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Catalysis in inverted potential energy profiles

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Abstract

When the activated state of a substrate molecule is chemically similar to the ground states of substrate or product, enzymes are limited in their ability to stabilize the activated state. In conventional schemes this leads to slow catalysis. We present a model, based on two postulates, that removes this limitation. Here catalysis is based on a vibrational excitation along the S → P transition, that is isolated from other dynamic modes of the protein. We discuss testable (verifiable) predictions of the model.

Keywords: Catalysis, Enzyme mechanisms; Brownian motion in potential wells; Chemical reaction rate theory; Catalytic inactivation

1. Introduction

The question how enzymes accelerate chemical reactions by orders of magnitude was explained in pioneering studies by Haldane [1] and by Pauling's principle of transition state stabilization [2]. Later Jencks [3], Fersht [4], and others [5,6] have made extensive surveys of kinetic and structural aspects of catalysis.

The workings of enzymes are commonly desdribed by transition state theory (TST) [7–9]. Here the catalytic action is explained as differential binding to the enzyme of substrates, transition structures and products. It is postulated that the binding free energy that favours the transition structure is used by the enzyme to reduce the free energy barrier that separates substrates from products. In TST the dynamic events occurring at the saddle point are often neglected or treated *ad hoc*. It is frequently assumed that once the system has crossed the surface normal to the direction of the reaction vector, it never comes back through the dividing surface. This notion has been challenged by a number of authors on the grounds that the non-recrossing condition will tend to make rate estimates based on TST biased towards too high values. Recently, Beece et al. [10] emphasized the role of viscosity in enzyme kinetics. They underlined the deep connections between diffusion theory and chemical reactions that follow from Kramers' [11] derivation of rate constants from a theory for Brownian particles in potential wells. Kramers' treatment contains aspects of transition state theory as a special case [10–13]. One aspect of Kramers' work, that is important in the present context, is the concept of a potential energy surface that defines the

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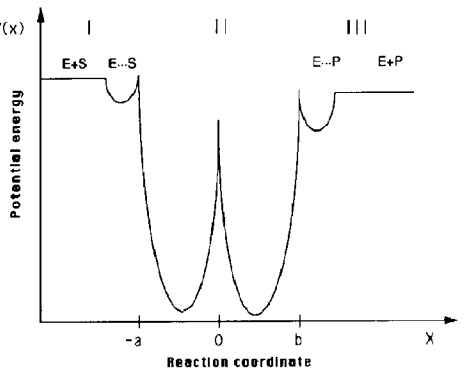

Fig. 1. Variation of potential energy $V(x)$ along the generic reaction coordinate x for a catalytic step on the enzyme in the present model (see text).

kinetics of the process as the system moves from one potential energy minimum to another.

In standard TST and in theories based on Kramers' approach the systems have to overcome activation barriers, and the simplest description of the role of enzymes is that they stabilize transition structures. In situations where the enzyme cannot discriminate well enough between substrates, products and transition structures, all these theories predict that catalysis is very slow.

In the present work we propose a new mechanism for catalysis where this fundamental constraint on the rates of enzymes is removed. We postulate, first, that the potential energies of substrates, products and transition structures in the active site are lower than the potential energies of the weak complexes at the entry to and exit from the enzyme. This leads to the characteristic inverted potential energy profile in Fig. 1. Our second postulate is that the system can move rapidly along the reaction coordinate with little exchange of heat with other parts of the protein (near-adiabaticity). It will pass freely over the activation barrier, since its total energy (kinetic plus potential) is always higher than the potential energy of the transition state. The major advantage of this new mechanism is that it makes catalysis fast also when the activation barrier between substrates and products remains high on the enzyme. One consequence of the model is that there is a small probability that the enzyme becomes inactivated every time it traverses its catalytic cycle. This feature may explain why some enzymes seem to be inhibited by their own catalytic action [26–32], and can be used to put our model to experimental tests.

In the next section (Section 2) we discuss in more detail some features of catalysis that will put the present work in its proper perspective. In Section 3, we describe diffusion in inverted potentials, where the movement is characterized by very small friction. Section 4 contains a short description of the steady state kinetics of catalytically inactivated enzymes. In Section 5 the Fokker–Planck equation for Brownian movement in inverted potentials is discussed in semiquantitative terms. Some aspects of optimal enzyme design are outlined in Section 6. Finally, a general discussion of the theory and its experimental implications is presented in Section 7.

2. Catalysis in and out of thermal equilibration

2.1 Standard descriptions of catalysis

Some further features of catalysis may be summarized as follows [4]. First, the reaction pathway for the conversion of a substrate S, free in solution, to a product P is compared to the corresponding enzyme catalyzed reaction (Scheme I):

$$\begin{array}{ccc} (E + S^*) & & \\ & \overset{k_u}{\rightleftharpoons} & \\ E + S & \underset{k_{-u}}{} & E + P \\ \Vert & & \Vert \\ (ES^*) & & \\ & \overset{k_c}{\rightleftharpoons} & \\ ES & \underset{k_{-c}}{} & EP \end{array}$$

Scheme I

The transition from S to P occurs over an activated state S* of the substrate. In the absence of the enzyme, the rate constant (k_u) for this transformation is determined by the difference in standard free energy (ΔG_u) between S* and S:

$$S \xrightarrow{k_u} P, \qquad k_u = \nu\, e^{-\Delta G_u / kT}.$$

Catalysis by an enzyme usually requires that the transition state S* is stabilized in relation to S and P:

$$ES \xrightarrow{k_c} EP, \qquad k_c = \nu\, e^{-\Delta G_c/kT}.$$

The standard free energy difference ΔG_c between ES* and ES is small ($\Delta G_c \ll \Delta G_u$) and the rate ($k_c$) is high. The magnitude of the rate increase from k_u to k_c depends therefore on how much stronger the enzyme binds S* in relation to S and P. Thus, fast catalysis poses a problem very similar to that of hyperselective enzymatic pathways. In transcription, aminoacylation, translation or DNA replication the biosynthetic enzymes must develop a much higher affinity for the cognate substrate than for the non-cognate ones (see (ref. [4], chap. 11). When the affinity difference is insufficient, Nature has developed subtle proofreading schemes with free energy driven discard steps, where the same affinity difference between right and wrong substrate is used several times, until the desired precision of the reaction is reached [14–20].

In a similar way catalysis requires that the protein has a much higher affinity for S* than for the ground states of S and P. Therefore, when the structure of S* is chemically similar to that of S or P the enzyme's ability to discriminate between S* and S or P is limited. Classically, this leads to slow catalysis since here the standard free energy difference ΔG_c always determines k_c.

Knowles [21] discusses three classes of reaction mechanisms: The first, and most primitive, contains enzymes that bind S, S* and P with about equal affinity. The second contains enzymes that even out standard free energy differences between S and P. In the third and most advanced class the enzymes stabilize S* in relation to S and P.

2.2 Catalysis by near-adiabatic movement in inverted potentials

In the present work we extend these views. First, we introduce a potential energy hypersurface associated with the transition from S to P. By relaxing the condition of thermal equilibration we show that high catalytic rates can be achieved also when the potential energy barrier between S and P is high. To do this, the enzyme must develop a very strong binding to S, S* and P, so that an inverted reaction potential develops for the S to P transition (Fig. 1). Such profiles are documented for gas phase ion-molecule reactions [22], and we postulate that they exist also in the active site of some enzymes (cf. [23]). A second requirement is that the strong binding of the substrate leads to a near-adiabatic movement along the reaction coordinate, loosely coupled to other dynamic modes in the enzyme–substrate complex. To evaluate the physical consequences of the model we describe the movement on the reaction path from S to P with a Fokker–Planck equation for position and velocity in the limit of very low friction (near-adiabaticity) [11,24,25].

3. Near-adiabatic diffusion in potential wells

The potential energy profile for an enzyme that binds S, S* and P with high affinity is sketched in Fig. 1. We adapt Kramers' [11] analysis to the series of chemical transformations that lead from the free state (E + S) through the shallow potential well (E ··· S) into ES in region II (Fig. 1) which is the catalytic site. Here, the actual chemical interconversion between S and P takes place. From region II the product EP moves to the shallow potential well (E ··· P) in region III and eventually enters the free state (E + P). We assume that the rates in regions I and III can be derived from a strongly overdamped diffusion equation [11]. Here, all vibrational modes of S and P are in thermal equilibrium with the surrounding protein at all points along the reaction coordinate x. At the entrance to region II ($x = -a$) ES has an exponentially distributed kinetic energy W_a (Boltzmann-distributed velocity). The probability that W_a is in a small interval δW_a around W_a is given by

$$P(W_a)\delta W_a = (1/kT)\, e^{-W_a/kT}\delta W_a \qquad (1)$$

Product molecules entering region II have an equivalent distribution of their kinetic energies.

The molecular design of region II is crucial. Here, the heat exchange with the rest of the protein must be poor as the system moves along the reaction coordinate from S to P.

The dynamics of these transformations follow from a diffusion equation in the limit of low friction [11,33]. A substrate molecule entering the catalytic site will in this limit move along x from $-a$ to b, rapidly exchanging kinetic and potential energies. The total energy (potential plus kinetic) is almost constant along x so that the enzyme is capable of rapid catalysis in spite of the two deep potential wells and the high barrier that characterize the catalytic site.

3.1 The Fokker–Planck equation

The movement through region II is determined by the shape of the potential energy $V(x)$, a friction parameter γ, and an effective mass m associated with the vibrational mode along the generic reaction coordinate x [11]. The initial conditions are determined by the exponentially distributed kinetic energy of the vibrational mode of the substrate at $x = -a$, or of the product at $x = b$ (eq. 1, Fig. 1). The vibrations can be characterized by their rate (v) and position (x). A full description of the dynamic process requires calculation of a probability distribution $P(x, v, t)$ for x and v, which is the solution to the Fokker–Planck equation [11,24]:

$$\frac{\partial P}{\partial t} = -v\frac{\partial P}{\partial x} + \frac{1}{m}\partial v/\partial x \partial P/\partial v + \frac{\gamma}{m}\left[\frac{\partial(vP)}{\partial v} + \frac{kT}{m}\frac{\partial^2 P}{\partial v^2}\right] \qquad (2)$$

Fundamental solutions to eq. (2) can be obtained with Dirac's delta initial conditions at the substrate entrance to region II, $P(x, v, 0) = \delta(x - x_a)\,\delta(v - v_a)$, or with initial conditions $P'(x, v, 0) = \delta(x - x_b)\,\delta(v - v_b)$ at the product entrance. These solutions, together with the kinetic energy distribution in eq. (1), can be used to calculate three important probabilities. The first, P_T, is the probability that a substrate entering region II goes right through the catalytic steps and into the complex E ··· P. The second, P_I, is the probability that so much total energy is lost during the passage that eventually the vibrational ground state of one of the deep potential wells in II is reached. The third probability, P_R, is the propensity of a movement starting at $x = -a$ to pass through region II and get reflected back through the catalytic site and into the starting configuration E ··· S. As a good approximation, these three probabilities add up to unity:

$$P_T + P_I + P_R = 1 \qquad (3a)$$

For a movement starting at the product side instead the corresponding probabilities are P'_T, P'_S, and P'_R respectively. Here we have the equivalent approximation

$$P'_T + P'_I + P'_R = 1 \qquad (3b)$$

The present mechanism differs from classical schemes for catalysis [4–6] in that a high barrier between substrate and product (Fig. 1) is compatible with rapid catalysis. The model is also associated with a certain rate of inactivation by the catalytic action of the enzyme. Inactivation occurs when heat dissipation in region II leads to stable ES or EP complexes. The low friction in region II leads, on one hand to a small probability of inactivation ($P_I \approx 0$, $P'_I \approx 0$) and makes, on the other, the spontaneous regeneration of the enzyme exceedingly slow. The presence of inactivation routes leads to special considerations concerning the steady state behaviour of this class of enzymes.

4. Steady state kinetics of catalytically inactivated enzymes

The properties of enzymes inactivated by their catalytic activity can be summarized as follows (Scheme II):

$$\mathrm{E} + \mathrm{S} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \mathrm{E}\cdots\mathrm{S} \underset{k'_c P'_T}{\overset{k_c P_T}{\rightleftharpoons}} \mathrm{E}\cdots\mathrm{P} \underset{q_1}{\overset{q_{-1}}{\rightleftharpoons}} \mathrm{E} + \mathrm{P}$$

$$\mathrm{E}\cdots\mathrm{S} \xrightarrow{k_c P_I}, \qquad \mathrm{E}\cdots\mathrm{P} \xrightarrow{k'_c P'_I}$$

Scheme II

Substrate (S) associates with the enzyme with the second order rate constant k_1 to form E...S from the free state E + S. S dissociates from E...S with the rate constant k_{-1}. From E...S the enzyme–substrate complex enters region II (Fig. 1) with the rate k_c. The rate of product (E...P) formation is $k_c P_T$ and the rate of inactivation is $k_c P_I$. In the reverse reaction P replaces S, q_1 replaces k_1, q_{-1} replaces k_{-1}, k_c' replaces k_c and P_T', P_I' replace, respectively, P_T and P_I. The probability of inactivation of the enzyme depends strongly on the product concentration. In the special case of a very large driving force for the reaction with [P] near zero and much below equilibrium with [S] the flow from substrate to product is given by (cf. [16])

$$J = \frac{A_E[S]R}{1 + [S]((k_1 - R)/k_{-1} + R/q_{-1})} \quad (4)$$

and the inactivation flow by

$$J_I = J((k_1/R - 1)k_c P_I/k_{-1} + k_c' P_I'/q_{-1}) \quad (5)$$

Equations (4) and (5) follow from elementary considerations. They were derived using the fact that according to our assumptions P_I and P_I' are much smaller than unity. A_E is the amount of active enzyme which is decreasing slowly with time.

The rate of inactivation per enzyme molecule is k_I, where

$$k_I = J_I/A_E \text{ and } dA_E/dt = -k_I A_E \quad (6)$$

R is k_{cat}/K_M for the reaction in Scheme II and is given by [16]

$$1/R = 1/k_1 + 1/(K_1 k_c P_T) + 1/(K_1 K_c q_{-1}) \quad (7)$$

where

$$K_1 = k_1/k_{-1} \quad \text{and} \quad K_c = k_c P_T/(k_c' P_T')$$

An efficient enzyme has J/A_E large and the inhibition ratio J_I/J small. These two conditions are partially in conflict as explained below. With the help of a simplified model we characterize in the next section in more detail the dynamics of region II (Fig. 2).

5. The Fokker–Planck equation for Brownian particles in harmonic potentials

The Fokker–Planck equation for Brownian motion in harmonic potential wells is particularly simple [11,24]. To illustrate the principles of the model we make the assumption that region II in Fig. 1 consists of two truncated harmonic potentials, with the same characteristic frequencies. One extends from $x = -a$, where the potential energy is V_a, to $x = 0$ where the potential energy is V_c. The other starts at $x = 0$ and ends at $x = b$, where the potential energy is V_b. A vibrational mode initiated at $x = -a$ has the total energy E_a, where $E_a = V_a + W_a$. The kinetic energy W_a is exponentially distributed according to eq. (1). A movement initiated at x = b has the total energy E_b, where $E_b = V_b + W_b$, and where w_b is exponentially distributed according to eq. (1) with b replacing a. The model requires that both V_a and V_b are larger than the catalytic barrier V_c. The special case where $V_a > V_b$ is favourable for enzymes working in the forward direction from S to P (see below). A movement initiated at $x = -a$, with the total energy E_a, gains kinetic and looses potential energy up to the middle of the left potential. Then the kinetic energy decreases and the potential energy increases as the barrier maximum V_c at $x = 0$ is approached. The average

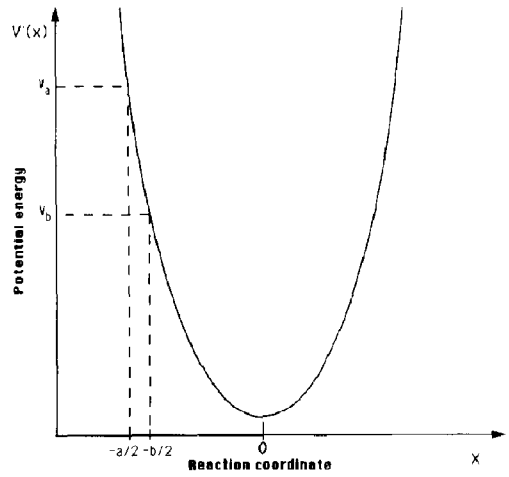

Fig. 2. Harmonic potential for obtaining the probability of successful catalysis and inactivation in inverted potential energy profiles (see text).

total energy is reduced during the passage from $x = -a$ to $x = b$ through dissipation of heat to the rest of the enzyme and there is a broadening of the distribution of energies due to the Brownian forces in region II. Movements with a total energy E_c larger than V_c will pass into the right side of region II and will finally reach the neighbourhood of $x = b$. Those with sufficient total energy at the turning point of their motion will reach the state EP in region III. In the case where all movements initited at $x = -a$ pass the barrier V_c, a movement from $x = -a$ to $x = b$ can be approximated by one full cycle in a single harmonic potential $V'(x)$, provided that V_a, V_b and V_c are not too different (Fig. 2). The potential $V'(x)$ in Fig. 2 has the shape

$$V'(x) = cx^2/2 = m\omega_0^2 x^2/2 \tag{8}$$

where $\omega_0^2 = c/m$, $V'(a/2) = V_a$ and $V'(b/2) = V_b$.

The solution to the Fokker–Planck equation for such a potential leads to periodic oscillations for the average rate and average position of the particle [11,24]. The characteristic frequency ω_1 is given by

$$\omega_1^2 = \omega_0^2 - (\gamma/m)^2/4 \tag{9}$$

The oscillations are attenuated with a characteristic rate constant γ/m. Our model requires that γ/m is much slower than the inverse of the time to complete one oscillation:

$$\gamma/m \ll \omega_1/2\pi \tag{10}$$

After one oscillation in $V'(x)$ (Fig. 2) a movement with the total energy E_a at $x = -a/2$ will have the average energy

$$\langle E \mid E_a \rangle_{t=2\pi/\omega_1} = E_a\, e^{-2\pi/\omega_1 \cdot \gamma/m} + kT(1 - e^{-2\pi/\omega_1 \cdot \gamma/m}) \tag{11}$$

as can be calculated from Wang and Uhlenbeck [24].

After two oscillations the average energy can be obtained from (11) if the time $2\pi/\omega_1$ is replaced by $4\pi/\omega_1$. The first term in Equation (11) shows how energy is dissipated as heat to the rest of the protein. The second term shows how the average energy approaches its equilibrium value kT irrespective of the starting value E_a

$$\langle E \mid E_a \rangle_{t \to \infty} = kT \tag{12}$$

The exponentials in eq. (11) are small and the thermal energy (kT) is much smaller than the three potential energy barriers in region II. The average energy losses after one cycle ($\langle \delta E \rangle_1$) can therefore be approximated by

$$\langle \delta E \rangle_1 = \langle \delta E \mid E_a \rangle_{t=2\pi/\omega_1} = \frac{2\pi}{\omega_1} \frac{\gamma}{m} \tag{13}$$

The fate of a movement starting at $x = -a$ in Fig. 1 can be assessed as follows. If the total energy after one oscillation in a well as in Fig. 2 is larger than V_b, then the product state E $\cdots$ P will be reached. This will happen with a proability near unity as long as $V_a - V_b$ is much larger than $\langle \delta E \rangle_1$. For movements initiated at the product side the situation is more intricate. First, a successful transition through region II to region I requires that the movement has sufficient kinetic energy at $x = b$ to compensate for the difference in potential energy between V_a and V_b as well as for the energy dissipated during the transition:

$$W_b > V_a - V_b + \delta E_1 \approx V_a - V_b \tag{14}$$

The probability for this inequality to be fulfilled follows from eq. (1) with a replaced by b.

Those movements that fail to lead to the comlex E $\cdots$ S are reflected into region II towards $x = b$. In order to escape into region III they must have a total energy larger than V_b at the turning point of their orbit. This corresponds to the probability that W_b is larger than the energy dissipation during two oscillations in the well in Fig. 2.

$$W_b \geq \langle \delta E \rangle_2 = V_b \frac{4\pi}{\omega_1} \frac{\gamma}{m} \tag{15}$$

and the probability for this is from eq. (1)

$$P(W_b \geq \langle \delta E \rangle_2) = \exp(-\langle \delta E \rangle_2 / kT) \tag{16}$$

If the dissipated energy is small in relation to kT the probability is near unity. To calculate more exactly the probabilities of being trapped in or

escaping from II, the above mentioned broadening of the energy distribution during the passage through II must be accounted for. However, the simplified treatment that we present here is sufficient to illustrate the most important aspects of the model. A more extensive discussion will be presented elsewhere.

These results imply that the transition from S to P through region II is favoured when the potential energy V_a at the entrance is larger than the potential energy V_b at the exit of the region. When $V_a - V_b$ is significantly larger than the average energy dissipation $\langle \delta E \rangle_1$ for one passage over region II, the probability of inactivation will be small.

The energy losses depend linearly on the ratio between the damping rate (γ/m) and the frequency (ω_1) of the harmonic potential.

For an enzyme with $V_a > V_b$ the back reaction from the configuration $E \cdots P$ into region II, is most hazardous. To avoid inactivation of the enzyme the movement must in this case deliver its energy back to $E \cdots P$ after a reflection near $x = -a$. The condition for this is that the energy dissipated during the passage is much smaller than the thermal energy kT.

Finally, for an enzyme where $V_a > V_b$ the probability of inactivation for the $S \rightarrow P$ transition (P_I) is much smaller than the probability of inactivation for the inverse $P \rightarrow S$ transition (P_I').

6. Optimal enzyme designs

The law of detailed balance relates the rate constants in eq. (5) to the equilibrium concentrations of S and P.

$$\left\{ \frac{[P]}{[S]} \right\}_{eq.} = \frac{k_1}{k_{-1}} \frac{k_c P_T}{k_c' P_T'} \frac{q_{-1}}{q_1} \tag{17}$$

Low rate of inactivation requires that k_c'/q_{-1} in eq. (5) is small. When the inactivation probability P_I for transitions through region II in the forward direction is negligible, it is the ratio k_c'/q_{-1} alone that governs the overall rate of inactivation. When this ratio is sufficiently small, the inactivation of the enzyme becomes insignificant. Equation (17) shows that this requirement is most easily fulfilled if P has a lower standard free energy than S. If the reaction is balanced, or if P has a higher standard free energy than S a small value of k_c'/q_{-1} leads to a small value of k_{cat}/K_M for scheme II. When the rate constants in eq. (5) are adjusted to minimize the inactivation losses of the enzyme, then a high substrate concentration may be necessary to compensate for a low k_{cat}/K_M to preserve a high turnover rate.

7. Discussion

A new type of catalytic mechanism has been examined. Here the potential energy along the reaction coordinate has one deep minimum on each side of the transition state energy barrier (Fig. 1).

We suggest that this type of energy profile may solve a fundamental rate problem, when the transition structure (S*) is chemically similar to the ground states of substrate (S) and product (P), and where the enzyme cannot easily reduce the energy barrier between S and P. The model requires that the exchange of heat between the movement (vibrational mode) along the reaction coordinate and other parts of the protein is slow. This makes possible a very rapid passage from S to P as compared to conventional schemes, where thermal energy has to be added from other parts of the protein or from the surrounding solvent to allow barrier passage. Thus the present hypothesis has nothing to do with ideas about enzymes working as energy funnels [34] where the kinetic energy of various vibrational modes is somehow directed to the catalytic site [35,36].

We have used a classical description of the present model, and this may lead to objections. First, we suggest a new way that covalent bonds are formed and broken, and any description that seriously attempts to apply our concept to dynamic calculations with realistic models of enzymes, must use quantum mechanical approaches. Second, the crucial condition of near adiabatic dynamics for the transition from S to P appears in our classical description as the requirement of a small friction term in the

Fokker–Planck equation. Again, to assess a realistic range for the viscosity along the reaction coordinate, a quantum mechanical formulation is necesary. In such a description the energy losses in region II will follow from the relative strength of the "allowed" vibrational couplings along the reaction coordinate, compared to the "forbidden" transitions to other vibrational modes of the enzyme–substrate complex. A quantum mechanical description is beyond the scope of the present work. However, we wish to point out that quantum mechanical formulations of the bivariate Fokker–Planck equation are described in the literature. A useful approach starts from Feynman's path integral formulation of the time development of quantum systems, as described in detail by Kleinert [37]. How these computational techniques can be applied to a quantum system moving in a potential well with position and momentum known at time zero, has been thoroughly treated by Ranfagni et al. [38]. Thus, we think that our classical approach is closely related to its quantum mechanical counterpart, and that our results are valid also for real systems.

One characteristic property of our model is the inverted reaction profile (Fig. 1). In models for the active site of carbonic anhydrase [23] the potential energy profile of carbon dioxide hydration to bicarbonate looks very similar to the one in Fig. 1. Although more realistic boundary conditions for the transition structure may influence the absolute values of the potential energies, the inverted character of the potential profile is likely to be a property of the real enzyme [23]. Carbonic anhydrase is designed to make bicarbonate dehydration remarkably fast at pH 7. The solution to these difficult kinetic problems, may have lead to evolution of the mechanism for catalysis that we discuss here and which is revealed by the chacracteristic inverted profile observed by Jacob et al. [23].

Another distinguishing feature of the model, is the possibility that the enzyme is inactivated every time that the catalytic region II in Fig. 1 is traversed. The cause of inactivation is irreversible losses of energy from the excited vibration along the reaction coordinate to other parts of the protein, so that the ground state of one of the wells in Fig. 1 is reached. As a result a high affinity complex between the enzyme and one of its ligands is created and detection of such putative complexes can be used to identify the mechanism experimentally.

This inactivation is a direct consequence of the physical properties of the proposed mehcanism. If the pathway in Scheme II is permitted to reach steady state with respect to the distribution between active and inactive enzyme configurations, then practically all enzyme molecules will end up in either one of the deep wells in Fig. 1. Here the enzyme will be in a strong complex with a substrate, a product or with a reaction intermediate. For the mechanism to work well, the loss of kinetic energy that leads to the groundstate of region II must be very slow in relation to the transition through this region. This means that Kramers' friction parameter must be very small. From detailed balance it follows that when the transition down into the wells is very slow, then the passage out from them is also very slow. This is so since a low friction for the vibrational movement through region II means that the exchange of energy with other vibrational modes of the protein is very poor. The escape out from a groundstate in region II requires that kinetic energy is supplied from other parts of the protein, so that the poor exchange of heat will also slow down the spontaneous reactivation of the enzyme. Therefore the mechanism can only work as long as the enzyme is kept out of steady state with respect to its inactivation pathways. This, in turn, makes the lifetime of the enzyme limited, and for continuous catalytic action either new enzymes must be made, or free energy driven enzyme regeneration pathways should exist, as will be discussed below.

One example of catalytic inactivation comes from a model of the active site of carbonic anhydrase, built up with β-cyclodextrins [39]. This construct has catalytic properties; It speeds up the hydration of carbon dioxide but is simultaneously deactivated in the course of the reaction. This is exactly what would be expected from a potential energy as derived by Jacob et al. [23].

In vitro studies of citrate–lyase, which catalyzes the conversion of citrate to oxalacetate,

indicate that this enzyme has a small probability of catalytic inactivation per cycle [26].

A tRNA synthetase mutant discussed by Anderson and Neidhardt [27,28] also seems to become inactivated by its own catalytic action.

Further studies of these enzymes are necessary to identify the exact cause of their inactivation, but these experimental results are so far consistent with the hypothesis put forward here. To keep the fraction of inactivated enzymes small under *in vivo* conditions requires the probability of inactivation per cycle to be quite low. This is illustrated by the following example: We assume that a bacterial population with growth rate k contains an enzyme with turnover rate v and inactivation probability per cycle α_p. The total rate of inactivation is then $v\alpha_p$, the rate of making new, active protein is equal to the growth rate k and the (steady state) fraction of active molecules is given by $A_E/E_0 = 1/(1 + \alpha_p v/k)$;With $k = 5\ 10^{-4}\ s^{-1}$ and $v = 50\ s^{-1}$, α_p must be smaller than about 10^{-5} for there to be at least 50% active enzyme molecules in the cell.

It is difficult at present to guess what the lower limits are for the friction parameters of optimally designed enzymes.

A large number of rapidly interchanging micro-states of proteins has been postulated by Frauenfelder and Wolynes [13]. This dynamic behaviour may tend to couple the vibrational mode along the S → P transition to other modes of the protein, and thereby increase γ. However, Bialek and Onuchic [40] have presented theoretical and experimental evidence strongly in favour of mode-specific chemistry in proteins; and this type of behaviour of enzymes is exactly what our model requires.

One may argue that our mechanism for catalysis can work only in enzyme systems that are extremely fast, like in the early stages of visual excitation. One may further argue that, since most biochemical reactions are catalyzed by enzymes with turnover times between 1 and 100 ms, vibrational excitation in inverted potentials can only be relevant for a few special cases.

However, it must be remembered that the model describes a single step, where the pathway from S to P may contain many additional steps. It is furthermore unlikely that the particular catalytic step that we are concerned with is rate limiting for the rate of turnover of the enzyme. On the contrary: if the mechanism works the way it should, then one can almost be certain that some other steps are limiting the rate of the enzyme. The turnover time of the enzyme has, in other words, nothing to do with the transition time through region II in Fig. 1.

The latter is determined by the equivalent mass of the vibrational mode and of the strength of the potential $V(x)$ according to eq. (8). We estimate that transition times involved in catalysis are typically in the range between 10^{-14} and 10^{-12} s. This means that the times that are relevant for the transition through region II, may be about ten orders of magnitude shorter than the turnover time of the enzyme.

For the mechanism to work efficiently in a bacterial cell, the calculation above shows that the probability for inactivation per cycle must be about 10^{-5} or smaller. Therefore, transitions to other vibrational modes than the ones along the reaction coordinate must be slower than about $10^8\ s^{-1}$.

This difference between the time scale of the catalytic vibrational transition, and the turnover time of the enzyme leads to an experimental problem. Thus, it is difficult to study with direct physical methods a vibrational mode with a duration of, say, 10^{-13} s in an enzyme system where the turnover time is 100 ms. The reason is that the fraction of time that the enzyme–substrate complex spends in an excited vibration state is not more than one part in 10^{11} of the total cycle time. That is why we emphasize biochemical rather than physical approaches to identify and characterize experimentally this new type of catalytic mechanism.

It seems reasonable to assume that mechanisms to regenerate catalytically inactivated enzymes have evolved. It is thus possible that a firmly bound substrate or product can be hydrolyzed or otherwise changed in another catalytic step that takes place at the bottom of a well in region II. This could facilitate dissociation of inactivating reactants from the enzyme, and make it ready for another cycle.

Another possibility is that there exists a regeneration pathway involving ATP or GTP hydrolysis to supply the necessary free energy to liberate the catalytic site after an inactivation event. Such an ATP-driven regeneration system has been postulated for carboxylase–oxygenase (Rubisco) [29,41,42]. This enzyme is inactivated by its catalytic action, with a probability of about 0.05% per cycle [30]. The inactivation is caused by two tightly bound inhibitors. One is xylolose-P_2 and the other unknown [29]. The Rubisco regeneration system, the activase protein, is able to remove these as well as other strong inhibitors in a reaction that requires ATP [29]. Further experimental investigation is required to decide whether these features of Rubisco reflect a mechanism for catalysis as suggested here, or whether the remarkable properties of this enzyme have other causes (cf. refs. [29,32]).

To sum up, the present paper tentatively identifies an upper limit to how much enzymes can accelerate chemical reaction rates. This bound has its origin in how well the enzyme can discriminate between the transition state on the pathway from substrate to product on one hand and the ground states of substrate and product on the other. We show that this limit, present in all classical descriptions, can be surpassed, provided that the conventional postulate of thermal equilibration along the reaction coordinate is relaxed. We have investigated a new type of mechanism of catalysis where there is an excited vibration along the reaction coordinate, and found it consistent with the laws of physics. This mechanism inevitably leads to a slow catalytic inactivation of the enzyme, which may be connected with free energy driven regeneration pathways. These predictions of the model are compatible with a set of observations concerning catalytic inactivation of enzymes, and can be used to put our hypothesis to severe experimental tests.

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