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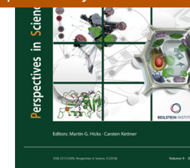

In search of lost time constants and of non-Michaelis—Menten parameters☆

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Summary Upon completing 100 years since it was published, the work *Die Kinetik der Invertinwirkung* by Michaelis and Menten (MM) was celebrated during the 6th Beilstein ESCEC Symposium 2013. As the 7th Beilstein ESCEC Symposium 2015 debates enzymology in the context of complex biological systems, a post-MM approach is required to address cell-like conditions that are well beyond the steady-state limitations. The present contribution specifically addresses two hitherto ambiguous constants whose interest was, however, intuited in the original MM paper: (i) the characteristic time constant τ_∞, which can be determined using the late stages of any progress curve independently of the substrate concentration adopted; and (ii) the dissociation constant K_S, which is indicative of the enzyme—substrate affinity and completes the kinetic portrayal of the Briggs—Haldane reaction scheme. The rationale behind τ_∞ and K_S prompted us to revise widespread concepts of enzyme's efficiency, defined by the specificity constant k_{cat}/K_M, and of the Michaelis constant K_M seen as the substrate concentration yielding half-maximal rates. The alternative definitions here presented should help recovering the wealth of published k_{cat}/K_M and K_M data from the criticism that they are subjected. Finally, a practical method is envisaged for objectively determining enzyme's activity, efficiency and affinity — $(EA)^2$ — from single progress curves. The $(EA)^2$ assay can be conveniently applied even when the concentrations of substrate and enzyme are not accurately known.
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Introduction

The year of 2013 marked the one hundredth anniversary of the publication of the classic Michaelis and Menten (MM) paper *Die Kinetik der Invertinwirkung* (Michaelis and Menten, 1913), which became the standard approach to quasi-steady-state (QSS) enzyme kinetics. Supported by the work of earlier authors, most notably Brown (1902) and Henri (1902, 1903), MM understood the significance of pH control in enzymatic experiments and acknowledged that initial rates were easier to interpret than time courses as they are not restrained by issues such as the reverse reaction, product inhibition or enzyme inactivation (Cornish-Bowden, 2012). Modern representations of the MM model use the Briggs and Haldane reaction scheme encompassing the reversible combination of free enzyme E and substrate S to form the enzyme—substrate complex ES followed by its irreversible transformation into product P and release of enzyme (Eq. (1)) (Briggs and Haldane, 1925)

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \overset{k_2}{\longrightarrow} E + P \tag{1}$$

where k_1 and k_{-1} are the rate constants of the reversible binding step and k_2 is the rate constant of the catalytic step. The evolution of the concentration of the different species with time t is mathematically described by the following system of first-order differential equations

$$\frac{d[S]}{dt} = -k_1[E][S] + k_{-1}[ES] \tag{2}$$

$$\frac{d[ES]}{dt} = k_1[E][S] - (k_{-1} + k_2)[ES] \tag{3}$$

$$\frac{d[E]}{dt} = -k_1[E][S] + k_{-1}[ES] + k_2[E] \tag{4}$$

$$\frac{d[P]}{dt} = k_2[ES] \tag{5}$$

subject to the initial conditions ([S], [E], [ES], [P]) = ($S_0, E_0, 0, 0$). Although the analytical solution of Eqs. (2)—(5) is not known (Berberan-Santos, 2010), a simplified alternative results from adopting the QSS approximation stating that, in the presence of a large excess of substrate, the concentration of the enzyme—substrate complex remains constant after the initial ES build-up period has ended (Briggs and Haldane, 1925). If, in addition, the duration of the transient period is short enough to assume invariant [S], the reactant stationary approximation is applicable (Hanson and Schnell, 2008), and the final form of the MM equation is obtained (Eq. (6))

$$v_0 = \frac{V_{\max} S_0}{K_M + S_0} \tag{6}$$

with v_0 being the initial reaction rate; $V_{\max}$, the limit reaction rate obtained for very high substrate concentration values; and K_M, the Michaelis constant. In the Briggs and Haldane notation $V_{\max}$ corresponds to k_2E_0 and K_M corresponds to $(k_{-1} + k_2)/k_1$; in practical terms, $V_{\max}$ is written as $k_{cat}E_0$ to extend its use to reaction schemes of higher complexity than Briggs and Haldane's, while K_M is commonly referred as the concentration of substrate for which $v_0 = 0.5V_{\max}$. The QSS and the reactant stationary approximations severely limit the applicability of the MM equation to the initial phases of enzymatic reactions that start with great substrate excess over the enzyme ($S_0 \gg E_0$) (Pinto et al., 2015; Segel, 1988; Hanson and Schnell, 2008). With the publication of the Pinto et al. (PEA) model in 2015, additional threats associated to the usage of the classical formalism were identified, at the same time that the ''whole picture'' of single active-site enzyme kinetics without inhibition was revealed (Pinto et al., 2015). The PEA model also uncovered new applications or ''hidden meanings'' in the Briggs and Haldane mechanism, of which the present contribution particularly focuses the cases of the characteristic time constant τ_∞ and of the dissociation constant K_S. These parameters were chosen as they help to answer some of the new problems posed by Systems Biology while studying increasingly realistic enzymatic networks. Not only that the following sections illustrate how τ_∞ and K_S can be used to characterize enzymatic activity, enzymatic efficiency and enzyme—substrate affinity in a straightforward and unambiguous manner.

Numerical procedures

The system of differential equations describing the Briggs and Haldane reaction scheme (Eqs. (2)—(5)) was expressed in normalized units as Eqs. (7)—(9) (Pinto et al., 2015)

$$-\left(1 - \frac{K_S}{K_M}\right)\frac{ds}{d\theta} = e_0 s - c\left(\frac{K_S}{K_M} + s\right) \tag{7}$$

$$\left(1 - \frac{K_S}{K_M}\right)\frac{dc}{d\theta} = e_0 s - c(1 + s) \tag{8}$$

$$\frac{dp}{d\theta} = c \tag{9}$$

where $s = [S]/K_M$, $c = [ES]/K_M$, $p = [P]/K_M$, $\theta = k_2 t$ and $K_S = k_{-1}/k_1$. Enzymatic reaction progress curves showing the evolution of scaled product concentration p over scaled time θ were simulated with Mathworks® MATLAB R2013b. A script was developed to this end in which a MATLAB ordinary differential equation (ODE) solver was employed to numerically solve Eqs. (7)—(9) over the scaled time. The specific ODE solver used to this effect was ode45, a one-step solver (i.e. when computing the solution for t_n, the solver only requires the solution at the immediately preceding time point, t_{n-1}) based on an explicit Runge—Kutta(4,5) formula, the Dormand-Prince pair (Dormand and Prince, 1980). Numerical solutions were obtained over different ranges of integration of θ for limiting values of the scaled dissociation constant K_S/K_M and for different sets of e_0 and s_0 initial conditions.

Results

The characteristic time constant (τ_∞) and the enzyme efficiency

The analytical solution describing single active-site enzyme kinetics without inhibition was obtained after introducing the ''pivotal variable'' $(S_0 - P)/v$ representing the concentration of product still to be formed $(S_0 - P)$ over the instant reaction rate v (Pinto et al., 2015). Fig. 1A illustrates the

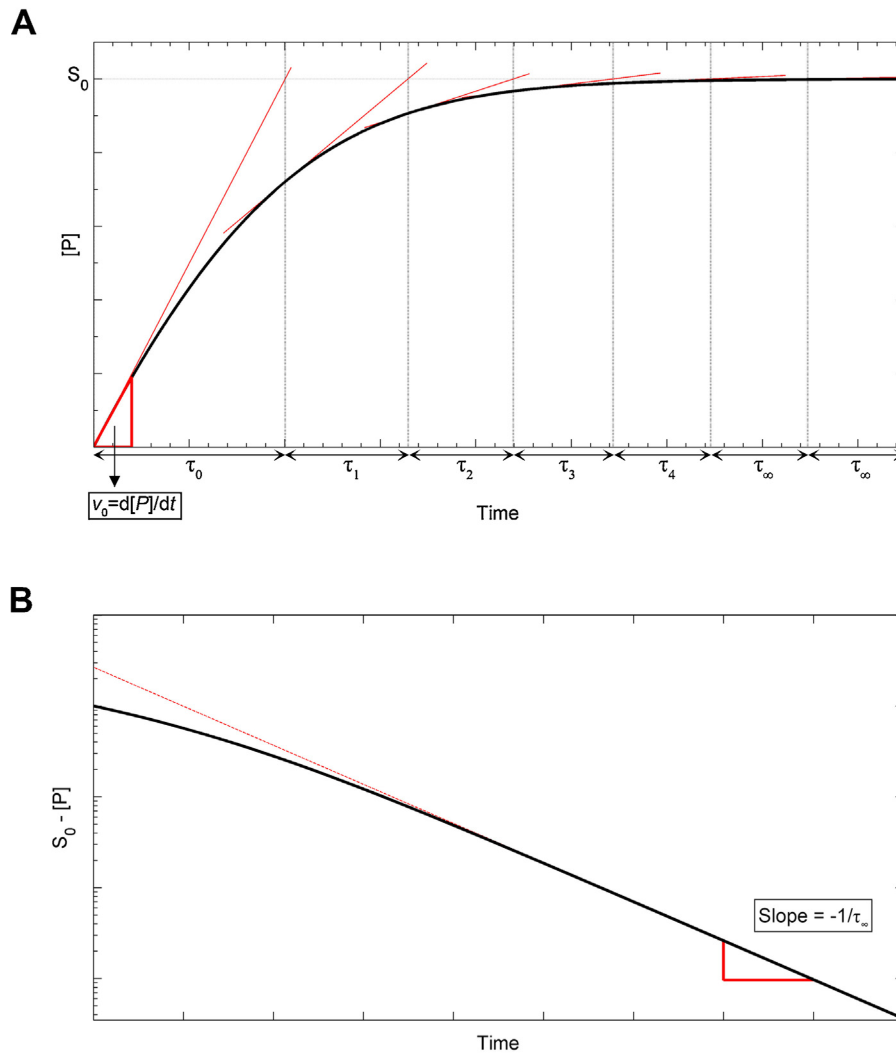

Figure 1 Different representations of the theoretical progress curve obtained from the numerical solution of the ODE system comprising Eqs. (7)—(9) using $S_0/K_M = 1$, $E_0/K_M = 0.01$ and $K_S/K_M = 1$. (A) Product concentration [P] represented over time t in a linear plot. Red tangent lines represent the period of time τ_n that would be required to complete the reaction if the instant reaction rates were maintained. For long reaction times this period of time tends to the value of the characteristic time constant τ_∞. The slope of the initial tangent corresponds to the value of the initial reaction rate v_0. (B) Log-linear plot of the concentration of product still to be formed ($S_0 - P$) as a function of time. The slope of final tangent (red dashed line) corresponds to the negative reciprocal of the characteristic time constant.

physical meaning of the pivotal variable as the period of time τ_n that would be required to complete the reaction if the instant reaction rate was maintained. Alternatively, the negative reciprocal of this variable is promptly computed as the instantaneous slope of the ($S_0 - P$) time—course curve represented in a log-linear scale (Fig. 1B). The asymptotic limit of $(S_0 - P)/v$ for late reaction phases is here defined as the characteristic time constant τ_∞ and corresponds to the reciprocal of the ''integration constant'' shown in the original MM paper to be independent of the initial substrate concentration (Michaelis and Menten, 1913). Later interpretation of QSS results identified the integration constant as the specificity constant k_2/K_M (or, more generically, k_{cat}/K_M) multiplied by the enzyme concentration (Johnson and Goody, 2011), while its reciprocal corresponds to the period of time τ needed to completely exhaust the existing substrate if the initial reaction rate is maintained and the enzyme is operating under first-order conditions (Cornish-Bowden, 1987). Despite the similarities between the latter definition and our own definition of τ_∞, the following differences should be noted: the time constant τ is defined in relation to the initial reaction rates under QSS conditions, whereas τ_∞ is concerned with the late reaction phases under whatever experimental conditions. From the definition of the pivotal variable for long reaction times given in the Supporting Information of the PEA paper (Pinto et al., 2015), the following relationship exists between τ_∞ and τ (Eq. (10)):

$$\tau_\infty = \frac{\tau}{2}\left(1 + e_0 + \sqrt{(1 + e_0)^2 - 4\left(1 - \frac{K_S}{K_M}\right) e_0}\right) \tag{10}$$

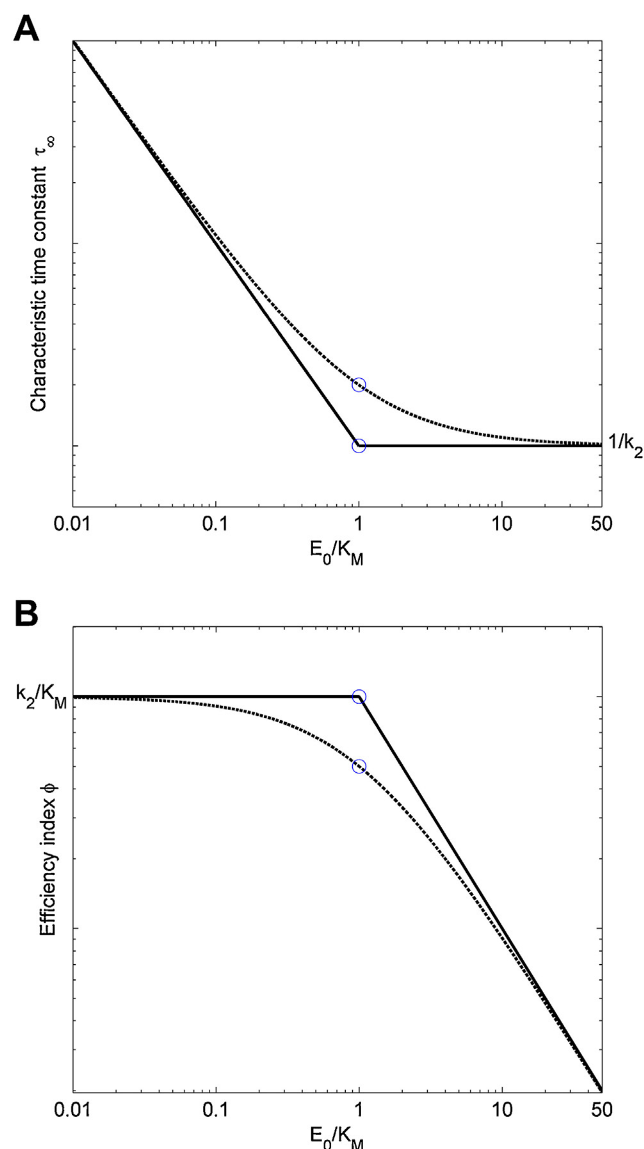

Figure 2 The characteristic time constant and enzyme efficiency. Log—log plots depicting the influence of the K_M-normalized enzyme concentration on the (A) characteristic time constant τ_∞ and on the (B) efficiency index ϕ for limiting values of the scaled dissociation constant $K_S/K_M = 0$ (solid lines) and $K_S/K_M = 1$ (dashed lines). The blue round markers show the point where the largest difference between both curves is observed. (A) The smallest value for the characteristic time is obtained for $E_0 > K_M$. (B) The maximal efficiency index ϕ_{max} is obtained for $E_0 < K_M$.

The representation of this function in Fig. 2A takes into account the alternative definition of $1/\tau$ as $k_2 e_0$ to show that the shortest characteristic time corresponds to $1/k_2$ and is obtained for enzyme concentrations above the Michaelis constant. This compromise between finishing reaction rates and enzyme concentration motivated us to propose an efficiency index ϕ balancing kinetic performance over the enzyme expenditure:

$$\phi = \frac{1/\tau_\infty}{E_0} \qquad (11)$$

Defined in this way, enzyme efficiency is exempted from the practical limitations of the specificity constant, whose application to compare the catalytic efficiency of different enzymes in the catalysis of the same substrate has been discouraged (Eisenthal et al., 2007). In fact, by attending to the final phases of the enzymatic reaction, the definition of ϕ is free from the ambiguities caused by the role of the substrate concentration on the initial reaction rates (Eisenthal et al., 2007). On the other hand, the fact illustrated in Fig. 2B that the maximum value of efficiency ϕ_{max} corresponds to the value of k_2/K_M (or, more generically to k_{cat}/K_M), might be extremely convenient so as to recover published k_{cat}/K_M data from any misgivings while comparing the efficiency of different enzymes. Finally, and as addressed more in detail in the discussion section, the efficiency index can be straightforwardly estimated from a single enzymatic assay using Eq. (11) and the values of τ_∞ determined as described in Fig. 1B.

The K_S/K_M ratio and the enzyme—substrate affinity

In the original MM paper, the now-called Michaelis constant K_M was defined as the protein—ligand dissociation constant (Michaelis and Menten, 1913), which for enzyme—substrate complexes is now commonly represented by K_S. Comparing their mathematical formulations given in the introduction part shows that the catalytic step (rate constant k_2) must be much slower than the unbinding step (rate constant k_{-1}) for K_M to be equivalent to K_S (Baici, 2015). In the PEA paper, K_S is referred to as a non-MM constant, which, together with K_M and V_{max}, completes the portrayal of the 3-parameter mechanism proposed by Briggs and Haldane (Pinto et al., 2015). Fig. 3 shows two sets of theoretical curves simulated for enzyme concentrations much lower than K_M (Fig. 3A) and equal to K_M (Fig. 3B) to illustrate the peculiar role of K_S in both situations. Fig. 3A partly explains the absence of K_S from QSS kinetic analysis, seeing that the enzyme—substrate affinity has a weak effect on the progress curves, which is only visible for product conversions below 5%, and considering substrate concentrations S_0 close to E_0. This does not mean that K_S is equivalent to K_M, only that the effect of K_S is masked under conditions of great substrate excess. In the other extreme, experimental conditions for which the enzyme concentration is of the same order of magnitude of K_M (and $S_0 \leq E_0$) are expected to clearly reveal the effect of K_S during initial and late phases of the progress curves (Pinto et al., 2015); for this reason, and because of the biological interest, this is considered a ''critical region of conditions'' that is potentially representative of an intracellular environment (Schnell and Maini, 2000; Tzafriri, 2003; Bersani and Dell'Acqua, 2011). Fig. 3B shows that asymptotically high affinities between enzyme and substrate ($K_S/K_M = 0$) should produce characteristic product accumulation curves with sigmoidal (rather than hyperbolic/linear) onsets. Since low K_S/K_M ratios mean much faster product formation rates than enzyme—substrate dissociation rates, it might be technically difficult to access the earlier phases of such kinetic curves and discern their shape, especially when high enzyme concentrations are involved. The PEA alternative to estimate the value of K_S/K_M is through the characteristic time constant τ_∞, which, as described in the previous subsection, can be straightforwardly obtained from a single enzymatic assay.

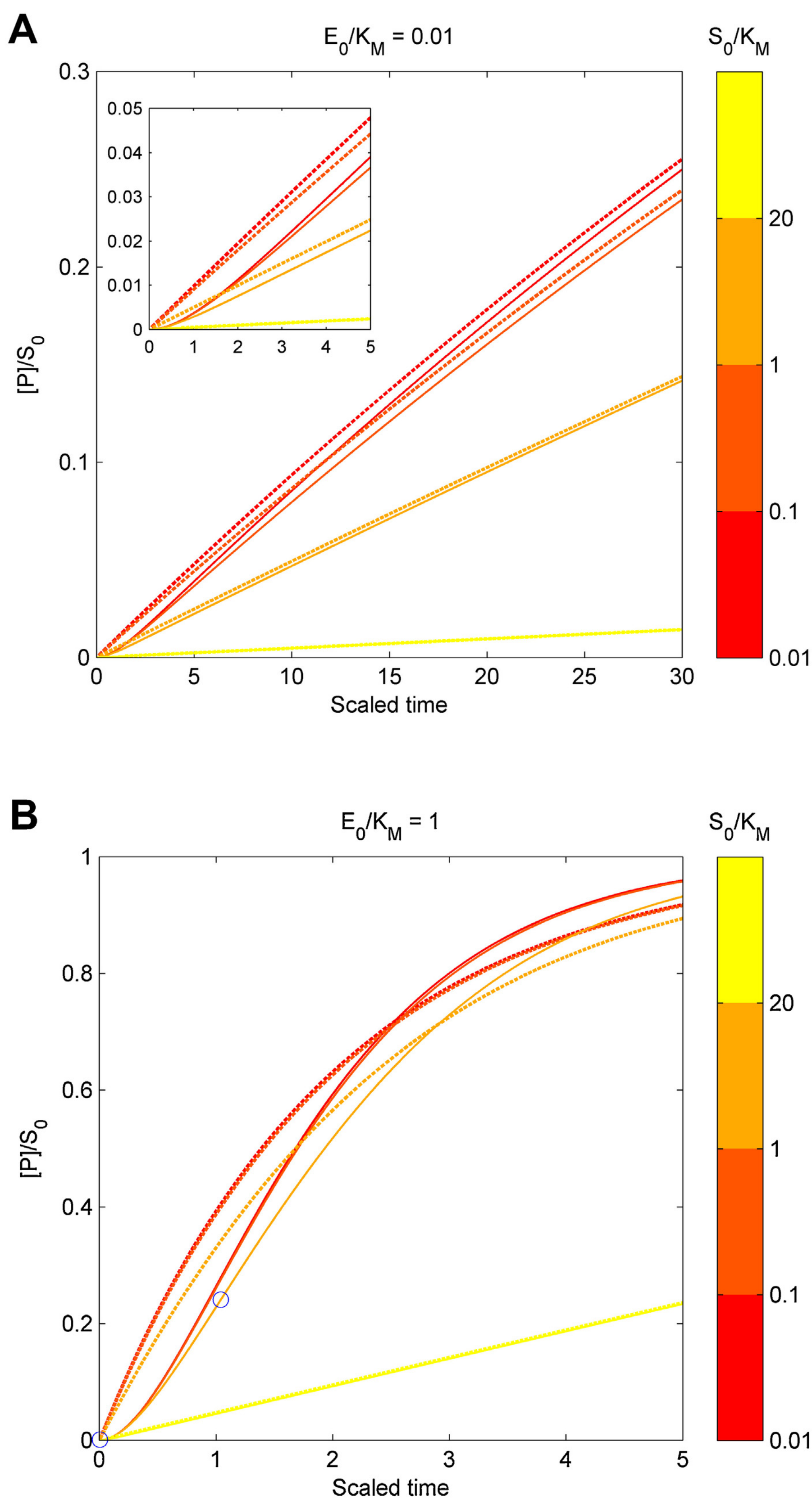

Figure 3 Major differences between theoretical progress curves calculated for limiting values of the dissociation constant $K_S/K_M = 0$ (solid lines) and $K_S/K_M = 1$ (dashed lines). Progress curves represented as the linear plots of the normalized product concentration $[P]/S_0$ over the scaled time $\theta = k_2 t$. The system of ODE comprising Equations 7—9 was solved using the set of S_0/K_M values indicated in the log-scaled color bars for (A) $E_0/K_M = 0.01$ and (B) $E_0/K_M = 1$. (B) The blue round markers on the curves obtained for $S_0/K_M = 1$ indicate the stationary moment for which the maximum reaction velocity is reached.

Given that the characteristic time constant is independent of the initial substrate concentration, values of S_0 as high as the solubility limit can be adopted in order to extend the duration of the catalytic reactions over technically accessible time periods. As previously represented in Fig. 2A, the influence of E_0 on τ_∞ is not significantly affected by the value of the K_S/K_M ratio, unless enzyme concentrations close to K_M are considered. This window of conditions is, therefore, recommended to estimate the dissociation constant from experimentally determined characteristic time constants. The K_S/K_M value follows directly from Eq. (10) rewritten as Eq. (12)

$$\frac{K_S}{K_M} = 1 + (k_{cat}\tau_\infty)^2 \frac{E_0}{K_M} - (k_{cat}\tau_\infty)\left(1 + \frac{E_0}{K_M}\right) \quad (12)$$

which requires previous estimations of the MM parameters using, for example, the PEA model equations (14) in the appendix section or the MM equation (Eq. (6) for QSS conditions only). In the Discussion section we anticipate some of the practical and fundamental consequences arising from the accurate knowledge of the parameter K_S.

Discussion

The present work is the first follow-up of the PEA model, which, as the acronym incidentally suggests, is envisaged to seed several other future applications in modern enzymology. Specifically, we took the opportunity at the 7th Beilstein ESCEC Symposium to expand the meaning and practical significance of the characteristic time constant τ_∞ and of the equilibrium dissociation constant K_S. The relevance of these parameters was already intuited in the 1915 paper of MM, seeing that $1/\tau_\infty$ and K_S correspond, in the limit cases, to the original ''integration constant'' and to the Michaelis constant, respectively. More than enlarging the QSS scope, our approach motivates a renewed interpretation of the fundamental meaning of MM and non-MM kinetic constants. For example, enzyme efficiency defined in relation to τ_∞ is not affected by the concentration of substrate and, therefore, it is free from the ambiguities associated to the specificity constant defined as the k_{cat}/K_M ratio extracted from initial velocity experiments (Eisenthal et al., 2007). A direct indicator of the enzyme's kinetic performance, the value of $1/\tau_\infty$ is also an apparent first-order rate constant that increases with the concentration of enzyme until the upper limit of k_2 is attained for $E_0 > K_M$ (Fig. 2A). Consequently, enzyme efficiency is here presented as the kinetic performance balanced over the total enzyme expenditure $\phi = 1/(\tau_\infty E_0)$. Fig. 2B showed that the efficiency index reaches a maximal value of k_2/K_M that is nearly invariant for enzyme concentrations below K_M. This value of ϕ_{max}, which operationally corresponds to k_{cat}/K_M, can be used to compare the catalytic effectiveness of different enzymes for technological applications or for enzyme evolution studies. As the differences summarized in Table 1 intend to illustrate, the numerical equivalence between ϕ_{max} and the specificity constant is circumstantial and does not imply a common underlying principle. Different fundamental definitions (#4 and #5 in Table 1) stipulate different methodological procedures for the determination of the two indicators (#1 to #3 in Table 1) which, nevertheless, should produce the same numerical results, provided that the Briggs and Haldane mechanism holds true. Reaction schemes involving product inhibition may originate different values of k_{cat}/K_M if estimated as ϕ_{max} or as a specificity constant (#6 in Table 1). Although product inhibition is not contemplated by the PEA model, the common usage of apparent rate constants (such as k_{cat}) as an approximation to true rate constants (such as k_2) might also be extended to the efficiency index, whose apparent value may help to characterize quantitatively the deviations from Briggs and Haldane kinetics.

Table 1 Different interpretations of k_{cat}/K_M in the light of the MM model (as a specificity constant) and in the light of the PEA model (as the maximal enzyme efficiency ϕ_{max}). Differences 1—3 concern parameter estimation methodologies; differences 4 and 5 concern kinetic and operational meanings, respectively; difference 6 concerns reaction schemes other than Briggs and Haldane's.

#	Specificity constant	ϕ_{max}
1	Estimated based on *initial* reaction rates v_0	Estimated based on the characteristic time constant τ_∞ during *late* reaction phases
2	*Limited* to QSS experimental conditions	Estimations of ϕ are *not limited* to any experimental condition; ϕ_{max} is reached for $E_0 < K_M$
3	Substrate concentration *influences* the v_0-based enzyme's efficiency (Eisenthal et al., 2007)	Substrate concentration *does not* influence τ_∞-based enzyme's efficiency
4	Corresponds to an apparent second-order rate constant	Corresponds to an apparent first-order rate constant expressed per units of enzyme concentration
5	Sets the *lower* limit for enzyme-substrate association rate constant (Fersht, 1999)	Sets the *upper* limit of the ratio enzyme performance/enzyme expenditure
6	It is *not* affected by product inhibition	It may be affected by product inhibition

Another MM parameter subject to a renewed PEA perspective is the Michaelis constant itself. Appointed as less important than the parameters k_{cat} and k_{cat}/K_M (Johnson and Goody, 2011), the value of K_M is frequently defined as the concentration of substrate producing $v_0 = 0.5V_{max}$; on the other hand, the formulation $K_M = (k_{-1} + k_2)/k_1$ indicates that the Michaelis constant is an overall/apparent dissociation constant of all enzyme-bound species (Fersht, 1999). The latter definition is directly concerned with the enzyme—substrate affinity, which can be characterized accurately using true dissociation constants (K_S) determined as described in the previous subsection. The PEA model additionally shows that the first definition of K_M (as the substrate concentration yielding half-maximal rates) loses its validity outside the region of QSS conditions (Pinto et al., 2015). For example, for $E_0 > S_0$ the initial reaction rate v_0

becomes linear dependent on the substrate concentration in the cases of very low enzyme—substrate affinity ($K_S/K_M \sim 1$) — see Eq. (14b) in the appendix section. Instead, Fig. 2 confers to parameter K_M the biophysical significance of a threshold enzyme concentration. According to Fig. 2A, K_M is the smallest enzyme concentration required to achieve the shortest completion time, i.e. required to conclude the enzymatic reaction at the fastest rates. Perhaps more useful for *in vivo* and *in vitro* kinetic analysis, Fig. 2B presents K_M as the maximum enzyme concentration that can be kept without losing catalytic efficiency — after this limit, increasing enzyme expenditure no longer accelerates the concluding reaction phases. Curiously enough, enzyme concentrations close to the value of K_M are also the most favorable to experimentally investigate the effect of the enzyme—substrate dissociation constant on the characteristic time constant (Eq. (10)). According to this new angle of approach, enzyme efficiency can be regulated by dynamically controlling the enzyme's abundance in the cell. Concentration levels close to the reference value of K_M are important for the enzyme to be critically sensible to the structural affinity of different metabolites. By systematically adopting QSS conditions, it is conceivable that *in vitro* enzymatic assays have been missing kinetic aspects of metabolic homeostasis that are important (Pinto et al., 2015), for example, in molecular systems biology (Finn and Kemp, 2014) and in drug discovery (Acker and Auld, 2014; Yang et al., 2009; Sols and Marco, 1970).

The enzyme—substrate affinity is important to define which catalysis occurs preferentially in a cellular environment crowded with multiple enzymes and substrates that possibly act as competitors toward each other. Therefore, the explanation for the apparent disregards of the dissociation constant K_S compared to k_{cat} or K_M resides in the lack of straightforward methods to estimate this non-MM constant. Existing methods for the determination of all individual rate constants require specific techniques designed to measure transient-state kinetics, the interpretation of which is not exempted from simplifying hypothesis such as the reactant stationary approximation during the pre-steady-state phases (Hanson and Schnell, 2008; Fersht, 1999) or the linearization of the reaction mechanism for time—relaxation analysis (Cornish-Bowden, 2012). These limitations are not present in the PEA method for the determination of K_S using the characteristic time constant and Eq. (12). By facilitating the characterization of enzyme specificity, we also expect to contribute to the understanding of enzyme evolution and enzyme promiscuity, upon which the design of novel biological functions is based (Pandya et al., 2014). A quantitative description of the enzyme response to alternative substrates is now possible using true dissociation constants as an alternative to entropic predictions based on the k_{cat}/K_M ratio (Nath and Atkins, 2008).

A single assay to estimate enzyme activity, efficiency and affinity (EA)^2

Estimating the MM parameters requires different enzymatic reactions to be carried out adopting substrate concentrations S_0 above and below K_M and in great excess over the enzyme ($S_0 \gg E_0$). Although the usage of a single progress curve to determine K_M and $V_{\max}$ is theoretically possible, this procedure is discouraged in practice in view of the undefined time span over which the QSS approximation is valid (Duggleby, 2001). The insights provided by the PEA model let us envisage a new method to determine the classic parameters from a single enzymatic reaction and in an unbiased manner. In addition, the information thus, obtained can be used to analyze a second progress curve to estimate the non-MM parameter K_S. Because this method characterizes enzyme activity, efficiency and affinity we call it the (EA)^2 assay. In principle, the (EA)^2 assay involves the following steps:

1. Measure the progress curve of the enzymatic reaction under typical QSS conditions ($S_0 \gg E_0$).
2. Determine the initial reaction rate v_0 as indicated in Fig. 1A.
3. Determine the characteristic time constant τ_∞ as indicated in Fig. 1B. Assume that $\tau_\infty = \tau$.
4. Estimate $V_{\max}$ from Eq. (6) (Equation MM) rewritten as $V_{\max} = v_0 S_0/(S_0 - v_0\tau)$.
5. Estimate $K_M = V_{\max}\tau$.
6. The condition $\tau_\infty = \tau$ in step 3 is only valid for $E_0 \ll K_M$ (Fig. 2A). Check if $E_0 < 0.1K_M$.

6.1. If not, restart with a more diluted enzyme solution.

7. Estimate enzyme's activity as $V_{\max}/E_0$ (equivalent to k_{cat}).
8. Estimate the maximal enzyme's efficiency $\phi_{\max} = 1/(\tau_\infty E_0)$ (corresponding to k_{cat}/K_M for the conditions of step 6).
9. Measure a new progress curve adopting $E_0 = K_M$ and determine a new value of τ_∞.
10. Estimate the dissociation constant K_S characterizing the enzyme—substrate affinity. Use the value of τ_∞ estimated in step 9 and Eq. (12) rewritten as $K_S/K_M = (1 - k_{cat}\tau_\infty)^2$.

Notably, this method does not require to know an accurate value of the substrate concentration S_0, provided that this value is assuredly much higher than the product $v_0\tau_\infty$ so as to obtain $V_{\max} = v_0$ in step 4. The MM parameters can alternatively be determined using the PEA model in Eqs. (14) in the appendix section or the MM equation (Eq. (6) for QSS conditions only). When the enzyme molarity is not accurately known, the (EA)^2 assay might also be useful to estimate the lower limit of the catalytic power taking into consideration that E_0 estimates such as absorbance readings at 280 nm are in excess, thus, yielding lower limits of enzyme activity $V_{\max}/E_0$ and of enzyme efficiency $1/(\tau_\infty E_0)$. In another instance, if only the amount of impure powdered enzyme is known, enzyme efficiency can be expressed in units of $s^{-1}(mg/l)^{-1}$ as an alternative to $s^{-1}M^{-1}$, similarly to what happens with the catalytic activity expressed as the amount of enzyme converting the substrate into product at a given rate (1 mol/s or 1 μmol/min for katal or international unit IU, respectively). It may occur that the (EA)^2 assay fails to produce useful data because of either too slow or too fast enzymatic reactions; in the first case, sample conditions may not be maintained with time (e.g. protein degradation leading to enzyme-activity loss); in the latter case, the reaction may finish before any valid measurement is performed — especially under the $E_0 = K_M$ conditions of step 9. The solution to these problems involves decreasing or increasing of

the substrate concentration values within the operational limits to prolong or shorten the reaction span to convenient limits. Obtaining enzyme samples as concentrated as the K_M-order of magnitude might also not be possible in practice. In those cases, the estimation of the K_S/K_M ratio is still possible using the initial phases of the progress curves measured using dilute enzyme solutions (Pinto et al., 2015). During the application of the PEA model and, in particular, of the $(EA)^2$ assay, the Briggs and Haldane mechanism is implicitly assumed to be valid. As previously discussed in Table 1, deviations from this mechanism can be identified by comparing the estimations of MM parameters obtained from initial and late phases of the enzymatic reactions using, in one case, Equation 14 in the appendix section, and in the other, the characteristic time constant τ_∞. We intend to keep developing the ideas organized in this paper by applying them on the characterization of enzymatic systems with biological and industrial interest.

Conclusion

Firstly published in the same year of the classic MM paper, Marcel Proust's novel *À la Recherche du Temps Perdu*, In Search of Lost Time (1913–1927), gives the motif for the title of the present contribution, in which we try to recuperate the fundamental meanings of the characteristic time constant τ_∞ and of the equilibrium dissociation constant K_S. This exercise is based on the recently published PEA model that provides, after a long wait, the closed-form solution of the Briggs and Haldane kinetic mechanism (Pinto et al., 2015). Although the Briggs and Haldane mechanism is the minimal reaction scheme needed to explain enzyme catalysis, it remained very incompletely described by the existing analytical solutions. The pivotal variable of the PEA model measured for late reaction phases gives a practical estimate of the characteristic time constant τ_∞, which in turn is helpful to clarify the concepts of enzyme efficiency and selectivity. The maximal enzyme efficiency $\phi_{\max}$ corresponds to the value of $1/(\tau_\infty E_0)$ measured for concentrations of enzyme below K_M (Fig. 2B). Parameter $\phi_{\max}$ is expected to help in recovering the wealth of published k_{cat}/K_M data from the criticism it has been voted as an efficiency standard: although both parameters are, in most cases, numerically equivalent, $\phi_{\max}$ is free from the conceptual limitations of k_{cat}/K_M (Table 1). The PEA framework also provides a renewed perspective of the somewhat obscure Michaelis constant K_M as a threshold enzyme concentration above which the catalytic efficiency starts to decrease. The practical definition of K_M as the substrate concentration yielding half-maximal rates should be adopted carefully as it loses accuracy under non-QSS conditions. The true dissociation constant K_S can now be straightforwardly determined from a single progress curve without requiring specific experimental arrangements or model simplifications. Besides completing the Briggs and Haldane portrayal of the catalytic cycle, this parameter objectively characterizes the affinity of the enzyme to different substrates, thus, contributing to the study of enzyme evolution and promiscuity. Summarizing our conclusions, a practical method to determine enzyme activity, efficiency and affinity from single progress curves is proposed, in which model parameters are rapidly estimated even if the concentrations of substrate and enzyme are not accurately known.

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Appendix A. Appendix

The following equations comprise the overall and stationary formulations of the PEA model as described by Pinto et al. in 2015. The overall analytical solution corresponds to Eq. (13a), where scaled variables are used, namely $\tau = K_M/V_{\max}$, $e_0 = E_0/K_M$, $s_0 = S_0/K_M$, $\theta = t/(e_0\tau)$ and $\beta = 1 - K_S/K_M$.

$$\frac{S_0 - P}{v} = \frac{\tau}{2}\left(1 + e_0 + \tilde{s} + \frac{\tilde{\lambda}}{\tanh(\tilde{\lambda}\theta/2\beta)}\right) \quad (13a)$$

The corresponding daughter variables $\tilde{s}$, $\tilde{\lambda}$, s^* and θ^* are given by Eqs. (13b)–(13e). The value of λ^* in Eq. (13e) corresponds to the value of $\tilde{\lambda}$ calculated by Eq. (13c) for $\tilde{s} = s^*$. The superscript asterisk is indicative of stationary conditions, occurring after the initial fast transient period of $[ES]$ build-up has taken place.

$$\tilde{s} = \omega(s^* \exp(s^* - e_0(\theta - \theta^*))) \quad (13b)$$

$$\tilde{\lambda} = \sqrt{(1 + e_0 + \tilde{s})^2 - 4\beta e_0} \quad (13c)$$

$$s^* = \frac{1}{2}\left(s_0 - 1 - e_0 + \sqrt{(s_0 + e_0 + 1)^2 - 4e_0 s_0}\right) \quad (13d)$$

$$\theta^* = \frac{2\beta \operatorname{arctan\,h}(\lambda^*/(1 + e_0 + s^*))}{\lambda^*} \quad (13e)$$

The choice of the stationary instant t^* is in order to simplify the usage of the PEA model given that the stationary pivotal variable $(S_0 - P^*)/v^*$ is independent of K_S. The stationary version of the PEA model can easily be used to estimate MM parameters through the application of linear regressions (Pinto et al., 2015):

$$\frac{S_0 - P^*}{v^*} = \begin{cases} \dfrac{K_M + S_0}{V_{\max}}, & S_0 > E_0 \\ \dfrac{K_M + E_0}{V_{\max}}, & S_0 < E_0 \end{cases} \quad (14a)$$

It should be noted that in the case of maximal dissociation constant ($K_S/K_M = 1$), the previous equation is reduced to the

MM equation for $S_0 > E_0$, and to the simplified Bajzer and Strehler equation (Bajzer and Strehler, 2012) for $S_0 < E_0$:

$$v_0 = \begin{cases} \frac{V_{\max} S_0}{K_M + S_0}, & S_0 > E_0 \\ \frac{V_{\max} S_0}{K_M + E_0}, & S_0 < E_0 \end{cases} \quad (14b)$$

References

Acker, M.G., Auld, D.S., 2014. Considerations for the design and reporting of enzyme assays in high-throughput screening applications. Persp. Sci. 1, 56—73, http://dx.doi.org/10.1016/j.pisc.2013.12.001.

Baici, A., 2015. Kinetics of Enzyme-Modifier Interactions. Springer, pp. 39, http://dx.doi.org/10.1007/978-3-7091-1402-5.

Bajzer, Z., Strehler, E.E., 2012. About and beyond the Henri—Michaelis—Menten rate equation for single-substrate enzyme kinetics. Biochem. Biophys. Res. Commun. 417, 982—985, http://dx.doi.org/10.1016/j.bbrc.2011.12.051.

Berberan-Santos, M.N., 2010. A general treatment of Henri—Michaelis—Menten enzyme kinetics: exact series solution and approximate analytical solutions. MATCH Commun. Math. Comput. Chem. 63, 283—318.

Bersani, A.M., Dell'Acqua, G., 2011. Asymptotic expansions in enzyme reactions with high enzyme concentrations. Math. Methods Appl. Sci. 34, 1954—1960, http://dx.doi.org/10.1002/mma.1495.

Briggs, G.E., Haldane, J.B.S., 1925. A note on the kinetics of enzyme action. Biochem. J. 19, 338—339, http://dx.doi.org/10.1042/bj0190338.

Brown, A.J., 1902. Enzyme action. J. Chem. Soc. Trans. 81, 373—388, http://dx.doi.org/10.1039/ct9028100373.

Cornish-Bowden, A., 1987. The time dimension in steady-state kinetics: a simplified representation of control coefficients. Biochem. Educ. 15, 144—146, http://dx.doi.org/10.1016/0307-4412(87)90048-3.

Cornish-Bowden, A., 2012. Fundamentals of Enzyme Kinetics, 4th ed. Wiley-Blackwell, Weinheim, Germany, pp. 25—45, http://dx.doi.org/10.1016/b978-0-408-10617-7.50007-9, 400—410.

Dormand, J.R., Prince, P.J., 1980. A family of embedded Runge—Kutta formulae. J. Comp. Appl. Math. 6, 19—26, http://dx.doi.org/10.1016/0771-050x(80)90013-3.

Duggleby, R.G., 2001. Quantitative analysis of the time courses of enzyme-catalyzed reactions. Methods 24, 168—174, http://dx.doi.org/10.1006/meth.2001.1177.

Eisenthal, R., Danson, M.J., Hough, D.W., 2007. Catalytic efficiency and k_{cat}/K_M: a useful comparator? Trends Biotechnol. 25, 247—249, http://dx.doi.org/10.1016/j.tibtech.2007.03.010.

Fersht, A., 1999. Structure and Mechanism in Protein Science, 2nd ed. W.H. Freeman and Company, New York, pp. 110—111, 143.

Finn, N., Kemp, M., 2014. Systems biology approaches to enzyme kinetics: analyzing network models of drug metabolism. In: Nagar, S., Argikar, U.A., Tweedie, D.J. (Eds.), Enzyme Kinetics in Drug Metabolism. Humana Press, pp. 317—334, http://dx.doi.org/10.1007/978-1-62703-758-7_15.

Hanson, S.M., Schnell, S., 2008. Reactant stationary approximation in enzyme kinetics. J. Phys. Chem. A 112, 8654—8658, http://dx.doi.org/10.1021/jp8026226.

Henri, V., 1902. Théorie générale de l'action des quelques diastases. C. R. Hebd. Séances Acad. Sci. 135, 916—919, http://dx.doi.org/10.1016/j.crvi.2005.10.007.

Henri, V., 1903. Lois Générales de l'action des Diastases. Hermann, Paris, http://dx.doi.org/10.1007/bf01692307.

Johnson, K.A., Goody, R.S., 2011. The original Michaelis constant: translation of the 1913 Michaelis—Menten Paper. Biochemistry 50, 8264—8269, http://dx.doi.org/10.1021/bi201284u.

Michaelis, L., Menten, M., 1913. Die Kinetik der Invertinwirkung. Biochem. Z. 49, 333—369.

Nath, A., Atkins, W.M., 2008. A quantitative index of substrate promiscuity. Biochemistry 47, 157—166, http://dx.doi.org/10.1021/bi701448p.

Pandya, C., Farelli, J.D., Dunaway-Mariano, D., Allen, K.N., 2014. Enzyme promiscuity: engine of evolutionary innovation. J. Biol. Chem. 289, 30229—30236, http://dx.doi.org/10.1074/jbc.r114.572990.

Pinto, M.F., Estevinho, B.N., Crespo, R., Rocha, F.A., Damas, A.M., Martins, P.M., 2015. Enzyme kinetics: the whole picture reveals hidden meanings. FEBS J. 282, 2309—2316, http://dx.doi.org/10.1111/febs.13275.

Schnell, S., Maini, P.K., 2000. Enzyme kinetics at high enzyme concentration. Bull. Math. Biol. 62, 483—499, http://dx.doi.org/10.1006/bulm.1999.0163.

Segel, L.A., 1988. On the validity of the steady state assumption of enzyme kinetics. Bull. Math. Biol. 50, 579—593, http://dx.doi.org/10.1016/s0092-8240(88)80057-0.

Sols, A., Marco, R., 1970. Concentrations of metabolites and binding sites. Implications in metabolic regulation. Curr. Top. Cell. Regul. 2, 227—273, http://dx.doi.org/10.1016/b978-0-12-152802-7.50013-x.

Tzafriri, A.R., 2003. Michaelis—Menten kinetics at high enzyme concentrations. Bull. Math. Biol. 65, 1111—1129, http://dx.doi.org/10.1016/s0092-8240(03)00059-4.

Yang, J., Copeland, R.A., Lai, Z., 2009. Defining balanced conditions for inhibitor screening assays that target bisubstrate enzymes. J. Biomol. Screen. 14, 111—120, http://dx.doi.org/10.1177/1087057108328763.