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Efficiency of Proton Transfer Catalysis in Models and Enzymes†

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Molecular Recognition of Transition States

We want to understand better the extraordinary efficiency with which enzymes make and break covalent bonds. In simple terms, what enzymes do is bind and thus stabilize selectively the transition states for their reactions;[1] so their primary, catalytic, role can be defined as the molecular recognition of transition states.[2] Though it is a major oversimplification to think of mechanism in terms of a single rate-determining step—a typical enzyme must service a succession of transition states—for purposes of analysis, understanding the most energetically demanding step of an intrinsically slow reaction of interest is sufficient challenge. Understanding in this context means defining and as far as possible quantifying the differences between the transition state in solution and in the enzyme active site. These will be greatest, and thus most interesting and most revealing, for intrinsically very slow reactions. So we have a special interest in the ways enzymes catalyze transfer reactions involving the extraordinarily stable groups of structural biology—peptides, glycosides, and phosphodiesters—often without the use of metals. Paradoxically, it may be just these intrinsically very slow reactions where the efficiency of proton transfer catalysis is most important.

If we think about enzyme catalysis specifically in terms of the molecular recognition of transition states, it becomes clear that more than one sort of recognition is involved. Most binding interactions are no different in kind or strength from those involved in recognition of the substrate ground state, but recognition *at the reacting center* is a dynamic process, responding to the changing distribution of charge as bonds are made and broken—and presumably optimal at the transition state. Compared with ordinary ("passive" [2]) recognition, dynamic binding is potentially stronger because it may include partial covalent bonding. For example,[3] the transition state for the initial step of a serine protease reaction involves up to five partial bonds (dashed in Figure 1), each of which contributes to transition state binding.[4] We would like to know how important such contributions might be. This Account outlines a possible approach.

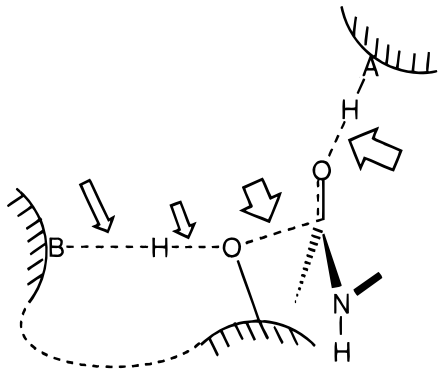
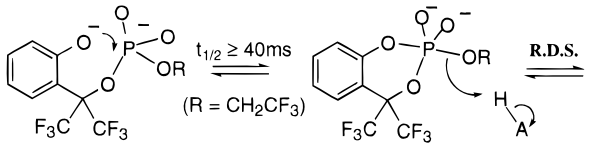


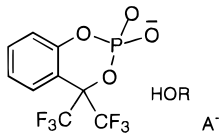
FIGURE 1. Dynamic binding interactions (arrows) in the transition state for the initial step of a serine protease reaction.

Intramolecular Reactions

Our chosen experimental tool is the intramolecular reaction: we bring functional groups together on the same molecule to model what goes on when an enzyme brings together the same functional groups in its active site. This procedure, like any based on models, has limitations, but it has the unique advantage that we can at least *see* the reactions of interest: we do not need activated and perhaps atypical substrate groups to set up a reaction fast enough to study in the absence of enzyme. For example, we have a special interest in nuclease mechanisms. The remarkable kinetic stability of phosphodiesters is well-known: the half-life, based on measurements for dimethyl phosphate,[5] is typically many hundreds of years (at least) in water at pH 7 and 37 °C. In the active site of the appropriate nuclease, where the attacking nucleophile is a hydroxyl group, this is reduced to a fraction of a second; the same is true of the simple intramolecular model **1**.[6]



1 **2**



In this way intramolecular models allow us to study the mechanisms of specific reactions of interest (the

Tony Kirby is Professor of Bioorganic Chemistry at Cambridge, where he was also a student. He spent an inspirational postdoctoral year (1963−1964) with W. P. Jencks at Brandeis: to whom—in this his 70th year—this paper is dedicated.

† This Account is a distillation from the work of a group of highly talented research students, and the odd post-doc, supported in most cases by the Engineering and Physical Sciences Research Council of Great Britain.

(1) Fersht, A. R. *Enzyme Structure and Mechanism*, 2nd ed.; Freeman: New York, 1985.
(2) Kirby, A. J. *Philos. Trans. R. Soc. (London), Ser. A* **1993**, *345*, 67.
(3) Kirby, A. J. *Angew. Chem.* **1996**, ***35***, 707−724.
(4) Some, e.g. electrocyclic, reactions which do not involve active site functional groups directly in catalysis do not involve this sort of interaction. It is however fundamental to reactions in which strong σ-bonds are made and broken: and an essential part of catalysis for intrinsically slow reactions, where the enzyme has to be particularly efficient.
(5) Wolfenden, R. *Science* **1994**, *267*, 90−93.
(6) Dalby, K. N.; Kirby, A. J.; Hollfelder, F. *J. Chem. Soc., Perkin Trans. 2* **1993**, 1269.

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reactions of system **1** are models for phosphodiesterases which use the OH group of tyrosine as the nucleophile) in systems simple enough to understand in detail. But that is only the beginning. Most relevant are the mechanisms of model reactions going at rates comparable with those in enzyme active sites, because rate-determining steps or even mechanisms may change as the absolute rate changes. So we routinely vary structure to maximize the rate of our intramolecular reactions, learning on the way about those factors—particularly geometrical factors—which control reactivity when functional groups are brought together.

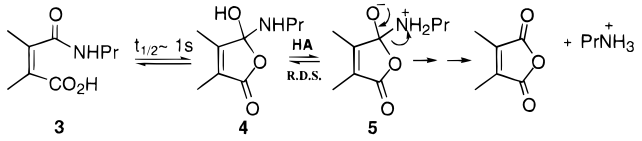
Efficiency of Catalysis

To do this systematically, we need an objective measure of efficiency which is characteristic of the system and as far as possible independent of the reaction. We use the effective molarity (EM),[7] simply measured as the ratio of the (first-order) rate constant for the intramolecular reaction divided by the second-order rate constant for the intermolecular process (going by the same mechanism under the same conditions). The EM is nominally the concentration of the equivalent external catalyst (e.g., unsubstituted phenolate anion in the case of **1**) needed to make the intermolecular rate match that of the intramolecular reaction. This concentration may be purely nominal (EMs are often greater than any physically attainable concentration), but its interpretation is simple: the higher the EM, the more efficient the intramolecular reaction.

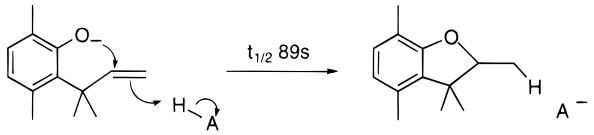
EMs have been measured over the years for many hundreds of intramolecular reactions. They fall into two main groups, depending on the type of reaction concerned. For intramolecular cyclizations to form stable 5- or 6-membered rings—which involve additions, and most substitution reactions—EMs are high, typically between 10^3 and 10^9 M in unstrained systems. By strategic use of ground-state strain, they can be raised as high as 10^{13} M[7] (worth 17−18 kcal mol^{-1} in terms of dynamic binding[3]). In sharp contrast, EMs for intramolecular general acid/base-catalyzed reactions, involving proton transfer rather than ring-formation, are typically less than 10 M. In fact, an EM of greater than 80 M was originally considered *prima facie* evidence for a nucleophilic mechanism.[7]

Insofar as intramolecular reactions are models for enzyme reactions, these generalizations carry conflicting messages. We are looking to explain very large rate accelerations in the case of enzymes catalyzing the reactions of unreactive substrates. Proteins are not particularly rigid structures, so enzymes cannot be expected to induce strain in a bound substrate to the extent that we can build it in by synthesis. Nevertheless, a potential EM of up to 10^{9-10} M for a nucleophilic group in an enzyme active site is persuasive evidence that "proximity" makes a major contribution to enzyme efficiency when covalent bonds are being formed.[1] (There was much discussion at one time about the origins of the "proximity effect" (summarized in reviews by Jencks[8] and Menger[9]). The simplest way of thinking about the problem is in terms of the free energy of the cyclization process. If this is strongly favorable and most of it available in the transition state, cyclization will be rapid. When a covalent bond is formed in an enzyme active site, where the angle of approach is unrestricted and may be presumed to be optimal, it seems entirely reasonable that the reacting centers should be brought together in such a way that bond formation is thermodynamically favored at least as well as in an unstrained cyclization process.)

Where no covalent bond is formed the conclusion is less self-evident. Most such reactions involve proton transfers. Indeed proton transfer is the most common enzyme-catalyzed reaction: enzyme reactions are generally heterolytic, and heterolytic reactions in water generate ions, which have to come to terms with physiological pH. Yet intramolecular proton transfer reactions are generally inefficient. This may not seem important for the intrinsically rapid, typically diffusion-controlled proton transfers between electronegative centers, but even these can become rate determining in reactions involving high-energy intermediates. An example is the rapid cleavage of the amide group of **3** by neighboring COOH,[10] where the rate-determining step is a (diffusion-controlled) proton transfer, catalyzed by an external general acid.



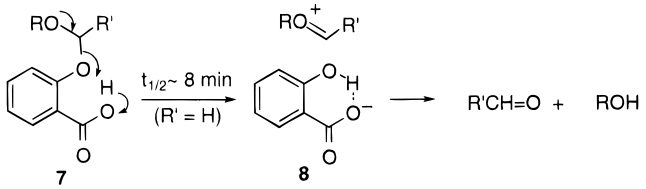
The efficiency of proton transfer is certainly relevant for (the much slower) classical general acid/base-catalyzed reactions, where it is concerted with the formation and cleavage of bonds between heavy atoms (e.g., the breakdown of the phosphorane **2** described above), and for proton transfer to and from carbon. A striking example embracing both classes is the nucleophilic addition of phenolate anion to the unactivated double bond of alkene **6**.[11]



Not surprisingly, a primary carbanion is not an intermediate in this reaction, and if no general acid is present in the encounter complex, the reaction shown does not proceed. There is clearly scope for more substantial assistance from a properly positioned proton donor group, and an enzyme in such a situation can rather confidently be expected to provide it. Yet our generalization based on results with model systems told us that making the proton transfers intramolecular does not make them very efficient. The outline of a solution to this problem that has emerged from our work in the past 10 years is the main message of this Account.

(7) Kirby, A. J. *Adv. Phys. Org. Chem.* **1980**, *17*, 183−278.
(8) Jencks, W. P. *Adv. Enzymol.* **1975**, 37, 219.
(9) Menger, F. M. *Acc. Chem. Res* **1985**, *18*, 28−33.
(10) Kirby, A. J.; Lancaster, P. W. *J. Chem. Soc., Perkin Trans. 2*, **1972**, 1206.
(11) Evans, C. M.; Kirby, A. J. *J. Chem. Soc., Perkin Trans. 2* **1984**, 1269.

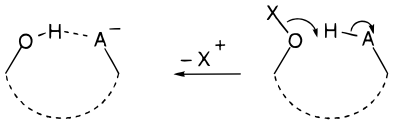
Scheme 1



Efficiency of Proton Transfer Catalysis

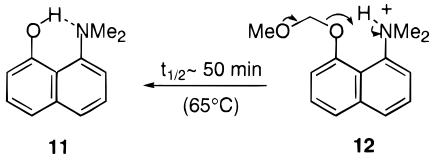
We started from the reasonable assumption that enzymes can catalyze proton transfer reactions efficiently, in which case the available models must be deficient. So it should be possible to devise systems which can do intramolecular general acid or general base catalysis with EMs substantially greater than the typical 1−10 M. Fortunately one such system was already in the literature. Two derivatives of salicylic acid—the O-acetals and the phosphate monoester—were known to undergo hydrolysis by a mechanism which involves the *o*-COOH group as a general acid (as shown in Scheme 1), and Buffet and Lamaty[12] estimated an EM of over 10^4 M [7] for this group catalyzing the rapid hydrolysis of the benzaldehyde acetal **7** (R = Et, R′ = Ph).

Our results with various structures derived from salicylic acid suggested that the unique feature responsible for the high efficiency of intramolecular general acid catalysis in this system is the strong intramolecular hydrogen bond in the salicylate anion (**8**) produced. This is known to be worth 4−5 kcal/mol (its stabilizing effect on the monoanion raises the pK_a of the phenolic OH to 12.95 at 25 °C[13]). However, there are enough special factors involved, in particular the direct conjugation between the catalytic and leaving groups, to make the salicylate structure a possible exception, so we looked for an independent system for corroboration. The simple requirement was a structure **9** in which an OH donor group forms a strong intramolecular hydrogen bond to a basic center A^-. A suitable derivative should rapidly lose a stable electrofuge X^+ from the conjugate acid by the mechanism shown in **10**. In our test systems, X is conveniently the alkoxyalkyl carbon of an acetal.



In practice, strong intramolecular hydrogen bonds that persist in water are not common because adjacent donor and acceptor groups are generally better solvated separately. We have by now devised several suitable systems, but the first was the hydroxynaphthylamine **11** (pK_a of OH group 14.9[14]), based on proton sponge. The methoxymethyl acetal **12** derived from **11** did indeed undergo efficient intramolecular general acid catalysis, with a pH−rate profile dominated by the pH-independent reaction of the cation up to the pK_a of 7.4.[15] The mechanism was as expected (Scheme 2) and the rate acceleration at pH

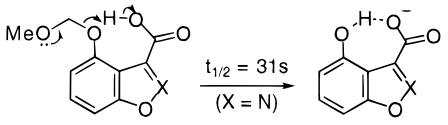
Scheme 2



7.4 on the order of 10^6 (compared with the specific acid-catalyzed hydrolysis of the acetal group; since the corresponding intermolecular reaction is not detectable, it is not possible to estimate an EM for this process.)

In this system, the leaving group oxygen and the dimethylammonium group are electronically independent, and we have no reason to doubt that efficient catalysis results, as predicted, from the development of strong hydrogen bonding in the product **11**, and thus also in the transition state leading to it. Efficiency is reduced because a (weaker) hydrogen bond is already present in the reactant **12**; as shown by the elevated pK_a of the Me_2NH^+ group (7.4, compared with a value near 5 expected for an (*N*,*N*-dimethylamino)naphthalene.[14] This factor, and the relative weakness of the general acid, means that the reaction of **12** (Scheme 2) goes at a convenient rate only at 65 °C.[15]

Our most reactive methoxymethyl acetal (so far) is the benzisoxazole derivative **13** (X = N), which has a half-life of 31 s at 39 °C.[16] Here the general acid is particularly strong (pK_a 1.55, ruling out strong H-bonding in the reactant), and again the catalytic and leaving groups are electronically independent; the decisive factor is the developing intramolecular H-bond in **14**. In terms of dynamic binding this is worth up to 9 kcal mol^{-1}.[3]

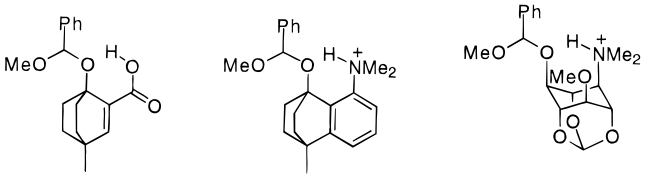


Thus far the acetals we and others have found to show intramolecular general acid catalysis have generally involved phenolic—and therefore activated—leaving groups. Enzymes, on the other hand, must almost invariably break bonds to alcohol oxygens; so we set out to design systems which would be expected to provide strong intramolecular hydrogen-bonding to a developing alkoxide anion. We have recently prepared three structurally unrelated model systems, based closely on the reactive systems **7** and **11** already described and using three different general acids. All three show efficient general acid catalysis in the hydrolysis of dialkyl acetals.

The rates of such reactions are sensitive to the basicity of the leaving group,[17] so changing to alkoxy derivatives will reduce reactivity sharply. To compensate, we use more reactive, benzaldehyde, acetals.[18] Two of our first generation systems (**15** and **16**) are based directly on **7** and **11**, while the third also incorporates the successful

(12) Buffet, C.; Lamaty, G. *Recl. Trav. Chim. Pays-Bas* **1976**, *95*, 1.
(13) Hermans, J.; Leach, S. J.; Scheraga, H. A. *J. Am. Chem. Soc.*, **1963**, *85*, 1390−1395.
(14) Awwal, A.; F. Hibbert *J. Chem. Soc., Perkin Trans. 2*, **1977**.
(15) Kirby, A. J.; Percy, J. M. *J. Chem. Soc., Perkin Trans. 2* **1989**, 907−912.
(16) Kirby, A. J.; Parkinson, A. *J. Chem. Soc., Chem. Commun.* **1994**, 707−708.
(17) Craze, G.-A.; Kirby, A. J. *J. Chem. Soc., Perkin Trans. 2*, **1978**, 354.

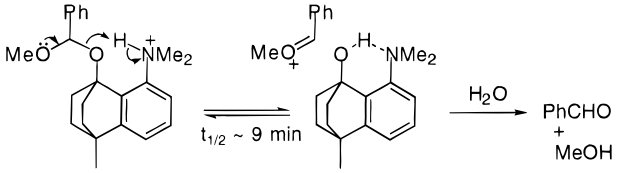
eclipsed geometry of the C−O and C−AH bonds, in the all-axial system **17** based on inositol.[19,20]



15 **16** **17**

All three of these dialkyl acetals show rather efficient intramolecular general acid catalysis, with EMs (intermolecular general acid catalysis by acetic acid has been measured for benzaldehyde dialkyl acetals[21]) on the order of 3000, 1000, and 10 000 M for the reactions of **15**, **16**, and **17**, respectively.[20] The mechanism appears in each case to be classical general acid catalysis (Scheme 3, **16***), as indicated by kinetic isotope effects (k_{H_2O}/k_{D_2O}) of up to 2.2.

Scheme 3



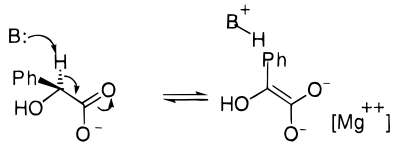
16* **18**

System **15** based on salicylate has a half-life of less than 1 s at 25 °C, while **17** is some 10^7 times less reactive. Thus the highest absolute reactivity does not correspond to the highest EM—for reasons that are simple but instructive. Efficiency depends on the difference in energy between ground and transition states, and reactant **16**, like **11**, is stabilized by intramolecular hydrogen bonding in the ground state (indicated by the pK_a of 6.93 for the Me_2-NH^+ group). Were it not for this effect, worth up to perhaps 2 orders of magnitude, **16** might have been the most efficient of the three systems.

Proton Transfer to Carbon

These results showed that EMs for intramolecular general acid−base catalysis can be at least 10^4 M, if the proton transferred ends up in the reaction product in a thermodynamically favored intramolecular hydrogen bond. This requires rather precise positioning of the donor and acceptor groups, which in these simple systems means bringing them together on rigid structures. The fundamental requirement for high efficiency is hydrogen bonding-type stabilization (dynamic binding) of the in-flight proton in the transition state: hydrogen bonding must be weaker (preferably absent) in the reactant ground state. This requirement is most easily met in reactions such as enolization, involving proton transfer to and from carbon.

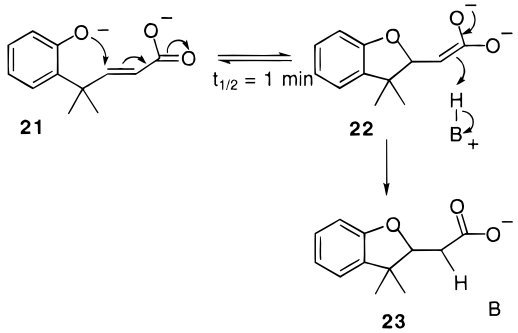
Of particular current interest is mandelate racemase, which catalyses the enolization of the mandelate anion **19** remarkably efficiently ($k_{cat} = 700\ s^{-1}$ [22]). The active site general bases which deprotonate the enantiomers of mandelate are a histidine imidazole and a lysine amino group. The product dianion **20**—and of course the transi-



19 **20**

tion state leading to it—are heavily stabilized by hydrogen bonding and metal coordination, but the overall rate acceleration is so great that there seems no doubt that the proton transfer part of the reaction (insofar as this can be considered in isolation) must itself be highly efficient. If so, any hydrogen-bonding-type stabilization of the in-flight proton must be expressed more or less exclusively in the transition state for this part of the reaction: H-bonding between the general base B and the α-proton in the reactants **19** is unlikely to be significant. (A strong H-bond from BH^+ to the double bond of the trienolate dianion **20** is less unlikely but would not affect the rate in the forward, thermodynamically unfavorable direction.)

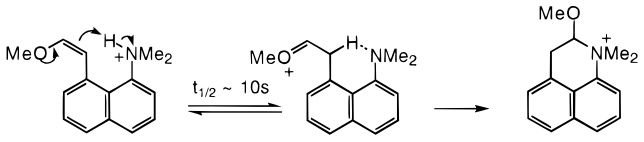
This reaction is too slow to measure in the absence of the enzyme, primarily because the product **20** is a high-energy species (not inaccessibly high, since we generated the dianion **23** of a carboxylic acid as a full intermediate in water[23,24] simply by the intramolecular cyclization of **21**).



21 **22** **23**

Although enolization is too slow to measure near pH 7 with a system like **19** (or **23**), we can conveniently characterize the transition state by studying the reaction in the reverse, thermodynamically favorable direction. Our most efficient model for this process is the enol ether **24**, in which an sp^2 carbon replaces oxygen as the hydrogen-bond acceptor in **11**.[25]

(18) As a rough guide, hydrolysis reactions of methoxymethyl, tetrahydropyranyl, and benzaldehyde acetals are faster than those of the corresponding glucopyranosides by 4, 6−7, and 9−10 orders of magnitude, respectively.
(19) Brown, C. J.; Kirby, A. J. *J. Chem. Soc., Chem. Commun.* **1996**, 2355−2356.
(20) Brown, C. J.; Kirby, A. J. *J. Chem. Soc., Perkin Trans. 2* **1997,** in press.
(21) Jensen, J. L.; Herold, L. R.; Lenz, P. A.; Trusty, S.; Sergi, V.; Bell, K.; Rogers, P. *J. Am. Chem. Soc.* **1979**, *101*, 4672−4677.
(22) Landro, J. A.; Gerlt, J. A.; Kozarich, J. W.; Koo, C. W.; Shah, V. J.; Kenyon, G. L.; Neidhart, D. J.; Fujit, S.; Clifton, J. R.; Petsko, G. A. *Biochemistry* **1994**, *33*, 635- 643.
(23) Amyes, T. L.; Kirby, A. J. *J. Am. Chem. Soc.* **1988**, *110*, 6505−6514.
(24) In this reaction, the intermediate **22** has the remarkable property of being protonated by water to give the product **23** of *anti* addition but by protonated amines to form the *syn* diastereoisomer. This is may be a function of the short lifetime of the intermediate, but the intrinsic chemistry is still not properly understood.
(25) Kirby, A. J.; O'Carroll, F. *J. Chem. Soc., Perkin Trans. 2* **1994**, 649−655.



24 **25** **26**

In sharp contrast to derivatives of **12**, which all have pK_a close to 7 for the dimethylammonium group, the pK_a of **24** is 4.0—good evidence for the absence of significant hydrogen bonding to the π-system of the enol ether in the ground state. And reactivity is indeed remarkably high: the half-life of **24** (and of its *E* isomer) is some 10 s at 39 °C, though the hydrolysis of PhCH=CHOMe is too slow to measure at convenient rates in dilute aqueous acid.[26] The closely similar rates of disappearance of the *E* and *Z* isomers of **24** suggest that the proton transfer step may not in fact be exclusively rate determining, as it generally is for the hydrolysis of enol ethers. This would be a logical consequence of a very efficient proton transfer step. (The likely alternative rate-determining step is the conformation change necessary for the conversion of **25** to **26**, the immediate product of the reaction. The direct addition of water to the C=OMe$^+$ group of **25** is a minor pathway and is clearly slower than the proton transfer step.[25])

Once again it is not possible to estimate the EM for the intramolecular reaction of **24** accurately because the corresponding intermolecular process is too slow to measure. A conservative estimate, based on proton transfer as the rate-determining step, is 60 000 M; as an order of magnitude the EM is 10^5 M. This makes the simple proton transfer process drawn as **24** (arrows) the most efficient known outside an enzyme active site.

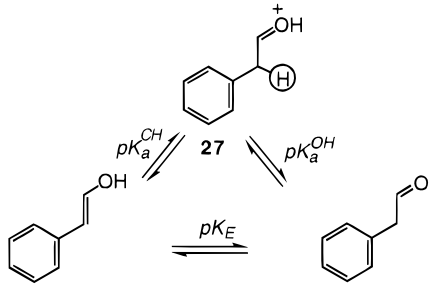
We arrived at this system by logical arguments based on transition state stabilization by intramolecular hydrogen bonding, as it develops in product structures known to form strong intramolecular hydrogen bonds. It is not immediately obvious that this is relevant to **25**, a high-energy intermediate, not accessible to structural investigation, in which the formal hydrogen bond donor is an sp^3-C—H. However, a simple calculation, based on the thermodynamic cycle shown as Scheme 4,[27] shows how this particular C—H bond is well-qualified to be involved in hydrogen bonding.

We take the protonated aldehyde **27** as the model for the CH=OMe$^+$ group. The pK_a of a protonated aliphatic aldehyde cannot be measured accurately because aldehydes are rapidly destroyed in strong acid, but a value between −5 and −8 is a best estimate.[27] The equilibrium constant for enolization, pK_E, on the other hand, is known, and is close to 10^{-3} for phenylacetaldehyde.[28] So we estimate a pK_a for the CH proton of **27** between −5 and −2, making it as strong a proton donor as H_3O^+ and certainly a well-qualified hydrogen bond donor.

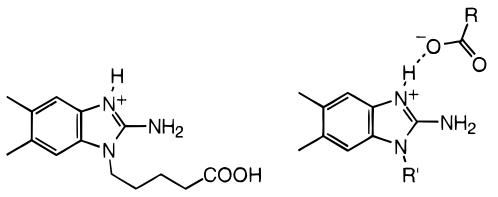
Proteins Designed and Recruited as Catalysts

This work has established that proton transfer catalysis can be efficient, given very precise positioning of the donor and acceptor groups—so precise that so far only a handful of carefully designed intramolecular model systems meet the requirements. Even high-resolution X-ray structures of enzymes allow no more than indications that such geometries are or are not possible in bound transition states. So it is of great interest to explore alternative ways of setting up the correct geometry. The most sophisticated way of bringing functional groups systematically together outside enzyme active sites is in the binding sites of antibodies. It is possible to elicit complementary charged groups on antibodies by the use of properly designed antigens (haptens) so that binding is reinforced by electrostatic attraction. In favorable cases such groups should be in well-defined positions with respect to the bound hapten, and thus also—in the case of haptens designed as transition state analogues—with respect to a bound transition state.

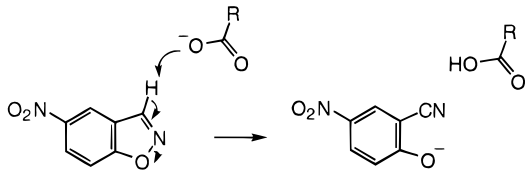
Scheme 4



Our attempts to raise antibodies capable of catalyzing proton transfer reactions have met with limited success, but Hilvert and co-workers[29] found that antibodies raised against the benzimidazolium hapten **28** catalyzed the Kemp elimination (**29** → **30**) rather efficiently.[30,31] The hapten carries a positive charge, so an antibody elicited against it might have a side-chain carboxylate anion close enough for a favorable electrostatic interaction, which would contribute to binding most effectively if it also involved a hydrogen bond to the NH$^+$ group (**31**). Such a carboxylate would then be correctly placed to act as a general base in the Kemp elimination of a suitable substrate bound in the same position.



28 **31**



29 **30**

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Two antibodies raised against **28** were remarkably efficient catalysts for the Kemp elimination of 5-nitrobenzisoxazole (**29**), and the catalytic base was in each case an active site carboxylate.[29] The rate acceleration for the more efficient of the two (comparing the second-order rate constant for the antibody-catalyzed reaction (k_{cat}/K_M) with that for catalysis by acetate) was over 10^8, corresponding to an EM of over 10^4 M.[29] This observation was clearly directly relevant and potentially of major significance for the efficiency of proton transfer catalysis, and it inspired the experiments described below.

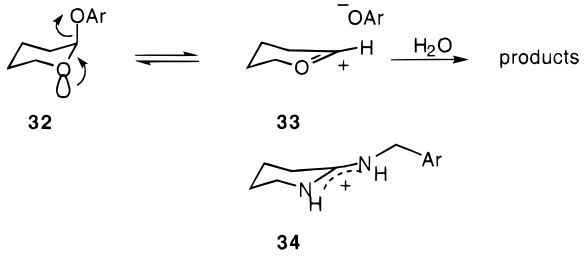
As pointed out by the original authors, there is more than one possible source for the high rate of the antibody-catalyzed reaction of **29**. Precise positioning of the general base could by itself account for the high apparent EM, but the Kemp elimination is also famously sensitive to the medium. The acetate-catalyzed reaction shown for **29**, for example, is over 10^7 times faster in acetonitrile than in water,[32] primarily a consequence of the stabilization of carboxylate anions by hydrogen-bonding solvation in water. So a medium effect, potentially of comparable magnitude, is an alternative explanation: the calculated EM of over 10^4 M is based on the second-order rate constant for catalysis by acetate in water, whereas the antibody carboxylate operates in an active site which is expected to be at least partly hydrophobic, to accommodate the aromatic system (and has its pK_a raised to 6.0 as a result[29]).

There is no obvious way to partition the observed rate acceleration between the two effects, so we tried a different tack. The basic requirement for an enzyme mimic that will catalyze the Kemp elimination of **29** is a hydrophobic binding site with a general base in close proximity. The combination is not particularly unusual, so we screened for catalysis of the reaction of **29** a few proteins where it is a known feature. And found some quite respectable catalysts.

Serum albumins such as BSA catalyze the conversion of **29** to **30** with efficiencies rivaling those of the catalytic antibodies (measured k_{cat} values are closely similar at the different pH optima).[33] Conveniently they use a lysine amino group rather than carboxylate as the catalytic general base. This allows an assessment of the contribution of the medium effect to catalysis because the amine-catalyzed Kemp elimination is relatively insensitive to medium effects. The EM for catalysis by the lysine amino group of BSA, based on the comparison with the reaction catalyzed by a comparable primary amine in water, is 14 M, and smaller by less than 1 order of magnitude if the rate constant for the comparison reaction is measured in acetonitrile. These values lie within the normal range of EMs established for intramolecular general acid/base catalysis: as might be expected for functional groups brought together in flexible systems.

On the other hand, the EMs calculated in the same two ways for catalysis by the carboxylate group of the most efficient anti-**28** antibody are 4.1×10^4 and 2.3×10^{-4} M, respectively. It seems certain that the "correct" value lies somewhere between, with a substantial contribution to the observed efficiency from a medium effect: that the true EMs for catalysis by the antibodies are unexceptional and that they do not achieve the very precise positioning of the general base that we believe is necessary for exceptionally efficient proton transfer catalysis.

This conclusion is reinforced by recent results with another quite different group of proteins which use an active site carboxylate as the general base. Various authors have tried, with no great success, to raise antibodies that will catalyze glycoside hydrolysis.[34] A favorite target reaction is the hydrolysis of 2-nitrophenyl tetrahydropyranyl acetal **32** (which, like the conversion of **29** to



30, is relatively rapid and readily followed by visible spectroscopy). The mechanism is well-understood and involves the spontaneous (and general acid catalyzed) rate-determining elimination of *p*-nitrophenolate.[17,35]

Tellier and co-workers raised monoclonal antibodies against an amidinium hapten **34**,[36] designed to mimic the half-chair conformation of the oxocarbocation **33** and to induce a complementary charged group, presumably carboxylate, in the antibody binding site. This group could act as the anion to stabilize the cation **33** or, in its conjugate acid form, as a proton donor to the exocyclic anomeric oxygen, or both. Several excellent hapten-binding monoclonal antibodies were identified and purified, but none accelerated the rate of hydrolysis of (aryloxy)tetrahydropyrans significantly.

These antibodies provide another group of proteins meeting the basic requirement—a hydrophobic binding site with a general base in close proximity—for catalysis of the Kemp elimination, and indeed, six of 20 hybridoma supernatants catalyzed the conversion of **29** to **30**.[37] Of two antibodies studied in detail, the more active was shown to act through a basic group, identified as carboxylate, with a pK_a of 5.7. Catalysis is moderately efficient, with k_{cat} about 20 times smaller than found for the very efficient antibody of Thorn et al. Particularly remarkable is the sensitivity of this antibody-catalyzed reaction to the medium: k_{cat}/K_m is reduced by 90% in water containing 10% acetonitrile and halved by just 1% of organic cosolvent (due almost entirely to an increase in K_m; hapten binding is unaffected). This cannot be a bulk medium effect: the pK_a of acetic acid and the rate

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of the acetate-catalyzed Kemp elimination show only small changes at low concentrations of added acetonitrile. Whatever the origin of the effect, this result reinforces the conclusion that the medium plays a dominant role in controlling reactivity in these systems.[38]

Conclusions

The approach described in this Account is based on the simple idea that partial covalent bonding makes an important contribution to the binding—and thus the stabilization—of the transition state in enzyme reactions. Such bonding interactions can be very strong in the intramolecular situation, depending on the way the groups concerned are brought together. Simple intramolecular model systems can provide the necessary firm platform for approximation but are limited by the geometrical restrictions intrinsic to bringing groups together side-by-side. The approach of reacting groups in enzyme active sites is in principle free from such restrictions. We regard the effective molarities achievable in intramolecular reactions as the best available guide to those available for similar enzyme-catalyzed reactions.

A nucleophilic component can make the major contribution (EM up to perhaps 10^{10} M) to the rate acceleration for a group transfer reaction if nucleophilic and substrate groups are brought together in such a way that bond formation is strongly thermodynamically favored. Proton transfer needs much more precise positioning of donor and acceptor to achieve this, but the EMs of 10^5–10^6 M achieved in our latest intramolecular systems show that this can make a substantial contribution to catalytic efficiency. Strong hydrogen bonding in the product is the key to this efficient catalysis: the H-bonds concerned in our systems are strong because they are intramolecular.

In the area of proton transfer catalysis there are obvious similarities between this approach and the ideas of Gerlt and Gassman[39] and Cleland and Kreevoy[40] on short, strong hydrogen bonds, but also important differences. We are concerned specifically with transition states; our results say nothing quantitative about the thermodynamic stabilization of high-energy intermediates. Experimentally, we can achieve EMs for intramolecular general acid–base catalyzed reactions up to about 10^6 M, corresponding to transition state stabilizations of 8–9 kcal mol^{-1}. We do not know whether this is a limit; most likely it is not (though we may be approaching the limit), so we continue to look for more efficient systems. In particular, with the availability of new efficient systems for proton transfer catalysis it becomes interesting to look for further efficiency—i.e., synergy—in the coupling of proton transfer processes with the making and breaking of bonds between heavy atoms.

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(38) It is important to stress at this point that the great majority of proteins do not catalyse the Kemp elimination!

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