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 catalyzed 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 (His19 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, and at least one textbook has incorporated this mechanism. 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 and additional results presented. 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). 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

enormously useful in sorting out and understanding mechanisms of enzymatic catalysis, and the structures of RNases A and other RNases are of very high precision. 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 database 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). 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, 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. 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. 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 pKₐ 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 pKₐ) but not when p-nitrophenolate is the leaving group (low pKₐ), as was observed. However, the possibility that the reaction pathway changes with the change in the nature of the leaving group cannot be excluded.

The classical and triester-like mechanisms are distinguished for RNase A herein upon the basis of previously reported thioeffects 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

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**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 drawn 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.
importance of a low-barrier hydrogen bond in catalysis by RNase A\textsuperscript{12} and a modified triester-like mechanism.\textsuperscript{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 observed in nonenzymatic reactions of phosphate diesters and phosphate triesters. A "thio-effect" is defined herein as the ratio of rate constants for reaction between diester- and triester-like mechanisms. The results of substitutions that perturb 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 is a RNase-catalyzed reaction is inconsistent with expectations for a triester-like mechanism and would therefore support the classical mechanism.\textsuperscript{14} The thio-effect on $k_{RNase}$ for the opening of the 2',3' cyclic phosphate of uridine (cUMP) by RNase A is $S$ for both the $R_P$ and $S_P$ thio-isomers.\textsuperscript{15} 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 $S$ 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 $\sim 200 (0.15 \text{N HClO}_4)$.\textsuperscript{16} This reaction is expected to include protonation of a nonbridging phosphoroyl 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 phosphorol 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 ethanol is 4 pH units lower than that of ethanol, representing a difference of 10$^9$ in proton affinity.\textsuperscript{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.\textsuperscript{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 phosphorothioate and for more negative charge localized on unprotonated sulfur than on unprotonated oxygen within thiophosphate and related compounds.\textsuperscript{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 $k_{RNase}$ in the RNase A reaction.\textsuperscript{16} This suggests that neither phosphorothioate nor phosphorothiyl 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.\textsuperscript{12} As proposed, matching of $pK_a$'s between hydrogen bond donors on the enzyme and the phosphoroyl 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 phosphoroyl 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 phosphoroyl oxygen atom. Thus, the modest thio-effects of $\sim 5$ in the RNase A-catalyzed opening of cUMP\textsuperscript{16} suggest that the proposed short, strong hydrogen bonds are not major contributors to catalysis by RNase A. Monitoring the effects of substitutions of reactants and products of $pK_a$ values of reactants and products of (17) Jencks, W. P.; Regenstein, J. In Handbook of Biochemistry and Molecular Biology; Fasman, G. D., Ed.; CRC Press: Cleveland, OH, 1976; Vol. 1, p 305-351.


(19) Frey, P. A.; Sammons, D. Science 1985, 228, 541-545.
intermediates may provide a test of the proposed involvement of short, strong hydrogen bonds in other reactions\(^\text{(2)}\) (e.g., via substitution of fluorine for hydrogen in carbamion 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.\(^\text{(1,13)}\)

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.\(^\text{(20,21)}\) 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

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 phosphonate 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.

(22) Burgers, M. J.; Eckstein, F. Biochemistry 1979, 18, 592–596.

(23) Despite the small thio-effect of ~5 on \(k_{\text{cat}}\) or \(k_{\text{cat}}/K_m\) for the \(\text{exo}\) thio-isomer of cUMP, there is a larger thio-effect of ~70-fold on \(k_{\text{cat}}\) or \(k_{\text{cat}}/K_m\) for cleavage of the \(\text{Sp}\) isomer of UpA.\(^\text{(22)}\) This difference occurs even though these reactants have the “same” phosphoryl oxygen atom substituted by sulfur (i.e., the \(\text{exo}\) thio-isomer of cUMP is the product from cleavage of the \(\text{Sp}\) thio-isomer of UpA).\(^\text{(22)}\) There is also a ~10-fold increase in \(K_m\) for the \(\text{exo}\) thio-isomer of cUMP, presumably reflecting weaker binding, whereas the thio-effect on \(k_{\text{cat}}\) with the \(\text{endo}\) thio-isomer.\(^\text{(24)}\) 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. 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. 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.

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.

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