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Ribonuclease Revisited: Catalysis via the Classical General Acid–Base Mechanism or a Triester-like Mechanism?

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Abstract: A general acid–base catalytic mechanism for ribonuclease A and other ribonucleases was previously widely accepted. However, an alternative to this mechanism was recently reintroduced in which attack by the 2′ hydroxyl group is facilitated by protonation of a nonbridging phosphoryl oxygen atom, instead of the leaving group oxygen atom, to give a triester-like mechanism of catalysis. Literature values for rate effects upon substitution of nonbridging phosphoryl oxygen atoms by sulfur (i.e., "thio-effects") for enzymatic and nonenzymatic reactions of RNA and for nonenzymatic reactions of other phosphate diesters and triesters are compared herein. The thio-effects observed in the RNase A-catalyzed reactions are consistent with predictions based on the classical general acid–base catalyzed mechanism but are inconsistent with predictions based on the triester-like mechanism. Thus, the results of this analysis support the classical general acid–base mechanism rather than the triester-like mechanism. In addition, the results suggest that short, strong hydrogen bonds do not contribute substantially to RNase A catalysis.

Most biochemistry textbooks cite a general acid–base pathway for ribonuclease action. In this "classical" mechanism, one group (His12 in RNase A) acts as a general base to remove the proton from the nucleophilic 2′ hydroxyl group and another group (His119 in RNase A) acts as a general acid to protonate the leaving group 5′ hydroxide ion in the transition state (Figure 1A). However, this mechanism has been called into question with the proposal of a "triester-like" pathway,[1] and at least one textbook has incorporated this mechanism.[2] In this alternative mechanism, a nonbridging phosphoryl oxygen atom is protonated to render the substrate triester-like (Figure 1B).

The recent data suggested to support this pathway in the nonenzymatic cleavage of RNA have been questioned[3] and additional results presented.[4] Regardless of the final resolution of this controversy, the triester-like mechanism should be considered as a possible catalytic route for the enzyme-catalyzed reaction. Protonation of one of the nonbridging phosphoryl oxygens, to render the transition state more like that for a triester reaction, provides a potential catalytic mechanism because phosphate triesters are typically more reactive than the corresponding phosphate diesters (~10^3–10^5-fold).[5,6] Thus, whether or not there is substantiated evidence for this mechanism in solution, it represents a chemically reasonable proposal and therefore cannot be summarily dismissed for the enzymatic reaction. Indeed, even if the classical mechanism is followed in solution, there would be no guarantee that this mechanism would hold for the enzyme-catalyzed reaction.

Information from X-ray crystallographic structures has been

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enormously useful in sorting out and understanding mechanisms of enzymatic catalysis, and the structures of RNase A and other RNases are of very high precision.[7] However, even these high-resolution structures cannot resolve the question of the classical vs triester-like mechanism. For example, inspection of several structures of RNase A with bound ligands from the protein data bank suggests that His12 can be positioned to either accept a proton from the nucleophilic 2′ hydroxyl group (classical mechanism, Figure 1A) or donate a proton to a phosphoryl oxygen atom (triester-like mechanism, Figure 1B).[8] Indeed, if one mode of interaction were clearly preferred in the ground state, this would still not establish the positioning in the transition state. Even structures with bound transition state analogs, despite their widespread usefulness, are limited in their ability to resolve mechanistic questions by the extent to which they mimic the actual transition state. For example, in the pentavalent uridine vanadate transition state analog/RNase A complex, the 2′ oxygen appears to make a full covalent bond to the vanadium, whereas in the actual transition state, this oxygen would make a partial covalent bond to phosphorus. Thus, although His12 appears to be positioned to hydrogen bond with an anionic nonbridging oxygen atom in the RNase complex with the transition state analog,[9] it may nevertheless serve as a general base in the actual transition state. This underscores the idea that information about transition states and catalytic mechanisms is implied from structures and not directly deduced.

Computer simulations also suggest that His119 can adopt the correct orientation to donate a proton to a nonbridging phosphoryl oxygen atom, as would occur in a triester-like mechanism.[10] Though such simulations are helpful in developing and exploring mechanistic proposals, they cannot yet replace experiments in distinguishing mechanistic proposals. Some of the current limitations include the short time scale of the simulations and the approximate nature of the molecular parameters used in the simulations.

A recent mutagenesis experiment in which His119, the putative general acid catalyst of RNase A (Figure 1B), was replaced by Ala is most simply consistent with the classical mechanism.[11] In the classical mechanism, His119 would be expected to donate a proton in the reaction with an adenosine leaving group but not in the reaction with a *p*-nitrophenolate leaving group. This is because His119 would act as a general acid only when there is a sufficient driving force for proton transfer from a leaving group pK_a that is significantly greater than that of histidine. Thus, the presence of His119 acting as a general acid would speed the reaction (relative to the Ala119 mutant) when adenosine is the leaving group (high pK_a) but not when *p*-nitrophenolate is the leaving group (low pK_a), as was observed. However, the possibility that the reaction pathway changes with the change in the nature of the leaving group could not be excluded.[11]

The classical and triester-like mechanisms are distinguished for RNase A herein upon the basis of previously reported thio-effects for enzymatic and nonenzymatic reactions. The results of this analysis strongly support the classical general acid–base catalytic mechanism. The results also argue against the

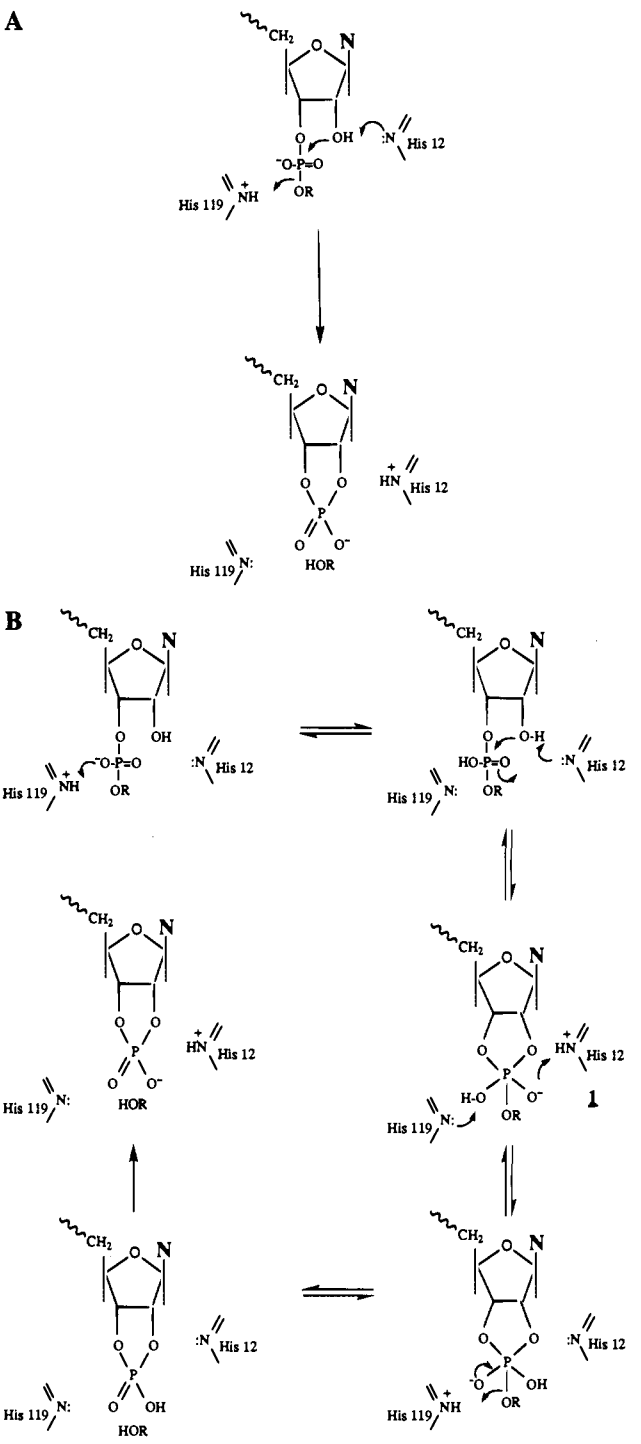

Figure 1. Two possible mechanisms for RNA cleavage by ribonucleases. (A) The classical general acid–base mechanism. In this mechanism, drawn for RNase A, His12 acts as a general base and His119 acts as a general acid in RNA cleavage. The second step of the reaction, opening of the 2′,3′ cyclic phosphate, is presumably analogous to the reverse of the reaction shown, with water (HOH) replacing the alcohol leaving group (ROH). The reaction is draw as concerted, though it is not known whether or not there is a pentavalent species on the reaction path that exists as an intermediate with a finite lifetime. (B) A triester-like mechanism for RNA cleavage. In this mechanism, a nonbridging phosphoryl oxygen atom is protonated to render the reactant "triester-like". Though one specific mechanism is drawn, there are several potential pathways and orders for the proton transfers (see e.g., refs 1 and 28, ref 22, and also Figure 2 and the text). Though the pentavalent species is drawn as an intermediate, the triester-like mechanism does not *a priori* require the existence of a pentavalent species with a finite lifetime.

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Table 1. Thio-Effects in Nonenzymatic Phosphate Diester and Phosphate Triester Reactions[a]

	thio-effect
A. Intermolecular Reactions (Non-RNA)	
phosphate diesters[b]	4−11
phosphate triesters[c]	10−160
B. Intramolecular Reactions (RNA)[d]	
UpA[e]	1.3
	1.0
Gp↓AGU[f]	1.1
UpU[g]	1.3
	0.8
2′,3′ cyclic UMP[h]	6

[a] Thio-effect = k_O/k_S. [b] From ref 15. [c] From ref 29. [d] All reactions are alkaline-catalyzed. [e] From ref 22. The values of 1.3 and 1.0 are for the R_P and S_P thio-isomers, respectively. [f] From ref 15. The S_P thio-isomer was substituted at the position of the arrow, and the thio-effect is reported for cleavage at just this position. [g] From ref 30. The values of 1.3 and 0.8 are for the R_P and S_P thio-isomers, respectively. [h] The thio-effect on the alkaline hydrolysis to open the cyclic phosphate or phosphorothioate.[16] This reaction is analogous to the reverse of RNA cleavage with the nucleophilic hydroxide ion replacing an alkoxide leaving group.

importance of a low-barrier hydrogen bond in catalysis by RNase A[12] and a modified triester-like mechanism.[1,13]

Classical and Triester-like Mechanisms Distinguished through Analysis of Thio-Effects

Two types of analyses are presented below that distinguish between the classical and triester-like mechanisms; each analysis is distinct, though both are based on comparisons of predicted and observed thio-effects. In the first, the different thio-effects for nonenzymatic reactions of phosphate diesters and triesters are used as a basis for predicting the enzymatic thio-effects. In the second, the differential proton affinities of oxygen and sulfur are used.

(1) Table 1 summarizes thio-effects obtained in nonenzymatic reactions of phosphate diesters and phosphate triesters. A "thio-effect" is defined herein as the ratio of rate constants for reaction of the phosphate compound relative to the corresponding phosphorothioate, in which a nonbridging phosphoryl oxygen has been replaced by sulfur; the thio-effect then represents how much the sulfur substitution *slows* the reaction. Thio-effects measured for phosphate diester reactions are smaller than those for phosphate triester reactions. Thus, a small thio-effect in a RNase-catalyzed reaction is inconsistent with expectations for a triester-like mechanism and would therefore support the classical mechanism.[14] The thio-effect on k_{cat} for the opening of the 2′,3′ cyclic phosphate of uridine (cUMP) by RNase A is 5 for both the R_P and S_P thio-isomers.[16] This is within the range observed for nonenzymatic reactions of phosphate diesters but smaller than observed for the triesters (Table 1). In addition, the thio-effects of 5 are remarkably similar to the thio-effect of 6 obtained for opening of cUMP in strong base (Table 1). In contrast, opening of cUMP in strong acid gives a large thio-effect of ~200 (0.15 N $HClO_4$).[16] This reaction is expected to include protonation of a nonbridging phosphoryl oxygen atom, thereby rendering the reaction phosphate triester-like. Thus, the different thio-effects in the alkaline- and acid-catalyzed reactions support the use of the thio-effect as a discriminator between diester- and triester-like mechanisms. The results suggest that the enzymatic reaction follows the classical mechanism rather than the triester-like mechanism.

(2) In the triester-like mechanism (Figure 1B), one of the phosphoryl oxygen atoms is protonated in the transition state. Thiolates are in general considerably more difficult to protonate than alkoxides. For example, the pK_a of ethanethiol is 4 pH units lower than that of ethanol, representing a difference of 10^4 in proton affinity.[17] Thiophosphate also has lower pK_a values than phosphate, with pK_a values of 1.7, 5.4, and 10.1 for thiophosphate and 2.1, 7.2, and 12.3 for phosphate.[17,18] These values represent lower limits for the differential proton affinity of sulfur and oxygen bound to phosphorus because protonation occurs preferentially on oxygen for thiophosphate; this occurs even though there is evidence for greater single bond character of P−S bonds relative to P−O bonds within a phosphoryl compound and for more negative charge localized on unprotonated sulfur than on unprotonated oxygen within thiophosphate and related compounds.[19] Thus, a large thio-effect would be predicted for at least one of the thio-isomers in the triester-like mechanism because it would be considerably more difficult to protonate the sulfur atom (Figure 1B); a significant thio-effect would also be predicted for the other thio-isomer because it would render the remaining nonbridging oxygen atom more difficult to protonate. However, it is difficult to predict precise values for these thio-effects. Again, both thio-isomers of cUMP give small thio-effects of 5 on k_{cat} in the RNase A reaction.[16] This suggests that neither phosphoryl oxygen is protonated in the transition state and further argues against the triester-like mechanism.

Each of the analyses above provides evidence against the triester-like mechanism. In combination, the evidence is stronger because the arguments are distinct and because the effects on protonation of the remaining nonbridging oxygen in a phosphorothioate to form the triester-like species [(2) above] and on intrinsic reactivity subsequent to protonation [(1) above] would both contribute to the thio-effect in the triester-like mechanism. Thus, the predicted thio-effect in the triester-like mechanism is even *larger* than that predicted from either effect alone.

Catalysis via Short, Strong Hydrogen Bonds? A third mechanism that has recently been proposed for RNases is one involving short, strong (or low barrier) hydrogen bonds.[12] As proposed, matching of pK_a's between hydrogen bond donors on the enzyme and the phosphoryl oxygen atoms in a pentavalent intermediate would facilitate the formation and breakdown of this intermediate. If such a mechanism provided a significant rate enhancement for RNase A, then substitution of a phosphoryl oxygen atom by sulfur would be predicted to greatly impede the reaction because of the poor hydrogen bonding ability of sulfur and because of perturbation of the pK_a of the nonsubstituted phosphoryl oxygen atom. Thus, the modest thio-effects of ~5 in the RNase A-catalyzed opening of cUMP[16] suggest that the proposed short, strong hydrogen bonds are not major contributors to catalysis by RNase A. Monitoring the effects of substitutions that perturb pK_a values of reactants and

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(13) Anslyn, E.; Breslow, R. *J. Am. Chem. Soc.* **1989**, *111*, 4473−4482. Breslow, R. *Acc. Chem. Res.* **1991**, *24*, 319−324.
(14) An important caveat to this is that, if the chemical step is not rate-limiting in the enzymatic reaction, then no mechanistic conclusion can be drawn from the thio-effect (see below). Further, the converse of the statement in the text, that a large thio-effect would be diagnostic of a triester-like mechanism, does not hold. This is because other factors, such as size, hydrogen bonding ability, and differential metal ion affinity, can augment the thio-effect beyond that resulting solely from effects on intrinsic reactivity (see ref 15 for a more complete discussion).
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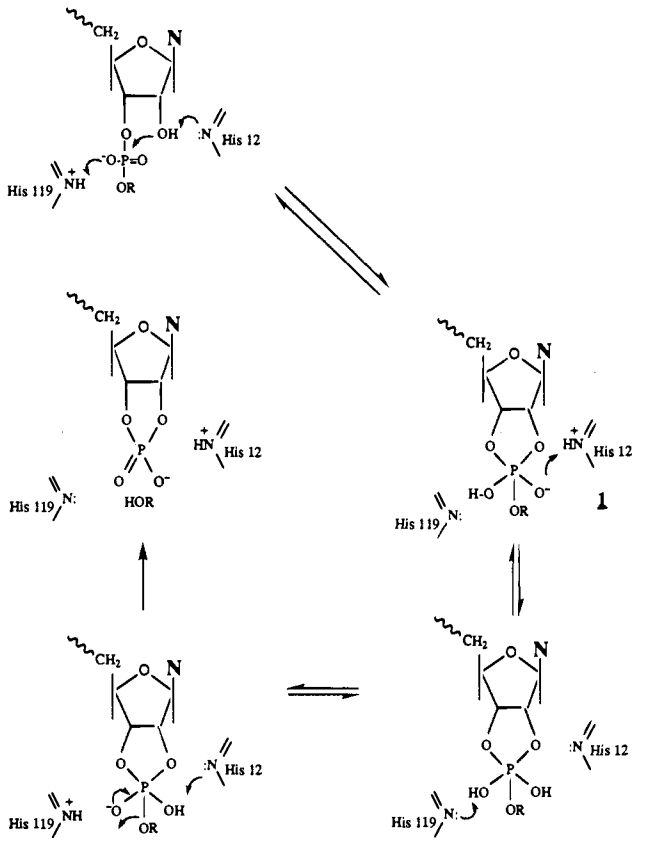

Figure 2. Modified "triester-like" mechanism previously proposed for RNases.[1,13] The initial steps of the triester-like mechanism (Figure 1B) have been suggested to be concerted because of prepositioning of the reactive groups by the enzyme. Thus, the second species in this pathway is the same as the third species in the triester-like pathway of Figure 1B (i.e., **1** in both figures).

intermediates may provide a test of the proposed involvement of short, strong hydrogen bonds in other reactions[12] (e.g., via substitution of fluorine for hydrogen in carbanion reactions).

A Modified Triester-like Catalytic Mechanism? It has been suggested that the RNase-catalyzed reaction follows a modified version of the triester-like mechanism, differing from the proposed solution reaction shown in Figure 1B. In the modified mechanism (Figure 2), the nucleophilic attack and two proton transfers, the protonation of the nonbridging phosphoryl oxygen atom and the deprotonation of the 2′ hydroxyl group that accompanies its attack at phosphorus, are suggested to occur in concert.[1,13]

The same line of analysis used above for the triester-like mechanism also holds for the modified triester-like mechanism so that the modest thio-effects observed in the RNase reactions do not support RNase catalysis via the modified triester-like mechanism either.[20,21] Briefly, according to the modified mechanism, there is partial protonation of a nonbridging phosphoryl oxygen atom in the transition state. As described in (2) above, protonation, or partial protonation, is expected to be more difficult both when the oxygen that is protonated is replaced by sulfur and also when the other nonbridging oxygen atom is replaced by sulfur. The modified triester-like mechanism, as proposed, also includes protonation of each of the nonbridging phosphoryl oxygen atoms (Figure 2.)[1,13] Thus, substantial thio-effects are predicted for *both* thio-isomers (see also Other RNase Reactions below). The thio-effects would be predicted to be further increased by the same features that result in larger thio-effects for phosphate triester reactions than for phosphate diester reactions (Table 1); these effects will be applicable to an extent reflecting the extent of proton transfer in the transition state, which is expected to be substantial.[21]

Other RNase Reactions. RNase T2 exhibits small thio-effects of ~2-fold in k_{cat} and ~4-fold in k_{cat}/K_m for the hydrolysis of both thio-isomers of UpA,[22] similar to the values obtained in nonenzymatic reactions (Table 1) and consistent with the classical general acid–base mechanism.

The thio-effects of k_{cat} and k_{cat}/K_m with RNase A are ~2- and ~4-fold, respectively, for the R_P thio-isomer of UpA, essentially the same as the values with RNase T2.[22] However, the thio-effects for the S_P thio-isomer are considerably higher with RNase A, ~70- and ~100-fold in k_{cat} and k_{cat}/K_m, respectively. As noted above,[14] large thio-effects can result from differences in the way that the sulfur interacts in the enzyme active site, so that these *large thio-effects cannot be taken as evidence for a triester-like mechanism.* Indeed, the small thio-effect for the R_P thio-isomer of UpA supports the classical mechanism for this cleavage reaction. This is because the triester-like mechanism predicts a large thio-effect for *both* thio-isomers. As outlined for cUMP hydrolysis above, a thio-effect at the position of protonation is predicted because protonation of sulfur is harder than protonation of oxygen, and a thio-effect at the other position is also predicted because sulfur substitution renders protonation of the remaining nonbridging oxygen more difficult and because the protonated diester is analogous to a triester, which exhibits large thio-effects. UpA cleavage is analogous to the reverse of cUMP hydrolysis, with the nucleophilic hydroxide ion replacing an alkoxide leaving group, so it would be expected that both reactions would follow the same mechanism.[23,26]

(20) The analogy between the two mechanisms can be seen as follows. First, the lowest energy ground state is the same in both the triester-like and modified triester-like mechanisms (Figures 1B & 2). Second, the mechanisms share a common intermediate, **1**, which is the third species in the triester-like mechanism (Figure 1B) and the second species in the modified triester-like mechanism (Figure 2). In addition, the transition states for attack by the 2′ oxygen atom in the triester-like and modified triester-like mechanisms share the common feature of (full or partial) protonation of a nonbridging phosphoryl oxygen atom. Thus, thio-effects originating from this protonation will be expressed in both mechanisms.[21] Although a protonated phosphate diester (the triester-like species) is formed as an intermediate in the triester-like mechanism (Figure 1B) and not in the modified triester-like mechanism (Figure 2), this species is an unstable intermediate; it is therefore expected to be kinetically invisible and not contribute to the observed thio-effect as rate constants reflect relative free energies of the lowest energy ground state and the rate-limiting transition state.

(21) The partial proton transfer to a nonbridging phosphoryl oxygen atom in the nucleophilic step of the modified triester-like mechanism (Figure 2), instead of complete proton transfer in the triester-like mechanism (Figure 1B), would reduce the expected thio-effect. However, the following suggests that this reduction would be small so that the thio-effects in the modified triester-like and triester-like mechanisms are predicted to be similar. The transition state in the modified mechanism is expected to resemble the high-energy phosphorane intermediate due to a Hammond effect so that substantial proton transfer is expected in this transition state. Analogously, electronic changes upon thio-substitution that affect the energy of the triester transition state should also be largely expressed in the transition state for the modified triester reaction.

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(23) Despite the small thio-effect of ~5 on k_{cat} for the *exo* thio-isomer of cUMP, there is a larger thio-effect of ~70-fold on k_{cat} for cleavage of the S_P isomer of UpA.[22,24] This difference occurs even though these reactants have the "same" phosphoryl oxygen atom substituted by sulfur (i.e., the *exo* thio-isomer of cUMP is the product from cleavage of the S_P thio-isomer of UpA.[25] There is also a ~10-fold increase in K_m for the *exo* thio-isomer of cUMP, presumably reflecting weaker binding, whereas the thio-effect on K_m for the S_P thio-isomer of UpA is <2-fold.[24] (There is no effect on K_m with the *endo* thio-isomer.[24]) These observations are consistent with the presence of a thio-sensitive contact with the enzyme at this position. It will be of interest to understand how the effect is differentially manifested in the binding and chemical steps for the two substrates.

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Caveats and Concerns

The Rate-Limiting Step. An alternative explanation for an observed small thio-effect is that there is actually a large thio-effect on the chemical step but that the observed thio-effect is attenuated because the chemical step is not rate-limiting. The following results suggest that the chemical step is indeed rate-limiting for opening of cUMP by RNase A reaction. (1) Several dinucleotide cleavage and cyclic phosphate hydrolysis reactions of RNase A follow the same pH dependencies for k_{cat}/K_m even though the absolute rates vary by $\sim 10^4$-fold.[26] This provides no indication of any kinetic complexities in the reaction that would render a step other than the chemical step rate-limiting. (2) Hydrolysis of cyclic phosphates by RNase, the reaction predominantly discussed above, is slower than dinucleotide cleavage; the ability of the enzyme to turn over much faster than cyclic phosphate hydrolysis renders it unlikely that this slower reaction is limited by a physical step. (3) If a large thio-effect on the chemical step were "masked" by an alternative rate-limiting step, then a much larger thio-effect might be expected from introduction of a second sulfur than from introduction of the first. (The first thio-substitution would greatly slow the chemical step but have little or no observed effect; introduction of the second sulfur to give the dithioate substrate would then have a much larger effect if the first thio-substitution had already rendered the chemical step rate-limiting or nearly rate-limiting.) However, k_{cat} for the dithioate of cUMP is only 5-fold slower than that for the cUMP and is similar to the values for the monothioates.[24] This again provides no indication of kinetic complexities that would render a step other than the chemical step rate-limiting.

An additional potential complexity is that there is not a single step in the triester-like and modified triester-like mechanisms (Figures 1B and 2). However, it would seem most likely that the P–O bond formation step or the P–O bond cleavage step would be rate-limiting, rather than the proton transfer steps; the thio-effects would be predicted to be expressed in both of these transition states as both would be expected to resemble the protonated phosphorane species.[21]

Other Possible Mechanisms? If the nonenzymatic phosphate diester and triester reactions are not adequate models for the enzymatic reactions, then the above conclusions based on a comparison of thio-effects would not necessarily hold. As our understanding of the details of both the nonenzymatic and enzymatic reactions is limited, this must be considered a formal possibility even in the absence of supporting data for such dramatic differences.

Conclusions

Analysis of thio-effects in RNase-catalyzed and nonenzymatic reactions strongly supports the classical general acid–base mechanism rather than a triester-like mechanism, a modified triester-like mechanism, or a mechanism involving short, strong hydrogen bonds. Despite the status of RNase as a "classical" enzyme, many mechanistic questions remain to be addressed. These will be especially important to understand given the increasing number of ribonucleases implicated in a variety of specific biological processes.[27]

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