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Commentary

A unified theory of enzyme kinetics based upon the systematic analysis of the variations of k_{cat}, K_M, and k_{cat}/K_M and the relevant $\Delta G^{0\neq}$ values—possible implications in chemotherapy and biotechnology

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Abstract

To elucidate the kinetic properties of critical enzymatic situations that have previously escaped classification, we performed a systematic analysis of all the possible variations of the kinetic constants $k_{cat,}$ $K_{M,}$ and $k_{sp} = k_{cat}/K_{M,}$ encompassing all aspects of enzymology. The equation gives a total of thirteen theoretically possible cases, comprising the reference case plus 12 different sets of variations, which can be divided into six principal cases and six specular ones. The six relevant cases are examined individually in the context of each of the main chapters of enzymology, i.e. as regards mechanism of action, specificity of substrate and isoenzyme, reversible and irreversible inhibition, and mutation of residues (enzyme evolution and enzyme engineering). Some critical cases where k_{sp} does not hold as a specificity index are classified for the first time. Interestingly, the six possible cases correspond to the five known cases of reversible inhibition (competitive, non-competitive, incompetitive, mixed competitive/non-competitive, and mixed incompetitive/non-competitive) plus an additional case of biphasic nature (activation-inhibition), which is crucial for a full understanding of specificity and which leads us to propose some modification to the definition of enzyme specificity. The systematic approach to enzymology outlined herein could find practical applications in various sectors of biotechnology, including chemotherapy.

Keywords: Enzyme specificity; Kinetic properties; Free energy; Enzyme evolution; Enzymatic engineering; Inhibitors and activators

1. Introduction

Herein we consider the basic kinetic parameters governing the rate equation $v_0 = f([S])$ of single substrate enzymes (Uni-Uni and Uni-Bi mechanisms) at constant *T, P,* and pH, and variations produced by the following conditions, all of which lead to the definition of relevant properties: (a) using different substrates with the same enzyme (substrate specificity); (b) using different isoenzymes with the same substrate (isoenzyme specificity); (c) natural mutations in the course of biological evolution (enzyme evolution and kinetic perfection); (d) site-directed mutagenesis by recombinant DNA techniques (enzyme engineering); and (e) using different types of inhibitors and activators (modulation of enzyme activity). Each of these items defines a different aspect of enzymology. One can ask if all the possible variations of kinetic constants have been taken into consideration in the study of the above-cited cases, or whether some type of variation, important in the study of new or rarely found effects, has been overlooked. Up to today, even in the most extensive thorough and clearly expressed published work in this area [1–3], no attempt has been made to calculate and review all the possible variations of the fundamental kinetic constants $k_{cat,}$ $K_{M,}$ and k_{cat}/K_M (i.e. k_{sp}) and to systematically examine all the consequences of these variations. This will be done in the present paper by considering all the possible variations (obtained by Combinatorial Analyses) of the fundamental constants of enzyme kinetics and the related standard free-energy variations ($\Delta G^{0\neq}$) appearing in the profiles of G^0 versus reaction coordinate in the light of the modern view of enzyme catalysis [1–5]. This systematic approach to enzymology should provide a tool capable of encompassing all cases, including certain rare ones that have hitherto escaped clas-

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Table 1
Scheme for combining the possible variations of the kinetic parameters for enzymes discussed in the text

k_{cat}	=	=	=	↑	↑	↑	↓	↓	↓
K_M	=	↑	↓	=	↑	↓	=	↑	↓
k_{sp}	=	↓	↑	↑	=↑↓	↑	↓	↓	=↑↓

Key: (=) invariance; (↑) increase; (↓) decrease; $k_{sp} = k_{cat}/K_M$.

sification. The unified theory set out herein could find practical applications in various sectors of biotechnology.

2. Possible variations of k_{cat}, K_M, and k_{sp}

Beginning with the most simple case, we shall limit our analysis to one-substrate enzymes obeying Michaelis–Menten kinetics under the so-called equilibrium approximation ($K_M \cong K_S$), according to the classical Henri–Brown reaction scheme ($k_{cat} = k_{+2}$). We will discuss all the physically possible variations of $k_{cat,}$ $K_{M,}$ and $k_{sp} = k_{cat}/K_M$. These will, in turn, be related to all the possible relative variations in the G^0 levels of the external (ES) or activated ($ES^{\neq}$) complexes [4].

A total of thirteen possible cases are obtained by the following reasoning. If we consider that each of the two kinetic parameters k_{cat} and K_M can increase, decrease, or remain constant (in symbols: ↑, ↓, =), we get nine possible combinations (Table 1). The third relevant kinetic parameter, $k_{sp} = k_{cat}/K_{M,}$ is dependent on the first two [6]. In some cases, only one type of variation is possible (e.g. k_{cat} ↑ and K_M ↓ implies k_{sp} ↑). However, parallel variations in k_{cat} and K_M can give rise to three possible variations of $k_{sp,}$ depending on whether the changes in k_{cat} and K_M are exactly proportional or are greater for k_{cat} than for $K_{M,}$ or vice versa [7]. Therefore, two of the nine combinations of k_{cat} and K_M are split into three by the possible variations of k_{sp} to give a total of thirteen cases (Table 1). One of these is, in fact, the reference case, when no variations in kinetic parameters occur.

As stated above, the variations of the fundamental kinetic constants are related to variations in the G^0 level of the ES (or Michaelis–Menten) and the $ES^{\neq}$ complexes in the standard free-energy profiles versus the reaction coordinate (Table 2). It is easily shown that with the approximations assumed by us $\Delta G^{0\neq}{}_{kcat} = G^0{}_{ES\neq}$ - $G^0{}_{ES.}$ Indeed, the apparent rate constant for enzymatic reactions under conditions of saturation of E with S (V_{max} conditions), which is given simply by $k_{cat,}$ is equal in our conditions to the elementary constant k_{+2} (rate constant of the elementary reaction ES $\xrightarrow{k+2}$ E + P), whose $\Delta G^{0\neq}$ is that indicated above: $G^0{}_{ES\neq}$ - $G^0{}_{ES.}$ On the other hand, under conditions in which [S]$\ll K_{M,}$ the apparent kinetic constant of an enzyme reaction is k_{cat}/K_M [8]. This constant can be expressed within our kinetic model as

$$\frac{k_{cat}}{K_M} = \frac{k_{+2}}{k_{-1}/k_{+1}} = \frac{k_{+2}k_{+1}}{k_{-1}} \tag{1}$$

Table 2
All the possible variations of the standard Gibbs free energy levels for the Michaelis-Menten complex (ES) and for the transition state ($ES^{\neq}$) of the enzyme reaction

$G^0{}_{ES^{\neq}}$	=	=	=	↑	↑	↑	↓	↓	↓
$G^0{}_{ES}$	=	↑	↓	=	↑	↓	=	↑	↓

Key: (=) invariance; (↑) increase; (↓) decrease.

Now, combining the $\Delta G^{0\neq}$ of the elementary reactions +2, +1, and -1, we immediately obtain on the basis of (1):

$$\Delta G^{0\neq}{}_{k_{cat}/K_M} = G^0{}_{ES^{\neq}} - G^0{}_{(E+S).}$$

Finally, we get:

$$\Delta G^0{}_b = G^0{}_{(E+S)} - G^0{}_{ES} = -RT\ln K_{ass} = RT\ln K_M$$

where the subscript “b” stands for “binding” and “ass” for “association.”

Thus, it is clear that all the aspects of enzymology listed above can be studied in a unified way by examining the effects of a chemical change in either the enzyme or substrate on $G^0{}_{ES}$ and $G^0{}_{ES\neq}$ values: that is to say in the affinities between the free enzyme (E) and substrate (S), as well as between the enzyme and the transition state of the reactant ($S^{\neq}$). For instance, an increase of the affinity of E for S will cause a decrease of the same order of magnitude (as an absolute value) in the $G^0{}_{ES}$ level. Likewise, an increase of the affinity of E for $S^{\neq}$ will cause a corresponding decrease (as an absolute value) of $G^0{}_{ES\neq.}$ If we further accept the usually adopted premise that the chemical changes of E and S do not modify $G^0{}_{(E+S)}$[1], then all of the variations of the kinetic constants reported in Table 1 can be retraced only to the relative variations of $G^0{}_{ES}$ and $G^0{}_{ES\neq}$ levels.

Following these premises, it seems natural to examine from this standpoint all the possible variations of $G^0{}_{ES}$ and $G^0{}_{ES\neq.}$ There are $3^2 = 9$ possible variations of these G^0 levels (Table 2).

Bearing in mind the connection of the three fundamental kinetic constants with the G^0 levels relative to E + S (k_{sp} increases when $G^0{}_{ES\neq}$ decreases, K_M increases when $G^0{}_{ES}$ increases, k_{cat} increases when the difference $G^0{}_{ES\neq}$ - $G^0{}_{ES}$ decreases), it is possible to obtain the variations for the constants reported in Table 3. If $G^0{}_{ES\neq}$ and $G^0{}_{ES}$ vary concordantly, it is possible to obtain three possible cases for $k_{cat.}$

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[1] Actually, chemical variations of E and/or S change μ^0 of the chemical species in question and, therefore, $G^0{}_{ES}{}^{\neq.}$ However, in many cases, it is possible to admit that a similar variation in the considered complexes can occur, and in this way the only remarkable variation in the differences would be that in affinity of E for S or $S^{\neq}$.

Table 3
All the possible variations of the kinetic parameters for enzyme reactions based on the variations of standard Gibbs free energy levels of ES and ES$^{\neq}$ complexes[a]

k_{sp}	=	=	=	↓	↓			↓	↑	↑	↑		
K_M	=	↑	↓	=	↑			↓	=	↑	↓		
k_{cat}	=	↑	↓	↓	=	↑	↓	↓	↑	↑	=	↑	↓
Case	1	2	3	4	5	6	7	8	9	10	11	12	13

Key: (=) invariance; (↑) increase; (↓) decrease.
[a] $G^0{}_{ES}$ and $G^0{}_{ES\neq}$ from Table 2.

Table 3 contains the same cases reported in Table 1; however, in contrast to Table 1, which was based on the experimental kinetic approach, in which k_{cat} and K_M were the primary constants, and k_{sp} was the one to be calculated, in Table 3 the primary variations due to the $G^0{}_{ES\neq}$ and $G^0{}_{ES}$ levels concern k_{sp} and $K_{M,}$ while the variation of k_{cat} comes as a consequence.

Having obtained the general systematic picture of all possible variations of the constants and of the two G^0 levels from which they derive, it is now possible to observe that case 1 represents the reference (no variation). The other twelve cases are in reality constituted by six pairs within each of which one case mirrors another (e.g. k_{cat} ↑, K_M ↓, k_{sp} ↑ is specular to k_{cat} ↓, K_M ↑, k_{sp} ↓). It is thus possible to report six cases only: we selected cases 3–8.

Figures 1-6 each show the curve $v_0 = f([S])$ for both the control and changed situations; they also report an insert containing the G^0 profile for the considered situations. It is necessary to remember that k_{sp} is proportional to the slope at the origin of the curve $v_0 = f([S])$. For each case, we report the consequences stemming from each of the cited aspects of enzymology. These will then be unified on the basis of the graphs. It must be noted that for reversible inhibition the variations of the constants are apparent, being due to a multiplicative factor depending also on the concentrations; in G^0 profiles, it is possible to speak of "apparent variations of the G^0 levels."

3. Enzymological consequences deriving from case 3

3.1. Specificity

This is an interesting case of two isoenzymes with the same k_{sp} for one substrate, catalyzing the reaction at very different velocities for [S] values out of the condition $[S] \ll K_M$: "breakdown" case of k_{cat}/K_M as the specificity index. Similar considerations are also true for two different substrates acted upon by one enzyme only.

3.2. Enzyme evolution and enzymatic engineering

The specular case of the one shown in Fig. 1 (the mutant enzyme is indicated by a dotted line) is important in the co-evolution of enzymes and the physiological concentration of the substrate ($[S]_{physiological}$). At constant $k_{cat}/K_{M,}$ a proportional increase of k_{cat} and K_M (by means of mutations leading to an increase of both $G^0{}_{ES}$ and K_M) occurs, up to $K_M \approx [S]_{physiological,}$ a condition that actually optimizes the reaction rate at that value of [S].

3.3. Inhibitors and activators

Situation of the "reversible" incompetitive inhibition: a combination between inhibitor and ES complex may occur, leading to an increase of the apparent affinity of E for S. This can also describe a situation of an "irreversible" inhibition impairing the binding of E to S, but not to $S^{\neq}$.

4. Enzymological consequences deriving from case 4

4.1. Specificity

This is a regular case, in which $k_{sp} = k_{cat}/K_M$ is a good specificity index in any experimental condition.

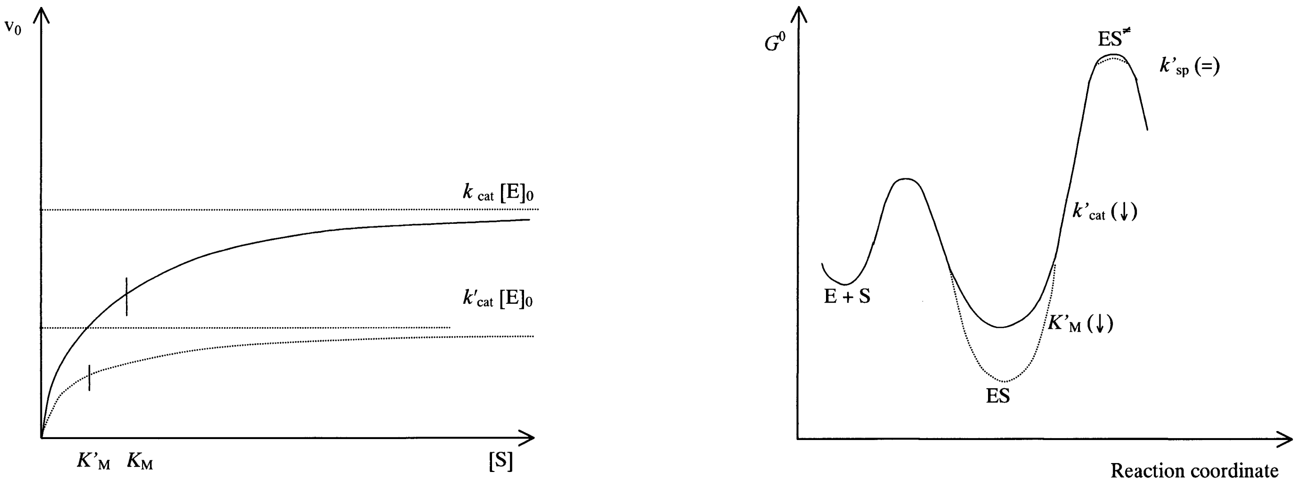

Fig. 1. Case 3, where $G^0{}_{ES\neq}$ (=), k_{sp} (=), $G^0{}_{ES}$ (↓), K_M (↓), and k_{cat} (↓); the decreases of k_{cat} and K_M are proportional.

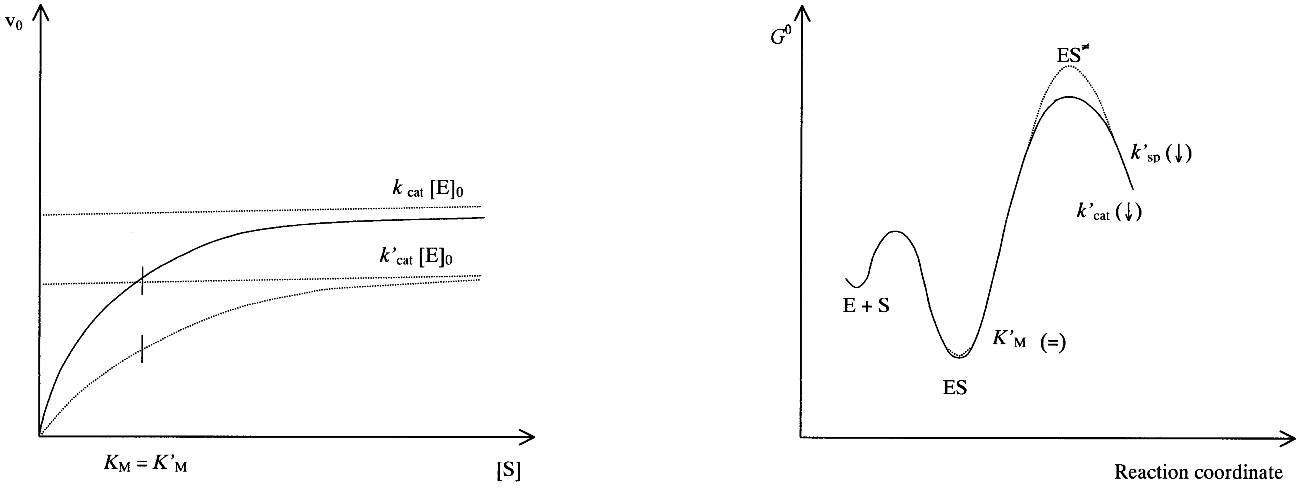

Fig. 2. Case 4, where $G^0_{\mathrm{ES}\neq}$ (↑), k_{sp} (↓), G^0_{ES} (=), K_{M} (=), and k_{cat} (↓).

4.2. Enzyme evolution and enzymatic engineering

The specular case of the one shown in Fig. 2 (the mutant enzyme is indicated by a dotted line) represents the evolution towards kinetic perfection: an increase in the affinity of E for $S^{\neq}$, with a consequent increase of $k_{\mathrm{sp}} = k_{\mathrm{cat}}/K_{\mathrm{M}}$.

4.3. Inhibitors and activators

Situation of “reversible” purely non-competitive inhibition: the affinity of E for S is unchanged, whereas a decrease of the apparent affinity for $S^{\neq}$ occurs (due to the formation of the ternary complex EIS, which, by not yielding the products, inactivates the complexed enzyme). This situation also represents a chemical modification of the enzyme (“irreversible” inhibition), which impairs the binding of E to $S^{\neq}$.

5. Enzymological consequences deriving from case 5

5.1. Specificity

Here, $k_{\mathrm{sp}} = k_{\mathrm{cat}}/K_{\mathrm{M}}$ is a good specificity index with the exception of V_{max} conditions (in experiments with substrates assayed one at a time) in which initial rates tend to be equal notwithstanding the different k_{sp} (Fig. 3).

5.2. Enzyme evolution and enzymatic engineering

The case is similar to the preceding one. The unmodified k_{cat} does not alter the evolution towards kinetic perfection, under the conditions in which the apparent kinetic constant is given by $k_{\mathrm{sp}} = k_{\mathrm{cat}}/K_{\mathrm{M}}$.

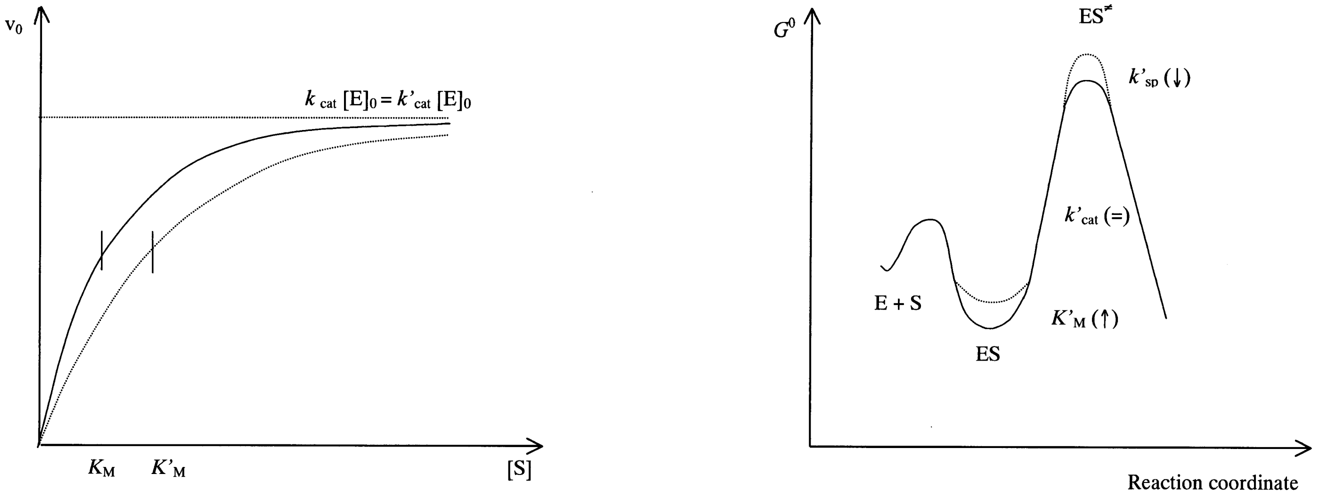

Fig. 3. Case 5, where $G^0_{\mathrm{ES}\neq}$ (↑), k_{sp} (↓), G^0_{ES} (↑), K_{M} (↑), and k_{cat} (=); $k_{\mathrm{cat}} = k_{\mathrm{sp}} \cdot K_{\mathrm{M}}$ is not changed, since the variations of k_{sp} and K_{M} are counterbalanced (equal variations of $G^0_{\mathrm{ES}\neq}$ and G^0_{ES}).

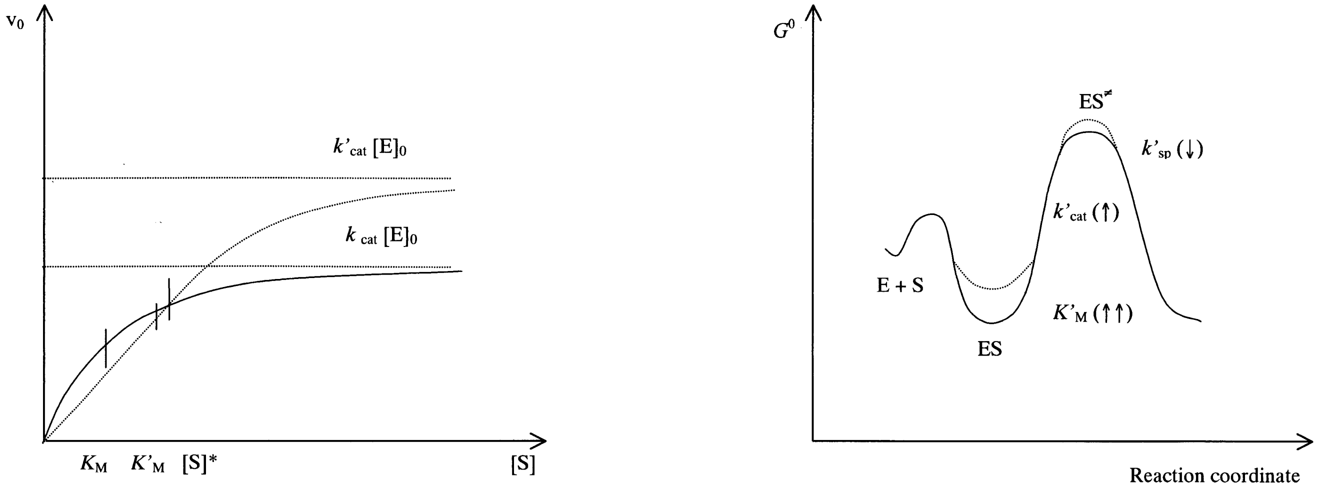

Fig. 4. Case 6, where $G^0_{ES\neq}$ (↑), k_{sp} (↓), G^0_{ES} (↑ ↑), K_M (↑ ↑), and k_{cat} (↑); $k_{cat} = k_{sp} \cdot K_M$ increases since K_M increases more than k_{sp} decreases (the levels of $G^0_{ES\neq}$ and G^0_{ES} are nearer). Note: the double arrow indicates a change greater than that with which it is compared.

5.3. Inhibitors and activators

Here we have a situation where there is "reversible" competitive inhibition, V_{max} is unchanged, and the apparent affinity of E for S decreases due to the competition with I. It is a situation of chemical modification to the enzyme ("irreversible" inhibition), which equally impairs the binding of the enzyme to both S and $S^{\neq}$.

6. Enzymological consequences deriving from case 6

6.1. Specificity

In this case (Fig. 4), experiments have been considered with substrates with different k_{sp} values assayed one at a time; here $k_{sp} = k_{cat}/K_M$ does not represent the specificity index at $[S] \geqslant [S]^*$ (concentration of "inversion"). The reaction rates are equal at $[S] = [S]^*$; at $[S] > [S]^*$ the rate is actually higher with substrates with low k_{sp} than those with higher $k_{sp.}$ Similar conclusions can be drawn by the comparison between two isoenzymes working on the same substrate and possessing the kinetic constant of case 6.

6.2. Enzyme evolution and enzymatic engineering

In addition to considerations on the evolution of cases 4 and 5, it is possible to observe that following an enzymatic mutation that does greater damage to the binding of S than of $S^{\neq}$, there is an increase in the catalytic power in the V_{max} condition. This phenomenon could be important in the use of engineered enzymes in biotechnology.

6.3. Inhibitors and activators

The effect of this variation is equal to that achieved by means of the biphasic effector (inhibitor for $[S] < [S]^*$, activator for $[S] > [S]^*$). A chemical that is an enzyme modifier would also act in this way (see the case of isoenzymes reported above in section 6.1).

7. Enzymological consequences deriving from case 7

7.1. Specificity

In this case (Fig. 5), k_{sp} represents a good index of specificity under all experimental conditions.

7.2. Enzyme evolution and enzymatic engineering

Considerations on the evolution are similar to those regarding cases 4–6. For the purposes of enzyme engineering, the specular case could be interesting in which an opportune mutation produces an increase of both k_{sp} and $k_{cat.}$ However, for reasons concerning the evolution of wild-type enzymes, such a mutation would be highly improbable.

7.3. Inhibitors and activators

This is a situation which occurs with mixed inhibition in which the apparent K_M increases and V_{max} decreases (mixed competitive/non-competitive inhibition).

8. Enzymological consequences deriving from case 8

8.1. Specificity

Under these conditions, k_{sp} represents a good index of specificity under all experimental conditions (Fig. 6).

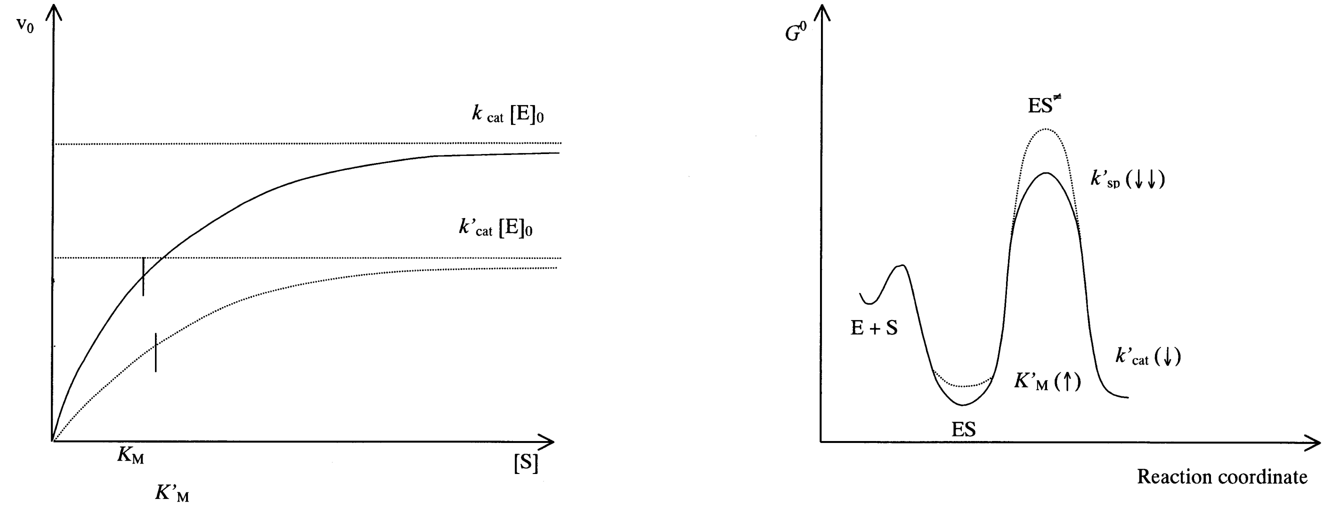

Fig. 5. Case 7, where $G^0_{ES\neq}$ (↑ ↑), k_{sp} (↓ ↓), G^0_{ES} (↑), K_M (↑), and k_{cat} (↓); $k_{cat} = k_{sp} \cdot K_M$ decreases, since the increase of K_M is lower that the decrease of k_{sp} (the levels of $G^0_{ES\neq}$ and G^0_{ES} becoming more different). See note in the legend of Fig. 4.

8.2. Enzyme evolution and enzymatic engineering

Conclusions are similar to those drawn in the preceding case. It can be observed that in this case, the mutation favors the binding with S and adversely affects binding with $S^{\neq}$.

8.3. Inhibitors and activators

This is an apparent situation of an inhibition similar to the incompetitive one. In this case, however, there is not a proportional decrease of K_M and V_{max} (namely mixed non-competitive/incompetitive inhibition).

Overall, all of these mathematically possible cases are realistic from the physico-chemical standpoint, as they are all related to realistic relative variations of G^0_{ES} and $G^0_{ES\neq}$ levels on the profiles of standard free energy [9]. Many of these cases can be readily found in the literature [10–25]. Moreover, all of the five known situations of reversible inhibition are included in five out of the six cases examined by us [3, 4]. Here, we will merely quote a reported case of the most unusual type (case 6), that of biphasic nature, which does not correspond to any of the five known types of inhibitors. Indeed, in a study on the NAD(P)H (quinone acceptor) oxidoreductase (DT-diaphorase) isolated from rat liver, the kinetic data regarding the two substrates daunorubicin and 1,4-dihydroxyanthraquinone showed that while the enzyme had a higher V_{max}/K_M value for 1,4-dihydroxyanthraquinone (8.14 vs 7.35 nmol NADPH oxidoreductase · mg protein^{-1} · min^{-1} · μM^{-1}), it had a greater V_{max} value for

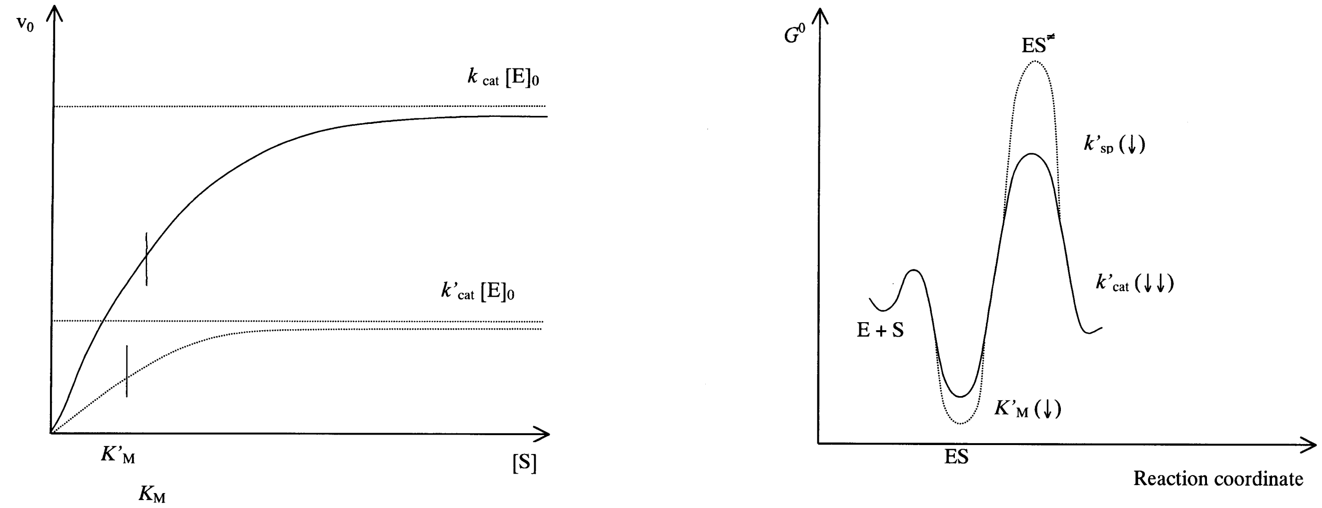

Fig. 6. Case 8, where $G^0_{ES\neq}$ (↑), k_{sp} (↓), G^0_{ES} (↓), K_M (↓), and k_{cat} (↓ ↓); $k_{cat} = k_{sp} \cdot K_M$ markedly decreases since both K_M and k_{sp} are lowered (the $G^0_{ES\neq}$ and G^0_{ES} levels becoming more different due to opposite variations); $k_{sp} = k_{cat}/K_M$ decreases since k_{cat} decreases more than K_M. See note in the legend of Fig. 4.

daunorubicin (380.1 vs 349.9 nmol NADPH oxidoreductase $\cdot$ mg protein^{-1} $\cdot$ min^{-1}) [26].

Finally, as regards the problem of the breakdown of k_{sp}, as the specificity index, analysis of all the cases examined by us gives rise to following rule: *for one enzymatic system to have a greater specificity (velocity) than another in any experimental condition, it is necessary (and sufficient) that the values of both* k_{sp} *and* k_{cat} *are greater in the first system than in the second.*

9. Conclusions

In summary, by examining all the possible types of variation of the three kinetic constants of enzymes, we have pinpointed a sixth possibility with distinct functional and kinetic properties, which had previously evaded classification. This novel enzymatic situation, which is biphasic, has practical relevance (we provide an example of it in relation to two substrates of a well-known chemotherapeutic agent) and could find applications in biotechnology and chemotherapy studies. Finally, our analysis of all the six possible enzymatic situations leads us to propose some modification to the commonly accepted definition of enzyme specificity.

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