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Binding and catalysis with flexible locks and flexible keys[1]

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Binding and catalysis with flexible locks and flexible keys[1]

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In the final structure of an enzyme-substrate complex, or of a related molecular complex, the two components may fit together in a lock and key relationship, but flexibility is often needed in the binding process. Furthermore, flexibility is needed in enzyme-substrate complexes and their mimics to accommodate the geometric changes that the reaction causes. Examples of such situations from enzyme mimic studies are described.

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INTRODUCTION

On this anniversary of the introduction of the lock and key concept into understanding binding by enzymes, and by antibodies and other biological receptors, it is important to recognize both the genius of this idea and its limitations. It seems clear that in the final structure of an enzyme-substrate complex there is close fitting of most parts of the two components, leading to good binding and the significant immobilization of the substrate. However, it is a mistake to extrapolate from the properties of every-day locks and keys.

For example, one could not design a key that was narrow at both ends but with a large bulge in the middle. Even if the lock were designed to accommodate the bulge in the final inserted structure, there would be no way to get the bulge past the narrow parts of the lock while inserting the key. However, lots of molecules that bind to proteins have fat middles and narrow ends. They are able to bind because of the flexibility of the protein, and often of the substrate.

A reasonable analog of a common situation in binding to proteins is the Venus flytrap. The protein originally has a rather open structure; when the substrate binds, the protein then folds around it to complete the lock surrounding the substrate key. Koshland—the first person to point out the general implications of such a process—calls this "induced fit".[2] There is much evidence for such a process in enzyme binding. Not only is the binding pocket constructed by such induced fit, the catalytic groups are often organized into their active arrangement only as the result of this process. This is how some enzymes avoid performing step one of a sequence until the appropriate substrate has been bound to carry out step two. Binding of the substrate for the second step induces a structural change in the enzyme so it can carry out the first step. This is true, for instance, for the enzyme that synthesizes serine from glycine and hydroxymethyl-tetrahydrofolate.[3]

Even after an enzyme binds a substrate, some flexibility is normally needed. The chemical reaction being performed always involves a geometric change on proceeding from substrate to product, and the enzyme must have the flexibility to permit this change.

In this paper, I will describe our work on mimics of enzymes and of antibodies in which the requirements and effects of flexibility are most evident. As will become apparent, some flexibility is often required, but too much is harmful.

ACYLATION OF CYCLODEXTRINS BY BOUND SUBSTRATES

A little over 15 years ago we reported the remarkable rate of acylation of β-cyclodextrin by the p-nitrophenyl ester **1** of ferrocenylacrylic acid.[4–10] The ferrocene ring binds into the cyclodextrin cavity, and the ester group is then attacked by an alkoxide ion of a C-2 hydroxyl group on the ring edge (Figure 1). The rate of this process was 360,000 times faster than was hydrolysis of the ester at the same pH, by hydroxide attack instead of alkoxide attack.

Bender had previously shown[11] that m-nitrophenyl acetate acetylated β-cyclodextrin with only a 10^2 or so acceleration over hydrolysis under the same conditions. Molecular models suggested a lock and key explanation of the difference. With m-nitrophenyl acetate the sub-

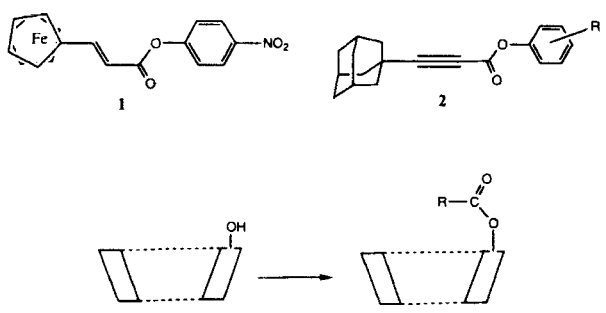

Figure 1 Acylation of β-cyclodextrin by substrates **1** and **2**. With **1** the ferrocene unit binds into the cyclodextrin cavity, holding the ester group in an ideal position for addition of the cyclodextrin hydroxyl to form a tetrahedral intermediate, but in the final product the ferroceneacrylate must be badly twisted to accomodate the ester stereochemistry. With the adamantanepropiolate esters **2**, flexibility allows a rotation to permit a smooth transition from tetrahedral intermediate to product ester.

strate fit well into the cyclodextrin cavity, but when it went to the tetrahedral intermediate the aromatic ring was pulled upward partly out of the cavity. With the ferrocene derivative the bound ferrocene ring could stay in the cavity even when the hydroxyl group had added to the ester, forming a tetrahedral intermediate. Since this process was the rate-limiting slow step (*vide infra*), the key fit the lock throughout this chemical reaction.

Esters related to 1 were also examined in which the acrylate ester group was rigidly held by ring fusion to the ferrocene unit.[6,7] In the best case an acceleration of the acylation reaction relative to hydrolysis under the same conditions was 5,900,000-fold. A rigid well-fitting key was giving very large accelerations of an intracomplex reaction.

However, with leaving groups poorer than p-nitrophenoxide ion the picture changed. We had seen, and reported in our first work,[4] that the N-acylimidazole analog of substrate 1 acylated the cyclodextrin with only a 1200-fold acceleration over the hydrolysis rate. As we pointed out, this made sense in terms of the geometrical changes involved in the entire acylation process. Going from bound substrate to bound tetrahedral intermediate involves little loss of the binding interaction (studies[8] of the effects of pressure on the reaction rates indicated that there was some change) but in the second step there are big problems. Decomposition of the tetrahedral intermediate to the acylated cyclodextrin product requires that the attacking oxygen atom—which originally attacked perpendicular to the ester group plane—now end up *in* the ester group plane. This requires that the product badly twist in the cavity, losing much of its good binding geometry.

An important addition to this story came from Menger and Ladika.[12] They reported a study of ferroceneacrylate esters of various phenols with varying pK_a's. Of course as the phenol pK was raised the hydrolysis rate slowed, but more importantly the rate of cyclodextrin acylation slowed much faster. As the leaving group phenoxide anion became less stable, the second step made a larger contribution to the overall rate, and the twisting required to accommodate the second step became more important. We,[9] and also Menger and Sherrod,[13] performed molecular mechanics calculations that supported this picture.

Final evidence came from a study[10] in which we purposely included more freedom that allowed the twisting to occur without loss of binding. We prepared a series of phenyl esters of adamantanepropiolic acid (2), in which the phenol pK's ranged from 6.1 to 13.2. We saw a break in the plot of hydrolysis rate vs. pK near 12, where the rate determining step changed from the first addition step to the second elimination step. However, the same thing was seen for the acylation reaction. Over the entire pK range, the acceleration of acylation compared with hydrolysis went from 2280—with a 2,5-dinitrophenoxide leaving group of pK 6.15—to 547 with a 2,4-dimethylphenoxide group of pK 13.2, only a four-fold decrease in the acceleration. Most of the problems with the second step were now gone.

In the ferroceneacrylate esters the sidechain and ester group are locked by conjugation, so the product acylated cyclodextrin must be twisted in the cavity. By contrast, the adamantanepropiolate group can rotate freely around the two single bonds of the sidechain, so it accommodates to the geometric needs of the product with little problem.

Of course this freedom means that the first step is not nearly so well accelerated as it was for the ferroceneacrylate ester case—if rotational freedom is not needed, its inclusion will slow the reaction. However, the usefulness of this rotational flexibility in the second step is so great that with the poorest leaving group the overall acceleration is actually better for the adamantane system than for the ferrocene system. A looser fitting flexible key can be important if a reaction sequence demands geometric changes.

CHANGING THE LOCK AND CHANGING THE KEY IN TRANSAMINASE MIMICS

We have been preparing and studying mimics of transaminase enzymes over many years.[14,15] These enzymes use pyridoxal phosphate and pyridoxamine phosphate to perform transamination, and many other transformations of amino acids. Some of our work has involved attaching basic groups to pyridoxamine or pyridoxal to perform the proton transfers that are catalyzed by basic enzyme groups, often with stereoselectivity. There is a significant lock and key aspect to this work, such as when the substrate ketoacid "key" binds covalently to the pyridoxamine amino group as a Schiff base, and then interacts

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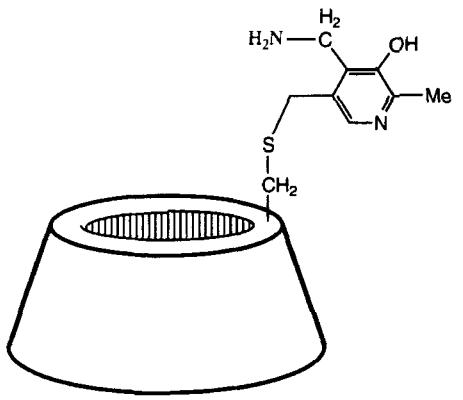
further with proton acceptor and donor groups held in a defined geometric place. However, with respect to flexibility the clearest story involves the attachment of binding groups to pyridoxamine.

Our first example involved attachment of the primary face of β-cyclodextrin to pyridoxamine.[14] The resulting compound 3 had a 100-fold preference for transaminating phenylpyruvic acid to form phenylalanine relative to the conversion of pyruvic acid to alanine. The preference reflected additional binding of the phenyl group into the cyclodextrin cavity; as expected from this, a better binding p-t-butylphenyl group has an even larger effect, and the preference for the formation of 4-t-butylphenylalanine from its ketoacid 4 now exceeds 15,000 times that for the amination of pyruvic acid.[15]



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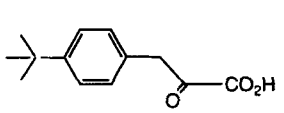

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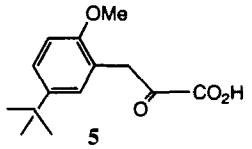
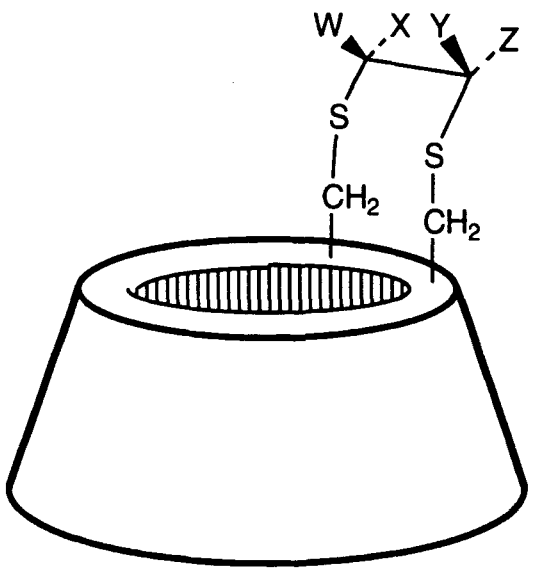


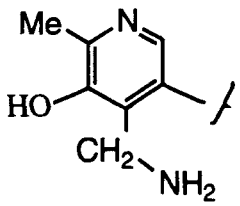
5

Changing the key causes a great loss of this preference, in spite of the flexibility of 3. With a m-t-butylated phenylpyruvic acid derivate 5, there is essentially no preference in competition between 5 and pyruvic acid.

We have set the geometry of the enzyme mimic more rigidly, by double linkage to the cyclodextrin.[15] The result is a set of four compounds, 6–9, two of which hold the pyridoxamine group over the cyclodextrin cavity while the other two hold it away from the cavity. With the endo pair 6 and 7 there is still a preference for phenylpyruvic acid over pyruvic acid as a substrate "key", and for the 4-t-butylphenylpyruvic acid 4 even more so, in which binding holds the ketoacid sidechain straight out from the cavity. Without the t-butyl group to lock this geometry, a simple phenyl group can swivel in the cavity. Also with the endo pair there is almost no selectivity between the m-t-butyl substrate 5 and pyruvic acid, since now the t-butyl lock group holds the ketoacid sidechain off at the wrong angle for reaction with the pyridoxamine.



6 W =
7 Y =
8 X =
9 Z =



All others = H

The exo pair of compounds 8 and 9 reverse this behavior. Now there is only a small (8 ± 2) preference for reaction with 4-t-butylphenylpyruvic acid 4 relative to pyruvic acid, since the optimal bound geometry of 4 does not fit the lock well at all. Probably the small preference reflects sub-optimal binding of this group in the transamination reaction. However, now the m-t-butyl substrate 5 fits the lock well, and shows a 40-fold preference over alanine.

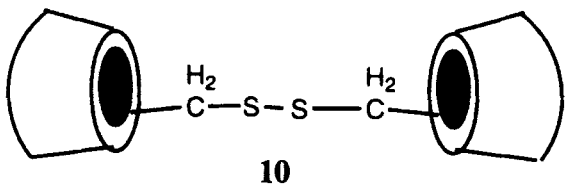
To summarize, when the lock 3 had a flexible link between the reactive group and the binding group, it could best adapt to the geometry of the rigidly held p-t-butyl substrate 4, so it had a large preference for this substrate. In spite of the flexibility of the lock 3, it did not readily adapt to the geometry of the m-t-butyl substrate 5, and showed little extra reactivity from substrate binding. When the lock was made more rigidly endo (6 and 7), with the extra link, it still showed a preference for the p-t-butyl substrate 4, but not by quite as much as with the flexible lock 3. However, with the rigidly held lock of alternative geometry (8 and 9), there was little preference for the p-t-butyl substrate 4, which points in the wrong direction on binding, but a real preference now

for the m-t-butyl substrate 5 that points off to the side, to fit the locks 8 and 9 better. Removing flexibility in the lock and the key has led to greater selectivity.

RIGIDITY AND FLEXIBILITY IN BINDING OF SUBSTRATES TO CYCLODEXTRIN DIMERS

We have been examining binding and catalysis by molecules in which two cyclodextrin units are linked. With a single linker group there is still considerable flexibility in the "lock", while with two linkers attached to adjacent glucose units it can no longer swivel around the linker unit. However, models suggest that such a double linker still acts as a strap that permits the two rings to open and close like a clamshell. With some substrates, it is clear that this opening and closing is the way that binding occurs.

We have reviewed our work in this area recently,[16] so it will just be summarized here. Some fairly subtle lock and key aspects were seen. For instance, with host 10 we saw that there was almost no binding of cis-stilbene derivative 11, even weaker binding than the 10^4 M^{-1} seen with a simple t-butylphenyl group.[17] Models show that the t-butylphenyl groups in 11 are too close together to permit them to enter the cavity of even one cyclodextrin, let alone two. However, the closely related cyclopropene derivative 12 binds very strongly ($K_a = 3.5 \times 10^8$ M^{-1}) to 10, with a binding free energy essentially twice that for simple t-butylphenyl bonding into a cyclodextrin cavity.



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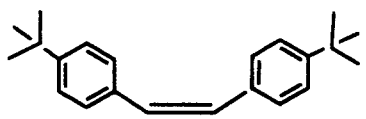

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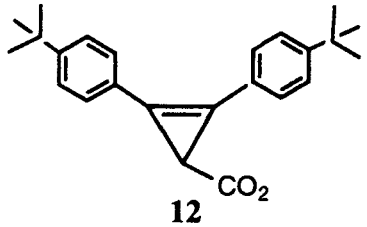
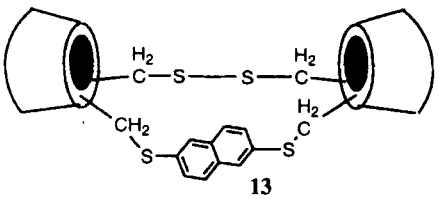


12

Perhaps the most striking effects were seen with dimer 13, which has one long link and one short link from neighboring glucose units. It can open and close like a clamshell, but because of the difference in lengths of the two linkers it prefers a substrate "key" that is bent. It could bind the linear isomer 14 with a substantial binding constant, as shown, but the binding of the bent isomer 15 was at least 4000 times stronger. The binding with such a rigid lock and rigid key, and with complementary geometries, is so strong that we have only a lower limit to the value so far with the available methods.



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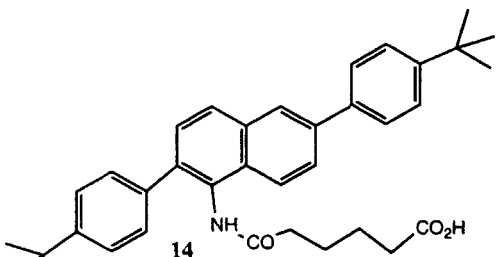
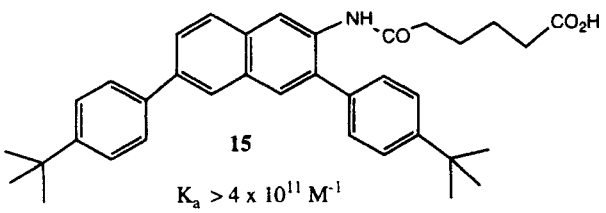


14

$K_a = (1.1 \pm 0.2) \times 10^8$ M^{-1}



15

$K_a > 4 \times 10^{11}$ M^{-1}

Molecular models show that 14 and 15 cannot thread through the cavities of dimer 13 in its closed geometry, since the naphthalene ring will not fit. Instead, binding must be by induced fit, in which an open structure of 13 folds up around the substrates. Thus, removing flexibility has led to large binding constants, but this one remaining degree of flexibility seems to be important to the binding process itself.

FITTING THE TRANSITION STATE KEY TO THE CATALYST LOCK WITH BIFUNCTIONAL CATALYSTS

Many enzymes use an acid and a base group in concert, performing simultaneous bifunctional catalysis. At pH 7 or so, the imidazole group of histidine is a useful catalyst: with its pK_a near 7, it is the strongest base that

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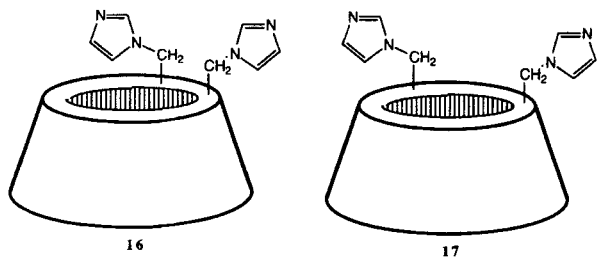
can exist unprotonated at neutral pH, while its conjugate acid is the strongest acid that can exist still protonated at that pH. If two imidazoles both had pK's of exactly 7.0, each would be 50% as the base and 50% as the acid at pH 7.0. In enzymes, the local environment can shift the pK of such groups; ideally, the base group should have a slightly lower pK so it exists unprotonated, while the acid group should have a higher pK so it can sit with an attached proton.

A classic example of such an enzyme is ribonuclease A. It catalyzes the cleavage of RNA, using the imidazole groups of His-12 and His-119 of the enzyme. The first stage involves conversion of the linear phosphate diester group of RNA to a 2′,3′-cyclic phosphate, by ester exchange with the free 2′ hydroxyl of the ribose ring (Figure 2). In the second stage, the enzyme catalyzes the hydrolysis of this cyclic phosphate, regenerating the 2′ hydroxyl group and leaving a phosphate monoester on the 3′ position. Both stages are performed by a bifunctional catalytic mechanism, using the two imidazole groups.



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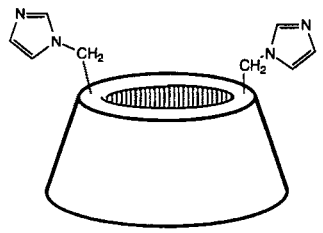

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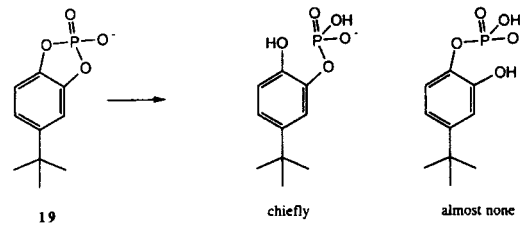


19 chiefly almost none

In mechanistic work that we will not describe here, we saw that we could duplicate this biochemistry with high concentrations of imidazole buffer,[18–20] and in detailed studies we were able to elucidate the mechanism of the process.[18–22] It served as an important guide to the mechanism of the enzyme itself, and as well to the design of an enzyme mimic that used two imidazole rings to catalyze the hydrolysis of a cyclic phosphate. In this account we will focus on the lock and key aspect of various isomers of the enzyme mimic. As we will see, one transition state fits a particular isomer best of all, but for another reaction quite a different isomer is preferred.

β-Cyclodextrin has seven glucose rings, that we letter A to G. We have attached two imidazole rings to the 6A and 6B positions in compound **16**, putting them on neighboring glucose units, but we have also made the 6A,6C isomer **17** and the 6A,6D isomer **18**.[23] The latter has the imidazoles as far apart as possible, attached 154° apart around the ring. In line with the classic mechanism for the enzyme, we thought that this would be the best isomer for catalyzing the hydrolysis of substrate **19**, a cyclic phosphate that can bind into the cyclodextrin cavity. It indeed was a bifunctional catalyst, showing a rate optimum at a pH where on the average there was one Im and one ImH^+ group. However, after our mechanistic work, it seemed likely that the classic enzyme mechanism was wrong.

In the classic mechanism, a base catalyst delivers a water molecule to the cyclic phosphate while the acid catalyst protonates the oxygen atom of the leaving group, which is the 2′ oxygen for the enzymatic cleavage of a 2′,3′-cyclic phosphate. Such a mechanism requires a 180° alignment of the attacking and leaving oxygens, and molecular models indicated that the catalyst groups should be far apart to bind to this transition state. Our new mechanistic work indicated that the first role of the acid group is probably to protonate the phosphate oxyanion oxygen, not that of the leaving group. The projection angle for this mechanism has the attacking oxygen only 90° from the oxygen to be protonated, quite different from the original suggestion. Models indicated that this mechanism could be catalyzed well by the AB isomer **16** of the catalysts. The imidazoles in this isomer are mounted only 51.4° apart, but the angles of hydrogen

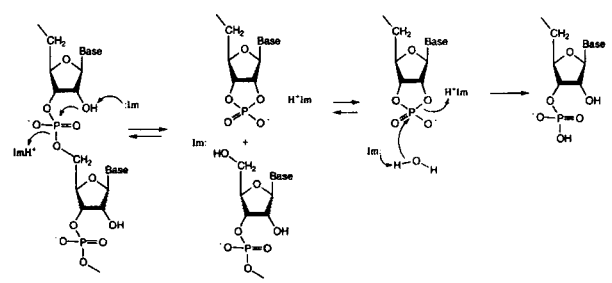


Figure 2 The sequence by which the enzyme ribonuclease first causes cyclization-cleavage of RNA, then hydrolyzes the cyclic ester. The mechanism shown is the classic mechanism found in textbooks; our work suggests that the detailed mechanism is different from that shown.

bonds are such that this is fine for a 90° projection angle between the oxygens themselves.

We saw[23] that indeed the 6A,6B isomer **16** of the catalyst was the best of all for the hydrolysis of our cyclic phosphate ester **19**. We also saw,[24] using a technique of kinetic isotope effects called proton inventory, that the two protons were transferred simultaneously just as they are in the enzyme. This confirms our mechanistic ideas, and indicates that here the lock and key requirements can be deduced from mechanistic work.

With the availability of three isomeric locks for bifunctional catalysis, it is attractive to examine other reactions and determine their geometric preferences. In the case that has been done so far, we have examined enolization. Many enzymes catalyze enolization reactions, and arguments can be made that they prefer a simultaneous bifunctional mechanism in which a base removes the C-H proton while an acid attaches the O-H proton. Such a mechanism bypasses the somewhat less stable enolate ion. Whether or not enzymes do this, we have seen that one of our isomeric catalysts can perform such a simultaneous reaction.

The reaction studied (Figure 3) was the enolization of ketone **20**, followed by deuterium exchange.[25] At pH 6.3 there is essentially no enolization in the absence of our catalysts, but all three of our isomers catalyze the enolization. However, the 6A,6B and 6A,6C isomers have rates not much above the rate of β-cyclodextrin 6-imidazole, so they are essentially monofunctional catalysts. Only the 6A,6D catalyst **18** is significantly faster, so it is the one that best fits the transition state for bifunctional catalysis of enolization.

This result was at first surprising, since models show that the 6A,6D isomer does not fit well a transition state in which the C-H bond is being broken by linear attack of the base. However, the non-linear attack that we deduce actually makes sense if one considers the stereoelectronics carefully. In this case, the information about which lock fits the reaction key tells us details about the mechanism that would not be otherwise known.

Recently we have studied another example of bifunctional catalysis in which lock and key factors are apparent.[26] Many enzymes use two metal ions to catalyze reactions, and this is particularly seen with some Zn(II)-containing enzymes that perform hydrolysis reactions. We have prepared a set of dimers carrying triaz-

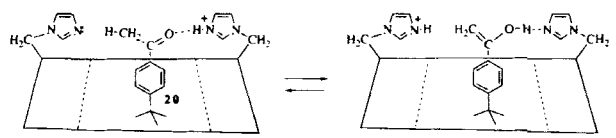
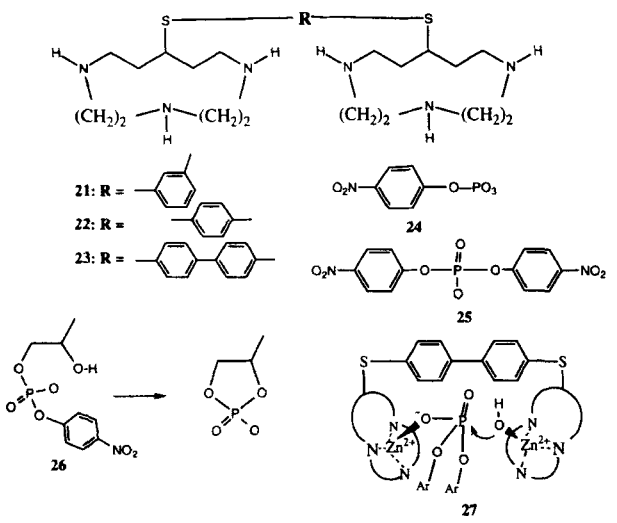


Figure 3 The enolization of substrate **20** catalyzed by the cyclodextrin bis-imidazole **18**. The geometry of the preferred catalyst isomer is quite different from that needed for the bifunctional catalyzed hydrolysis of substrate **19**.



amacrocycles that are well-known Zn(II) ligands. Compounds **21**, **22**, and **23** are among the compounds made; they may be considered to be "locks" of various shapes. We then examined them as catalysts for the hydrolysis of p-nitrophenyl phosphate **24** and of bis-p-nitrophenyl phosphate **25**, and also for the cyclization cleavage of compound **26** and of 3′,5″-uridyluridine, an RNA segment. The transition states of these reactions are the "keys". The results were striking.

With substrate **24**, only the short dimers **21** and **22** were better hydrolysis catalysts than was Zn(II) alone, or some monomeric Zn(II) complexes of the macrocycle. In particular, the long dimer **23** did not evidence bifunctional catalysis, in the sense that it had no rate advantage over monomers. However, this was not the case for the hydrolysis of the phosphate diester **25**. Here the shortest dimer **21** was no more effective than were monomeric Zn(II) complexes, the longer dimer **22** was about 2 times better, but the longest dimer **23** was 50 times better than the best monomeric complex, and 150 times better than Zn(II) alone. A similar set of preferences for the longest dimer was seen for the cyclization of substrate **26**, and for the cyclization-cleavage of 3′,5″-UpU.

Apparently the catalyzed hydrolysis of **24** can best be achieved by the coordination of *two* metal ions to the phosphate group, a mechanism that fits the shorter dimers better. However, for the phosphate diester **25**, models suggest mechanism **27** in which the two Zn(II) ions play different roles. A similar mechanism can be written for the cyclization of **26** and of uridyluridine.

CONCLUSIONS

In the area of molecular recognition and catalysis it seems clear that congruence of shape, as in the lock and key analogy, is important. However, it is also clear that

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the shape does not always have to be preorganized. Flexibility can permit congruence, and in some cases such flexibility is critical to the binding and catalysis processes. Thus the locks and the keys must be flexible, at least in part, in contrast to the situation with real locks and keys.

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