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RIBONUCLEASE MIMICS

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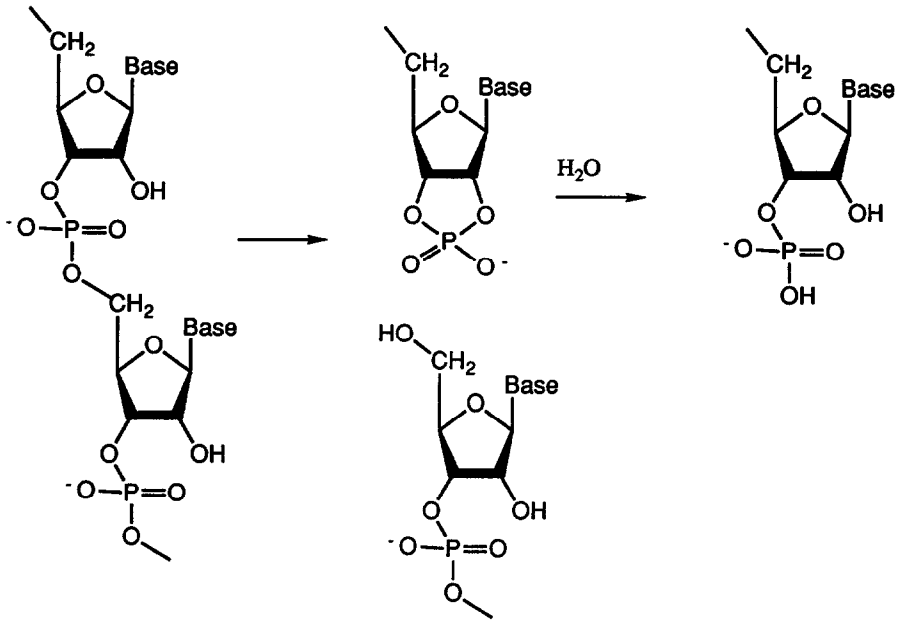
ABSTRACT - *The cleavage of the ribonucleotide UpU by imidazole buffers shows kinetic behavior that indicates a sequential bifunctional mechanism, in which the imidazolium ion acts first. Similar results are obtained for the cleavage of ApA. This mechanism is related to the simultaneous bifunctional cleavage of RNA by the enzyme ribonuclease, and has guided us to the synthesis of an improved cyclodextrin-bis-imidazole enzyme mimic that also uses a simultaneous bifunctional mechanism.*

The enzyme ribonuclease A catalyzes a two-step cleavage of RNA (Scheme 1).[1] In the first step the linear phosphate 3'-5" diester is converted to a 2',3'-cyclic phosphate diester by attack on the phosphate by the C-2' OH group, with loss of the attached nucleoside and resultant chain cleavage. In the second step the cyclic phosphate ester is hydrolyzed to form a C-3' phosphate monoester and regenerate the C-2' OH group. Extensive studies on the enzyme[1] have made it clear that the principal catalytic groups for both steps are the imidazoles of His-12 and His-119, with additional catalysis by the terminal ammonium group of Lys-41. The pH dependence indicates that one imidazole is used as a base, while the other acts as the acidic imidazolium group.

It was of interest to see what the preferred mechanism is for the cleavage of RNA by imidazole and imidazolium groups, as present in imidazole buffer. Kinetic methods are available for elucidating mechanisms with such freely moving species that are not applicable to enzymatic reactions; the chemically preferred mechanism might furnish insight into the mechanisms used by the enzyme, and the contrasts between simple buffer catalysis and catalysis by the same groups fixed in an enzyme could also be instructive. Finally, we had a program to synthesize catalysts that could mimic ribonuclease, and knowledge of the best

chemical mechanism could help guide this program. All of these hopes were fulfilled by the results of the studies.



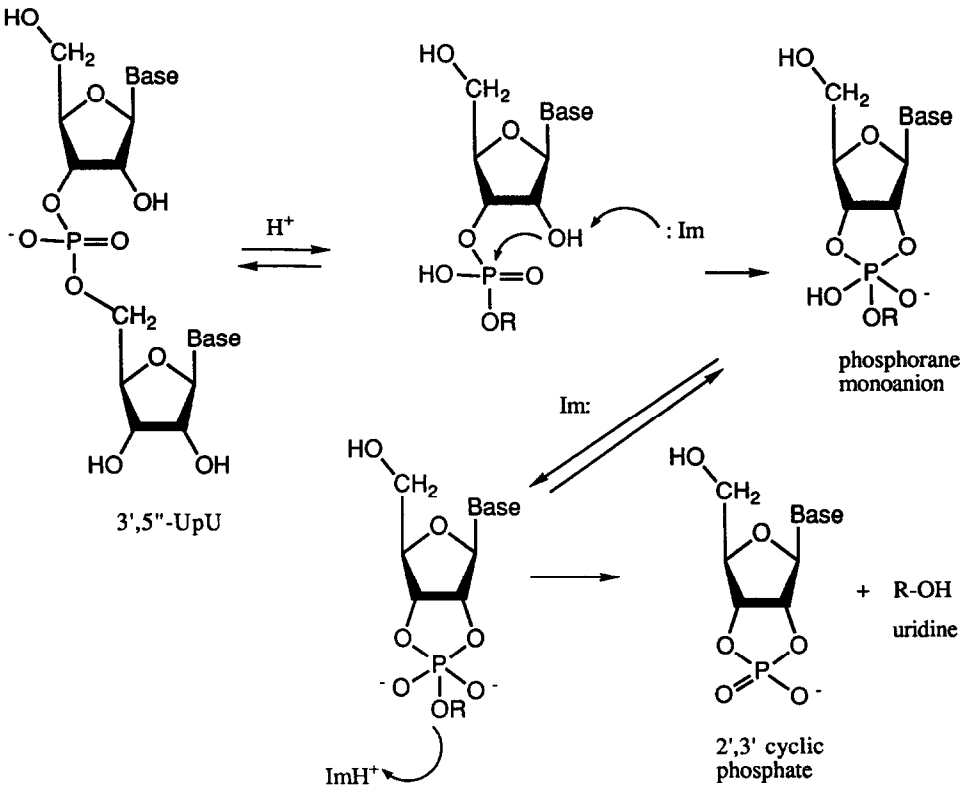
Scheme 1

The first experiments used homogeneous RNA, a polymer of ribouridylic acid (polyU). With an analytical technique developed for this purpose,[2] it was possible to follow the cleavage of this RNA by imidazole buffer, a cleavage that produced a cyclic phosphate ester just as in the first step of the enzymatic process.[3] The reaction exhibited a bell-shaped pH *vs.* rate profile for buffer catalysis (correcting for pH effects alone); both the basic imidazole and the acidic imidazolium ion were participating, so the maximum rate was seen when they were both present. However, the rate was simply first order in total buffer concentration, indicating that both the base and the acid were not present at the same time in the transition state. As we have described,[3] this means sequential bifunctional catalysis, with one catalyst in the first step and the second species in the second step. With two steps there must be an intermediate, and the only sensible intermediate between the phosphate diester starting material and the cyclic phosphate product is a phosphorane. We showed that this must be a phosphorane monoanion to fit the kinetics.[3]

To clarify the mechanism further, we examined the imidazole buffer catalyzed cleavage of 3',5"-uridyluridine (3',5"-UpU), a simple dimeric analog of polyU, and of the 2',5" isomer.[4] Both cyclize to the same 2',3' cyclic phosphate on cleavage of uridine, and

under some conditions (*vide infra*) the two isomers interconvert. Both dimers undergo cyclization-cleavage with the same type of kinetics seen with polyU: a rate maximum when both imidazole and imidazolium ion were present, but only a first order dependence on the buffer concentration. Thus the findings with polyU do not reflect any special properties of a polymer. The same type of behavior is also seen with ApA, adenyl(3',5")adenosine, so there is nothing special about the uridine system.[5] ApA also prefers the sequential bifunctional catalytic mechanism.

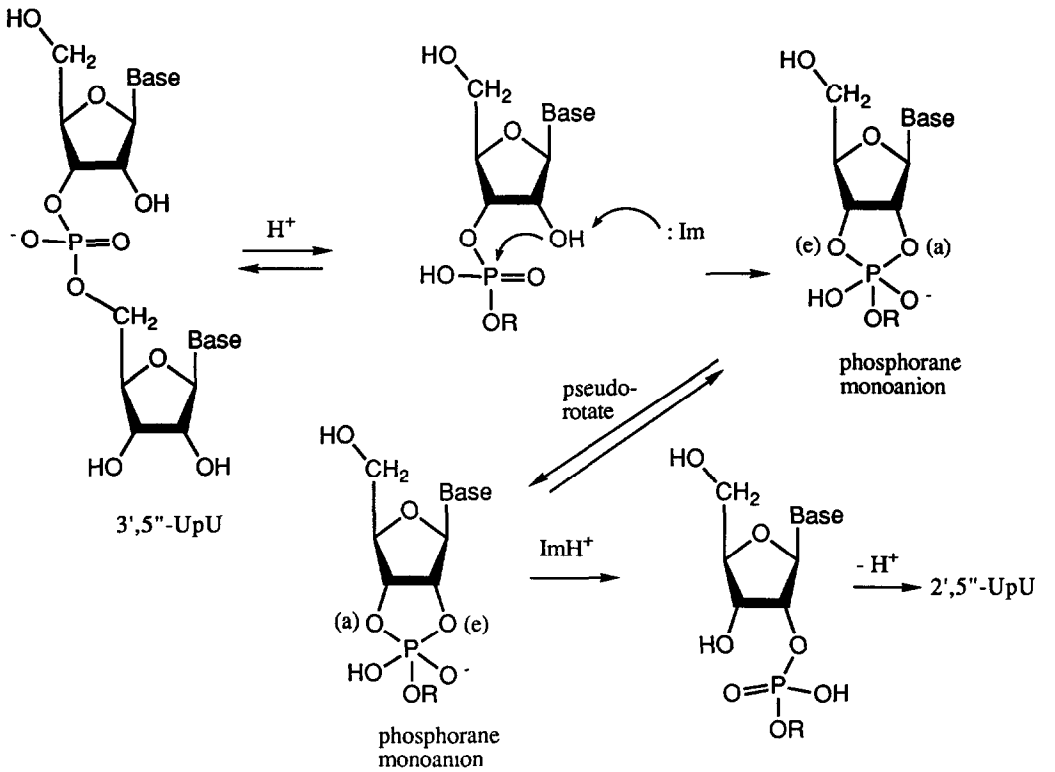
As we have discussed,[3,4] the basic imidazole is catalyzing one step and the acidic imidazolium ion the other step, but the kinetics does not tell which is the first step and which the second. This was answered by examining the rearrangement reactions interconverting 3',5"-UpU with 2',5"-UpU. We found that these rearrangements are catalyzed by imidazolium ion alone, not by imidazole.[4] In fact, increasing the imidazole concentration at a given concentration of imidazolium ion actually slows the rate, "negative catalysis." This is all uniquely consistent with the idea that each substrate is converted to the phosphorane monoanion by imidazolium ion catalyst in the first step. Then the phosphorane can fragment



Scheme 2

to form the cyclic phosphate in a second step with imidazole catalysis, or alternatively the intermediate can undergo an uncatalyzed pseudorotation to interconvert the two phosphorane stereoisomers initially formed from the 3',5" and 2',5" isomers respectively. After this pseudorotation there would be a subsequent ring opening with imidazolium ion catalysis, in a fast step that would not be seen in the kinetics. This is all shown in Schemes 2 and 3.

The apparent negative catalysis by imidazole shows that the cyclization-cleavage and the migration-isomerization reactions branch off a common intermediate.[5] The imidazole catalyzes the cleavage, diminishing the steady state concentration of the intermediate and thus the rate of isomerization. Such negative catalysis could not have been seen under our conditions (initial rates with little conversion of starting material) if the two paths had not branched from a common intermediate with low concentration. Thus the catalysis of rearrangement by the acid imidazolium ion indicates that this is also the catalyst in the first step for cyclization (Scheme 3). This is confirmed by the finding[4] that whereas with these buffers the fragmentation is faster than isomerization, with a more acidic acetic acid buffer the reverse is true. The stronger acetic acid performs the first step even better, but the

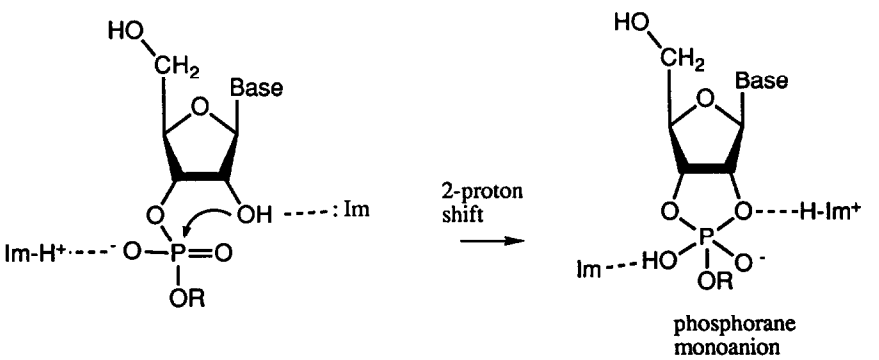


Scheme 3

weaker base acetate ion is not as good a catalyst for fragmentation in competition with pseudorotation.

If imidazolium ion acts in the first step by which a phosphate anion is converted to a phosphorane anion, it must be reversibly protonating the phosphate anion (Scheme 2). Then imidazole can catalyze the attack on the resulting phosphoric acid by the 2' OH group, by removing the proton. This automatically produces the phosphorane monoanion that the kinetics require, in a "first step" that is actually two sequential steps. Reversible protonation, followed by catalysis by imidazole, is kinetically equivalent to the involvement of imidazolium ion; it is the "specific acid/general base" version. The involvement of imidazole as a catalyst of the next step, the fragmentation of this phosphorane to form the cyclic phosphate, means that it must deprotonate the phosphorane anion to form a dianion. This can then fragment with protonation of the leaving group by the just formed imidazolium ion, and the kinetics will show this sequence as imidazole catalysis. We have discussed elsewhere[6] the kinetics seen when the catalyst of one step is formed in a prior step, as in this case. Thus as hoped, the kinetic methods available for the reactions of such free solution species has let us determine the preferred mechanism for RNA cleavage (or isomerization) by imidazole/imidazolium ion. The cleavage mechanism is shown in Scheme 2, that for isomerization in Scheme 3.

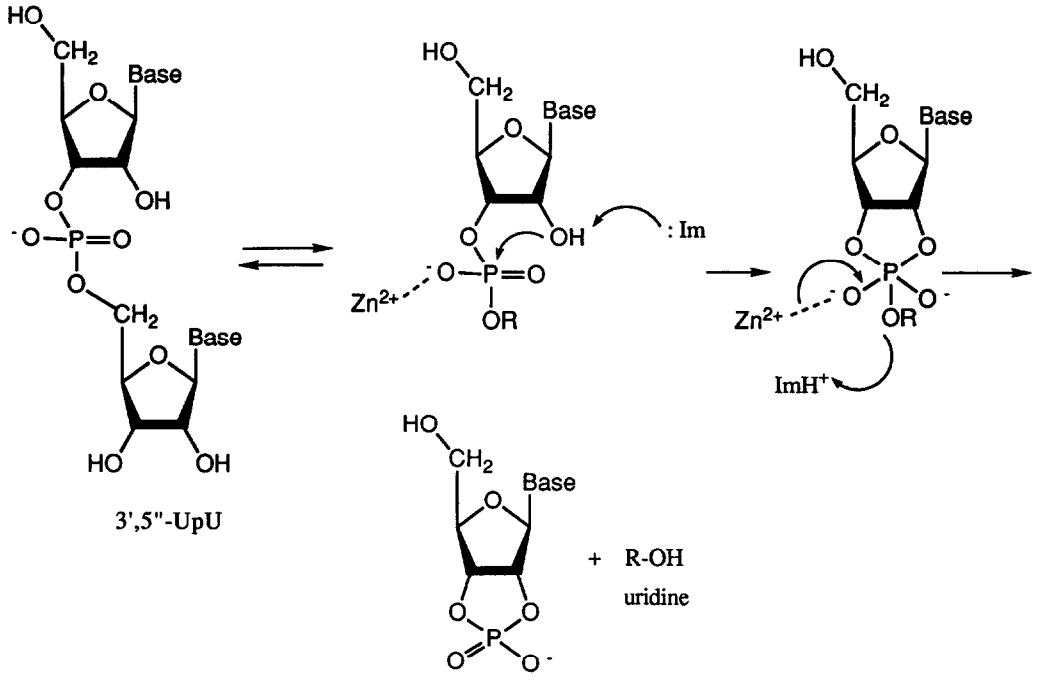
How might this cleavage mechanism be related to that used by the enzyme ribonuclease? First of all, the enzyme differs in an important respect: both catalytic groups are held in place, so it could perform simultaneous bifunctional catalysis. The sequential mechanism used by the model is more probable with freely moving solution species, acids and bases that are unlikely to bind all at once. Proton inventory studies on the enzyme (the proton inventory method will be discussed later in this paper) suggest that indeed two protons are moving in the rate determining step, as expected if bifunctional catalysis is simultaneous. However, one might think that it could still be catalytically useful to form a phosphorane anion intermediate. If so, the acidic catalyst should protonate the phosphate anion oxygen of the substrate as in the model reaction, not just protonate the leaving group during cleavage.



Scheme 4

The first step would be as shown in Scheme 4, which directly forms a phosphorane monoanion by two-proton simultaneous transfer (the protons are vibrating in indeterminate positions, but the positions of the energy minima in the potential surfaces of the hydrogen bonds both move simultaneously). As we have pointed out, X-ray and spectroscopic evidence on ribonuclease indicates that the imidazolium ion of His-119 indeed has the hydrogen bond to the phosphate anion oxygen of the substrate that this mechanism requires.[7] It seems very likely that the enzyme indeed uses a version of our mechanism, but one in which the first two steps are compressed into the one simultaneous step of Scheme 4.

Ribónuclease A is not a metalloenzyme, but many enzymes use metal ions to assist in hydrolysis reactions, including the hydrolysis of phosphate esters. Thus we have done several studies of metalloenzyme mimics. In one directly related to the above work, we have examined[7] the cleavage of 3',5"-UpU by imidazole buffer containing Zn^{2+}. The situation is complicated by the fact that imidazole can bind to Zn^{2+}. However, the kinetic studies indicated clearly that the metal ion and imidazole could cooperate in bifunctional catalysis of UpU cleavage in which the imidazole was acting as a base, not just as a metal ligand.



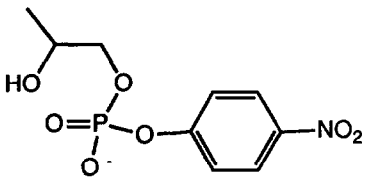
Scheme 5

In contrast to the situation without the Zn^{2+}, there was no catalysis by imidazolium ion. Thus the Zn^{2+} has replaced the role of imidazolium ion; it coordinates to the phosphate group, activating it similarly to the activation by protonation in our previous work.

The mechanism we have deduced for this Zn^{2+}/imidazole system is shown in Scheme 5. Again later catalysis by imidazolium ion is shown, protonating the leaving group after the phosphorane is formed, but again it must be kinetically invisible; imidazolium ion is formed in an earlier step when the imidazole acts as a base. It is interesting that there is no rearrangement-migration to form the 2',5"-UpU with the Zn^{2+} system.[7] The phosphorane intermediate is not a monoanion but instead a zinc-coordinated dianion. The zinc may even bind both oxyanions. As we have pointed out,[7] this will make the pseudorotation that leads to migration slower, while the fragmentation can be faster since no deprotonation is required.

These results help confirm our previous mechanistic conclusions, and they also suggest mechanisms for metal catalyzed enzymatic reactions. They also point to a particularly effective system for synthetic catalysts: 1 mM Zn^{2+} with 10 mM imidazole proved to be almost 50 times as active as was 10 mM imidazole alone.

We also examined another cyclization reaction, the closure of the propylene glycol phosphate ester **1** to form a cyclic phosphate with loss of p-nitrophenoxide ion.[7] In this case imidazole alone can act as a catalyst, with no need for imidazolium ion, since with a p-nitrophenyl substituent the phosphate is more reactive than that of UpU and does not require prior protonation for cyclization. However, Zn^{2+} is still helpful. With 1 mM imidazole, the addition of 1 mM Zn^{2+} increases the rate by ca. 400 fold.



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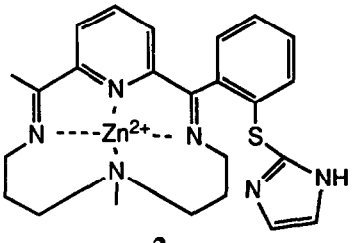
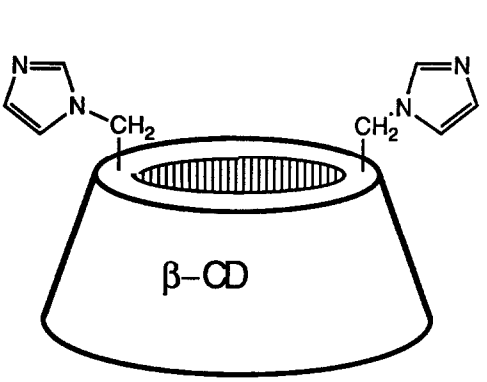


2

In these studies the relative geometries of the Zn^{2+} and the imidazole are not controlled, so imidazole can not only play the useful catalytic role as a base but it can also decrease the rate by coordinating to the Zn^{2+}. The results were a compromise between these two effects. In another study, we made a catalyst **2** with Zn^{2+} and imidazole linked so that they cannot directly coordinate.[8] This also catalyzed the cyclization of **1**, although not with much real rate improvement over the unlinked systems at reasonable concentrations. Other catalysts related to **2** could prove to be more interesting.

Of course in ribonuclease the two imidazole groups are also linked, by the rest of the enzyme. We made a mimic of this some time ago,[9] the β-cyclodextrin-bis-imidazole **3**. Since at that time it was widely believed that ribonuclease uses a mechanism in which the imidazole delivers the C-2 OH group but the imidazolium ion just protonates the leaving group, the geometry of **3** was set up to optimize this mechanism. That is, the two imidazole groups were put as far apart as possible so they could be on the two opposite faces of a trigonal bipyramid intermediate or transition state. Since β-cyclodextrin has seven glucose rings, we mounted the imidazoles on the C-6 carbons of rings four units apart, producing the 6A,6D isomer of β-cyclodextrin-bis-imidazole (compound **3**).



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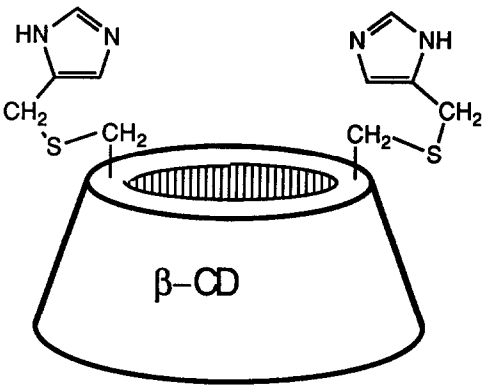

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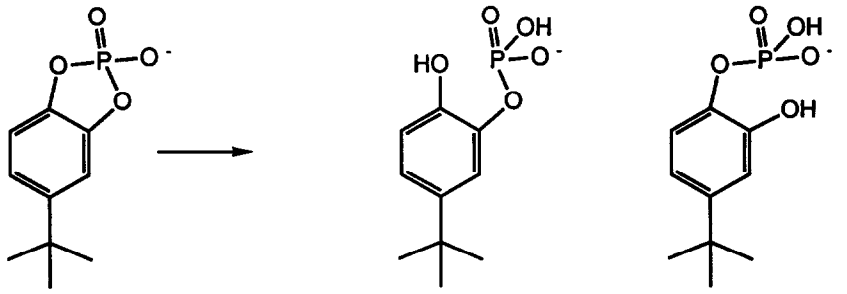
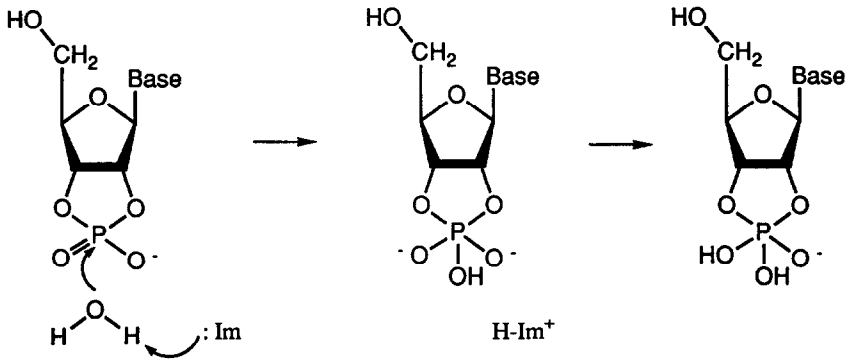
The substrate selected was the cyclic phosphate ester **4** of 4-t-butylcatechol. Its hydrolysis would mimic the second step of RNA hydrolysis, in which a cyclic phosphate is hydrolyzed to form the phosphate monoester. The t-butylphenyl group binds well into the cavity of β-cyclodextrin, and the cleavage of a cyclic phosphate involves a substrate with well defined geometry. Models showed that the phosphate group in a complex of **3** with **4** could lie between the two imidazoles and be hydrolyzed if one imidazole delivered a water molecule to the phosphorus while the other, as an imidazolium ion, protonated the leaving oxygen. Of course this catechol ester **4** is somewhat more reactive than is a 2',3' cyclic phosphate of a nucleoside, but the catechol group also makes it easy to follow the hydrolysis reaction in the u.v.

We found[9] that the hydrolysis followed Michaelis-Menten kinetics, indicating formation of a molecular complex between the substrate **4** and the catalyst **3**, and that it showed a bell-shaped pH *vs.* rate profile with a rate maximum near pH 6.2. This is very close to the pH at which ribonuclease shows its rate maximum. The substrate **4** was hydrolyzed almost exclusively to monoester **5**, with a few percent of the isomer **6** also formed. This specificity is exactly that predicted from models of the geometry of water delivery by imidazole.



4 5 6

A related enzyme mimic 7 was also made, in which the imidazole groups were attached flexibly and could reach further from the cyclodextrin ring.[10] As expected this catalyst hydrolyzes substrate 4 also, and with a bell-shaped pH *vs.* rate profile. The data indicated that the imidazolium component of the catalyst was hydrogen bonded to the phosphate anion group, just as discussed above for ribonuclease. The imidazole component could now reach over a larger area, and direct the hydrolysis to form more of isomer 6 than with the original catalyst 3. Because of the flexibility of the system, this hydrolysis was not as specific as was the previous one.[11]

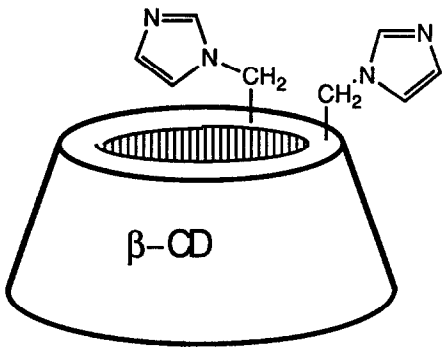


Scheme 6

With the finding that the preferred mechanism of RNA hydrolysis by imidazole/imidazolium involves use of the imidazolium to protonate the phosphate oxygen, the geometric arguments for using the A,D isomer 3 of cyclodextrin-bis-imidazole faded. Of course in the mechanistic work with imidazole buffers we had examined the first step of RNA cleavage, formation of the cyclic phosphate, while the cyclodextrin-bis-imidazole catalysis was mimicking the second step in which the cyclic phosphate is hydrolyzed. However, the mechanism of this process should be analogous to the reverse of the mechanism for

cyclization; the difference is that a water molecule attacks, instead of nucleoside hydroxyl. Looking at the mechanism of Scheme 2, but running it backward, it can be seen that the imidazole should first deliver a water molecule to the phosphate group to form a phosphorane dianion, and that this should then pick up a proton from an imidazolium ion (Scheme 6). If a true bifunctional catalyst such as an enzyme or a cyclodextrin-bis-imidazole is involved, the two steps could well be simultaneous and thus resemble the first step in the forward direction.

A simultaneous mechanism in which the imidazolium ion is hydrogen bonded to the phosphate and delivers its proton as the imidazole delivers a water molecule requires a very different geometry from one in which the imidazolium ion is just protonating the leaving group. Molecular models indicate that the simultaneous hydrogen-bonding mechanism can be performed by the A,B isomer **8**, in which the two imidazoles are located on adjacent glucose units. We examined this, along with the A,C and A,D isomers, and were gratified to see that the A,B isomer is not only functional, it is the best catalyst of the three.[12] It cannot use the mechanism we have excluded, but can use the simultaneous mechanism with protonation of the phosphate group. This mechanism is essentially the reverse of the mechanism of Scheme 2, but with two steps merged into one.



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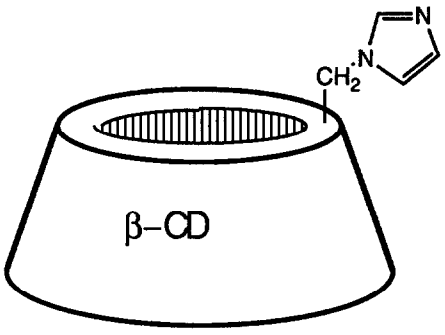

9

It seemed very likely that the two proton transfers would be simultaneous, but a proton inventory was done to establish the point. In this technique[13] a reaction rate is determined in a series of solvents ranging from 100% H_2O to 100% D_2O. Corrections must be made for pH changes and the like, and the corrected data then can be used to establish the number of exchangeable protons that are moving in the transition state. If one proton is moving the rate *vs.* isotopic composition plot will be linear, but if two protons are moving there is a curved plot whose shape can be calculated theoretically. In a sense converting a deuterium to a protium is a form of catalysis, as the rate increases because of the isotope effect. The kinetic plot indicates whether this "catalyst" appears to the first power or to a higher order. We found that the hydrolysis of **4** by the A,B isomer **8** showed a two-proton inventory;[14] the data were very similar to those for the enzyme ribonuclease.[15]

Usually there is no way to check the conclusions of this method, but we could examine the proton inventory for a related reaction. The cyclic phosphate **4** is also hydrolyzed by β-cyclodextrin-monoimidazole **2**, in a reaction whose rate increases with higher pH as the catalyst is converted into its imidazole form. It cannot show a two-proton inventory unless some solvent species comes in to take the place of the missing imidazolium ion, and apparently this did not happen. Catalysis by **2** showed only a one-proton inventory.[14] This confirms our interpretation of the curved plot with bifunctional catalyst **8**, and helps to validate the proton inventory method itself.

A recent paper by Karplus[16] reports calculations on the enzyme ribonuclease A. He supports the concerted mechanism we have proposed.

Summary.- 1. This work has established the preferred mechanism for the cleavage of RNA by imidazole buffers, a sequential bifunctional process in which the first step is protonation of the substrate phosphate anion. It has also established the mechanism for the accompanying rearrangement of a 3',5" phosphate diester in the substrate to the 2',5" diester.

2. An examination of previous data on the enzyme ribonuclease A suggests that it also protonates the phosphate anion of the substrate with a catalytic imidazolium ion in the first step.

3. In model systems, imidazole can cooperate with Zn^{2+} to catalyze RNA cleavage, and models for it, that are more effective than with imidazole alone. The Zn^{2+} can substitute for the proton of an imidazolium ion, with some changes in the details of the mechanism.

4. Cyclodextrin carrying two imidazole groups can catalyze the hydrolysis of a cyclic phosphate in a model of the second step of ribonuclease action. The dependence of rate and selectivity on the geometry of the catalyst indicates that it also uses a mechanism related to that of the imidazole buffer model system, in which an imidazolium ion is protonating the substrate phosphate group in the first step.

5. In contrast to the sequential bifunctional catalysis by the imidazole buffer model system, both the enzyme and the cyclodextrin enzyme mimic use simultaneous bifunctional catalysis, in which two protons are moving at the same time and the phosphate protonation accompanies nucleophilic attack on the phosphorus. Except for this subtlety, the mechanism used by the model buffer system is indeed preferred by the enzyme, and by the cyclodextrin-based enzyme mimic.

Experimental Section

We have described elsewhere[2,3,4] the techniques involved in our kinetic assay for the cleavage of polyuridylic acid, and of uridyluridine. The cleavage and rearrangement of 3',5" adenyladenosine (ApA) was followed in a fashion similar to that for UpU. Internal standards were prepared containing known amounts of adenosine and of 2',5" adenyladenosine, and

HPLC was used to follow the kinetics with an early elution using ca. 5% MeOH in 4.5 mM pH 7.0 phosphate buffer, followed by a 12 % methanol eluent with this buffer. All buffers were prepared by careful weighing, and precautions were taken to avoid contamination by adventitious ribonuclease. The techniques involved in our study of Zn^{2+} catalyzed reactions have also been described.[7]

We have described the synthesis of β-cyclodextrin imidazole catalysts,[12] and their use in the cleavage of the cathecol cyclic phosphate. The experimental methods used in our proton inventory studies have also been described,[14] as has the synthesis and study of bifunctional catalyst **2**.[8]

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