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# Do enzymes bind their substrates in the ground state because of a physico-chemical requirement?

Robert Pascal*

*UMR 5073, CC 017, Département de Chimie, Université Montpellier 2, Place Eugène Bataillon, F-34095 Montpellier cedex 5, France*

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**Abstract**

Transition state theory has provided no convincing explanation for the nearly universal observation of complexes of enzymes with substrates. Bimolecular catalytic reactions are assumed here to take place through reactive encounter complexes defined as the subset of reactant state species able to proceed directly to low lying energy levels at the transition state. By assessing the probability of these complexes from the maximum efficiency of intramolecular reactions, an upper limit for the rate constants promoted by hypothetical catalysts unable to bind substrates is deduced. This limit, which is below the ordinary range of bimolecular rate constants ($k_{cat}/K_M$) for enzyme reactions, results from a kinetic limitation in the formation of reactive encounter complexes. Exceeding this limit requires a stabilization of these complexes. Using the terminology of transition state theory, the need for enzymes to form complexes with substrates is then expressed as a necessity to restore Boltzmann distribution at the transition state.
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## 1. Introduction

Many catalytic reactions involve prior equilibrium association of catalysts with substrates and catalysis by enzymes is typically characterized by Michaelis–Menten

* Fax: +33-4-6763-1046.
*E-mail address:* rpascal@univ-montp2.fr.

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kinetics resulting from the occurrence of complexes with substrates and products. These complexes can either be considered as a source of catalytic power or, on the contrary, as a manifestation of the inhibitory effect of substrates and products. In the field of enzymatic catalysis this observation led to the "lock and key" analogy [1]. But, following the success of transition state theory, Pauling [2] analyzed the power of enzymes as resulting from their complementarity to transition states, which then led to a description that only uses the language of transition state (TS) stabilization to account for catalysis [3] (cf. [4] for a recent discussion). The discoveries of efficient inhibitors designed by analogy with transition states [5] and catalytic antibodies [6] have supported this description. Accordingly, the occurrence of enzyme–substrate complexes ($E \cdot S$) on the reaction path is predicted to have no favorable influence on the overall rate and the usual similarity between RS and TS is often considered to be responsible for the stability of $E \cdot S$ complexes [7]. But Wolfenden has earlier proposed the idea that enzymes could bind substrates in their ground state because encounters of activated forms of substrates with enzyme active sites would be too improbable [8,9]. In an alternative, but not incompatible, description, enzymes have been proposed [10] to use interactions with non-reacting portions of substrates to compensate for the loss of entropy at the TS. But this description has now become less popular and the entropy loss has even been considered as overestimated [11,12]. However, using three-dimensional free energy diagrams [13], the presence of complexes of catalysts with substrates has been analyzed as a manifestation of *induced intramolecularity* [14]. In this scheme (derived from the thermodynamic cycle introduced by Kurz [15]) any bimolecular catalytic reaction can be divided into two stages (Fig. 1A). The first stage leads to an unstable state defined as an encounter complex ($C \cdot S^*$) in which motions that are incompatible with entering the TS have been lost. This notion is related to that of a Boltzmann distribution of substrate molecules in states preceding the TS bottleneck [7] or that of near attack conformers (NACs) [16], but it includes further dynamic constraints that are more accurately defined here. Induced intramolecularity can then be assigned to the stabilization process in which the free energy levels of the encounter complexes and the TS are lowered by *uniform binding* [17] (Fig. 1B). In this way the entropy loss at the TS can be compensated for by binding energy corresponding to non-reacting portions of the substrate [10] or prior binding of reacting centers [18]. However, the description of Fig. 1A is dependent on the assumption that approximation and chemical processes are not concerted and proceed sequentially via $C \cdot S^*$ in the bimolecular catalytic reaction. This assumption is demonstrated here by analyzing the approximation stage leading to $C \cdot S^*$ complex in purely bimolecular processes with rates that are shown to be inadequate to account for the usual rates of enzyme reactions. The occurrence of $E \cdot S$ complexes is therefore needed merely to supply reactive encounter complexes at sufficient rates.

## 2. Occurrence of reactive encounter complexes

The entropy loss is an intrinsic consequence of TS stabilization, as shown in Fig. 2 using the potential energy profile corresponding to a vibrational–translational

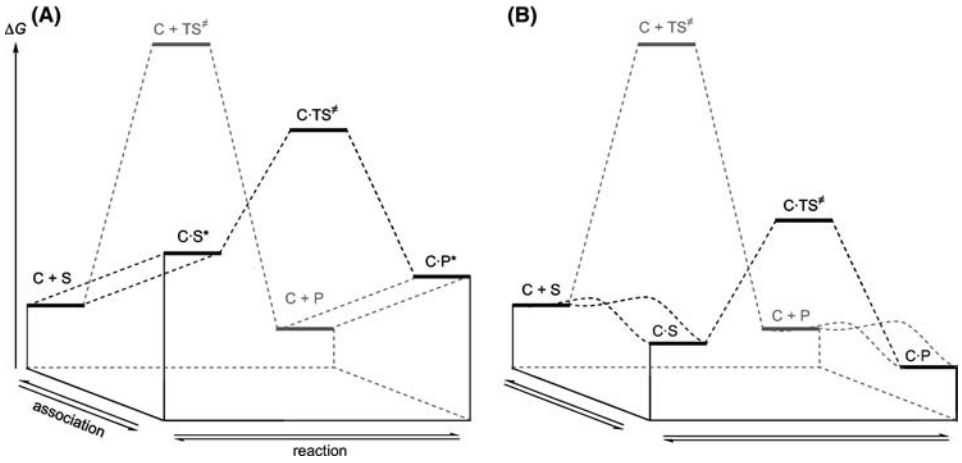



Fig. 1. Representation of a catalytic reaction by a three-dimensional free energy diagram as a two stage process: first the approximation step in which reactants are brought together into exactly the right position to react and second the activation process to reach the TS. (A) Purely bimolecular process, $C \cdot S^*$ and $C \cdot P^*$ encounter complexes are not stabilized. (B) Catalysis through induced intramolecularity: uniform binding is involved to stabilize the encounter complexes and the TS.

motion[1] associated with the binding interaction. Fig. 2 is based on an illustration introduced [19–21] to analyze the importance of deep potential energy wells in explaining enthalpy–entropy compensation and in quantifying binding cooperativity [20,22]. We can first consider an "ideal" catalyst promoting catalysis by tightly binding the TS as enzymes (binding free energy values of 50–130 kJ $mol^{-1}$ [23], which corresponds to a significant fraction of the energy of a covalent bond), but without interactions with the substrate (Fig. 2A). In the RS a huge number of levels can be occupied by the system, entropy is high. By contrast, the probability of low energy levels corresponding to the realization of binding energy with the altered substrate at the TS is high, which corresponds to low entropy.[2] The important point is that downhill transitions are needed to reach low energy levels at the TS. But a concerted process can be ruled out by applying the principle of microscopic reversibility. The breakdown of the stabilized TS ($C \cdot TS^{\neq}$) back to the free reactants ($C + S$) would on the one hand correspond to bond-making and/or bond-breaking along the reaction coordinate and on the other hand to the weakening of interactions of the "ideal" catalyst with the altered substrate. This means that thermal (Boltzmann) activation through collisions with solvent molecules or groups of the active site would have to assist the system in leaving low energy levels for repopulating higher levels. But, such transitions are uphill processes,

[1] Similar schemes could be built for overall rotational motions converted into internal rotations involving energy barriers separating conformers in the complex, but the conclusions may be less obvious since they correspond to less deep potential energy wells.

[2] As pointed out in [10] these changes are usually not observed in experimental entropies or enthalpies because of changes in the structure of solvent molecules in the environment. See also [19] for a discussion of enthalpy–entropy compensation.

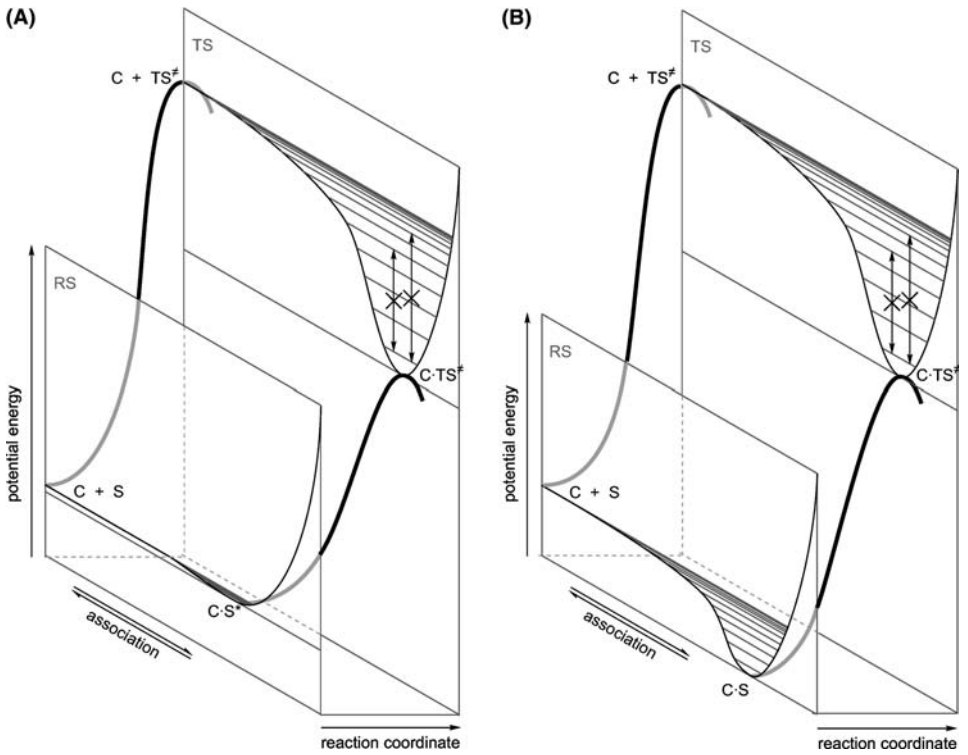



Fig. 2. Schematic potential energy profiles for a vibrational degree of freedom corresponding to different affinities of the catalyst for the substrate and the TS. (A) "Ideal" catalyst: weak binding interactions between the catalyst and the substrate; energy levels are close to each other, entropy is high because of the huge number of states available for the system. Strong binding interactions of the catalyst with the TS: energy levels are distant from each other, entropy is low, binding energy is fully expressed at the zero point energy. Transitions during the activation process are ruled out by applying the microscopic reversibility principle and considering that transitions to higher energy levels are uphill processes than cannot take place during TS breakdown back to RS. (B) Catalysis involving a complex with the substrate: strong binding interaction with the TS; intermediary interactions in the RS, energy levels are distant and lower energy levels are thermally populated, the probability of energy levels corresponding to strong binding at the transition state is substantial (the loss of entropy can approach the value obtained for strong interactions at the transition state since there is a limit to the adverse entropy of a bimolecular association [19]).

intrinsically slower than TS breakdown, which rules out any concerted pathway. Furthermore coupled mechanisms are unlikely because the corresponding degrees of freedom would not be independent of the reaction coordinate and fast intramolecular energy transfers between vibration modes are unlikely, as reported for gas phase reactions [24]. Such transitions are not consistent with the assumption that motions other than the reaction coordinate are adiabatic, i.e., that their quantum number are preserved as the reaction proceeds [25]. Therefore, in the forward direction, binding interactions with the catalyst fully realized in low energy levels at the TS are only accessible from a subset of species associating the substrate and the catalyst in the corresponding levels in the RS. Splitting the reaction into two steps through $C \cdot S^*$ (Fig. 1) is therefore

the only realistic and simple possibility to reach low energy levels at the TS. However, the ability of a mechanism via C · S* complex to account for the usual rates of enzyme reactions is uncertain because of the low probability of this encounter complex. The need for enzymes to form complexes with their substrates would consequently result from a stabilization of C · S* by uniform binding (Fig. 1B) and then binding energy with the TS can be fully realized. In this way, the entropy loss can be compensated by using binding energy as a driving force in a distinct stage leading to the formation of an intermediate having a lifetime compatible with a thermal distribution and sharing similar properties with respect to entropy (Fig. 2B). At first glance, this view seems to be compatible with the formulation of NACs as "turnstiles through which substrate molecules must pass to arrive at the lowest energy TS" [11]. But it is clearly attributed here to a dynamic limitation in the RS for the "ideal" catalyst that real enzymes easily avoid by forming E · S complexes. In contrast to Bruice's proposals [11], this view is neither in conflict with the need to pay for the entropy loss to account for enzyme power nor with a description using the language of TS theory as shown below. Although the statement that there is no transition between binding states at the TS seems to challenge the assumption that transition states correspond to a Boltzmann distribution, it must be emphasized that a thermal distribution at the TS does not imply a direct equilibration. It is a more general "consequence of Liouville's theorem by which a system in thermal equilibrium in one region of phase space will evolve into a thermal equilibrium system in other regions of space. Thus if reactants are at equilibrium, transition states species originating as reactants will be in equilibrium" [26]. Then, a thermal distribution at the TS is not predicted if reactants are not in thermodynamic equilibrium because of the depletion of reactive states by a very fast reaction. Therefore, the conclusions of the present discussion are not in conflict with transition state theory.[3] They simply result from the fact that, if there were no affinity of the catalyst for the substrate, the rates could be limited by the formation of states in which these species are coupled and designed here as reactive encounter complexes however high the affinity for the TS may be.

This approach can be compared to the idea that enzymes binding altered forms of their substrate would have their rates limited by the access of low populated species to the active site, "simply because encounter is too infrequent" [8]. This idea led Wolfenden to the conclusion that very efficient enzymes with $k_{cat}/K_M$ reaching the diffusion limit must bind nearly unaltered forms of substrates. But, the present approach is more general since it is the low probability of reactive encounter complexes with unactivated forms of the substrate that is shown to limit the efficiency of much less efficient "ideal" catalysts.

## 3. Reaction rates

The above hypothesis is supported by an assessment of the concentration of the non-stabilized C · S* complex by having recourse to non-enzymatic intramolecular

[3] The equilibrium assumption of transition state theory is not valid if the reaction is fast compared to the internal relaxation process that repopulates the reactive states [26].

reactions. These reactions, which can rival their enzyme-catalyzed counterparts [27,28], correspond to the formation of cyclic intermediates or products. Rate increases as compared with a reference bimolecular reaction are usually measured by the effective molarity (EM, Scheme 1) of the intramolecular reagent [28]. In strain free systems, the conversion of $C \cdot S^*$ state into products would be independent of its inter- or intramolecular nature ($k^*_{inter} = k^*_{intra}$) and EMs would only depend on the relative concentrations of reactive encounter complexes. To estimate the population of the non-stabilized $C \cdot S^*$ state of the bimolecular process, we can first consider that in a perfect intramolecular reaction all molecules would be present as $C \cdot S^*$ ($K^*_{intra} \gg 1$ then $EM_{max} = 1/K^*_{inter}$). Second, the maximal advantage of chemical intramolecular reactions in the absence of strain release has been assessed to a factor of $10^8$ M [27][4] and, for reactions at electrophilic centers, values of EMs consistent with this theoretical estimate have been observed [28]. This factor is also consistent with the probability of reactive conformers estimated by molecular dynamics [16]. Finally, for the "ideal" catalyst devoid of interactions with the substrate, the concentration of $C \cdot S^*$ state can be assessed using the equilibrium constant $K^*_{inter}$ ($K^*_{inter} = 1/EM_{max} \approx 10^{-8}\,M^{-1}$). But a catalyst of that kind is not able to bring about rates faster than the formation of $C \cdot S^*$ complex, a limit that would be reached if $C \cdot S^*$ reaction is faster than its dissociation ($k^*_{inter} > k^*_r$).[5] The rates of dissociation of the unstable $C \cdot S^*$ state must be smaller than a vibration frequency ($k^*_r \leqslant 10^{12}$–$10^{13}\,s^{-1}$).[6] Then: $k_{inter} \leqslant k^*_f = k^*_r K^*_{inter} \approx 10^4$–$10^5\,s^{-1}\,M^{-1}$.

This means that our "ideal," but not very efficient, catalyst cannot ensure bimolecular rate constants above this physically achievable limit, which is several orders of magnitude below the usual values [23,31,32] of $k_{cat}/K_M$ for real enzymes,[7] which are included in a narrow range of $10^6$–$3 \times 10^8\,s^{-1}\,M^{-1}$.

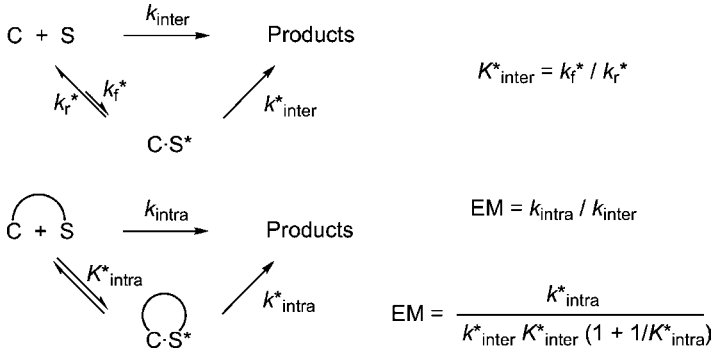
Moreover, enzyme reactions involve chemical barriers separating substrate and product complexes. These barriers correspond to the first order rate constants $k_{cat}$ (if a single chemical step is rate-determining), which are generally [31] comprised

[4] Values largely exceeding $10^8$ M found when strain is released during the cyclization process [29] are not considered here.

[5] In fact, this situation could be achievable only if there were no significant potential energy barrier between reactant and product complexes: the potential energy well would then be so deep that zero point energy at TS would be below the energy level of $C \cdot S^*$ complex. This means that the rates would be limited by the association step. Most reactions involving potential energy barriers, it is unlikely to be experimentally observed. Since many solution reactions are accompanied by solvation changes, this situation would not be perceptible from negative entropy values because of enthalpy–entropy compensation.

[6] First order dissociation constant values of ca. $10^{13}\,s^{-1}$ have recently been calculated for the breakdown of contact pairs by molecular dynamics [30]. But the dissociation of $C \cdot S^*$ encounter complex might be slower since it corresponds to the fraction of contact pairs having lost motions incompatible with the reaction.

[7] The largest values of $k_{cat}/K_M$ (fumarase and superoxide dismutase) are observed for enzymes reaching diffusion-controlled rates. In these cases, $E \cdot S$ complexes cannot be observed because their reaction is faster than their dissociation back to reactants. This means that there is a free energy barrier for dissociation, which is consistent with a stabilization of $E \cdot S$ complex and with an active site that is open to substrate access [8].




Scheme 1. Dissociation of bimolecular catalysis and intramolecular catalysis of a chemical reaction into two stages. The higher probability of reactive encounter complexes is assumed to be responsible for the efficiency of the intramolecular chemical process. This scheme does not apply to enzymatic catalysis because of the preorganization of the active site to accommodate for the transition state.

within a range of 50–$10^6\,s^{-1}$. Supposing again a catalyst that does not use uniform binding to stabilize bound species, the rate would be limited to:

$$k_{inter} < k_{cat}K^*_{inter} \approx 10^6 \times 10^{-8} = 10^{-2}\,s^{-1}\,M^{-1}.$$

Uniform binding [17] would then contribute to enzyme efficiency by a factor greater than $10^8$ (but that could exceed $10^{14}$) to account for the values of second order rate constants ($k_{cat}/K_M$) measured at very low substrate concentrations (below $K_M$). This factor is also a rough estimate of the contribution of induced intramolecularity to the affinity of enzymes for transition states (corresponding to a hypothetical dissociation constant of $10^{-8}$–$10^{-23}$ M [23]). The importance of binding interactions of enzymes with non-reacting portions of their substrates (uniform binding) was recently illustrated by studies of triose phosphate isomerase (TIM) [33] and orotidine 5′-monophosphate decarboxylase (OMPase) [32]. Removing the phosphoryl group of glyceraldehyde-3-phosphate and the phosphoribosyl group of orotidine 5′-monophosphate dramatically reduces the reaction rates of TIM and OMPase by factors of $10^9$ and $10^{12}$ (on $k_{cat}/K_M$), respectively, which are in agreement with the above estimation.

The present proposal may have consequences on strategies aimed at developing efficient artificial enzymes. The activity of catalytic antibodies remains several orders of magnitude below that of natural enzymes. Now, it can be understood that a strategy based on TS stabilization would not regularly yield catalysts that form complexes with substrates in which induced intramolecularity compensate for the entropy loss. If the affinity for the TS is not the only factor limiting enzyme efficiency, there is a possibility that an artificial catalyst with a high affinity for the TS may not be a highly efficient catalyst because of its low affinity for the corresponding state in the RS. On the contrary, this proposal has no consequence on the design of inhibitors by analogy with transition states since lifetime limitations do obviously not apply to stable species.

## 4. Conclusion

Enzymes commonly use several interactions of moderate strength cooperating to bring about catalysis [34]. These interactions can be classified into two categories. The first one corresponds to specific stabilization of the TS (changes in hydrogen bond strength, active site preorganization, electrostatic interactions...) that makes an essential contribution to catalysis, which is not challenged here. The other one involves the utilization of binding energy corresponding to interactions with non-reacting portions of the substrate (induced intramolecularity, ground state destabilization) [10]. In addition, the need for enzymes to form complexes with their substrates, which corresponds to the notion of induced intramolecularity, is now clearly related to a dynamic limitation during the binding step, which is more severe than previously proposed [8]. The "fundamentalist" position "that the entire source of catalytic power is the stabilization of the transition state; that reactant state interactions are by nature inhibitory and only waste catalytic power" [3] assumed to formulate catalysis in the language of transition state theory must be corrected. The second sentence must be changed into: *that reactant state stabilization is only required in so far as the Boltzmann distribution of the transition state has to be restored*. But, in addition to this requirement, interactions with non-reacting portions of the substrate or prior binding of reaction centers are also the more straightforward ways by which enzymes can enhance reaction rates to an extent that is considerable, although it is not unlimited [10,13,35].

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