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## How Do Enzymes Work?

### Joseph Kraut

The principle of transition-state stabilization asserts that the occurrence of enzymic catalysis is equivalent to saying that an enzyme binds the transition state much more strongly than it binds the ground-state reactants. An outline of the origin and gradual acceptance of this idea is presented, and elementary transition-state theory is reviewed. It is pointed out that a misconception about the theory has led to oversimplification of the accepted expression relating catalysis and binding, and an amended expression is given. Some implications of the transitionstate binding principle are then explored. The amended expression suggests that internal molecular dynamics may also play a role in enzymic catalysis. Although such effects probably do not make a major contribution, their magnitude is completely unknown. Two examples of recent advances due to application of the transition-state binding principle are reviewed, one pertaining to the zinc protease mechanism and the other to the generation of catalytic antibodies.

VEN A CASUAL SURVEY OF THE CURRENT BIOCHEMICAL literature reveals a rising interest in enzymes. This upsurge is due in part to the advent of site-directed mutagenesis methods, which have now been reduced to an almost standardized collection of laboratory procedures (1) whereby the amino acid sequence of a given enzyme molecule (or any other kind of protein molecule) may be altered by deliberately and precisely mutating the cloned gene encoding that molecule. As a tool for investigating structure-function relations, site-directed mutagenesis is made still more powerful by the use of x-ray crystallography to redetermine the three-dimensional structure of the mutated enzyme molecule and thereby define exactly what has been changed. The large amount

techniques, together with steady advances in methods for preparing hybrid enzymes, semi-synthetic enzymes, and even totally synthetic enzyme-mimetic compounds, and most recently for the production of catalytically active antibodies (3), has given birth to a burgeoning new discipline with the optimistic name of enzyme engineering. Reasons for this growing interest are not hard to find. Among

The increasingly widespread application of site-directed mutagenesis

them are the practical possibilities of putting engineered enzymes to work in industrial and medical applications. Also, since most drugs act by modifying or blocking the activity of some enzyme or another, a deeper understanding of suitably chosen target enzymes should lead to major advances in rational drug design. But most compelling is our sheer curiosity about these ingenious molecular machines, operating at the boundary where chemistry just becomes biology.

The phenomenal rate accelerations and specificities of enzymes have intrigued investigators ever since the 1830s when enzymic activity was first observed [see page 8 of (4)]. Over the years numerous hypotheses and ad hoc explanations have been advanced to account for enzymic catalysis, many of them tagged with imaginative names by their proponents. Page lists no fewer than 21 hypotheses (5). But only gradually has it come to be accepted that the most profitable way to think about the problem is the one first clearly stated by Pauling some 40 years ago (6). The basic idea, as simple as it is elegant, results from a straightforward combination of two fundamental principles of physical chemistry: absolute reactionrate theory and the thermodynamic cycle. In this view an enzyme is essentially a flexible molecular template, designed by evolution to be precisely complementary to the reactants in their activated transition-state geometry, as distinct from their ground-state geometry. Thus an enzyme strongly binds the transition state, greatly increas-

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ing its concentration and accelerating the reaction proportionately. This description of enzymic catalysis is now usually referred to as transition-state stabilization. In fact, as was convincingly documented by Schowen (7), almost all of the 21 hypotheses mentioned above simply amount to alternative statements of transition-state stabilization or suggested factors contributing to it. In any event, because enzymology is evidently poised to enter upon a period of renaissance, it seems appropriate to review the elements of transition-state theory as applied to the problem of enzymic catalysis.

#### A Brief Chronology

The history of the transition-state stabilization principle in enzymology provides an interesting example of how scientific thought usually develops—by slow evolution rather than by sudden revolution. Moreover, a brief chronological outline helps to illuminate the subject.

Modern theories of enzymic catalysis can probably be said to begin with Haldane's treatise titled "Enzymes" (4). He introduced the idea that an enzyme-substrate complex requires a certain additional energy of activation before reacting [chapter 10 in (4)] and suggested that Fischer's famous lock-and-key simile be amended to allow that "the key does not fit the lock quite perfectly but exercises a certain strain on it." The notion of substrate strain or distortion has been a part of enzymology ever since. Soon thereafter, Eyring (8) initiated the development of contemporary theories of the activated transition-state complex, now usually termed transitionstate theory or absolute reaction-rate theory. Eyring's approach was based on the simplifying idea of treating the transition-state complex as though it were in equilibrium with the reactants. In my judgment, transition-state theory has long since made the language of strain or distortion obsolete, although it is still commonly used in enzymology textbooks and is arguably equivalent in principle (9).

Eyring's theory laid the groundwork for the later suggestion by Pauling (6), already mentioned above, that the catalytic powers of enzymes result from their highly specific binding of the transition state. It is unclear why it took so long for this idea to gain widespread acceptance. Perhaps one reason was the manner in which it was first presented, more or less buried in two articles aimed primarily at communicating Pauling's enthusiasm for chemistry to a general scientific audience. In any event, the principle of transition-state stabilization resurfaced in various forms over the next 20 years, but failed to become part of the mainstream of enzymological thought. In 1955 Ogston (10) used it in a discussion of enzyme activation and inhibition. In 1959 Bernhard and Orgel (11) theorized that specific inhibition of serine proteases by certain phosphoric acid esters is due to the resemblance of the enzymeinhibitor complex to the transition state, and suggested that the phenomenon might be general.

An expression relating reaction-rate acceleration by any catalyst, without particular reference to enzymes, and the relative strength of binding of transition state versus ground state (in fact, Eq. 5 below) was first given by Kurz in 1963 (12). Kurz combined a thermodynamic cycle argument with Eyring's equation, leading immediately to a quantitative formulation of Pauling's assertion, although apparently Kurz was unaware of the latter. In 1966 Jencks (13) first suggested the existence of transition-state–analog inhibitors and cited several possible examples from the literature. Still, although transition-state stabilization was certainly not ignored, it continued to play only a minor role in enzymological thinking and, as Schowen (7) has pointed out, it was usually treated as just one more factor contributing to enzymic catalysis.

Beginning in 1969 and through the early 1970s transition-state

theory began to have more impact on enzymology. In a series of especially lucid articles, Wolfenden (14, 15) and Lienhard (16, 17), writing independently, cogently emphasized its broad generality and power. They argued that compounds structurally resembling the transition state, transition-state analogs, should bind many orders of magnitude more strongly than substrates, collected numerous examples, and proposed that such transition-state analogs could furnish important clues to the catalytic mechanisms of individual enzymes.

In the meantime, with the first three-dimensional structure of an enzyme determined by x-ray crystallography, that of hen egg-white lysozyme (18), the complementarity of a catalytic site to the transition-state geometry actually became visible. Using the new xray structure, together with difference-Fourier maps showing the binding of several oligosaccharide inhibitors and information about cleavage patterns in oligosaccharide substrates, Phillips and his colleagues deduced how substrates interact with the lysozyme molecule. Model-building studies led to the conclusion that a sugar residue occupying subsite D, where hydrolysis occurs, would be strongly bound only when in the half-chair (or sofa) conformation and not in the normal chair conformation. The half-chair, it was immediately realized, is precisely the conformation expected for a transition state resembling a glycosyl oxocarbonium ion at ring D, although the description was still framed in the language of strain and distortion.

The period immediately following saw x-ray structures of new enzymes being reported in rapidly increasing numbers, but they were rarely interpreted in terms of transition-state binding. For example, the serine proteases chymotrypsin, trypsin, elastase, and subtilisin were among the first few protein structures determined, and many years of prior enzymology had uncovered a bewildering assortment of inhibitors. But it was not until 1977 (19) that a common feature was noticed among a half-dozen especially potent covalent inhibitors: although otherwise chemically unrelated, they resemble the expected tetrahedral transition state for substrate hydrolysis and they bind in the same complementary oxyanion binding pocket. As an indication of how difficult it has been to sort out the details of enzyme function, it may be noted that although the serine proteases are probably the most intensively investigated class of enzymes, debate still continues about the role of various structural features in catalysis (20).

### **Transition-State Theory**

As presented in most enzymologically oriented reviews and textbooks, derivation of the basic equation of elementary transitionstate theory (Eq. 2 below) is deceptively simple. In fact, such derivations are usually incorrect, or at best misleading (21). The same is true even in some older elementary physical chemistry texts, which presumably explains why certain misconceptions have been so persistent. Fortunately, these errors probably do not much matter for the principle of transition-state binding that flows from the basic equation, since exact quantitative computation is never required. But a corrected expression of the principle does introduce some interesting further possibilities into the discussion of how enzymes function (see below).

An important point is that transition-state theory, unlike thermodynamics, is not exact or rigorous, but is instead based on certain assumptions and approximations (22). Nevertheless it works, and works very well. During its 50-year history the theory has undergone extensive refinement and is now widely accepted as conceptually accurate. Moreover, it has been tested both against experiment and against more rigorous computational results (22–24). Thus the theory predicts, with excellent accuracy, gas-phase reaction rates for Fig. 1. Thermodynamic cycle relating substrate binding and transitionstate binding: E, enzyme; S, substrate; P, product. Superscript double daggers ( $\ddagger$ ) denote transition states. The upper pathway represents the non-catalyzed reaction and the lower pathway represents the enzyme-catalyzed reaction.



certain bimolecular atom-diatom and diatom-diatom reactions when variational and tunneling corrections are included. Chemically simple test cases like these are important because it is possible to calculate accurate potential energy surfaces for them, which is not true for more complex types of reactions (25). Transition-state theory has also proved immensely fruitful in furnishing the basis for semiempirical correlations of rates and equilibria in several areas of chemistry.

The theory rests on two assumptions, a dynamical bottleneck assumption and an equilibrium assumption (22). The first asserts that the reaction rate is controlled by decomposition of an activated transition-state complex, and the second asserts that the system can be treated as though the transition-state complex is in equilibrium with the reactants. The resulting fundamental equation, essentially the working hypothesis, is

$$k = \kappa \nu K^{\ddagger} \tag{1}$$

where k is an experimentally observable reaction-rate constant,  $\kappa$  is the transmission coefficient,  $\nu$  is the frequency of the "normalmode" oscillation of the transition-state complex along the reaction coordinate (more rigorously, the average frequency of barrier crossing), and  $K^{\ddagger}$  is the equilibrium constant for formation of the transition-state complex from reactants (26, 27).

The meaning of the transmission coefficient  $\kappa$  as used here requires some explanation, since different treatments adopt different conventions. For present purposes one can lump all "correction factors" together under  $\kappa$ , including tunneling, the barrier recrossing correction, and solvent frictional effects (22). Their precise definitions are not important here, but it should be noted that although  $\kappa$  can in general differ dramatically from unity (in some cases by orders of magnitude), it is thought to fall in the range from 0.1 to 1 for reactions in solution at ordinary temperatures. Much current investigation is concerned with accurately assessing the numerical values of these factors (24).

In the next step of the elementary textbook derivation, the equilibrium constant  $K^*$  is written in terms of partition functions and a factor within the resulting expression, corresponding to the unique reaction-coordinate normal mode (with frequency  $\nu$ ), is extracted and approximated by  $k_B T/h\nu$ . Here  $k_B$  is the Boltzmann constant, T is the absolute temperature, and h is Planck's constant. This approximation holds if  $k_B T/h\nu >> 1$ , which applies in this case because  $\nu$  corresponds essentially to vibration of a loose, partially formed bond that is the defining feature in the nature of any transition-state complex. The  $\nu$ 's now appearing in both numerator and denominator of the recast Eq. 1 cancel, giving one form of the Eyring equation

$$k = \kappa \frac{k_{\rm B}T}{h} K^{\dagger'} \tag{2}$$

Notice that  $K^{\dagger'}$  is a quasi-equilibrium constant; it includes all possible modes in which the transition-state complex may contain energy except for the one just factored out.

Two points should be particularly emphasized about Eq. 2. One is that  $k_{\rm B}T/h$  does *not* correspond, as is often erroneously stated, to some universal frequency at which all transition-state complexes

decompose into products. The second is that  $K^{\ddagger'}$  is *not* the true thermodynamic equilibrium constant  $K^{\ddagger}$ . The former may actually be quite different from  $K^{\ddagger}$ , since  $k_{\rm B}T/h\nu$  has been factored out. That factor may not only be far from unity, but, more importantly, it is variable and depends on  $\nu$ , and there is no reason why  $\nu$  should be the same from one transition-state complex to another (28). I do not use Eq. 2 in the following discussion, but rely instead on Eq. 1, for which the definition of terms is less confusing.

#### The Theory Applied to Catalysis

As first explicitly shown by Kurz (12) and later in more detail by Wolfenden (14, 15, 29) and Lienhard (16, 17), elementary transition-state theory can be applied to enzymic catalysis by using the conceptual device of the thermodynamic cycle. In so doing one is just restating, in quantitative symbolic terms, Pauling's verbal description of transition-state binding. The appropriate thermodynamic cycle is depicted in Fig. 1.

Comparing the first-order rate constants for an elementary singlesubstrate enzyme-catalyzed reaction,  $k_e$ , and for the same reaction in the absence of enzyme,  $k_n$ , and using Eq. 1 above

$$\frac{k_e}{k_n} = \frac{\kappa_e \nu_e K_e^{\ddagger}}{\kappa_n \nu_n K_n^{\ddagger}}$$
(3)

The subscripts e and n refer to the enzymic and non-enzymic reactions, respectively; note that for this simplest possible case,  $k_e$  is the same as the conventional enzyme-kinetic parameter  $k_{cat}$ . Then, by the thermodynamic cycle argument, one can equate the ratio of transition-state formation constants in Eq. 3 to the ratio of dissociation constants for the substrate,  $K_S$ , and for the transition state,  $K_T$ , so that

$$\frac{k_{\rm e}}{k_{\rm n}} = \frac{\kappa_{\rm e}\nu_{\rm e}K_{\rm S}}{\kappa_{\rm n}\nu_{\rm n}K_{\rm T}} \tag{4}$$

Magnitudes of  $k_e/k_n$  are exceedingly large for typical enzymes; rate enhancements of  $10^{10}$  to  $10^{14}$  are not uncommon (30), and although difficult to measure, some may be much greater still (31, 32). Focusing on the right-hand side of Eq. 4, it is unlikely that the factor  $\kappa_e \nu_e/\kappa_n \nu_n$  differs from unity by many orders of magnitude, although there are no data whatsoever on this point. This question is further considered below. In the meantime, to the degree of approximation implied, one may provisionally write

$$\frac{k_{\rm e}}{k_{\rm n}} \approx \frac{K_{\rm S}}{K_{\rm T}} \tag{5}$$

This is the central result of the present section. It says that the transition state must bind enormously more strongly to the enzyme E than does the substrate S in its ground state—that is, than the substrate binds in the Michaelis complex ES—by a factor roughly equal to the enzymic rate acceleration.

#### Some Implications and Questions

The implications of Eq. 5 and its variants have been extensively analyzed, annotated, and interpreted (14–17, 19, 29, 30, 32–35). It is surprising how much substance can be extracted from such an elementary relation, and how much it simplifies the discussion of enzyme function. The following paragraphs attempt to summarize some of the major points that can be made.

1) The principle of transition-state binding, embodied in Eqs. 4

Fig. 2. Schematic representation of the postulated tetrahedral transition state for carboxypeptishowing interacdase, between enzyme tions and substrate in the S<sub>1</sub> and S<sub>1</sub> subsites. Dashed lines indicate hydrogen bonds and dotted lines indicate coordination to the zinc ion. [Reprinted from (55), with permission © American Chemical Society]



and 5, is *not*, as often erroneously implied, an independent theory, hypothesis, or premise about the nature of catalysis. Given the validity of transition-state theory and, of course, thermodynamics, it follows immediately from the observation that catalysis occurs (34). The value of Eqs. 4 and 5 is that they provide an agreeably parsimonious conceptual framework for thinking about enzyme action.

2) The line of argument leading to Eq. 5 can be readily extended to the more realistic cases of multisubstrate reactions (17) and covalent intermediates (17, 33), but in the latter instance experimental evaluation of certain equilibrium constants occurring in the resulting equations becomes problematical. The essential content of Eq. 5, however, remains unaltered.

3) Aided by the transition-site binding principle, it is easy to see through an apparent paradox that has evidently puzzled investigators in the past (36, 37). Many enzymes exhibit greatly increased activity toward extended substrates as compared with smaller substrates, for example, substrates with larger amino acid side chains, or with additional amino acid residues or sugar residues on either side of and at some distance from the peptide bond or glycosidic bond to be hydrolyzed. The puzzle was this: increased activity is usually manifested more by an increase in  $k_{cat}$  than by a decrease in  $K_m$ , that is, by an increase in maximum rate rather than by an increase in substrate binding (38, 39). Consideration of the common occurrence of this phenomenon led to introduction of the "induced fit" concept (36). One can now see that this is just the expected result when the template is designed to bind the extended transition-state geometry but not the ground-state geometry; distal portions may contribute substantially to overall binding of the transition state but not of the ground state. Thus it is not necessary to postulate conformational changes in the enzyme-template to explain the extended substrate effect. Nevertheless, conformational changes upon binding and catalysis often do occur as indicated by many other lines of evidence, and some authors also refer to these as "induced fit" [for example, see (40)]. Possible reasons for the existence of such phenomena are suggested below.

4) All of the above points immediately suggest that any molecule bearing a resemblance to the substrate in its transition state should bind much more strongly than the substrate itself. Hundreds of such transition-state analogs have now been reported (32, 34). Some are naturally occurring antibiotics; many were deliberately designed as a way to investigate the mechanism of a particular enzyme; and some were the result of efforts to synthesize potent inhibitors for use as drugs. For multisubstrate enzymes, even the simple ploy of uniting two substrate-like moieties in a single molecule can yield inhibitors that are bound much more strongly than either substrate alone, as must of course follow from entropic considerations (41). Such multisubstrate-analog inhibitors may be considered transition-state analogs of the most elementary kind.

5) Transition-state analogs never bind as strongly as might be estimated from enzymic rate accelerations, but then no stable molecule is likely to resemble a transition-state complex very closely.

6) How good can an enzyme be? Rearranging Eq. 4, and identifying  $k_e$  with  $k_{cat}$ , the resulting expression is

$$\frac{k_{\text{cat}}}{K_{\text{S}}} = k_{\text{n}} \left( \frac{\kappa_{\text{e}} \nu_{\text{e}}}{\kappa_{\text{n}} \nu_{\text{n}}} \right) \left( \frac{1}{K_{\text{T}}} \right)$$
(6)

The quantity on the left is just the usual second-order rate constant for reaction of free enzyme and substrate to give free enzyme and product, and thus cannot be larger than the diffusion-controlled limit, about 10<sup>9</sup>  $M^{-1}$  s<sup>-1</sup> (42). Thus a "perfectly evolved" enzyme will have reduced  $K_{\rm T}$ , that is, strengthened transition-state binding, until this limit has been reached for the reaction in the thermodynamically favored direction. (Continue to set aside the factor  $\kappa_e v_e$ for the moment.) Albery and Knowles (43) have given a detailed and perceptive analysis of how enzyme efficiency would be optimized by evolution.

7) Why is there a Michaelis-Menten complex? The existence of an ES complex is postulated in order to account for the phenomenon of substrate saturation in steady-state kinetics experiments. However, one can derive Eq. 6 without reference to the ES complex at all (29), in which case  $k_{cat}/K_S$  could have been written from the outset as  $k_e'$ a second-order rate constant (42). Thus the introduction of an ES complex and K<sub>S</sub> were prompted by an additional item of kinetic information, external to basic transition-state theory. Just why this saturation phenomenon is an almost universal feature of enzyme kinetics is not immediately obvious. Fersht [see pages 324 to 331 of (39)] has convincingly argued that, in general, evolution favors maximization of the turnover rate per enzyme molecule, and thus ought to result in Michaelis constants that are much larger than intracellular substrate concentrations. In fact, Km values are broadly distributed but typically in the range of 1 to 10 times [S]. Probably the ES complex represents binding of a Boltzmann distribution of substrate molecules in states preceding the transition-state bottleneck. That these bound states are well populated may be due simply to the inevitable fact that the transition state of the usual substrate molecule does not look very different from its ground state. Thus a template designed to bind the transition state strongly must also bind the ground state to some extent. Or perhaps ground-state binding is a manifestation of constraints on the physically attainable velocities of molecular motions, that is, on diffusion and vibrations and hence elementary reaction rates.

8) There is some degree of arbitrariness in any distinction between catalytic groups and binding groups in an enzyme molecule (15). This point is nicely illustrated by the recent report of Carter and Wells (44) on mutagenesis experiments with a bacterial serine protease, subtilisin. Mutants were constructed in which residues of the catalytic triad Ser<sup>221</sup>, His<sup>64</sup>, and Asp<sup>32</sup> were replaced by Ala in all seven possible combinations. Even for the triple mutant, with none of the catalytic side chains remaining, the residual catalytic activity still produced a reaction rate of more than 1000 times the uncatalyzed rate. In other words, the rest of the enzyme molecule still binds the transition state more strongly than the ground state. The authors point out that this residual activity is in the range achieved by catalytic antibodies (see below).

9) Optimum binding of the transition state is a cooperative phenomenon. That is, the numerous individual binding interactions between transition state and enzyme are synergistic, so that interfering with one adversely affects the others. Or put more succinctly, the fit is very precise. This cooperativity can be seen as a strategy for amplifying enzyme specificity; a small perturbation in the chemical structure of a substrate can then cause a large decrease in binding of

its transition state. The mutagenesis experiments just described on the catalytic triad of subtilisin (44) also reflect this property, although here it is the enzyme that is perturbed. For example, replacing any one of the three residues with alanine causes a large decrease in  $k_{cat}/K_m$ , by as much as  $10^{-6}$ , but the product of these individual replacement effects would be  $5 \times 10^{-17}$ , far more drastic than the  $7 \times 10^{-7}$  actually observed on replacing all three simultaneously. It is as though the enzymic activity created by juxtaposing these three residues in more nearly an all-or-none phenomenon than an additive one.

10) Like any other molecule that contains more or less freely rotating bonds, enzyme molecules are conformationally mobile, and a variety of observations point to the common occurrence of conformational isomerizations in the course of enzyme catalysis (45). What, if anything, can transition-state stabilization tell us about the possible role of conformational changes in enzyme function? I exclude from consideration such complex phenomena as allosteric effects and continue to focus only on simple catalysis by a hypothetical single-subunit enzyme.

Initially it might appear that a maximally efficient enzyme molecule should be a rigid template completely enclosing the transition state. Such a conclusion would seem to follow, inasmuch as any free energy expended to convert the enzyme molecule to a less stable conformation required for binding would decrease the overall free energy of binding; also, a wrap-around template would be able to provide more numerous favorable contacts. However, rapid diffusion in and out by the substrate and product requires a more open binding site (29). Evidently, then, evolution may well have arrived at an optimal compromise that uses relatively minor, low-energy conformational changes in the course of catalysis. As yet, no enzyme reaction is sufficiently well characterized to enable us to depict a full sequence of such events with confidence.

A closely similar line of reasoning explains why, in an ES complex, the substrate might be bound in a conformation that is unfavorable with respect to its predominant solution conformation—to allow more favorable enzyme-substrate interactions in the transition state. Examples are the nicotinamide nucleotides, which are folded in solution but extended when bound to oxidoreductases (46).

Another obvious reason to expect conformational flexibility to play a role in enzyme catalysis is that most enzyme-catalyzed reactions have more than one transition state. Since they will differ in geometry somewhat, the template must adjust to accommodate each one. An example may occur in the serine protease reaction, in which during the acylation step  $\text{His}^{57}$  accepts a proton from Ser<sup>195</sup> and donates a proton to the leaving group of the substrate (19). However, in the tetrahedral transition state, O $\gamma$  of Ser<sup>195</sup> is at least 2.4 Å from the leaving group, and the side chain of  $\text{His}^{57}$  is not positioned to swing by the amount required to make good hydrogen bonds with both. In all likelihood a small internal adjustment of the whole enzyme molecule accommodates the required shift, although the existence of such a conformational isomerization has not yet been established to my knowledge.

11) Up to this point it has been convenient to temporarily ignore the factors  $\kappa_e$  and  $\nu_e$  in Eq. 4, but evidently these may also be at the disposal of enzyme evolution. For example, coupling of appropriate enzyme-molecular vibrations to the reaction-coordinate mode of the bound transition state might cause  $\nu_e$  to become greater than  $\nu_n$  and thereby yield a further catalytic advantage. Little can be said at present beyond merely pointing out this possibility.

There is more scope for conjecture concerning  $\kappa_e$ , which includes corrections for barrier-recrossing and tunneling effects. In this connection, Bergsma *et al.* (47) have shown by molecular dynamics simulation of a model  $S_N2$  reaction in water that barrier-recrossing effects result in  $\kappa$  values of about 0.5. The recrossing phenomenon

in aqueous solution is due to the solvent structure being effectively frozen on the time scale of the barrier-crossing event, thus influencing the trajectory of the reacting atoms by opposing their shifting charge distribution. Although the magnitude of such effects is certainly not large for ordinary reactions in solution, the authors suggest that significantly smaller  $\kappa$  values are likely to result when barriers are lower, and that is precisely the case for enzyme-catalyzed reactions. Thus there may be evolutionary pressure for the enzyme to overcome this adverse effect by dynamically facilitating barrier crossing. Here is another way in which enzyme molecular dynamics may enter the catalysis picture.

Could tunneling play a role in hydrogen-atom transfers within enzyme-catalyzed reactions? The possibility has long aroused considerable curiosity, and evidence is accumulating that it may indeed (48). Klinman (49) and her co-workers have recently observed isotope effects, which have temperature dependencies that strongly suggest tunneling in the yeast alcohol dehydrogenase and plasma amine oxidase reactions.

#### **Examples of Recent Progress**

A major objective of structural enzymology is to describe the transition states for the various categories of enzymic reactions and to characterize the stabilizing molecular interactions between transition state and enzyme. This is no easy task, because transition states are by definition the least stable, most transitory species along the reaction coordinate. Moreover, it is not always obvious just what the transition state for a particular reaction should look like. Progress is nevertheless being made on many fronts. In some ways the mechanisms of enzyme-catalyzed reactions are easier to investigate than mechanisms of reactions in solutions, because the geometrical arrangement of the participants can be visualized with the aid of xray crystallography. In these remaining few paragraphs I conclude by pointing out only two illustrative examples among the many that might be cited. Both relate to the tetrahedral transition state characteristic of acyl transfer reactions, one of the simplest and most thoroughly examined reactions in enzymology.

A proposed transition state and mechanism for zinc proteases. The zinc proteases carboxypeptidase  $A_{\alpha}$  and thermolysin were among the first enzyme structures to be determined crystallographically (50). Carboxypeptidase is a digestive enzyme of the vertebrate pancreas, whereas thermolysin is produced by the thermophilic bacterium Bacillus thermoproteolyticus. The two molecules bear no resemblance to one another in either amino acid sequence or three-dimensional structure and have distinct substrate specificities. Thermolysin is an endopeptidase with specificity determined primarily by a large hydrophobic residue following the peptide bond to be hydrolyzed. Carboxypeptidase is an exopeptidase with specificity toward a large hydrophobic carboxyl-terminal residue. Despite such differences, however, both molecules have a similarly coordinated  $Zn^{2+}$  ion at their reactive centers, with its first coordination sphere donated by two His side chains, a Glu side chain, and a water molecule. These two zinc proteases may therefore represent another example of molecular evolution converging to a common chemical mechanism, comparable to the well-known subtilisin-chymotrypsin example within the serine protease class.

Carboxypeptidase and thermolysin have been intensively studied for many years, and now there is a consensus emerging regarding a common mechanism based on a common transition-state geometry. This perhaps unsurprising development follows upon recent binding and structural investigations that used a variety of inhibitors, many of which were deliberately designed as transition-state analogs (51–56). Aldehyde and ketone analogs of substrates were used in the **Fig. 3.** Stereoscopic pair depicting ball-and-stick model of the substrate binding region in the complex between thermolysin (open bonds) and the transition-state analog Cbz-Phe<sup>P</sup>-Leu-Ala (solid bonds; Cbz, carboxyben-zyl). The model is based on a high-resolution crystal structure (*52*). [Figure courtesy of D. Tronrud and B. W. Matthews]



carboxypeptidase studies, and phosphonic acid analogs were used in the thermolysin studies. The aldehyde or ketone moiety can be hydrated, either in solution or when bound to the enzyme, to give a tetrahedral gem-diol, whereas the phosphonic acid group is already tetrahedral. Thus both are capable of mimicking the transition-state geometry expected for the attack of water on the carbonyl carbon of the peptide bond, and both bind very tightly to the respective enzymes.

However, tight binding in itself is insufficient evidence of analogy with the catalytic transition state, as many enzyme inhibitors are known that bind tightly but cannot be, on the basis of their chemistry, related in any way to the transition state. This objection was convincingly countered for the phosphonamidate and phosphonate tripeptide analogs (53). Specificity constants  $k_{cat}/K_m$  for thermolysin-catalyzed hydrolysis of a series of amide and ester substrates were compared with inhibition constants  $K_i$  for the corresponding phosphonyl derivatives, and good linear correlations were observed. Reference to Eq. 6 shows that this is precisely the expected result if the inhibitors are indeed analogs of the transition state. One has only to make the reasonable assumptions that the factors preceding  $1/K_T$  remain constant throughout a series of closely related substrates, and that the same proportionality between  $K_T$  and  $K_i$  is maintained throughout the series.

High-resolution x-ray structures of the enzyme-inhibitor complexes revealed, for both carboxypeptidase and thermolysin, a fivecoordinated zinc ion with two of the zinc ligands donated by the tetrahedral group. That is, the tetrahedral moiety straddles the zinc ion as a bidentate ligand, with one of its oxygen atoms replacing the zinc-coordinated water molecule. Additionally, the carboxylate group of a nearby Glu side chain (Glu<sup>270</sup> in carboxypeptidase or Glu<sup>143</sup> in thermolysin) hydrogen bonds to that intruding oxygen atom. The enzyme-inhibitor structures in the neighborhood of the zinc ion are shown in Figs. 2 and 3; carboxypeptidase is represented schematically in Fig. 2, and thermolysin is shown as a ball-and-stick model in Fig. 3.

Mechanistic proposals based on these structures and other findings depict the following sequence of events. The carbonyl oxygen of the peptide bond being hydrolyzed coordinates to the zinc ion, forcing the zinc-liganded water toward the carboxylate group of the nearby Glu residue. The water molecule, activated by both the carboxylate and the zinc ion, then attacks the peptide carbonyl carbon to form a tetrahedral species with the geometry described above while transferring a proton to the carboxylate. The latter acts as a proton shuttle, donating the proton it received from the water molecule to the leaving-group nitrogen, leading to bond cleavage. Other enzyme-substrate interactions are different in the two enzymes, but similar in function. The role of Tyr<sup>248</sup>, formerly thought to be the proton donor in carboxypeptidase, is now believed to be nonessential substrate binding. This view is consistent with sitedirected mutagenesis studies showing that changing  $Tyr^{248}$  to Phe has only a relatively minor effect on activity (57).

It remains to be seen if this mechanistic scenario is supported by further evidence. The script may well be still more complicated, as spectroscopic and chemical data suggest that hydrolysis of at least certain substrates, in cryosolvents and at temperatures near  $-70^{\circ}$ C, proceeds by way of a mixed anhydride with Glu<sup>270</sup> (58). It may be, for example, that transient formation of a covalent bond to Glu<sup>270</sup> precedes attack by the activated water molecule.

Catalytic antibodies. A recent development that has attracted much attention was the demonstration, by two groups working independently (59-65), that antibodies can have catalytic activity. More importantly, it was shown to be quite feasible, by cleverly designing the eliciting antigen, to generate catalytic antibodies with a more or less predictable specificity. What makes this feat possible is the remarkable ability of the immune system to recognize almost any conceivable configuration of atoms on the surface of an invading foreign antigen, and on demand to produce immunoglobulins (antibodies) with the ability to bind that configuration strongly and specifically. If the immune system can be induced to make an antibody that binds some chemical grouping resembling the transition state for a given reaction, then that antibody should catalyze the reaction. The trick is to use a protein antigen to which the appropriate transition-state analog is coupled as a hapten. Among the monoclonal antibodies raised in this way there can then be found a few that strongly bind the haptenic group and also have weak (usually) but clearly enzyme-like catalytic properties (66).

One of the first successes in this area was the demonstration by Schultz and co-workers (61) of enzyme-like activity on the part of a naturally occurring immunoglobulin A (IgA), MOPC167. This IgA belongs to a structurally well-characterized class having a high affinity for phosphorylcholine esters, and MOPC167 in particular strongly binds *p*-nitrophenylphosphorylcholine (67). Recognizing the latter as analogous to the transition state for hydrolysis of the corresponding carbonic acid diester, the Schultz group looked for hydrolytic catalysis on the part of MOPC167 toward *p*-nitrophenyl *N*-trimethylammonioethyl carbonate chloride as a substrate. They observed clear enzyme-like specific activity, with  $k_{cat} = 0.4 \text{ min}^{-1}$ and  $K_m = 0.2 \text{ m}M$ . Indeed, the antibody accelerates OH<sup>-</sup>-mediated hydrolysis of this carbonic acid diester by a factor of 770.

Not only can enzyme-like activity be demonstrated on the part of naturally occurring antibodies, but monoclonal technology can be applied to elicit antibodies with deliberately tailored catalytic specificities. To this end, Tramontano, Lerner, and co-workers used phosphonate monoaryl esters as haptens to generate immunoglobulin G (IgG) antibodies with hydrolytic activity toward cognate carboxylic esters. Initially, with a carboxylic ester of 7-hydroxycoumarin as the substrate, only single turnover kinetics was observed, and it was concluded that some group within the binding site of the antibody is catalytically acylated (59). Shortly thereafter, other less labile phenyl esters were found to be hydrolyzed in truly enzymic fashion (60). One such antibody, 50D8, accelerated the hydrolysis of a particular phenyl ester by more than 10<sup>6</sup>, with kinetic parameters  $k_{cat} = 20 \text{ s}^{-1}$  and  $K_m = 1.5 \text{ mM}$ , not far from those of known esterolytic enzymes (65). A different tetrahedral phosphonate hapten, the *p*-nitrophenyl ester of an alkylphosphonate, was used by Schultz and co-workers to generate catalytic monoclonal IgG's (63). Hydrolysis of the homologous carbonate ester was accelerated 16,000 fold by one such antibody. In all instances described, catalytically active antibodies had the expected kinetic properties of enzymes: substrate specificity, saturation kinetics, and competitive inhibition by transition-state analogs. A curious feature common to esterolytic antibodies obtained by both groups of investigators is the apparent involvement of a Tyr residue. If preliminary findings are confirmed, these protein catalysts belong to a new class as far as their chemical mechanism is concerned. All well-known peptidase-esterases are members of one or another of four established classes with either Ser, Cys, zinc, or a pair of Asp residues as their essential functionality, but none involve Tyr.

A similar approach was applied to generate an antibody that catalyzes a stereospecific intramolecular cyclization reaction (62). Enzyme-like specificity was thus demonstrated quite dramatically. As for the esterolytic reactions just discussed above, the design strategy here is also based on analogy with the tetrahedral transition state for an acyl transfer reaction. In these experiments the transition-state mimic was a diastereoisomeric cyclic phosphonate ester, whereas the substrate was a racemic mixture of the corresponding open-chain phenyl ester bearing a chiral δ-hydroxyl group. Accordingly, an antibody was found that preferentially catalyzed the cyclization of just one enantiomer of the open-chain ester to the  $\delta$ lactone. A rate acceleration of about 170-fold was observed, and initial analyses indicated that enantiomeric differentiation probably is close to absolute.

#### Postscript

It will not be enough to catalog what all of the biological macromolecules do. We need to know how they do it. One question is about biology, the other chemistry. Enzymes have been perfecting their skills for more than 3 billion years and they surely have a great deal of sophisticated chemistry to teach us. Happily, there is some hope that the number of distinctly different lessons is merely finite, for we already see examples of enzyme molecules, unrelated by evolution, but with almost identically arranged working parts. In each such case, nature has twice faced the same biochemical problem and twice found the same optimum solution. Can we understand what the enzymes are trying to tell us?

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- 67. For purposes of comparison note that the phosphorus-containing moiety here is a phosphoric acid diester, in which a central phosphorus atom is tetrahedrally bonded to four oxygen atoms, whereas the phosphonic acid esters and amides featured in the preceding and following discussion are organophosphorus compounds in which a phosphorus atom is tetrahedrally bonded to carbon and three heteroatoms.

### **Research Articles**

# ІкВ: A Specific Inhibitor of the NF-кВ **Transcription** Factor

PATRICK A. BAEUERLE AND DAVID BALTIMORE

In cells that do not express immunoglobulin kappa light chain genes, the kappa enhancer binding protein NF-KB is found in cytosolic fractions and exhibits DNA binding activity only in the presence of a dissociating agent such as sodium deoxycholate. The dependence on deoxycholate is shown to result from association of NF-kB with a 60- to 70-kilodalton inhibitory protein (IkB). The fractionated inhibitor can inactivate NF-kB from various sourcesincluding the nuclei of phorbol ester-treated cells-in a specific, saturable, and reversible manner. The cytoplas-

N EUKARYOTIC CELLS, THE RATE OF TRANSCRIPTION OF MANY genes is altered in response to extracellular stimuli. Changes in expression of genes transcribed by RNA polymerase II in response to such agents as steroid hormones, growth factors, interferon, tumor promoters, heavy metal ions and heat shock are mediated through distinct cis-acting DNA-sequence elements (1). Most important are those called enhancers (2), which display great positional flexibility with respect to the gene they control (3), and promoters, which are confined to the 5' noncoding region of the gene (4). Both cis-acting elements contain multiple binding sites for sequence specific DNA-binding proteins (1, 5). The demonstration

mic localization of the complex of NF-kB and IkB was supported by enucleation experiments. An active phorbol ester must therefore, presumably by activation of protein kinase C, cause dissociation of a cytoplasmic complex of NF-kB and IkB by modifying IkB. This releases active NF- $\kappa$ B which can translocate into the nucleus to activate target enhancers. The data show the existence of a phorbol ester-responsive regulatory protein that acts by controlling the DNA binding activity and subcellular localization of a transcription factor.

of protein-DNA interaction in vivo (6), competition experiments in vitro (7) and in vivo (8), and the definition of protein binding sites by mutational alteration of regulatory DNA sequences (9, 10) suggested that occupation of cis-acting elements by trans-acting factors is crucial for the transcriptional activity of constitutive and inducible genes. There is increasing evidence that inducible transcription of genes is mediated through induction of the activity of

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