Kinetics and mechanism in RNA cleavage
(steady state/phosphorane/imidazole/partitioning)

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ABSTRACT Experimental studies on the cleavage of various RNA molecules—poly(U), 3',5'-UpU, 2',5'-UpU, and 3',5'-ApA—catalyzed by imidazole buffers show that there is a sequential bifunctional mechanism. One catalyst species converts the substrate to an intermediate phosphorane, and the second catalyst converts the phosphorane to cleavage products. Detailed steady-state kinetics are presented to determine all mechanisms that are consistent with the findings. Choice among these possibilities can be made considering other experimental evidence about the catalysis of an isomerization reaction that accompanies cleavage. It is concluded that all acceptable mechanisms involve action of the second catalyst on a phosphorane monoanion; it can be formed directly from the substrate in the first step or by proton equilibrations involving either the substrate or the phosphorane intermediate. The relationship of these conclusions to the likely mechanism of action of the enzyme ribonuclease is briefly discussed.

A series of studies have been performed on the cleavage of RNA catalyzed by imidazole buffers (1-4) and more recently by morpholine buffers (5, 6). With imidazole buffers, a bell-shaped rate vs. protonation state was seen for the cleavage of polyuridylic acid—forming a 2,3-cyclic phosphate while cleaving the chain—and for 3',5'-UpU, for 2',5'-UpU, and for 3',5'-ApA. In the latter cases the products of the cleavage reaction are uridine (or adenosine) and the 2',3'-cyclic phosphate of uridine (or adenosine).

The bell-shaped curve was seen whether or not one corrected for uncatalyzed reaction at the same pH, since the correction was small (ref. 1 and R.B. and R. Xu, unpublished data). The rate of reaction as a function of total buffer concentration was seen to be linear in buffer concentration, again whether or not the observed rates were adjusted by the small correction for uncatalyzed reaction at the same pH (ref. 1 and R.B. and R. Xu, unpublished data). As was pointed out, these two facts—a bell-shaped curve vs. protonation state, with an optimum when both buffer components are present, but a linear dependence on buffer concentration—are consistent with sequential bifunctional catalysis. The substrate is converted in a first step to an intermediate catalyzed by one buffer component, and in the second step this intermediate is converted to the product with catalysis by the other buffer component. Since the product is a cyclic phosphate, the intermediate must be a phosphorane.

It was asserted (1-5) that the intermediate phosphorane must be a monoanion, isomeric with the starting phosphate monoanion, for the kinetics to fit the observations. It is the purpose of this paper to develop the arguments in more depth and to see how steady-state kinetics, in the elegant partitioning form devised by Cleland (7), can clarify the possibilities.

Definitions and Assumptions

I define the starting material, whether poly(U) or a dinucleotide, as SH-. The H represents the proton of the neighboring hydroxyl group that must be lost to form the cyclic phosphate or the intermediate phosphorane; under the reaction conditions (1-5) the phosphate diester group is in its stable state as a monoanion. I define the phosphorane intermediate as I and indicate as its three possible forms the dianion FI-, the monoanion IH-, or the fully protonated phosphorane IH2. The product of the cleavage reaction is simply called P; it is a mixture of uridine and the 2',3'-cyclic phosphate of uridine when UpU is the substrate, for instance, as shown in Fig. 1.

I assume that imidazole Im and imidazolium ion ImH+ are the only two acting forms of these catalysts, and in particular I ignore any possible role of the imidazole anion. The pKa value of imidazole is quite high, 14.4, so its anion is unlikely to play a role, and as discussed by Corcoran (8) and Kool (9), N-methylimidazole—which cannot form an anion—is similar to imidazole as a catalyst. I also assume that no simultaneous termolecular reactions occur. For instance, one cannot have a step catalyzed simultaneously by Im and OH-. Termolecular reactions are quite improbable unless there is prior association of the components.

Mechanism and Kinetics

I first consider the situation in which whatever intermediate phosphorane is formed undergoes cleavage, or reversal to starting material, without proton equilibration in the intermediate. I will then consider the case in which the phosphoranes can equilibrate rapidly; since the pKa value of IH2 is estimated (10) to be 9, this means that such equilibration with imidazole buffers in the pH region near 7 will cause IH2 to be the dominant intermediate species, however it is formed.

I assume that any phosphorane is a steady-state intermediate, and I use the partitioning method of Cleland (7) to solve the kinetic equations. By this method the rate of formation of the product is (the rate of formation of the steady-state intermediate) times (the fraction of that intermediate that partitions forward to product, rather than back to starting material). By the steady-state approximation, it must do one or the other. Consider first two reaction schemes in which the intermediate is IH-, an isomer of the substrate. If the first catalyst is the imidazole B and the second step is catalyzed by BH+ (mechanism 1), the steady-state equation is Eq. 2.

\[ SH \xrightarrow{k_B} IH \xrightarrow{k_{BH}^+} P \]  

\[ \frac{dP}{dt} = k_3[B^+] \cdot \frac{k_1[B]}{k_2[B^+] + k_{-1}[B]} \]  

where \( P \) is product and \( t \) is time.
Eq. 2 has a maximum when B and BH⁺ are both present, but the rate is first order in buffer concentration; dimensionally, the numerator is the concentration squared, and the denominator is concentration.

With the alternative mechanism 3, Eq. 4 is almost the same.

\[ \text{SH} \xrightarrow{k_{BH}^-} \text{IH} \xrightarrow{k_B} \text{P} \]  

\[ \frac{dP}{dt} = \frac{k_1[\text{BH}^+]}{k_B + k_{BH}[\text{BH}^+]} \]  

For this reason it was at first (1) impossible to select between these two reaction schemes, and only later (refs. 2-5 and R.B. and R. Xu, unpublished data) was evidence found that the second catalyst is B, not BH⁺. For this reason I now favor mechanism 3, in a somewhat expanded form (see mechanism 8).

Consider another possibility, mechanism 5, in which the intermediate is instead IH₂. The steady-state equation for this is Eq. 6.

\[ \text{SH} \xrightarrow{k_{BH}^-} \text{IH}_2 \xrightarrow{k_B} \text{P} \]  

\[ \frac{dP}{dt} = \frac{k_1[\text{BH}^+]}{k_B + k_{BH}[\text{BH}^+]} \]  

It is apparent that this does not fit the bell curve, since [B] cancels and the rate depends only on [BH⁺]. The reason for this is that the first transition state has the composition B-IH₂ in the reverse direction and so does the second transition state in the forward direction. The bell curve requires that they differ by one proton in composition, as in reactions 1 and 3. The same argument excludes mechanism 7, whose equation would have no term in [BH⁺].

\[ \text{SH} \xrightarrow{k_B} \text{BH}^+ \]  

\[ \frac{dP}{dt} = \frac{k_1[\text{BH}^+]}{k_B + k_{BH}[\text{BH}^+]} \]  

However, the substrate SH⁻ could be in rapid equilibrium with the less-stable SH₂, and such a prior equilibrium allows another possibility. Mechanism 8 has IH⁻ as an intermediate, and it is the mechanism (shown in detail in Fig. 1) I favor. It is kinetically equivalent to mechanism 3, just the specific acid/general base version of the general acid catalysis in the first step of mechanism 3. Note that the pKₐ of the phosphate group is quite a few units below the operating pH of the buffer, which is near pH 7. Under those circumstances, [SH⁻] is essentially pH independent—equal to [S_total]—and [SH₂] is simply \( K_{eq}[\text{H}^+][\text{SH}^-] \).

\[ \text{SH} \xrightarrow{k_{1BH}} \text{SH}_2 \xrightarrow{k_B} \text{IH} \xrightarrow{k_B} \text{P} \]  

\[ \frac{dP}{dt} = \frac{k_1[\text{BH}^+]}{k_B + k_{BH}[\text{BH}^+]} \]  

Since [H⁺][B] is proportional to [BH⁺], this scheme also fits the observations.

However, a modification of mechanism 8 in which the intermediate is instead IH₂ is not possible. The catalysts in the k₁ and the k₋₁ steps would now have to be the same, since the conversion of SH₂ to IH₂ involves no proton loss or gain. If they were both B, then [B] would cancel from the parti-
tioning expression. If they were both BH\textsuperscript{+}, mechanism 10 would lead to Eq. 11.

\[
\text{SH} \xrightleftharpoons[k_+]{k_-} \text{SH}_2 \xrightarrow[k_+]{k_-} \text{IH}_2 \xrightarrow{k_B} \text{P} \tag{10}
\]

\[
\frac{dP}{dt} = \frac{K_{eq}[\text{H}^+]}{[\text{SH}^-]} \cdot \frac{k_B}{k_B}[\text{H}^+] + k_-[\text{BH}^+] \cdot [\text{OP}]. \tag{11}
\]

This has only [BH\textsuperscript{+}]	extsuperscript{2} in the numerator (because of the product [B][H\textsuperscript{+}]), so it has a maximum rate when [B] is zero. Similar arguments exclude the even less likely equilibration of SH\textsuperscript{-} with S\textsuperscript{2}-, then conversion to I\textsuperscript{2}. The only possible mechanism involving such a prior equilibration is mechanism 12, whose Eq. 13 is equivalent to Eq. 2. Again the intermediate must be IH\textsuperscript{+}, even if there is prior proton equilibrium of the substrate.

\[
\text{SH} \xrightleftharpoons[k_+]{k_-} \text{S}^- \xrightarrow[k_+]{k_-} \text{IH} \xrightarrow{[\text{BH}^+]} \text{P} \tag{12}
\]

\[
\frac{dP}{dt} = \frac{K_{eq}[\text{SH}^-]}{[\text{SH}^-]} \cdot \frac{k_B}{k_B}[\text{H}^+] + k_-[\text{BH}^+]. \tag{13}
\]

Thus I conclude that if there is only one intermediate that does not equilibrate with other protonation states during its short lifetime that intermediate must be IH\textsuperscript{+}, an isomer of the substrate. The situation is somewhat different if the phosphoranes are in fact able to equilibrate rapidly.

The pKa value of a phosphorane has been estimated (10) to be 9. Under those circumstances, the stable form of the intermediate at the operating pH of the buffers, near 7.0, is IH\textsubscript{2}. Thus if the phosphorane intermediates are in rapid proton equilibrium during their lifetime, which could be true, other schemes must be considered. For instance, mechanism 14 leads to Eq. 15, which fits the requirement for a bell-shaped rate vs. protonation state curve but a linear dependence on buffer concentration.

\[
\text{SH} \xrightarrow{k_B} \text{IH} \xrightarrow{H^+} \text{IH}_2 \xrightarrow{k_B} \text{P} \tag{14}
\]

\[
\frac{dP}{dt} = \frac{k_B}{k_B}[\text{H}^+] \cdot \frac{k_B}{k_B} + K_{eq}[\text{SH}^-]. \tag{15}
\]

Note that [H\textsuperscript{+}] does not appear in the numerator of the first equation, in which IH\textsubscript{2} is taken as the steady-state intermediate that partitions, since forming IH\textsuperscript{+} automatically forms IH\textsubscript{2}—the stable protonation state—if there is rapid proton equilibrium. The second equation just involves multiplying top and bottom by [H\textsuperscript{+}]; K\textsuperscript{'} is a composite equilibrium constant produced by this procedure.

Other schemes are possible in which IH\textsubscript{2} is the first intermediate. For instance, mechanism 16 leads to Eq. 17.

\[
\text{SH} \xrightarrow{k_B} \text{BH} \xrightarrow{H^+} \text{IH} \xrightarrow{k_B} \text{P} \tag{16}
\]

\[
\frac{dP}{dt} = \frac{k_B}{k_B}[\text{H}^+] \cdot \frac{K_{eq}[\text{SH}^-]}{K_{eq}[\text{SH}^-]}. \tag{17}
\]

Again the second equation is simply obtained from the first on multiplying top and bottom by [H\textsuperscript{+}].
Conclusions

It is sometimes believed that kinetics relates the starting materials to the transition state but cannot furnish information about the product of a reaction. This is normally true, but the steady-state situation is special. The kinetics reflect not just the rate of formation of an intermediate but also the rate at which it is converted back to starting material. Thus this rate ratio reflects an equilibrium constant connecting the starting material with the steady-state intermediate. It lets one deduce the composition of that intermediate, at least how its protonation state relates to that of the substrate. In the present case, it shows that the intermediate that is acted on by the second catalyst is IH⁻, an isomer of the substrate. This concept is incorporated in the detailed mechanism proposed for these reactions.

The mechanism for the enzyme ribonuclease A has been discussed (refs. 2–5, 11, and R.B. and R. Xu, unpublished data) that is proposed by analogy with the mechanism of this model system and that is supported by other evidence on the enzyme. Of course in the enzyme the acid and base group can act simultaneously, not sequentially, and there is evidence (12) that they do so. This is also true (13) for a bifunctional enzyme mimic (6) in which both imidazole groups are covalently linked to a cyclodextrin binding group. However, there are two aspects of the simple imidazole buffer model system that probably do carry over to the enzyme: a phosphorane monoanion is the intermediate, and it is formed by a reaction in which the acid catalyst BH⁺ acts just as it does in the model. This is a different role from that which has been assigned to BH⁺ in the past. Thus the detailed kinetic studies of this system have not only unraveled the mechanistic mystery of an interesting chemical catalysis, they have pointed the way to the solution of the enzymatic mystery as well.

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