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COMMENTARY

THERMODYNAMIC STUDIES OF ENZYME CATALYSIS

WHAT CAN WE LEARN ABOUT SPECIFICITY AND MECHANISM?

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Thermodynamic studies of drug- and xenobiotic-metabolizing enzymes often involve measurement of the temperature dependence of rate or equilibrium constants. While such experiments are technically quite easy to perform, the interpretation of results deriving from them is, not infrequently, subject to confusion. This may be due to the fact that thermodynamic studies of enzyme catalysis require in-depth understanding of topics as different as chemical thermodynamics, enzyme kinetics and enzyme mechanisms. In this paper, no attempt is made to review the literature on the topic. Instead, our purpose is to discuss the basic relevant concepts, and, by selected examples, show what kind of information can be obtained.

On what enzymes do

In nature, the presence of enzymes is intimately linked with living organisms, drawing our attention to their most fundamental feature and *raison d'être*: the acceleration of chemical reactions by their overwhelming catalytic power. Indeed, the relative chemical stability of many organic molecules under earth's atmospheric conditions would, paradoxically, make life impossible, and we only need to think about the most common human activities (e.g. breathing, eating, moving) to fully realize the significance of the innumerable enzymes involved. Energy intake, the conversion of energy into work and self-reproduction of organisms, all depend on cascades of enzyme-catalyzed reactions. Not infrequently, enzyme action enhances reaction rates by factors of 10^8 to 10^{14}.

Thus, the study of enzyme catalytic mechanisms has been one of the main topics of biochemistry from its very beginning. Faced with the impossibility of directly studying enzymes by, for example, X-ray diffraction, early enzymology focused on substrate selectivity and the temperature and pH dependence of enzyme catalysis. The first cell-free enzyme extract was prepared by Büchner in 1897, whereas the first crystallization of pepsin, trypsin and chymotrypsin had to wait until the early 1930s, providing material to prove, for the first time, that enzymes are proteins. However, the original idea of complementarity between the stereochemical structure of enzyme and substrate, embodied in the "lock and key" model, was published as early as 1894 by Emil Fischer [1].

On how they do it

Since Fischer, enzymology and theories of catalytic activity have evolved, and very explicit and accurate explanations of enzyme action now co-exist. Still, and fortunately, most of them call upon a limited number of mechanistic concepts. Pauling [2] postulated enzyme structure to match the substrate in its transition state, rather than its ground state, an idea ultimately leading to the development of transition state inhibitors. Entropy effects, also referred to as proximity effects, constitute another important aspect of enzyme catalysis [3]. The rate accelerations brought about by these effects can be compared to the rate difference between an *intramolecular reaction* and the analogous *intermolecular reaction*. For example, the rate of the intramolecular formation of succinic anhydride from succinic acid is about 10^5 M greater than that of its intermolecular counterpart. Because a unimolecular reaction is being compared with a bimolecular one, the rate enhancement has the unit of concentration, and can be thought of as the concentration of one of the reactants required for the intermolecular reaction to proceed at the same rate as that of the intramolecular reaction.

Other important theories of enzyme catalysis include Koshland's hypothesis of *induced fit* [4], which postulates that the three-dimensional structure of the binding site does within limits adapt itself to substrate geometry, thereby raising the energy released by enzyme–ligand binding to a maximum. An extensive discussion of concepts of enzyme catalysis is beyond the scope of this commentary, and excellent reviews have been published [5, 6]. Here, we will turn to the realm of chemical kinetics and discuss a theory, as simple as it is elegant, that leaves us with striking insights into the molecular mechanisms underlying enzyme catalytic power.

On how we can mathematically describe how they do it

Since the essence of enzyme action *is* the

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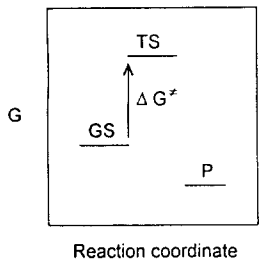

Fig. 1. Energy diagram for a unimolecular reaction. Key: GS: ground state; TS, transition state; P, product; and $\Delta G^{\neq}$, Gibbs free energy of activation.

enhancement of reaction rates, the use of the equations of chemical kinetics has made a major contribution to the understanding of enzyme mechanism, catalysis and specificity. One theory of particular importance in this respect is the *transition state theory* (TST)*.

TST describes the rates of chemical reactions in terms of the energies of reagents in their ground state and the energy of the last stable molecular species on the reaction pathway—the transition state. Their energy differences are commonly visualized in diagrams like Fig. 1.

The rate of the reaction is derived from the assumption that, while the reaction proceeds at a constant rate, the ground and transition states are in thermodynamic equilibrium with each other. In this situation, the concentration of the transition state can be calculated from its energy difference with the ground state. The well-known equation for chemical equilibrium (Eq. 1):

$$\Delta G = R \cdot T \cdot \ln K_{eq} \tag{1}$$

(ΔG = standard Gibbs function, R = molar gas constant, T = absolute temperature, K_{eq} = equilibrium constant) thus becomes Eq. 2 for a unimolecular reaction

$$[TS] = [GS] \exp\left(\frac{-\Delta G^{\neq}}{R \cdot T}\right) \tag{2}$$

with $[TS]$ being the concentration of the transition state, $[GS]$ the concentration of the ground state, and $\Delta G^{\neq}$ the Gibbs free energy difference between the two (and the modern counterpart of Arrhenius' "energy of activation," E_A).

Now, the rate of product formation will equal the frequency at which the transition state decomposes, which in turn is tantamount to the vibrational frequency of the breaking bond. This rate can be obtained from the equivalence of the energies of an excited oscillator, calculated from quantum theory ($E = h \cdot \nu$) and classical Newtonian physics ($E = \kappa \cdot T$).

$$\nu = \frac{\kappa \cdot T}{h} \tag{3}$$

where ν is the frequency, κ is Boltzmann's constant (1.38×10^{-23} J K^{-1}) and h is Planck's constant (6.63×10^{-34} J · sec).

Thus, the rate constant of decomposition of GS is given by

$$\frac{-d[GS]}{dt} = \nu[TS] = [GS]\left(\frac{\kappa \cdot T}{h}\right) \exp\left(\frac{-\Delta G^{\neq}}{R \cdot T}\right) \tag{4}$$

and the first-order rate constant k becomes

$$k = \left(\frac{\kappa \cdot T}{h}\right) \exp\left(\frac{-\Delta G^{\neq}}{R \cdot T}\right) \tag{5}$$

or, with the Gibbs free energy separated into enthalpic and entropic terms:

$$k = \left(\frac{\kappa \cdot T}{h}\right) \exp\left(\frac{-\Delta H^{\neq}}{R \cdot T}\right) \exp\left(\frac{\Delta S^{\neq}}{R}\right). \tag{6}$$

The importance of Equation 6 appears more obvious when we write it in a logarithmic form,

$$\ln k = \ln\left(\frac{\kappa \cdot T}{h}\right) - \frac{\Delta H^{\neq}}{R \cdot T} + \frac{\Delta S^{\neq}}{R} \tag{7}$$

and realize that the first term on the right side of Equation 7 is as good as constant over a considerable temperature range. The equation then shows that the enthalpy and entropy changes accompanying a chemical process can be determined from the slope and intercept, respectively, of a plot of the logarithm of the rate constant ($\ln k$) for that process against $-1/RT$. For statistical reasons, it is preferable to center the reciprocal temperatures around their mean value and obtain $\Delta H^{\neq}$ and $\Delta G^{\neq}_{hm}$ (Gibbs free energy of activation at the harmonic mean of experimental temperatures) by plotting the data according to the following modified Arrhenius equation [7]:

$$\ln k_e = \ln\left(\frac{\kappa \cdot T_e}{h}\right) - \frac{\Delta G^{\neq}}{R}\left\langle\frac{1}{T}\right\rangle - \frac{\Delta H^{\neq}}{R}\left(\frac{1}{T_e} - \left\langle\frac{1}{T}\right\rangle\right). \tag{8}$$

On binding and catalysis

One of the central features of enzyme action is the energy released upon the binding of the substrate to the enzyme binding site. *How* this energy is being used to accelerate the subsequent reaction can be appreciated by looking at the reaction

$$A + B \rightleftharpoons AB^{\neq} \longrightarrow C$$

in which A reacts with B to form a high-energy intermediate $AB^{\neq}$ before being transformed into product C. The association of A and B occurs with significant losses in translational and rotational entropy which have a negative effect on the rate of the reaction. In the corresponding *intramolecular* reaction

* Abbreviations: TST, transition state theory; SSK, steady-state kinetics; and RRK, rapid reaction or pre-steady-state kinetics.

this negative entropy factor is absent, giving rise to a reaction rate up to 10^8 times greater than that of the intermolecular reaction. So, in enzyme-catalyzed reactions, the negative entropy effects accompanying the encounter of the reactants are compensated for by the enzyme–substrate binding energy. Subsequently, the reaction can proceed as an intramolecular process.

The application of TST to enzyme catalysis inherently implies the assumption that enzymes bind substrates in their transition state rather than in their ground state structure. In this way, enzymes stabilize transition states, increase their probability of existence, and accelerate reactions. In contrast, carrier proteins and antibodies, which also bind small molecules with high affinity, can be expected to bind and stabilize substrates in their ground state structure, thereby *preventing* them from being metabolized or being harmful to the host organism. The importance of the binding energy released upon the association of an enzyme and a substrate in its transition state was most elegantly emphasized by Jencks [8], who referred to it as:

> *"the currency to pay for substrate destabilization through distortion, electrostatic interactions, and desolvation, for bringing about the necessary loss of entropy by freezing the substrates in the proper position for reaction, and for binding to the transition state. The maximum binding energy is [. . .] not realized directly in the binding of the substrate, but is more completely realized in the transition state."*

So, it appears that in the study of enzyme action, *binding* and *catalysis* cannot be considered as mutually independent events, separated in time and space. The high-affinity binding of substrates in their transition state is, in fact, the quintessence of enzyme catalysis. Therefore, making a distinction between the two may allow kinetic equations to be drawn, but remains questionable from a mechanistic point of view.

On the role of the solvent

Let us take a look at the vast field of enzyme kinetics and see if, and how, the study of the rates of enzyme-catalyzed reactions can give a clue as to the origin of enzyme specificity.

Enzyme kinetics can be divided into two main areas: *study-state kinetics* (SSK) and *rapid reaction* or *pre-steady-state kinetics* (RRK). These two types of investigations yield different, and often complementary, types of results. In general, the results of SSK are particularly relevant to enzyme *function*, whereas RRK studies provide information pertinent to *mechanism*. This has to do with the very different experimental conditions: SSK makes use of enzyme concentrations in the physiological concentration range, whereas RRK often utilizes very high concentrations, allowing the monitoring of the build-up and breakdown of intermediate structures along the reaction path. RRK studies involve measurements on a millisecond time scale, necessitating sophisticated equipment. The types of data provided by the two techniques, as indicated by Engel [9], can be compared to the information that is obtained from the two following ways of studying a country's postal system: (1) by posting a large number of different letters at different times and seeing whether and how soon they reach the intended destination, and (2) by going to the post office to watch the sorting.

Studies on steady-state kinetics constitute the bulk of the literature on enzyme kinetics and have generated most of our current knowledge in the field. Such studies are based on the Michaelis–Menten equation:

$$v_0 = \frac{k_{cat} \cdot [E]_0 \cdot [S]_0}{K_m + [S]_0} \qquad (9)$$

which relates the initial velocity of the enzyme-catalyzed reaction (v_0) to the concentration of substrate $[S]_0$ and enzyme $[E]_0$ and two kinetic parameters, i.e. K_m and k_{cat}. The Michaelis constant, K_m. is often considered to reflect enzyme–substrate binding affinity. Likewise, k_{cat}, the catalytic rate constant or turnover number can be regarded as the rate constant for the conversion of the enzyme–substrate complex into products. However, except for special cases, the kinetic parameters in the Michaelis–Menten equation are composites of several rate constants, each of which relates to the formation or breakdown of an intermediate along the reaction path. Therefore, a straighforward interpretation of steady-state kinetic data in terms of enzyme mechanism is generally difficult.

Yet, sound steady-state studies have yielded a better understanding of the determinants of enzyme specificity in some cases. For example, in the alkaline mesentericopeptidase-catalyzed hydrolysis of *p*-nitrophenyl carboxylates [10], both the activation enthalpy and entropy for enzyme deacylation showed linear dependence on the number of carbon atoms (n) in the aliphatic side-chain, with a break in the $\Delta H^{\neq}$ vs n and $\Delta S^{\neq}$ vs n plots at $n = 4$. Moreover, enthalpy–entropy compensation was observed for deacylation of the enzyme, and the authors explained the phenomenon by a prominent contribution of solvation-related terms to the energy of activation. By forming hydrogen bonds with the enzyme–substrate complex, water molecules stabilize the transition state of the deacylation step. The energy released upon formation of these bonds lowers $\Delta H^{\neq}$. However, as these molecules adopt a non-random spatial orientation with respect to the complex, they contribute to a decrease in the entropy of the enzyme–substrate–hydration water complex. Such a primary role for hydration in enzyme specificity in water putatively explains, at least in part, the dramatically altered properties of enzymes in organic solvents [11].

We measured, at seven temperatures, the steady-state kinetic parameters for the pig liver carboxylesterase catalyzed hydrolysis of the prochiral substrate dimethyl phenylmalonate, showing product enantioselectivity, and the separate enantiomers of

three chiral 2-phenylpropionic acid esters, showing substrate enantioselectivity [12]. (+)-(*S*)-Methyl-2-phenylpropionate, (+)-(*S*)-4-nitrophenyl 2-phenylpropionate and both enantiomers of phenyl 2-phenylpropionate showed very similar activation enthalpies and entropies (approximately 50 kJ mol^{-1} and -50 J K^{-1} mol^{-1}, respectively), but differences were observed for (−)-(*R*)-methyl 2-phenylpropionate ($\Delta H^{\neq} = 30.2$ kJ mol^{-1}, $\Delta S^{\neq} = -123$ J K^{-1} mol^{-1}) and (−)-(*R*)-4-nitrophenyl 2-phenylpropionate ($\Delta H^{\neq} = 60.6$ kJ mol^{-1}, $\Delta S^{\neq} = -17$ J K^{-1} mol^{-1}). Whereas entropies of activation of all 2-phenylpropionates were negative, positive entropies of activation were measured for the formation of monomethyl phenyl-malonate enantiomers from dimethyl phenylmalonate. Enthalpy–entropy compensation analysis of the data suggests a *common mechanism* of pig liver esterase substrate- and product-enantiospecificity in these reactions, possibly the stereospecific binding of the substrate acylmoiety to enzyme binding pockets.

For selected enzymes, rapid reaction kinetic techniques offer the advantage of yielding data that can be related to separate steps in the enzyme process with little ambiguity. In the case of the enzymes belonging to the serine hydrolases, these studies allowed the monitoring of the time course of the pre-steady-state formation of tetrahedral enzyme–substrate intermediates, giving rise to biphasic (exponential-linear) progress curves. A striking example of the power of rapid reaction kinetics as a tool in the study of enzyme mechanism was published by Kuramitsu *et al.* [13], who studied transamination reactions catalyzed by *Escherichia coli* aspartate aminotransferase for a series of amino acid substrates. The Gibbs free energy change for the rate-determining step, probably proton abstraction, as a function of the accessible surface area of substrates bearing uncharged side-chains, yielded a linear plot, suggesting enzyme–substrate binding to play a key role in the efficiency of the catalytic process.

These last two examples draw our attention, again, to the influence of the solvent on the rate and selectivity of enzyme-catalyzed reactions. For, wherever a correlation is found between the "goodness" of a substrate and its accessible surface area, its interpretation in terms of *hydrophobic interactions* is obvious. By this, it is generally meant that the optimization of the spatial orientation of solvent molecules with respect to each other is an important driving force in the reaction under investigation. This indeed is a very well-known phenomenon in all kinds of biochemical processes. However, the solvent influences enzyme catalysis in other ways as well. Thus, the enantioselectivity in the transesterification between the chiral alcohol *sec*-phenethyl alcohol and vinyl butyrate catalyzed by the enzyme subtilisin Carlsberg was determined in nine anhydrous solvents, and found to be inversely proportional to the solvent's dielectric constant and its dipole moment [11]. Moreover, the observed decrease of enantioselectivity in the more polar solvents was due to a higher reactivity of the *R* enantiomer rather than a lower reactivity of its *S* counterpart. This led the authors to interpret their data as evidence for the importance of conformational rigidity, imposed on the enzyme protein by non-polar solvents. In this view, water would act as a molecular lubricant, leaving greater conformational freedom to the enzyme and reducing enantioselectivity. Recently, a complete reversal of enzyme enantioselectivity upon a change in the solvent (acetonitrile → tetrachloromethane) was reported for the first time [14].

Concluding remarks

Sound thermodynamic studies can lead to significant conclusions on the inter- or intramolecular mechanisms underlying enzyme specificity. When studying kinetics, it is important to know how the different steps in the enzymatic process relate to the parameters observed. Where possible, the study of rapid reaction kinetics offers major advantages in this respect. Thermodynamic studies reveal the importance of enzyme–solvent and substrate–solvent interactions and their influence on enzyme rate and equilibrium constants.

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