# Enzyme mimics

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Abstract. Binding both ends of a substrate to a catalyst can lead to selective functionalization reactions, and also to very strong binding. Attachment of coenzymes such as pyridoxamine or thiamine derivatives to binding groups produces catalysts that show enzyme-like selectivities. When two functional groups are attached to a binding group the resulting catalysts are good mimics of enzymatic transaminases and ribonucleases. Studies on enzyme mimics show the importance of limiting the degrees of freedom in a catalyst, but retaining some flexibility.

DITOPIC BINDING The binding of a substrate to an enzyme in general involves several interactions, so that the substrate is fairly well immobilized and held near the catalytic functional groups. In many enzyme mimics relatively simple binding schemes are used, and considerable freedom is left unrestricted.

We have examined several systems in which a substrate is tightly held next to a catalyst or reactant by more than one binding interaction. Our earliest case involved the photofunctionalization of a flexible chain molecule by a bound benzophenone system. We had looked at flexible chains that were covalently linked to one end of a benzophenone, and found that they were functionalized over many carbons on photolysis, since the flexible chains could assume many conformations (ref. 1). By contrast, the photoinsertion of a benzophenone into flexible chains with two ion-pair links, in system **1**, was quite selective for the central carbons of the substrates (ref. 2). When there was only one ion-pair interaction in a related system, no functionalization occurred. Thus the double binding led to both selectivity and efficiency of reaction.
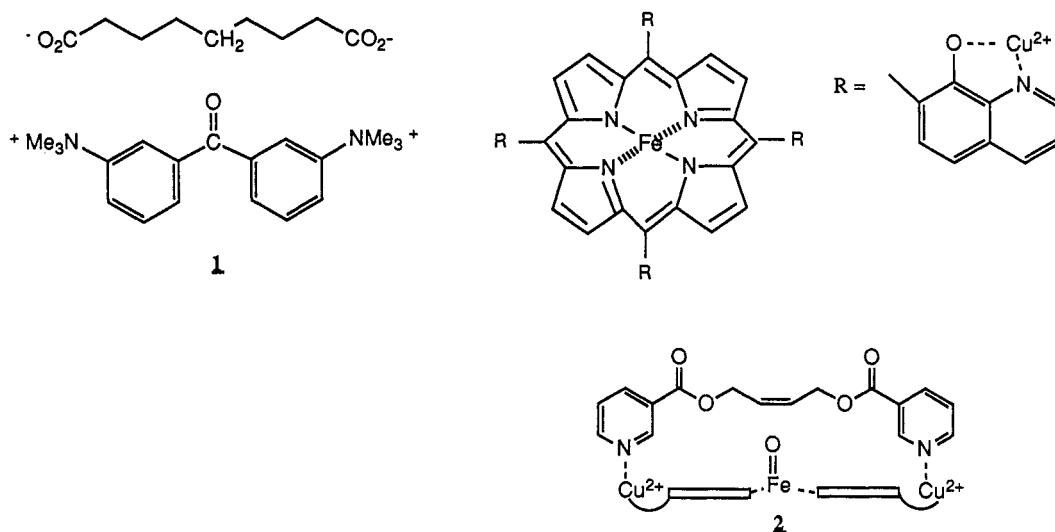

A related situation was seen in our mimic of cytochrome P-450 enzymes. When a substrate could doubly coordinate to the porphyrin catalyst (see **2**) we saw effective catalytic epoxidation of the substrate, but with only one coordination a related reaction was quite inefficient (ref. 3). In subsequent unpublished work, we have found that this porphyrin system can also hydroxylate the saturated carbons of doubly coordinated steroids in a catalytic turnover process.

These systems use ion pairing or metal coordination as binding forces, but both kinds of interactions require functional groups in the substrate. Enzymes can utilize hydrophobic binding of unfunctionalized substrate segments. Since we have been examining enzyme mimics that utilize such hydrophobic binding to cyclodextrins or to synthetic hydrophobic cavities, it was natural to examine the properties of dimers that could bind two substrate segments into conformationally restricted complexes. Indeed we examined the first cyclodextrin dimer system many years ago, but have recently returned to this area more intensively.

A series of cyclodextrin dimers have been prepared and examined with substrates that can utilize both binding sites (ref. 4). Typically, good hydrophobic binding into a cyclodextrin can have a binding constant of the order of 10^4 M^{-1}, similar to some enzyme-substrate binding constants. The dimers should have binding constants of the order of 10^8 M^{-1} or greater, since the free energies of binding should add and translational entropy does not have to be paid for twice. We indeed find that some of our dimers show such strong binding, a strength so high that we have some trouble determining the values. The data are published for many of our cases; an additional example is dimer **3**, which binds substrate **4** with a binding constant of 10^9 M^{-1} (S. Halfon, unpublished).


3

4
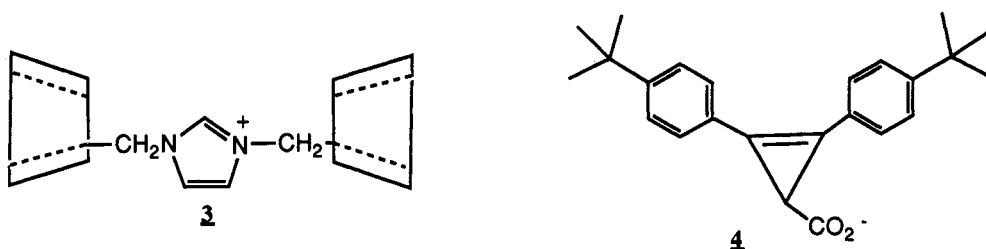
The cylodextrin dimers we have reported previously, including **3**, have a single flexible linkage joining the two rings so they can adopt many conformations. We have also prepared a series of cyclodextrins joined by two linkages, so they cannot pivot. For instance, linking two adjacent glucose rings of β-cyclodextrin with imidazolium rings produces two isomers, **5** and **6** (R. Zarzycki, unpublished). In **5** (with the occlusive or "clam shell" configuration) the two rings can cooperate to bind a substrate such as **4**, while in **6** (with the aversive or "love seat" configuration) they cannot. The symmetries of the systems are such that these geometries can be distinguished by NMR. In one example we have been able to separate the two isomers; the "love seat" shows a normal 10^4 M^{-1} binding constant for a ditopic substrate, since it can bind only one end, but the binding constant for the "clam shell" is $>10^9$ M^{-1} (S. Chung, unpublished). New methods will be needed to fix this number precisely.

5   **6**   **7**
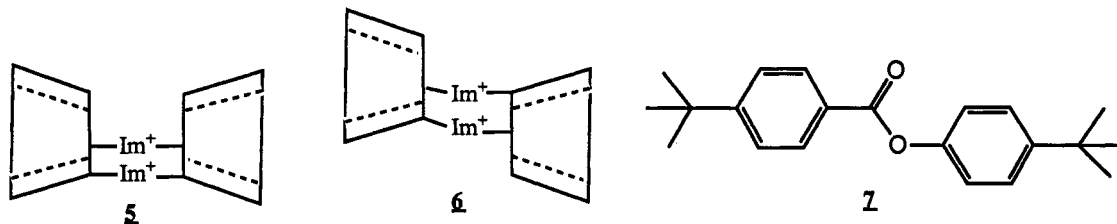
Of course the linkage between the two binding rings is an ideal place to mount a catalytic function. We have some systems in which the linkage is the type of functionality that we can use to direct hydrocarbon functionalization reactions (ref. 4), and others in which metal ions are present that can catalyze hydrolysis reactions (S. Halfon, unpublished). Some substrates that can bind to both rings and stretch across the interface do indeed react with these intervening catalytic groups; for instance, we see metal ion catalyzed hydrolysis of ester **7** by a catalytic doubly-binding molecule linked by a metal ligand group (S. Halfon, unpublished).

The potential for doubly binding catalysts to fix the geometry of a bound substrate has another interesting aspect. Many enzymes have been shown to use some of their binding energy to distort the substrate along the reaction path. Another description is that many of them bind the transition state of a reaction more strongly than they bind the substrate. In a general sense this is a trivial statement: binding results when the interaction between two species lowers their overall energy, and catalysis occurs when the energy of the transition state for a reaction is lowered by interaction with the catalyst. Thus all catalysis is a form of binding, but the situation with many enzymes is special. The preferential binding of the transition state occurs not just at the reaction center, by hydrogen bonding of developing anionic groups and the like, but also at segments of the substrate remote from that center. In a sense the substrate acts as a lever, in which pushing on a remote section causes changes at the reaction center. There are few non-enzymatic examples of this concept.

We have made a doubly linked β-cyclodextrin dimer that has two arms of unequal length, compound **8** (S. Chung, unpublished). Molecular models show that this should preferentially bind the bent transition state of a tetrahedral intermediate, in ester or amide hydrolysis, better than it can bind the linear substrate. If we can get such geometric torque to result in a substrate binding constant of only 10^4 M^{-1}, but a transition state binding constant of 10^{10} M^{-1}, the 10^6 difference could show up as a rate acceleration.

$$\underline{\mathbf{8}}$$

X: CH_2S-⬡⬡-SCH_2

Y: $-CH_2\text{-}S\text{-}S\text{-}CH_2-$
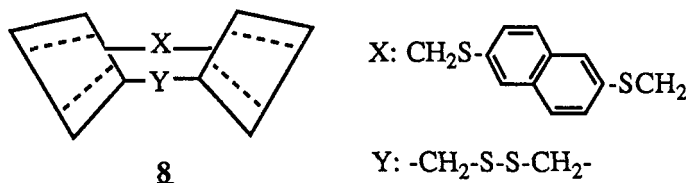
Obviously the field of ditopic binding catalysts is still in its infancy, but the potential seems extremely attractive.

MIMICS OF ENZYME–COENZYME COMPLEXES Much of the special chemistry of enzymatic reactions is performed with the assistance of coenzymes, since the chemical versatility of normal amino acid sidechains is limited. We have been interested in this special chemistry for many years, starting with a study of the chemical mechanism used by thiamine pyrophosphate (ref. 5). More recently we have attached such coenzyme groups to binding groups and to other functionalities, to imitate the full enzyme system. For example, we have studied catalysis by cyclodextrins carrying thiazolium rings (the active portion of thiamine coenzymes). When a thiazolium ring is linked to β-cyclodextrin, in compound 9, the catalyst can preferentially bind and activate a benzaldehyde molecule toward oxidation (ref. 6). However, the β-cyclodextrin cavity is too small to hold <u>two</u> benzaldehyde molecules, so the benzoin condensation that thiazolium salts catalyze is actually inhibited by the cyclodextrin. By contrast, with the larger cavity of γ-cyclodextrin (8 glucose units) two benzaldehydes can indeed both bind, and the benzoin condensation is strongly accelerated (ref. 7). A related catalyst based on a synthetic binding group has also been examined by Diederich (ref. 8).
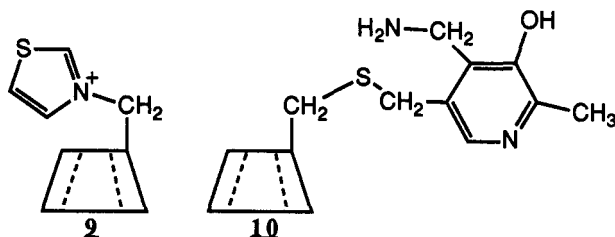

Much of our work in this area has focussed on the attachment of binding groups to the pyridoxal/pyridoxamine coenzyme groups. These coenzymes are involved in catalyzing most of the special chemistry of amino acid metabolism.

Our earliest example was compound **10**, with pyridoxamine covalently linked to the primary face of β-cyclodextrin (ref. 9). This was able to transaminate α-

ketoacids into amino acids, as pyridoxamine itself can do, but it showed selectivity for substrates that can bind into the cyclodextrin cavity. For example, it selectively transaminates phenylpyruvic acid and indolepyruvic acid to form phenylalanine and tryptophane, respectively, while having only normal reactivity toward the non-binding pyruvic acid itself. In recent work (ref. 10) we have found that with even more hydrophobic ketoacids this selectivity can be very high indeed.

We have made other analogs of **10**, including one with the pyridoxamine attached to the other face of β-cyclodextrin (ref. 11) and one in which a synthetic hydrophobic binding cavity is attached to pyridoxamine (ref. 12). They also show good selectivity for substrates that can bind into the cavity during transamination (ref. 13).

**11:** endo diastereomers
12: exo diastereomers
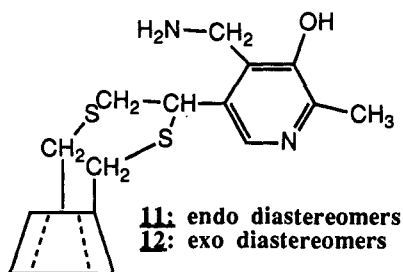
In all these cases, the pyridoxamine was attached to the binding cavity by a single thioether linkage. The resulting flexibility allows the molecule to adopt many conformations, including those that permit reaction, but excessive flexibility is never desirable in an enzyme mimic. Thus we have recently made two new molecules, **11** and **12**, in which a second link is present (ref. 10). In **11** this link holds the two components so as to promote very efficient transamination of bound substrates, while in the other stereoisomer **12** the geometry is very different and most substrates cannot utilize it. The difference is related to the "clam shell" and "love seat" isomeric arrangements that we saw in compounds **5** and **6** when we added an additional linkage to singly linked dimers such as **3**.

In transaminations and other pyridoxal/pyridoxamine catalyzed reactions, proton transfers are involved. Thus in a true enzyme mimic we must also incorporate proton transfer groups. Our earliest work involved the attachment of such proton transfer links to a pyridoxamine unit, without an additional binding group (ref. 14). We saw effective catalysis, and with chiral versions we saw good induction of optical activity in the product amino acids (ref. 13,15). Thus we have also combined these two lines into molecules that have pyridoxamine linked to both cyclodextrin binding groups and chirally mounted proton transfer catalyst chains (ref. 16). The resulting artificial transaminases show very interesting rates and selectivities.

RIBONUCLEASE MIMICS Of course many enzymes have no coenzymes to assist catalysis, but perform their function using amino acid sidechains alone. One very good example is the enzyme ribonuclease A, which uses imidazole groups of the amino acid histidine to catalyze the hydrolytic cleavage of RNA. The enzyme actually catalyzes two discrete steps: a cleavage of the RNA chain in which a linear phosphate diester group is transesterified to form a cyclic phosphate diester, and the subsequent hydrolysis of that cyclic phosphate diester to form a phosphate monoester.

Much work on the enzyme indicates that the principal catalytic groups are the two imidazole rings of His-12 and His-119, with additional help from the ammonium group of Lys-41. Thus many years ago we attached two imidazole groups to β-cyclodextrin, to try to mimic the principal catalytic functionalities of the enzyme (ref. 17). It was believed that one imidazole acts as a base to deprotonate a hydroxyl group as it attacks the phosphorus atom, and the other acted, as the protonated imidazolium ion, to assist departure of the leaving group. In the first step the attacking hydroxyl is the O-H group on C-2 of ribose, forming the 2,3-cyclic phosphate; in the second step it is the O-H group of a water molecule that cleaves the cyclic phosphate to regenerate the C-2 O-H group. For this mechanism it is important that the acid and base group be on opposite sides of the substrate phosphate, so we mounted the imidazole rings on glucose residues A and D of β-cyclodextrin (the seven glucose rings are lettered A through G).

13: A,D isomer
15: A,B isomer
16: A,C isomer

14
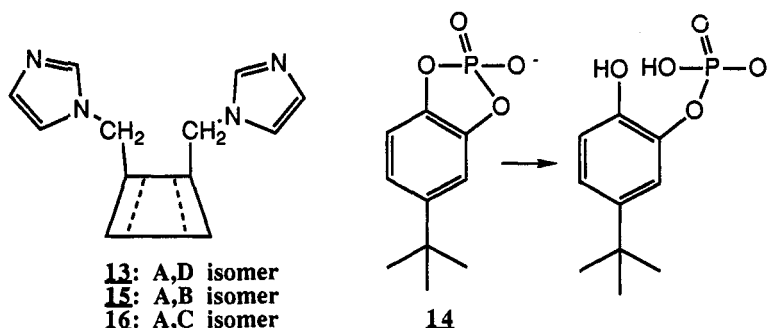
The resulting catalyst **13** was able to cleave a cyclic phosphate ester **14** that binds to the cyclodextrin ring in water so as to put its phosphate ester group in the right spot for cleavage. It also showed some activity toward normal ribonucleotides. Most important, it showed a bell-shaped pH *vs*. rate profile that indicates bifunctional catalysis like that performed by the enzyme, and the cleavage of the cyclic phosphate was selective just as the enzymatic hydrolysis is. That is, the hydrolysis of the cyclic phosphate **14** proceeded to give principally the O-H group on C-1 with the phosphate ester on C-2 of the 4-t-butylcatechol, with only a few percent of the other isomer. By contrast, ordinary chemical hydrolysis of **14** gives both isomers, with a little preference for the 1-phosphate.

These results were consistent with our mechanistic ideas, based on the accepted mechanism for the enzyme ribonuclease. The bifunctional catalysis presumably involved the imidazole ring acting as a base to deliver a water molecule while the imidazolium ion acted as an acid to protonate the leaving group. The selective hydrolysis of the substrate **14** in one direction was consistent with molecular models for this mechanism: delivery of water by a nearby imidazole ring has a geometry that favors the cleavage direction observed. As expected from this, we find that when an analog of **13** is prepared with the imidazole rings more loosely attached, and further from the cyclodextrin, more of the other cleavage isomer is seen (ref. 18).

We decided to do a mechanistic study of the cleavage of RNA catalyzed by imidazole buffer. It seemed likely that high concentrations of the buffer could duplicate to some extent the high effective concentration of the catalytic species in the enzyme itself, and that this study could guide us in the synthesis of even more effective catalysts. The results were striking (ref. 19,20). We saw that RNA is indeed catalytically cleaved by imidazole buffer, as is the simple RNA fragment UpU, but that the kinetic behavior was indicative of a special mechanism. There was a rate maximum when both the basic imidazole and the acidic imidazolium ion were present, but the kinetics showed that only one of these species was present in the transition state for the reaction. That is, in spite of the indication that both the base and the acid played a catalytic role, the kinetics were first order in buffer concentration.

These results indicate a bifunctional mechanism, but one in which there are two sequential steps and each catalyst plays a role in one of those steps. For instance, the substrate RNA could be converted to an intermediate phosphorane by base in the first step, and that intermediate could go on to form the product cyclic phosphate ester in the second step with acid catalysis. The kinetics required that the intermediate be a phosphorane monoanion, but did not indicate whether the first catalyst was the base or the acid (with the other species coming into the second step).

This ambiguity was solved by looking at another reaction that accompanies the cleavage process (ref. 20). Under some conditions the 3,5-UpU can rearrange to a 2,5-UpU; if this starts off with the same phosphorane intermediate then the first step should be the same as that for the cleavage reaction. The kinetics confirmed this hypothesis, and showed which catalyst operates in the first step. We saw that only the imidazolium ion catalyzed the migration rearrangement, and that the rate of this rearrangement was diminished by the base component. Thus the first step is acid catalyzed, the second step of the cleavage is base catalyzed, and addition of more of the basic component diverts a common intermediate to the cleavage pathway. This lowers the steady state concentration of the intermediate, so the rearrangement rate is

lowered; the effect would not have been seen if the cleavage and the migration did not proceed through a common intermediate. We have seen similar kinetics for the cleavage and migration of ApA (D.-L. Huang, unpublished).

These kinetic results indicate a fundamentally different mechanism from the one usually considered for the cleavage of RNA by the enzyme ribonuclease. In the conversion of a linear phosphate diester to a phosphorane monoanion intermediate, the only function that an acid catalyst can play in the first step is to protonate the phosphate group. Then the resulting phosphoric acid unit is attacked by the C-2 O-H group, assisted by imidazole. In the second step, base catalysis must involve deprotonation of the phosphorane to a dianion, whose fragmentation is probably assisted by imidazolium protonation of the leaving group (ref. 20).

In this mechanism we do indeed use imidazole to deprotonate the C-2 hydroxyl group, as previously believed, and we probably use an imidazolium ion to protonate the leaving group. The difference from previous mechanisms is the prior use of the imidazolium ion to protonate the phosphate group.

It seems unlikely that the enzyme uses precisely this mechanism: an intracomplex bifunctional catalytic mechanism can involve simultaneous catalysis by the two groups which act sequentially when they are not linked (ref. 21). That is, a third order reaction is unlikely in solution so the catalysis is sequential, but in the enzyme/substrate complex there is no such problem with simultaneous catalysis. Proton inventory studies of the enzyme indicate that indeed two protons are in flight in the enzymatic transition state, as expected for simultaneous acid-base catalysis (ref. 22). However, we have pointed out elsewhere (ref. 21) that the data on the enzyme suggests that with it as well the first function of the acid group is to protonate the phosphate, not the leaving group.

This mechanism has significant geometric consequences. If the acid and the base operate simultaneously in an enzyme mimic, as they do in the enzyme, then they must be close together if the acid is first protonating the phosphate oxyanion. The A,D isomer of β-cyclodextrin-bis-imidazole is not the optimum one for this mechanism. Guided by models, we predicted that the two imidazoles should be on neighboring glucose residues, in the A,B isomer, if the imidazolium ion were to hydrogen bond to the phosphate anion and transfer the bridging proton as the phosphorus were attacked by a water molecule with imidazole assistance.

Synthesis of the A,B isomer **15** was accomplished from the known diiodide (ref. 23). We found that it is indeed an excellent catalyst for the cleavage of phosphate ester **14**, considerably better than the A,D isomer **13** or the A,C isomer **16**. It also shows a bell shaped pH *vs.* rate profile, and highly selective cleavage of **14** to form the 2-phosphate. Furthermore, proton inventory studies (ref. 24) showed that it is using <u>simultaneous</u> two-proton transfer, just as the enzyme does, so it probably starts off with a hydrogen bond between the phosphate anion and the imidazolium cation. This study also helped validate the proton inventory method, by showing that only one proton transfer can be detected with a catalyst lacking the imidazolium ion group.

Compound **15** is quite a good enzyme mimic, with many of the characteristics of the enzyme itself. Its design illustrates the interaction between mechanistic work and synthetic work in this field. The work has also illuminated the probable mechanism of the enzyme.

Many enzymes use metal ions to perform catalyses. We have examined the RNA mimics in which we substitute a Zn^{2+} for the proton of the imidazolium ion (ref. 21). The cleavage is faster, showing that Zn^{2+} does an even better job of activating the phosphate group toward addition, but interestingly no migration rearrangement accompanies the cleavage of 3,5-UpU. We have discussed the reason for this difference from the proton catalyzed case.

CONCLUSION Studies of model systems for enzymatic reactions frequently give us insight into the enzyme itself. As an early example, the mechanisms of enzymatic reactions catalyzed by the coenzyme thiamine pyrophosphate were learned from model system studies, as were the mechanisms used by pyridoxal/pyridoxamine coenzymes. Recently, our studies of models for the Zn^{2+} enzyme carboxypeptidase led to a proposal for double proton transfer in the model

system that also seems to best explain some data on the enzyme (ref. 25). Of course model systems have limits. An obvious example is the use of sequential catalysis by imidazole buffer as a model for the enzyme ribonuclease, whereas the enzyme seems to use simultaneous catalysis. With care, such differences can be anticipated and are instructive.

The major finding with the systems we have described here is that we can get interesting increases in the effectiveness and selectivities of catalyses by introducing further elements of rigidity into our model systems. This is shown strikingly in the case of the dimeric cyclodextrins, but it is also clear in the pyridoxamine examples and implicit in the differences in effectiveness of the three different cyclodextrin-bis-imidazole isomers. Thus one might wonder how important rigidity is, and whether it is ever harmful.

We have examined this point in some studies of the acylation of β-cyclodextrin by bound substrates, models for the first step in serine protease reactions. With a very rigid substrate based on the ferrocene nucleus we observed very large accelerations relative to the hydrolysis rate of the same substrate, but these accelerations fell off as the leaving group activity was diminished. We concluded that the rigidity was very useful in promoting the first step of the reaction, formation of a tetrahedral intermediate, but that the same rigidity got in the way in the second step in which the tetrahedral intermediate is transformed to product cyclodextrin ester. The process is related to the two step conversion of RNA to the cyclic phosphate via a phosphorane intermediate.

Computer modelling made it clear that the problem was the need for motion in the second step (ref. 26,27). Thus as the substrate was made less reactive, with a poorer leaving group, this second step made a more important contribution to the overall rate and the rigidity got in the way. To confirm this, we made an analogous system in which the substrate had one extra degree of freedom introduced (ref. 28). Now the rigidity was less, so the first step was not as favorable, but the motion was allowed so no special problem arose in the second step. The result is that this more flexible substrate series is actually more reactive than the rigid one with poor leaving groups, but less reactive with good ones.

This example shows that rigidity is useful in the construction of catalysts so long as it simply restricts the geometry to the one needed for reaction. However, it must not inhibit the geometric changes needed for the reaction to occur.

An additional question is how rigid the system should be for a simple elementary step. That is, what are the quantitative geometric requirements for the transition state of a reaction--how much does the rate drop off if the geometry deviates from the optimum? This matter has been of wide interest for a long time. Koshland suggested (ref. 29) that "orbital steering" might require that a transition state be even more rigidly defined than is a normal molecule, but this idea has been widely discounted (ref. 30,31). However, a number of recent findings have caused us to rethink this situation (ref. 32).

It is true that any sensible calculation shows that the bending force constants for the partial bonds of a transition state will be weaker than those for the full bonds of the substrate or product. Thus one might think that bending these weakened bonds cannot have large energetic, or rate, consequences. However, in a chemical reaction it is necessary to supply energy so that the activation barrier can be surmounted, and the efficiency of energy transfer will also have a geometric dependence. Described simply, a head-on collision will put the translational energy into vibrations, and thus into the bond making and breaking needed for reaction, but a glancing collision will transfer the energy much less effectively.

If this is important, as we have argued that it could be (ref. 32), then geometric control could have large effects on the rates of reactions. It will be interesting to see whether enzyme mimics with such geometric control prove to imitate much more effectively the remarkable catalyses achieved by real enzymes.

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