the donors of the Petroleum Research Fund, administered by the American Chemical Society, from the National Science Foundation (CHE8511999), and from IBM Corporation Research Division.

Registry No. Pt, 7440-06-4; cyclohexane, 110-82-7; benzene, 71-43-2; cyclohexene, 110-83-8; 1,5-hexadiene, 592-42-7.

Geometric Evidence on the Ribonuclease Model Mechanism

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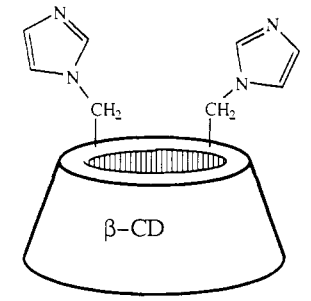
Received April 7, 1989

We have described the use of cyclodextrin-bis(imidazole) compounds (**1**) as catalysts for the hydrolysis of 4-*tert*-butylcatechol cyclic phosphate (**2**).[1,2] The reaction exhibited a bell-shaped profile for the plot of V_{max} (the rate at kinetic saturation) vs pH. There was also excellent selectivity for cleavage of one bond of **2**, related to the expected direction of approach



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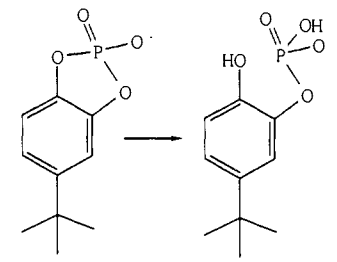

2

of a water molecule assisted by the catalytic imidazole group.[1] This selectivity was reversed to some extent when we changed the geometry of the catalyst by use of a mercaptomethylimidazole, moving the catalytic group further from the binding site.[2]

The bis(imidazoles) were prepared by imidazole displacements on β-cyclodextrin disulfonates difunctionalized by the Tabushi procedure,[4] by using a rigid disulfonyl capping reagent. We showed that this procedure actually gave a mixture of 6A,6C and 6A,6D isomers (the glucose rings are lettered A to G), different mixtures with different reagents.[2] Tabushi later[5] developed reagents that are rather selective for the three possible

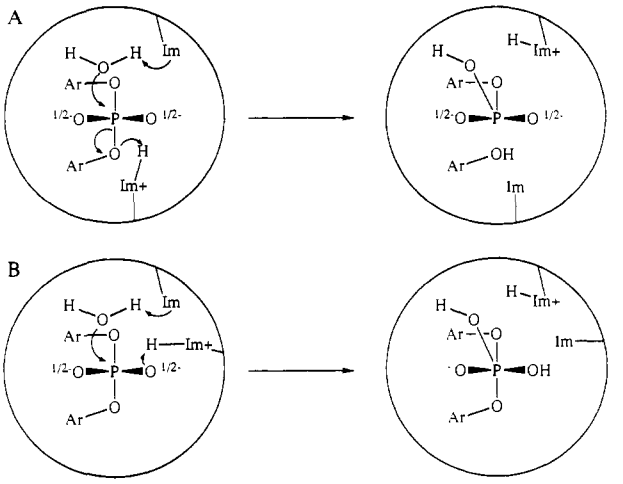

Figure 1. A top view of the bifunctional-catalyzed addition of water to substrate **2**, by the direct displacement mechanism (A) or the proton-assisted addition mechanism (B), forming a phosphorane that cleaves in a later step.

isomers—A,B and A,C and A,D—and we have used these procedures to prepare the corresponding bis(imidazoles) by displacement of the sulfonate groups with iodide ion and then imidazole. For the preparation of the A,B disubstituted isomer we cap with 1,3-dimethoxybenzene-4,6-disulfonyl chloride, which we find[6] to be better than the original Tabushi reagent. All three β-cyclodextrin-6,6′-bis(1-imidazole) compounds were carefully purified[7] and evaluated as catalysts for the hydrolysis of **2**.

In our original work[1] we invoked a mechanism (Figure 1A) in which one imidazole ring of the catalyst delivers H_2O to the phosphate as the ImH^+ group protonates the leaving phenoxide ion. Models showed that this mechanism was possible with both the 6A,6C and 6A,6D isomers of the catalyst, and it would explain the pH maximum for k_{cat} at catalyst half protonation. The mechanism of Figure 1A is also related to that commonly written[8] for ribonuclease A; an imidazole acts as base, while the ImH^+ is suggested to protonate the leaving group. However, we have recently questioned this picture.[9,10]

The cleavages of RNA (polyU)[11] or of the dimer UpU by imidazole buffers[9] also show bell-shaped pH vs rate plots, but we

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(7) Compound characterizations. Primed atoms represent imidazole atoms. A,B bis(imidazole)-β-cyclodextrin: ^1H NMR (D_2O, 297 K) δ 7.51 (1 H, s, H2′), 7.36 (1 H, s, H2′), 6.97 (1 H, s, H4′), 6.82 (1 H, s, H4′), 6.80 (1 H, s, H5′), 6.40 (1 H, s, H4′), 4.97 (1 H, d, H1), 4.87 (3 H, d, H1), 4.84 (1 H, d, H1), 4.80 (1 H, d, H1), 4.72 (1 H, d, H1), 2.90-3.90 (35 H, m with fine structure, H2 & H3 & H4 & H5 & H6); $^{13}C\{^1H\}$ NMR δ 47.03, 47.03 (C6 with N attached), 50.36, 60.42, 60.83, (C6), 70.41, 71.53, 71.98, 72.74, 73.33 (C2-C5), 80.62, 81.47, 81.87, 83.43 (C4), 101.15, 102.09 (C1), 120.72 (C5′), 127.80 (C4′), 138.42 (C2′); MS *m/z* 1236 $(M + 1)^+$; Anal. Found (Calcd for $C_{48}H_{74}N_4O_{33} + 6H_2O$) C, 43.12 (42.92), H, 6.42 (6.45), N, 4.05 (4.17). A,C bis(imidazole)-β-cyclodextrin: ^1H NMR (D_2O, 297 K) δ 7.53 (2 H, s, H2′), 7.02 (1 H, s, H4′), 6.99 (1 H, s, H4′), 6.88 (1 H, s, H5′), 6.67 (1 H, s, H5′), 4.87-4.82 (7 H, m, H1), 2.90-3.90 (35 H, m with fine structure, H_2 & H3 & H4 & H5 & H6); $^{13}C\{^1H\}$ NMR δ 47.64 (C6 with N attached), 59.58, 60.34, 60.66 (C6), 70.99, 71.92, 73.00, 73.19 (C2-C5), 80.88, 81.30, 81.69, 82.94, 83.16 (C4), 101.40, 101.59, 102.06 (C1), 120.54 (C5′), 127.88 (C4′), 138.54 (C2′); MS *m/z* 1236 $(M + 1)^+$; Anal. Found (Calcd for $C_{48}H_{74}N_4O_{33} + 4H_2O$) C, 43.93 (44.07), H, 6.27 (6.35), N, 4.11 (4.28). A,D bis(imidazole)-β-cyclodextrin: ^1H NMR (D_2O, 297 K) δ 7.53 (2 H, s, H2′), 7.07 (2 H, s, H4′), 6.89 (1 H, s, H5′), 6.86 (1 H, s, H5′), 4.87-4.82 (7 H, s, H1), 2.90-3.90 (35 H, m with fine structure, H2 & H3 & H4 & H5 & H6); $^{13}C\{^1H\}$ NMR δ 47.71 (C6 with N attached), 59.72, 60.39, 60.72 (C6), 70.98, 71.94, 72.46, 73.39 (C2-C5), 81.08, 81.41, 81.85, 83.10, 83.21 (C4), 101.55, 102.17, C1), 120.89 (C5′), 127.85 (C4′), 138.50 (C2′); MS *m/z* 1236 $(M + 1)^+$; Anal. Found (Calcd for $C_{48}H_{74}N_4O_{33} + 4H_2O$) C, 44.19 (44.07), H, 6.32 (6.35), N, 4.05 (4.28). The differing imidazole separations in the three isomers lead to characteristic ^1H NMR signals and p*K*'s reflected in Figure 2.
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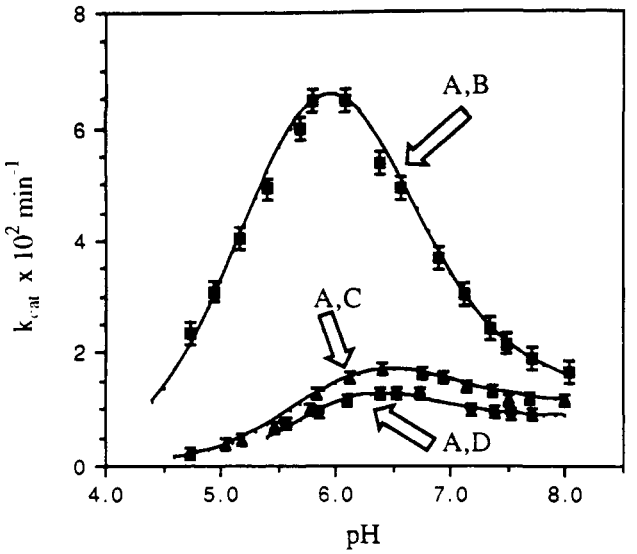

Figure 2. The k_{cat} at 25.0 °C for the hydrolysis of **2** (1.0 mM) complexed within the cavity of 5.0 mM β-cyclodextrin A,B (■), A,C (▲), and A,D (●) bis(imidazoles).

showed[9] that the ImH^+ acts to protonate the phosphate anion group of the substrate, not just the leaving group. Much evidence in the literature is consistent[9,10] with a similar mechanism for ribonuclease A itself: the ImH^+ group of the enzyme first hydrogen bonds to the O^- of the substrate phosphate, and the proton is transferred as the enzyme Im delivers a nucleophile to form an intermediate phosphorane *monoanion*. The key step is related to that shown in Figure 1B.

This led us to wonder about the mechanism of catalyzed hydrolysis of **2** by cyclodextrin-bis(imidazoles). Did they indeed perform bifunctional catalysis by the mechanism of Figure 1A, or was Figure 1B the true mechanism as we suggest for the enzyme? The geometric differences among the isomeric cyclodextrin-bis(imidazoles) allow us to choose between these possibilities. They demonstrate that in this system, too, the function of the ImH^+ is to protonate the phosphate anion so as to promote the formation of an intermediate phosphorane.

The mechanism of Figure 1A requires the Im and ImH^+ to interact with groups that are 180° apart, the attacking H_2O and the leaving O^-. Although the angles made by groups attached to oxygen mean that the catalytic groups need not themselves be 180° apart on the cyclodextrin framework,[2] the 6A,6D isomer would be well suited to this mechanism. By contrast, there seems to be no way for the 6A,6B isomer to use the mechanism of Figure 1A; the two catalytic groups are next to each other, not on opposite sides of the bound substrate. The mechanism of Figure 1B requires the Im and ImH^+ to interact with groups that are ca. 90° apart, the attacking H_2O and the phosphate O^-. This seems possible (models) for all three isomers but particularly easy for the 6A,6B bis(imidazole) and difficult for the A,D isomer. The A,C isomer can fit either mechanism of Figure 1.

We have now studied the kinetics of cleavage of **2** by all three isomers of β-cyclodextrin-bis(imidazole). All show kinetic saturation with increasing concentration of catalyst and a bell-shaped pH vs rate profile for k_{cat}. As Figure 2 shows, the A,B isomer is much more effective than is the A,D isomer, with the A,C bis(imidazole) in the middle. The preference for the A,B isomer means that this system indeed uses the mechanism of Figure 1B, just as we have suggested for the enzyme.

The A,B isomer is quite a good catalyst. At the pH optimum the k_{cat} for the hydrolysis of **2** is 0.0014 s^{-1}, and K_m is 0.18 mM. The resulting specificity constant k_{cat}/K_m (7.8 s^{-1} M^{-1}) is only 230 times smaller than that (1800 s^{-1} M^{-1}) for the hydrolysis of cytidine-2,3-cyclic phosphate by ribonuclease A;[13] of course the substrates are different.

We are studying the product selectivities and isotope effects in those hydrolyses to characterize them further. However, it is already clear that the geometric catalyst preference we have discovered is further support for our proposal that the best way for bifunctional catalysts to hydrolyze substrates related to RNA is by protonation of the phosphate anion group. This may well apply to the enzyme ribonuclease also. In any case this mechanistic idea has guided us to a particularly effective enzyme mimic.[12]

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Rates for 1,2 Migration in Alkylchlorocarbenes

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Received March 24, 1989

Alkylcarbenes, in cases where they have a singlet ground state, can undergo rearrangement with migration of alkyl or hydrogen, and much work has been expended on the studies of carbene rearrangements.[1-4] The isomerizations of alkylcarbenes are so rapid that the additions to multiple bonds are precluded, and, consequently, there has been no report on the intermolecular capture of dialkyl carbenes. Moss and co-workers[5-7] demonstrated that the presence of a chlorine atom on the carbene markedly affects the stability and reactivity, enabling intermolecular reactions to compete with intramolecular 1,2 shift. To our knowledge, there has been no direct measurements of rate constants for 1,2-H migration in alkylcarbenes at ambient temperature because they do not absorb intensively in a region where they can be monitored. We now wish to report the 1,2-H migration rate constants for a series of alkylchlorocarbenes in an effort to understand the migratory aptitudes in this rearrangement. Semiempirical MINDO/3 calculations for the same series of carbenes are in agreement with the observed migratory aptitude.

The alkylchlorodiazirines are synthesized by Graham's method.[8] The decomposition products are well-known and have been reported previously.[5-7,9,10] Laser flash photolysis of diazirines in isooctane at 25 °C in the presence of pyridine produces transient species with a maximum absorption in the 360-380-nm range. These transients are not present in the absence of pyridine and are attributed to pyridinium ylides. Plots of the observed pseudo-first-order rate constants for the growth of the absorption of ylides, k_{growth}, vs [pyridine] are linear and allow the measurements of the rate constant for 1,2-H migration, k_i, in alkylchlorocarbenes. The slopes give the rate constants for reaction of the carbenes with pyridine, k_y, and the intercepts yield k_i. The values of $\tau = 1/k_i$ are given in Table I as τ(exp). The analysis of k_{growth} vs [pyridine] gives only approximate values of k_i (≈10 ns) for ethyl-, propyl-

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‡ U.A. 503 du CNRS.
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