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REVIEW

THE PRINCIPLES OF ENZYMATIC CATALYSIS

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Some 100 yr after the introduction of the word "enzym" (Kühne, 1976) and some 50 yr after the demonstration that urease is a protein (Sumner, 1926) there are still many unsolved problems in understanding the effectiveness of enzymes as catalysts. However, in the last decade or so the belief has been growing that the efficiency of enzymic catalysis may be rationalized by known chemical and physical principles.

Individual enzymes *in vivo* have different constraints and requirements and it is justified to enquire whether there can be a unified theory of enzymic catalysis. Generally, intracellular enzymes are required to maintain a constant concentration of the various substrate metabolites and this is achieved by having a wide variation in the reaction flux of material through the various metabolic pathways. Extracellular enzymes, however, are often faced with dramatic changes in the substrate level and yet are required to maintain a steady flow of material for adsorption and use by the cell (Albery & Knowles, 1976). A low value of the enzyme-substrate dissociation constant,

$$E + S \underset{K_m}{\rightleftharpoons} ES \xrightarrow{k_{cat}} \text{products} \qquad (1)$$

K_m, (equation 1) ensures that the extracellular enzyme acts mostly under saturation conditions and thus easily maintains a constant rate of production despite changes in the substrate concentration because the rate is independent of substrate concentration. Intracellular non-regulatory enzymes that maintain a constant level of substrate concentration may act by binding the substrate *weakly*, i.e. have a high K_m value, which ensures that the enzyme-catalysed reaction occurs below saturation at ambient substrate concentrations and that the rate is given by the second-order rate constant k_{cat}/K_m. (Fersht, 1974).

Despite the evolutionary pressure (Fersht, 1974; Albery & Knowles, 1976) for individual enzymes to maximise their efficiency depending upon the requirements of the organism it appears that the fundamental principles of enzymic catalysis are similar whether one is considering intracellular or extracellular enzymes. Some enzymes e.g. carbonic anhydrase (Kernohan, 1965) and triosephosphate isomerase (Knowles & Albery, 1977) are so efficient that the rate is limited by the diffusion-controlled encounter of the enzyme and substrate i.e. k_{cat}/K_m is *ca.* 10^8–10^9 M^{-1} s^{-1}. If a reaction is diffusion-controlled in one direction then, as long as the ambient substrate concentration is below K_m, the rate of the reverse direction is limited by the rate of loss of product from the enzyme. This is known to be the case for several enzyme-catalysed reactions e.g. the dehydrogenases (Cleland, 1975). It is possible for the rate to be faster than that of a diffusion-controlled encounter of substrate and enzyme if the enzyme is operating in a multienzyme aggregate (Ginsburg & Stadtman, 1970). When substrate binding or product release limit the rate of an enzyme-catalysed reaction the actual chemical catalytic steps must be very fast. Despite the wide variation in the behaviour and requirements of individual enzymes, several questions are pertinent to all enzymes. This brief review will address itself to the following questions:

1. How do enzymes increase the rate of chemical reactions?
2. Why do small non-specific substrates that bind to the active site of the enzyme react so slowly?
3. Why is the binding energy resulting from the non-covalent interaction of the enzyme and the substrate so large?
4. Why are enzymes of such high molecular weight?

Classically the substrate binding process has been treated separately from the catalytic steps involving chemical reaction. It is now clear that these two processes cannot be treated separately if one wishes to understand the energetics of enzymic catalysis (Jencks & Page, 1972).

The combination of an enzyme with its substrate to produce the Michaelis catalytic complex is an exergonic process. The overall negative free energy change accompanying complex formation arises from a balance of favourable and unfavourable interactions. The unfavourable interactions may lower the activation energy for the subsequent chemical steps and thus contribute to catalysis. Only a few enzyme catalysed reactions occur without the direct chemical involvement of functional groups on the enzyme. However, although chemical catalysis may often account for a large proportion of the rate enhancement brought about by the enzyme this is not always the case and furthermore the contribution of chemical catalysis is not always separable from the contribution attributable to the enzyme-substrate binding energy. Two aspects of the involvement of functional groups on the enzyme should be distinguished: (1) the rate enhancement brought about by "chemical catalysis" relative to the "uncatalysed" or "solvent-catalysed" reaction—this usually represents catalysis by water, the hydronium-ion or hydroxide-ion (2) the rate enhancement brought about by "chemical cata-

lysis" occurring within the enzyme-substrate complex and being of lower kinetic order than an analogous intermolecular reaction.

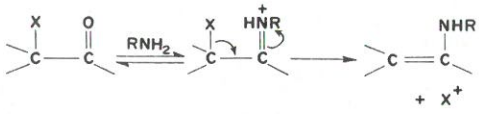
THE CONTRIBUTION OF CHEMICAL CATALYSIS

There appears to be nothing unusual in the pathways by which covalent bonds are formed and broken in enzyme-catalysed reactions; the mechanisms used to account for ordinary chemical reactions are also applicable to nature's reactions. Chemical catalysis can occur by the substrate being converted to a reactive intermediate or by stabilising, through bonding, an intermediate that is formed in the uncatalysed reaction or by a combination of these two processes.

(a) *Covalent catalysis*

Several enzymes have been shown to react chemically with their substrates by forming a covalent bond between a functional group on the enzyme and a group within the substrate to form a reactive intermediate which may be detected in several ways (Jencks, 1969; Fersht, 1977).

A classic example of covalent catalysis is the conversion of a carbonyl group to an iminium ion using an ϵ-amino group of a lysine residue of the enzyme.

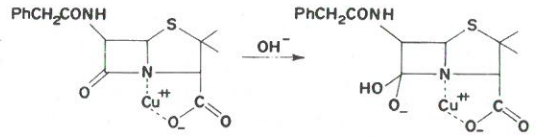


(2)

This facilitates the removal of a group X in the α-position, equation (2). Such processes are prevalent in enzyme catalysed reactions e.g. aldolases (X = H) and decarboxylases ($X = CO_2^-$). It has been shown that β-imino acids decarboxylate 10^5–10^7 times as fast as the corresponding β-keto acid and a large fraction of the rate enhancement brought about by acetoacetate decarboxylase is attributable to this chemical catalysis (Taguchi & Westheimer, 1973; Hupe *et al.*, 1973). The main driving force for catalysis appears to be simply the conversion of the carbonyl oxygen to a nitrogen atom which is more basic so that at neutral pH the nitrogen is protonated which greatly facilitates removal of X compared with the unprotonated carbonyl compound (by a factor of *ca.* 10^9 when X = H), (Williams & Bender, 1966).

(b) *Metal-ion catalysis*

The metal-ion of metallo enzymes may serve a variety of functions such as those of a catalyst, a binding agent for the substrate or an electron carrier (Mildvan, 1970). Generally the catalytic role is attributed to electrophilic catalysis by the metal-ion or to the metal-ion binding a ligand which acts as a nucleophile. When the metal-ion acts as an electrophile it coordinates to the substrate, which normally acts as a monodentate ligand, at a weakly basic site. When chemical reaction occurs this coordination site is changed to a more basic ligand and hence the metal-ion stabilizes this development of electron density and lowers the activation energy. The metal-ion binds to the transition-state more tightly than it does to the ground state. For example, copper(II)-ions increase the rate of the hydroxide-ion catalysed hydrolysis of benzylpenicillin(I) by stabilising the tetrahedral intermediate (II), the formation of the latter is accompanied by an increase in the basicity of the β-lactam



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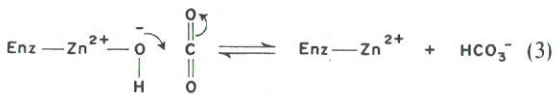
nitrogen of at least 12 pK_a (Gensmantel *et al.*, 1978). However, extrapolation from model systems to enzymes must be treated with caution. Metalloenzymes do not use high concentrations of metal-ions to bring about rate enhancements. For those cases studied the substrate usually acts as a monodentate ligand when direct coordination occurs between the metal-ion and the substrate e.g. the carbonyl oxygen of the amide linkage to be cleaved is coordinated to the zinc atom of carboxypeptidase (Lipscomb, 1974). Most of the driving force for binding must come from the interaction of the substrate with the protein.

Ligands attached to the metal-ion may act as effective nucleophiles (Buckingham & Englehardt, 1975). The acidity of a ligand with an ionisable proton is increased upon coordination to a metal-ion and consequently the deprotonated coordinated ligand is of intermediate reactivity between that of the protonated and of the deprotonated ligand. If the rate of reaction of the substrate is not very dependent upon the nucleophilicity of the attacking nucleophile then metal-bound nucleophiles may be effective catalysts.

For example carbonic anhydrase (Kannan *et al.*, 1977) is an enzyme which catalyses the hydration of carbon dioxide and has a molecular weight of *ca.* 30,000 and contains one zinc atom. A zinc-bound hydroxyl-ion is thought to be less solvated than hydrated hydroxide-ion (Jonsson *et al.*, 1978) and it is thought that the mechanism shown in equation (3)



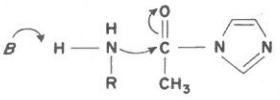
(3)

can account for a large fraction of the rate acceleration brought about by the enzyme (Woolley, 1977). In such cases it is, of course, of interest to know the function of the 30,000-odd molecular weight of protein.

(c) *General acid-base catalysis*

The formation of products from reactants often requires the transfer of a proton to or from the substrate which may occur from or to water or from any other acidic or to a basic species available. If this proton transfer occurs to or from a species other than water catalysis is observed. This pathway is, of course, less favourable than proton transfer to hydroxide-ion or protonation by the hydronium-ion but at neutral pH it can provide a considerable rate enhancement compared with catalysis by water (Jencks, 1972, 1976). For example, the aminolysis of acetylimidazole, requires the removal of a proton from the attacking amine, equation (4). When the catalysing

base is a second molecule of amine catalysis is *ca.* 10,000 times more effective than when water is the catalyst based on a molar scale (Page & Jencks, 1972).



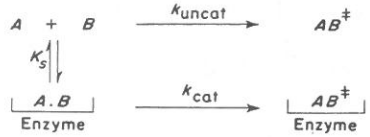
(4)

However, 1M amine base catalyst is only about 200 times more effective than the solvent water of 55.5 M. This is typical of the rate enhancement brought about by general acids and bases. It will be seen later that no great further rate enhancement is obtained when the general acid or base catalyst of an enzyme acts within the enzyme-substrate complex. Although general acid-base catalysis makes a contribution to the rate enhancement brought about by the enzyme it is generally a minor factor.

It is apparent that chemical catalysis alone cannot explain the rate enhancement brought about by enzymes. It appears that the intrinsic binding energy resulting from the non-covalent interaction of a substrate with an enzyme is responsible for a large fraction of the difference in the rates of enzyme and non-enzyme catalysed reactions (Jencks & Page, 1972; Jencks, 1975). The binding energy manifests itself in different ways such as compensating for unfavourable entropy or solvation changes, and geometric or electrostatic destabilisation.

THE CONTRIBUTION OF APPROXIMATION, ENTROPY AND BINDING ENERGY

It has long been thought that enzymes are effective by bringing the reactants together which is referred to as approximation or proximity. However, it is only recently that the magnitude of this effect has been understood (Page & Jencks, 1971). The importance of this effect may be illustrated simply by considering a reaction between two molecules A and B to give a transition state $AB‡$ and comparing this with the



(5)

same reaction occurring at the surface of an enzyme but without involving chemical catalysis by the enzyme (equation 5). Bringing two molecules together to form a transition state in a bimolecular reaction is generally accompanied by a large loss of entropy as the transition state is a more ordered system. The formation of the transition state from the independently moving and rotating reactants is accompanied by a large loss of translational and rotational entropy (Page & Jencks, 1971; Page, 1973). For a standard state of $1\,mol\,l^{-1}$ this entropy change amounts to about $-150\,J\,K^{-1}\,mol^{-1}$ which, at 25°C, makes bimolecular reactions unfavourable by a factor of about 10^8. Although the evidence for the magnitude of the entropy change is both theoretical and experimental (Page & Jencks, 1971; Page, 1973, 1977) it is important to emphasize that *observed* entropy changes in solution are dominated by solvent effects and therefore do not necessarily reflect this value.

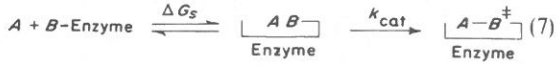
It the reactants A and B are bound to the enzyme tightly and in close proximity (equation 5) there will be little loss of entropy upon forming the transition state. Reaction within the enzyme-substrate complex therefore has an entropic advantage over the uncatalysed reaction and k_{cat} may be 10^8 times greater than k_{uncat} even though there is no chemical catalysis by the enzyme. However, initial binding of the reactants A and B to the enzyme is, of course, entropically unfavourable and any increase in the rate of reaction brought about by the enzyme is given by the enthalpy of binding the enzyme and the transition state, $AB^{‡}$, less the entropy of association of the transition state and the enzyme (Jencks & Page, 1972; Jencks, 1975). Any excess of the intrinsic binding energy between the enzyme and substrates over this entropy loss will appear as catalysis of the reaction.

Recently the idea of the binding energy being directly responsible for the rate enhancement brought about the enzyme has been substantiated by the observation that β-galactosidase catalyses the S_N1 hydrolysis of β-galactopyranosyl pyridium salts. The enzyme increases the rate of reaction by a factor of 10^{10} and yet there is *no chemical catalysis* by the enzyme (Jones *et al.*, 1977).

(a) *Covalent catalysis*

The advantage of covalent catalysis, where an electrophilic or nucleophilic group on the peptide chain of the enzyme forms a covalent bond with the substrate, is immediately apparent by considering the difference in entropy changes between the uncatalysed (equation 6) and enzyme catalysed reaction (equation 7). In equation (7), one of the reactants, B, is covalently bonded to the enzyme and a comparison of this reaction with that of equation (6) illustrates the

(6)



(7)

advantage of binding the substrate to the enzyme even if the chemical reactivity of B in the enzyme may be similar to that of B in intermolecular reaction, equation (6). It is easily shown (Jencks, 1975) that the amount of catalysis by the enzyme is given directly by the binding energy between the enzyme and substrate.

The assumption involved in this derivation is that the entropy of activation in the intermolecular reaction (equation 6) is approximately equal to the entropy change of binding the substrate A to the enzyme, equation (7) (Jencks, 1975). This will be true for good substrates that bind tightly. However, poor substrates which have a smaller enthalpy of binding will bind more loosely to the enzyme and the formation of the transition state will consequently be accompanied by a negative entropy change. Much or all of the more favourable intrinsic binding energy of good substrates may therefore appear as a rate acceleration, increased k_{cat}, equation (7), rather than as an increase in the binding constant. This illustrates one of the disadvantages of treating the binding pro-

cess, which is often described as some sort of "lock and key" model, as a separate process from the catalytic rate acceleration, which is often erroneously explained in solely chemical terms (Jencks & Page, 1972; Jencks, 1975).

(b) *General acid-base catalysis*

The rate enhancement that can be brought about by having a general acid or general base catalyst as part of the protein structure, equation (8), as opposed to the chemically equivalent intermolecular mechanism, equation (9) appears to be minimal (Page & Jencks, 1972; Morris & Page, 1978). Of course, it may be necessary for the proton acceptor or proton donor

$$S + H{-}A{-}\text{Enzyme} \rightleftharpoons \underset{\text{Enzyme}}{[S.HA]} \longrightarrow \underset{\text{Enzyme}}{[S\cdots H\cdots A^{\ddagger}]} \quad (8)$$

$$S\cdots H\cdots A \longrightarrow S\cdots H\cdots A^{\ddagger} \quad (9)$$

to be at the active site as the equivalent intermolecular catalyst may be sterically prevented from reaching the enzyme-bound substrate and therefore in the absence of the general acid or general base on the enzyme a slower rate of reaction would result. However, the fact that the general acid or base is *part* of the enzyme apparently makes little contribution to the enormous rate enhancement brought about by the enzyme. The reason for this is that proton transfer reactions involve "loose" transition states so that in intermolecular reactions such as equation (9) there is only a small loss of entropy in bringing the reactants together (Page, 1973; 1977). There is therefore little or no advantage in having the catalyst in close proximity to the substrate as in equation (8).

THE MAGNITUDE OF THE BINDING ENERGY

Observed free energies of binding substrates to enzymes are often less than the enthalpic intrinsic binding energy because much of the binding energy is "used up" in bringing about the required loss of entropy and in inducing any destabilization of the reactants to enable binding to take place (Jencks & Page, 1972). Neither are intrinsic binding energies easily estimated from observed enthalpies of binding because these also reflect solvation changes. However, it is now apparent that the interaction energy between proteins and other molecules is very much larger than is generally believed. Not only are there many examples of proteins forming complexes with dissociation constants as low as 10^{-13}–10^{15} mol l^{-1}, which are the result of a very large number of interactions, but there are indications that even relatively small substituents may make a large contribution to the binding energy (Jencks 1975; Page, 1977).

Intrinsic binding energies may be estimated from the increase in binding energy that results from adding a small substituent *A* to the larger molecule *B* (Jencks & Page, 1972). If the entropy of binding *B* and *A-B* are similar then the difference in free energies of binding *A-B* and *B* gives the true binding energy of *A* (Jencks, 1975). Binding energies estimated in this way indicate that the interaction between substituents and protein is often very favourable. For example, an additional methylene group in a substrate may increase the binding energy to an enzyme by as much as 12–16 kJ mol^{-1} (Fersht, 1977; Jencks, 1975; Page, 1977). Kinetic studies (Fersht, 1977; Jencks, 1975) also indicate that it is far more favourable to transfer a methylene group from water to an enzyme (12–16 kJ mol^{-1}) than it is from water to a non-polar liquid (*ca.* 4 kJ mol^{-1}). An enzyme is *not* like oil-droplet but is a close-packed array of covalently linked atoms which gain rigidity from disulphide links, hydrogen bonds, van der Waals and hydrophobic interactions. The fraction of space occupied by the atoms in a molecule of protein is large, *ca.* 0.75, whereas that in liquids is very much lower, *ca.* 0.3–0.5 (Klapper, 1971; Richards, 1974). In a liquid, a solute is surrounded by molecules separated by their van der Waals radii and hence a large fraction of the surface of a solute molecule is surrounded by empty space (Grunwald & Price, 1964). There is a greater surface area of contact between a bound molecule and a protein, compared with the situation in a liquid, which gives rise to a much greater interaction energy.

One of the reasons why enzymes are so large is simply that a large molecular weight is required to provide the favourable energy of folding the protein into a fairly rigid three-dimensional structure. Another reason is that this *prevents* the binding of certain molecules, a smaller enzyme would be more easily distorted and therefore a "wrong" molecule could bind to the enzyme if the favourable free-energy of binding was greater than the free-energy change accompanying distortion of the enzyme.

ENZYME-SUBSTRATE COMPLEMENTARITY, RIGID VS FLOPPY ENZYMES

The important contribution of enzyme-substrate binding energy to the efficiency of enzymic catalysis has been recognised for many years (Haldane, 1930). However, there has been much discusssion as to whether the binding energy between the enzyme and substrate, between the enzyme and product or between the enzyme and some intermediate is the most important. According to Transition-State *Theory* reactants are converted into products by passing through a state or configuration of maximum energy in which some of the covalent bonds are usually, but not always, partially broken or formed. This configuration or state is referred to as the Transition State and although it has a very short lifetime (*ca.* 10^{-13} sec) it is usually regarded as having a fixed geometrical molecular structure, i.e. the state is a "normal" molecule apart from the bonds undergoing bond breaking and making. This concept has enabled chemists to describe and conjecture transition state structures which define the relative amounts of bond-breaking and bond-making that have taken place.

Recently there have been many suggestions (Fersht, 1974, 1977; Wolfenden, 1972, 1976; Lienhard, 1972, 1973) which are not new, (Haldane, 1930; Pauling, 1946) that it is catalytically advantageous for the enzyme to be complementary in structure to that of the transition state rather than to the ground state reactant or product structure. A discussion as to at what stage during the reaction is the maximum bind-

ing energy between the enzyme and the substrate reached is not separable from the problem of "rigid" and "flexible" enzymes. *If* the enzyme is rigid and *remains undistorted* during the catalytic process the enzymes can be complementary in structure to only one particular molecular geometry during the conversion of the substrate to product.

The concept of the maximum binding energy occurring between the enzyme and the initial substrate structure was really introduced 85 yr ago by Fischer with his "lock and key" analogy (Fischer, 1894). However, if the enzyme is rigid and remains undistorted during the reaction it is simple to show that it is catalytically advantageous for the enzyme to be complementary to the structure of the transition state rather than to that of the ground state of the substrate (Fersht, 1977). The idea that the enzyme is complementary in structure to that of the transition state has been described previously in terms of the enzyme inducing strain in the substrate (Wolfenden, 1972; Lienhard, 1973) or by introducing electrostatic or solvent destabilisation (Jencks, 1975). These are the mechanisms by which the enzyme may work but the driving force to pay for these effects is the binding energy between the enzyme and substrate. In the case of the strain mechanism, it is probably better to consider that the transition state binds better to the enzyme than does the substrate (which increases k_{cat}, equation (1)) rather than to consider that the enzyme distorts the substrate (which increases K_m).

According to the induced fit model (Koshland & Neet, 1967) the native conformation of the enzyme is changed upon binding of the substrate. Induced fit *cannot* be important for catalysis by non-allosteric enzymes because part of the binding energy of the substrate is used to distort the enzyme, which increases K_m (Fersht, 1977; Jencks, 1975).

Ideas about the flexibility of enzymes are often dominated by one's preference for space-filling or skeletal models. In order to bring about the maximum loss of entropy of substrates, and provide optional alignment of substrates with catalytic groups at the active site, it is desirable that an enzyme be as rigid as possible (Jencks & Page, 1972). Rigidity of the enzyme is also important for transition state complementarity. This does not mean that groups within a protein cannot undergo internal rotation, in fact there is much evidence, particularly from nmr studies, that the interior of some proteins is mobile and that some proteins "breathe", i.e. the protein as a whole undergoes some vibronic motion (Campbell *et al.*, 1975; Dobson & Williams, 1975; Wyethrich & Wagner, 1975; Cooper, 1976). Part of the protein may be mobile while another part is not and these parts may be of diverse sizes for different proteins. Proteins may be classified into those exhibiting relatively rigid, flexible and mixed behaviour, but it appears that enzymes, generally, show the least conformational change of the main frame.

Although the kinetics of binding may control the reaction if enzyme complementarity is too good and the mobility of the enzyme is too restricted (Burgen *et al.*, 1975; Becker *et al.*, 1976) this is unlikely to be a general situation. Even if, because of weak binding, the rate of dissociation of an initially formed enzyme-substrate complex was fast, say $10^8\ s^{-1}$, there is still plenty of time for conformational changes which usually take place in less than 10^{-10} s. This is compatible with those enzyme-catalysed reactions where the rate-limiting step is diffusion-controlled encounter of the enzyme and substrate.

Because of the way that we make models and "draw" reaction mechanisms we often exaggerate the differences in geometry of ground-state and transition state structures whereas the actual distance that atoms move to reach a transition state is not *tremendously* more than that experienced by normal vibrations. For example, at 298 K mean vibrational amplitudes commonly range from about 0.05 to 0.10 Å and bending amplitudes of $\pm 10°$ are not rare. Formation of the pancreatic trypsin inhibitor-trypsin complex is accompanied by minor distortions in the substrate and enzyme (Bode *et al.*, 1976). However, an important factor contributing to the increased binding energy of the transition state to the enzyme is that formation of the transition state is often accompanied by large changes in the electron density surrounding atoms which provides the increased favourable interaction between the substrate and enzyme (see, for example, page 2).

The major driving force for enzymic catalysis appears to be the binding energy resulting from a very close packed interaction between the substrate and enzyme.

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