.

(i) The structured environment of the ribozyme perturbs a pKₐ of a ribozyme functional group that must be deprotonated for the reaction to proceed (B in Scheme 1A). Such a group would be a prime candidate for a general base that could remove a proton from the 3'-hydroxyl of G, the attacking group in the reaction.

(ii) The observed pKₐ of 6.9 does not reflect an actual titration; rather it is a kinetic pKₐ that arises from a change in the rate-limiting step that coincidentally occurs at pH 6.9. Scheme 1B shows one such model that can account for the observed pH dependence. According to this model, there is a proton lost in the chemical cleavage step \((k_e)\), but there is also a pH-independent conformational step \((k_{\text{conf}})\). This results in a break in the pH dependence at the pH where \(k_{\text{conf}} = k_e/[H^+]\), as is depicted in Figure 3A (Jencks, 1987; Knowles, 1976; Fersht, 1985). The rate of the conformational step sets a limit which cannot be exceeded, even though the chemical cleavage step continues to increase in rate with increasing pH.

Using Alternative Substrates To Test the Origin of the Apparent pKₐ of 6.9

Models i and ii have been distinguished by comparing the pH dependencies of substrates that vary in their intrinsic reactivities. According to model i, the pKₐ represents an actual titration so that the same step is always rate-limiting \((k_e\) in Scheme 1A). Thus, the ratio of reactivities of different substrates is predicted to be the same above and below this pKₐ (Figure 3B, solid vs dashed lines). However,
according to model ii, the ratio of reactivities is predicted to change with pH because a less reactive substrate would be expected to react more slowly than an intrinsically more reactive substrate at low pH where the chemical step is rate-limiting, but not necessarily at high pH, where a common conformational step is rate-limiting (Figure 3B, solid vs dotted lines).

It was previously shown that substitutions at the 2' position of U(-1), the site of the reactive phosphoryl group, have profound effects on reactivity; these effects were consistent with effects expected on the basis of differences in the intrinsic chemical reactivities of these substrates (Herschlag et al., 1993b). Thus, alternative oligonucleotide substrates that vary in reactivity are available for the ribozyme reaction. Figure 4A shows the pH dependence for three such substrates of varying reactivity. Although -1F,rS and -1d,rS (Chart 1) react 10- and 500-fold slower than rS at the low pH values, these slower substrates react nearly as fast as rS at the highest pH values. This is shown most clearly in the plot of the ratio of reactivities in Figure 4B. The change in the ratio with pH suggests that the apparent pKa arises from a change in the rate-limiting step (model ii) rather than from an actual titration (model i) (Figure 3B and above text). Further, the observation that the reactions with the different substrates approach similar limiting values at high pH is consistent with a common rate-limiting step at high pH that is distinct from the chemical cleavage step.

For several protein enzymes, an identical limiting reaction rate for substrates of varying intrinsic reactivity has provided evidence for a common rate-limiting step. For example, the similar kcat value for hydrolysis of various phosphate esters by Escherichia coli alkaline phosphatase suggests a common rate-limiting step, which, at neutral pH, involves dissociation of inorganic phosphate from a common enzyme/phosphate complex (Garen & Levinthal, 1960; Heppel et al., 1962; Hull et al., 1976). Analogously, the similar rates of reaction of various ester substrates with chymotrypsin suggest that decylation of a common acylenzyme intermediate is rate-limiting (Zerner et al., 1964; see also Fersht and Requena (1971), Caldwell et al. (1991), and Herschlag et al. (1993b)).

The Tetrahymena ribozyme reaction proceeds with inversion of configuration about the reactive phosphoryl group, strongly suggesting that there is no covalent intermediate, common or otherwise (McSwiggen & Cech, 1989; Rajagopal et al., 1989). Note also that single-turnover reactions have been followed, so that release of the second product (CCCCUCU, Figure 1) cannot be rate-limiting. It is suggested that the common rate-limiting step above pH 6.9 is a conformational change such as that depicted in Scheme 1B (see What Step Is Rate-Limiting above pH 7? below). Further support for a rate-limiting conformational step arises from results with phosphorothioate substrates presented in the next section.

pH Dependence of the Thiо Effect as a Probe for a Change in the Rate-Limiting Step. Substitution of a sulfur atom for a nonbridging phosphoryl oxygen atom at the cleavage site of the oligonucleotide substrate provides an independent means to lower the substrate's intrinsic reactivity. This substitution has been shown to decrease the nonenzymatic reactivity of a phosphate diester ~4-10-fold, and substitution of the pro-Rp phosphoryl oxygen atom at the substrate cleavage site by sulfur has been shown to decrease (kcat/Km)G for the ribozyme reaction ~2-fold at pH 6.7 (Herschlag et al., 1991). If the chemical step remains rate-limiting at all pH values, this "thio effect" would be expected to remain constant (model i; Scheme 1A), whereas the thio effect would be expected to disappear, approaching a value of 1, if the conformational step becomes rate-limiting at high pH (model ii; Scheme 1B).

As predicted from model ii, the thio effect for rS does approach 1 at high pH (Figure 5, closed symbols). Further, the change in the thio effect with pH is centered at pH ~7,
consistent with the apparent pK_a of 6.9 for this reaction (Figure 2B). These results provide additional support for an apparent pK_a that arises from a change in the rate-limiting step, with a common conformational step that is rate-limiting at high pH (model ii, Scheme 1B). In addition, the pH dependence of the thio effect argues against an alternative model in which the variations in the pH-rate profiles for the 2'-substituted substrates arise from effects of the 2'-substituents on the pK_a of an active site residue within the E-S complexes and coincidentally match the predictions from model ii.

A further test of model ii is that the thio effect for a slower substrate should not follow the same pH dependence as the thio effect for rS; instead the thio effect is predicted to be maintained until higher pH values. This is because a higher pH would be required for the rate of the chemical step for the slower substrate to overcome the rate of the conformational step (Figure 3). The open symbols of Figure 5 show that the thio effect obtained from reaction of the slower substrates -1d,rS and -1d,1r(P-S),rS is maintained over the entire pH range. This is expected because the rate of the -1d,rS reaction does not reach the limiting rate for the rS reaction even at the highest pH values investigated (Figure 3). Thus, the change in the thio effect for reactions of rS is again suggested to arise from a change in the rate-limiting step with a kinetic rather than an actual pK_a of 6.9.

In previous work with protein enzymes a change in an isotope effect with changing pH was utilized to distinguish between an actual pK_a (model i above) and a kinetic or apparent pK_a [model ii above; e.g., Cook and Cleland (1981a,b) and Fierke et al. (1987)]. Use of a change in the thio or elemental effect with changing pH provides an analogous approach for phosphoryl transfer reactions.

Thio effects have been shown to vary with substrate identity in the T7 DNA polymerase, DNA polymerase III exonuclease, and calf spleen phosphodiesterase reactions (Griep et al., 1990; Wong et al., 1991; Niewiarowski & Uznanski, 1985). This could arise in two ways: (1) Varying contributions from two partially rate-limiting steps, a chemical step with a thio effect that remains constant regardless of substrate identity and a conformational or other step that is unaffected by thio substitution, would result in observed thio effects that vary with substrate identity. (2) Alternatively or additionally, the thio effect on the chemical step could differ with different substrates; the thio effect in nonenzymatic reactions varies, and geometrical and steric contributions to the thio effect in nonenzymatic reactions varies, and geometrical and steric contributions to the thio effect within an active site might also differ with different substrates [see Herschlag et al. (1991) and references therein]. These alternative explanations for variation in observed thio effects might be distinguished by determining the effect of pH on the thio effect for each substrate, provided that the chemical and conformational steps have different pH dependencies.

**What Step Is Rate-Limiting below pH 7?** The initial observation of a pK_a of ~7 in the pH profile for $(k_{cat}/K_m)^G$, Figure 2B) raised the possibility that this proton was lost from a general-base catalyst with pK_a of 7 in the E-S complex. The above results with alternative substrates provide very strong evidence that the observed pK_a of 6.9 represents a kinetic or apparent pK_a rather than an actual titration and remove the basis for this suggestion.

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2 Note that the chemical and conformational steps are each partially rate-limiting near pH 7. For simplicity, we refer to one or the other step as rate-limiting throughout this discussion.

We have previously argued that the actual chemical cleavage of the oligonucleotide substrate is rate-limiting below pH 7 because the observed thio effect below pH 7 is similar to that obtained in model reactions (Herschlag et al., 1991) and because the relative reactivity of the substrates with 2'-substitutions at the cleavage site below pH 7 follows a linear free energy relationship expected on the basis of differences in intrinsic reactivities (Herschlag et al., 1993b). These previous conclusions are supported and extended by the current findings.

The slope of 1 for the pH dependence of $(k_{cat}/K_m)^G$ below pH 7 (Figure 2B) suggests that a proton is lost from the E-S-G ternary complex prior to cleavage of the oligonucleotide substrate. This is based on the above conclusion that the cleavage step is rate-limiting below pH 7 and on the absence of an effect of pH on the binding of G (McConnell et al., 1993; D. Knitt, G. Narlikar, and D. Herschlag, unpublished results), which is the other step in the $(k_{cat}/K_m)^G$ reaction (eq 2). The observed rate constant for reaction of the E-S-G ternary complex that incorporates both the deprotonation and actual chemical cleavage steps can be represented as $(k_{chem})_{obsd} = \frac{(k_{chem})}{[HO-]},$ with $(k_{chem}) = \sim 2 \times 10^9$ M$^{-1}$ min$^{-1}$; this holds below pH ~7 where the chemical step is rate-limiting and gives $(k_{chem})_{obsd} \sim 200$ min$^{-1}$ at pH 7 as in Figure 1. The deprotonation represents an additional step in the kinetic pathway that is not shown in Figure 1.

The simplest model to account for the pH dependence involves loss of a proton from the 3'-hydroxyl of G, as this proton must be lost in the course of the reaction and its removal increases the nucleophilicity of G. It is appealing to posit that Mg$^{2+}$ aids in removing this proton and thus in activating the G nucleophile because of the following: the 3'-oxygen of U(-1011) which is the nucleophilic atom in the reverse reaction, is stabilized by interaction with Mg$^{2+}$ and not by general-acid/base catalysis (Herschlag et al., 1993b; Piccirilli et al., 1993); metal ion activation of nucleophiles is observed in model reactions of phosphate esters and in reactions of phosphate esters catalyzed by protein enzymes (Herschlag & Jencks, 1990; Steitz & Steitz, 1993, and references therein); and there is no obviously suitable candidate for a general-base catalyst on RNA [see, e.g., Yarus (1993)]. However, there are currently no data beyond those presented herein that help distinguish between general- or specific-base catalysis; the data herein provide evidence against a general-base catalyst of pK_a $\leq 7$, but do not rule out a mechanism involving a general-base catalyst of higher pK_a or a mechanism in which a different proton is lost in a rapid but unfavorable conformational step prior to the chemical step [e.g., Kao and Crothers (1980)].

**What Step Is Rate-Limiting above pH 7?** As the chemical step has been implicated as rate-limiting below pH 7, a physical step is implicated as rate-limiting above pH 7. However, there are several possible physical steps, and these possibilities have yet to be distinguished. The physical step could in principle be binding of G or release of the first product, GAAAAA, steps in Figure 1 other than the chemical step that can contribute to $(k_{cat}/K_m)^G$. However, the limiting value of $(k_{cat}/K_m)^G$ of $\sim 10^{10}$ M$^{-1}$ min$^{-1}$ is far below the diffusion rate of $\sim 10^{14}$ M$^{-1}$ min$^{-1}$, suggesting that a simple binding event is not rate-limiting; $(k_{cat}/K_m)^{GAAAAA}$ in the reverse reaction is...
similarly far below the diffusional rate, suggesting that simple
diffusional binding does not limit the reverse reaction and, by
microscopic reversibility, that dissociation of GAAAA does
not limit the forward reaction (T. S. McConnell, T. R. Cech,
and D. Herschlag, unpublished results). Thus, if the G
binding step or GAAAA release step is rate-limiting above pH 7,
the multistep binding process is implicated.

It is possible that binding of an essential Mg$^{2+}$ represents
an additional step involved in the ($k_{\text{cat}}/K_m)^0$ reaction
not shown in Figure 1. However, there is no significant dependence
of ($k_{\text{cat}}/K_m)^0$ on Mg$^{2+}$ concentration above pH 7, arguing
strongly against rate-limiting binding of Mg$^{2+}$ (3-50 mM
Mg$^{2+}$, data not shown).

A remaining alternative is that a conformational rea-
arrangement of the E-S-G ternary complex is rate-limiting above
pH 7. One conformational rearrangement in the ribozyme
reaction has already been identified (Herschlag, 1992;
Bevilacqua et al., 1992). However, this conformational
rearrangement is involved in binding of the oligonucleotide
substrate (Figure 1), whereas ($k_{\text{cat}}/K_m)^0$ represents
the reaction subsequent to this binding step (eq 2). Thus,
the rate-limiting step for ($k_{\text{cat}}/K_m)^0$ above pH 7 must
represent a distinct conformational step. It is possible that
this conformational step is related to the coupled binding
between the oligonucleotide substrate and G (McConnell et al., 1993).

According to this model, after binding of G to form the E-S-G
ternary complex a rearrangement would occur that creates
additional binding interactions that are required in the
chemical step. A conformational change subsequent to binding
of G is also consistent with the observation that changes in
the 2'-substituents of G have much larger effects on reactivity
than on binding (Bass & Cech, 1986; Tanner & Cech, 1987;
Moran et al., 1993; G. Narlikar and D. Herschlag, unpublished
results). Finally, conformational rearrangements could pre-
cede or follow the deprotonation that occurs prior to the
chemical step.4

Further Implications. The kinetic approach utilized herein
of probing the pH dependence of substrates of varying intrinsic
reactivity has revealed that the observed $pK_a$ of $\sim$7 arises
from a change in the rate-limiting step and not from the
titration of a group on the ribozyme. This has identified a
new conformational step in the Tetrahymena ribozyme
reaction. Interestingly, this approach has been used in the
study of a different group I ribozyme, derived from a tRNA
intron of Anabaena, and has similarly revealed a change in
the rate-limiting step above pH 7, consistent with the
occurrence of an analogous conformational step (J. Davila-
This raises the possibility that this conformational change is
a fundamental step on the reaction pathway of all group I
introns. It is hoped that the future study of this and other
conformational steps will help uncover general principles of
RNA structure and dynamics. The catalytic RNAs offer a
unique opportunity for such investigations as the catalytic
reaction can provide a probe of structure and dynamics.

The pH dependence and the relative reactivity of the thio-
and 2'-substituted substrates provide probes for distinguishing
individual steps in the more complex self-splicing of group I
introns. The self-splicing reaction involves two chemical steps
with at least two structural transitions required between the
two chemical steps, replacement of the exogenous G at the

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4 The proton transfer itself, while potentially rate-limiting in principle,
is not expected to be rate-limiting because proton transfer from an acid
of $pK_a > 7$ would be expected to be fast and first-order in hydroxide ion
rather than pH-independent in this pH range (Jencks, 1987).

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REFERENCES
