Dissection of enzymatic kinetics and elucidation of detailed parameters based on the Michaelis-Menten model. Kinetic and thermodynamic connections

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Abstract
A computational procedure based on the numerical integration of the Michaelis-Menten model of enzyme action, free of any restrictions of steady-state conditions and substrate/enzyme ratios is proposed. The original Michaelis-Menten data for invertase (Michaelis and Menten, 1913, Biochem Z. 49:333-369) were reanalyzed. The surface and contour plots that were generated for substrate, free enzyme, complex, and product confirmed the model's usefulness. All energy potentials \( G \) and the “conformational drift parameter” \( \delta \) involved in the enzymatic reactions were determined. Our findings indicate that at \( s_0 = 0.0052 \text{M} \) the enzyme-substrate (ES) complex present an energy of dissociation of \( G_E + S \rightarrow ES = 15.0 \text{ kJ/mol} \) and as \( s_0 \) increases to 0.333 M, the \( G_E + S \rightarrow ES \) value decreases to 5.0 kJ/mol, thereby decreasing its presence in solution. Overall, the ability to determine \( G \) and \( \delta \) for each transition suggests a relationship between kinetics and thermodynamics. The analysis proposed here can be directly applied to chemical and biological situations, as well as industrial processes.

KEYWORDS
computer modeling, enzyme kinetics, invertase, Michaelis-Menten model, numerical modeling, Runge-Kutta method, substrate effect

1 | INTRODUCTION

The optimization of catalytic processes is a very important aspect of developing a more competitive product in different areas of industry. Models to describe enzyme activity have been proposed for more than a century, with an emphasis on a “general proposal” that can predict a large number of conditions and explain the catalytical phenomenon microscopically. Since the early studies by Sullivan and Tompson,1 Brown,2 and Henri,3,4 biochemical reactions catalyzed by enzymes
have been some of the most studied cellular processes. The subsequent steady-state description proposed by Briggs and
Haldane\(^5\) and the graphical method described by Lineweaver and Burk\(^6\) for estimating the kinetic parameters of reaction
represented important practical developments. Early discussions and the possible role of protein structural changes in
mediating enzymatic activity were broadly based on studies of oxygen binding to human hemoglobin. In particular,
reports by George and Pauling\(^7\) descriptions of the classic two-state model of Monod-Wyman-Changeux\(^8\) and studies
by Koshland et al\(^9\) and Weber\(^10\) pointed to such an interpretation of microscopic parameters, while studies involving
photochemical analysis\(^11\) and laser photolysis\(^12\) demonstrated experimental approaches for understanding the structural
heterogeneity and dynamics of proteins. A similar analysis of the early studies of Monod\(^13,14\) regarding fermentation
revealed that the equation proposed by this author was identical to those obtained by Michaelis-Menten for the velocity
of reaction of a single enzyme. Cornish-Bowden\(^15\) and Xie\(^16\) have highlighted past and recent advances, including statistical
interpretation on kinetics. In the present mathematical models for describing distinct topics in biology are very often,
and in general is associated with differential equations and computational tools.\(^17\)

Considerable efforts have been devoted to developing models of enzyme kinetics applicable to more general condi-
tions and comparing these models to one of the original steady-state propositions. A crucial problem in describing
heterogeneous systems is related to the fact that the analytical solution of the set of differential equations involving sec-
ond or higher-order kinetics is usually complex. In such cases, one approach usually adopted involves the assumption
that one of the reaction components, such as the substrate in the steady-state description of the Michaelis-Menten model,
is in excess in solution and its concentration is virtually unchanged during the reaction. One of the approaches used
to address this issue include the “quasi steady-state approximation” that considers the first part of an enzyme reaction
before the system reaches steady-state condition. Heineken et al\(^18\) developed an equation that apparently dealt with
such a condition, but, as pointed out by Walsh et al\(^19\) the “quasi steady-state approximation” failed to describe the time course of enzymatic reaction in relation to temporal changes in substrate concentration. Saa and Nielsen\(^20\) have discussed some other kinetic models and fitting systems that account for simplifications involving con-
straints or assumptions that allow more compact rate laws.\(^21-23\) In a recent report, we used Laplace transforms to obtain
a kinetic solution of the Michaelis-Menten model for situations other than the steady-state condition (equation 36 in
Reference 24). The simple absence of a steady-state constraint significantly increased the difficulty of the analytical solu-
tion of the velocities of reaction, even with an excess of substrate. In this context, combinations of methods involving
the theory of structural transitions proposed by Monod-Wyman-Changeux have been used to overcome the absence of
analytical solutions for enzyme kinetics in heterogeneous systems,\(^25\) virus dissociation,\(^26\) drying,\(^27,28\) yeast grown,\(^29\) and
cell damage.\(^30\) This approach led to a good interpretation of the observed phenomenon from a stochastic point of view,
but was unsatisfactory for determining the intrinsic kinetic parameters of individual species. Further work involving
numerical simulation of the Michaelis-Menten rate equations by using the fourth-order Runge-Kutta method confirmed
this point.\(^31\)

To fit complex dynamic systems such as described in the present work, computer simulation has been the most widely
used approach, with descriptions of as pioneering programs as KINSIM,\(^32\) FITSIM,\(^33\) and DYNAFIT.\(^34\) In addition, fast
dynamic simulations have been applied to explore enzyme kinetics by using initial parameters or parameter space as
search conditions. The latter authors proposed the use of a new program known as Global Kinetic Explorer that aimed
to simplify the user interface. The programs FITSIM, DYNAFIT and Global Kinetics Explorer use, respectively, Euler
numerical integration of the rate equation, a Newton-Raphson method for approximation and nonlinear regression of
exponential function involving the time course of product formation, with distinct results among these methods. The
results provided by equations based on exponential decay, as in the case of Global Kinetics Explorer, may obscure the
relationships between reaction rates and mechanisms.\(^19\) Indeed, the results of such type of approach correlate poorly with
the physical constants related to the kinetic process. FITSIM and DYNAFIT provide a suitable relationship between the
kinetic parameters and the approach adopted since both models are based on kinetic equations directly related to the rate
constants. However, the latter two programs require the input of time dependence curves for several species in solution.

The goal of the present work was to describe a numerical/computational method based on a direct numerical inte-
gration of the rate constants. Our strategy was to solve the kinetic equations by using a fourth-order Runge-Kutta method
and the concept of extent of reaction. This procedure allowed us to obtain the kinetic parameters of reactions in a model
based on data from conventional kinetic experiments at any enzyme/substrate ratio and completely free of steady-state
restrictions. As part of the validation of our proposal, we used this approach to reassess the original data of Michaelis
and Menten for the action of invertase. Our analysis yielded a much more detailed and precise description of the kinetics
and thermodynamics of this enzyme compared with previous reports. With appropriate adjustments, the algorithm and
program described here can be adapted to use in different kinetic models allowing to evaluate them.
2 | THEORETICAL CONSIDERATIONS AND COMPUTATIONAL PROCEDURES

Previous programs for solving enzymatic kinetics generally expressed the derived rate equations that involve the interaction of different species as the sum of exponential terms with an exponential phase for each step of the reaction. Consequently, the velocity equations derived from these exponentials will usually show some deviations that can lead to significant errors, especially when these functions are used to express certain rate parameters. In addition, independently of the differential equations that describe the kinetic system, the numerical integration of this system should be done with tools such as Euler’s method and the fourth-order Runge-Kutta method, that is, there is no need to use approximating equations. Although the use of approximations based on exponential considerations should allow rapid computation compared to numerical integration, such approximations are generally not very precise in expressing the proposed mechanism. Another problem with previous methods is the need for a second approximation fitting function (such as equations (2) and (3) of Johnson et al.) to obtain the approximate rate constants. This in turn increases the error propagation and constrains the quality of information obtained by the numerical approach.

To address these limitations, we propose that the use of differential equations in the Michaelis-Menten model should be analogous to the treatment proposed by Briggs and Haldane to encourage the use of numerical integration. In this case, we used the extent of reaction (\(x\)), as previously described. The classic kinetics of enzyme reactions can be represented by:

\[
E + S \overset{k_1}{\underset{k_2}{\rightleftharpoons}} ES \rightarrow E + P,
\]  

(1)

where \(S\) is the substrate, \(E\) the enzyme, \(P\) the product and \(ES\) the enzyme-substrate complex, with the terms \(k_{1+}\), \(k_{1-}\), and \(k_2\) representing the respective reaction rate constants (the subscripts indicate the reaction direction). In this view, the concentration \(C\) of the different species in solution over time is expressed in terms of the extent of reaction (\(x\)). For the model in Equation (1), this corresponds to:

\[
C_S(t) = s_0 - x_{1+}(t) + x_{1-}(t),
\]

(2)

\[
C_E(t) = e_0 - x_{1+}(t) + x_{1-}(t) + x_2(t),
\]

(3)

\[
C_{ES}(t) = x_{1+}(t) - x_{1-}(t) - x_2(t),
\]

(4)

\[
C_P(t) = x_2(t),
\]

(5)

where \((+)\) represents the amount of substrate (in mol/L) that forms \(ES\) and \((-)\) represents the amount of \(ES\) that dissociates to yield free substrate and enzyme, and \(s_0\) and \(e_0\) are the respective initial substrate and enzyme concentrations. The individual velocities of reaction are given by the following relationships:

\[
\nu_{1+}(t) = \frac{dx_{1+}(t)}{dt} = k_{1+}(s_0 - x_{1+}(t) + x_{1-}(t))(e_0 - x_{1+}(t) + x_{1-}(t) + x_2(t)),
\]

(6)

\[
\nu_{1-}(t) = \frac{dx_{1-}(t)}{dt} = k_{1-}(x_{1+}(t) - x_{1-}(t) - x_2(t)),
\]

(7)

\[
\nu_2(t) = \frac{dx_2(t)}{dt} = k_2(x_{1+}(t) - x_{1-}(t) - x_2(t)),
\]

(8)

where \(\nu_{1+}(t)\) is the velocity of complex formation, \(\nu_{1-}(t)\) is the velocity of complex dissolution into free enzyme and substrate and \(\nu_2(t)\) is the velocity of complex dissolution into product and free enzyme. Note that Equations (6) to (8), that are expressed in term of extent of reaction, come, respectively, from \(\nu_{1+}(t) = k_{1+} C_S(t) C_E(t)\), \(\nu_{1-}(t) = k_{1-} C_{ES}(t)\) and \(\nu_2(t) = k_2 C_{ES}(t)\) relationships.
As mentioned before, Equations (6) to (8) actually do not allow an analytical solution. The system is therefore usually solved by assuming an excess of substrate in solution and steady-state conditions. In these restrictive conditions, $C_S(t)$ in Equation (2) is assumed to be virtually unchanged with time ($C_S(t) \sim s_o$). Another consideration of steady-state conditions is the virtually constant concentration of the complex. With these simplifications, Equations (6) to (8) can be solved analytically, as proposed by the Briggs-Haldane solution:

$$\nu_2(t) = \frac{dx_2(t)}{dt} = \frac{V_M s_0}{K_M + s_0}$$

(9)

where $K_M = \frac{k_{-1} + k_2}{k_{1+}}$ is the Michaelis-Menten constant and $V_M = k_2 e_0$ is the maximum, or more correctly, limiting velocity.

In Equation (9), the contour condition at time zero is $x_2 = 0$, since there is no product in solution. Inspection of this equation shows that the parameter on the right side is constant with time. Thus, the direct integration of Equation (9) with respect to time furnishes a straight line with the intercept at zero, as follows:

$$x_2(t) = \frac{V_M s_0}{K_M + s_0} t$$

(10)

This equation presents restrictions related to steady-state conditions such as, for example, for any significant change in substrate concentration. The replacement of $s_0$ by $s(t)$ in Equation (9), that usually is done using Lambert’s W function, can be done by furnishing an equation different from Equation (10), but the resulting equation is applicable only until the steady-state is reached. When the steady-state condition is not considered and only the higher substrate concentration is assumed, it is possible to solve Equations (6) to (8) by using the Laplace transform. With this approach, Equations (9) and (10) become, respectively:

$$\nu_2(t) = \frac{V_M s_0}{K_M + s_0} t (1 - e^{-k_{1+}(K_M + s_0) t})$$

(11)

$$x_2(t) = C_P(t) = \frac{V_M s_0}{k_{1+}(K_M + s_0)^2} + \frac{V_M s_0}{K_M + s_0} + \frac{V_M s_0}{k_{1+}(K_M + s_0)^2} e^{-k_{1+}(K_M + s_0) t}.$$  

(12)

Equations (11) and (12) are a true mathematical solution of the Michaelis-Menten model for conditions until the steady-state is reached. Interestingly Heineken et al. developed a kinetic treatment that leads to an identical solution of Equation (11) (equation 53 in their report). The steps these authors used in solving the problem were very different from ours. Based on the proposal by Johnson et al. for the Michaelis-Menten data, the time-dependence of product concentration, $x_2(t)$, is expressed as:

$$x_2(t) = C_P(t) = A_1 \exp(-\lambda_1 t) + C,$$

(13)

or, from a general point of view, as:

$$x_2(t) = C + \sum_{i=1}^{n} A_i \exp(-\lambda_i t).$$

(14)

Compared to Equation (12), Equations (13) and (14) should provide a good fit to the experimental results. However, the parameters $A$, $C$, and $\lambda$ will furnish large deviations from the true rate constants if one uses the secondary fitting of equations 1 and 2 of Johnson et al. In addition, Equations (12) to (14) best represent the condition of substrate excess.

Since the mathematical solution of kinetic systems is very difficult and approximating equations generally increases the imprecision of the results as the order of reactions increases, we have developed a numerical method to solve kinetic systems directly from the rate equations (Equations (6) to (8)). The basis of the program needed to characterize any kinetic system is shown in Figure 1 and can be implemented directly in matrix programs such as MATLAB or Mathematica (see Appendix S1).
FIGURE 1 Flow diagram of the numerical procedure used to characterize Michaelis–Menten kinetic data. The numerical integration was done using the fourth-order Runge-Kutta method (see Appendix S1).

3 RESULTS AND DISCUSSION

We applied the approach described above to the original Michaelis-Menten data (table 1 in Reference 1). As shown in Figure 2A, six experimental results \((\text{num} = 6)\) were obtained for \(s_0 = 0.333, 0.167, 0.083, 0.0416, 0.0208, \) and 0.0052 M. Based on Figure 1, the program fits each experimental result individually from \(d = 1\) (first experimental result) to \(d = \text{num}\) (last experimental result). Thus, if \(d = 1\) then the algorithmic will first consider \(k_{j+} = k_{j-} = k_2 = n_i\); since the amount of enzyme used is not reported, we should consider in addition \(e_0 = n_i\) or any other starting value up to the final value \(n_f\). With these initial parameters the program uses a fourth-order Runge-Kutta method to integrate Equations (6) to (8) to obtain the time values for \(x_{1+}, x_{1-}\) and, more importantly, the product \(x_2\). Subsequently, a second routine calculates the fitting statistical parameters \(\text{SSE}\) (sum of squares error), \(r^2\) (correlation coefficient) and \(\chi^2\) (chi-square) for the first
Figure 2  Experimental data from Michaelis-Menten and SSE$_{\text{min}}$/SSE (sum of squares error) and $r^2$ (correlation coefficient) at distinct values of the kinetic constants and initial enzyme concentration. (A) Symbols: Michaelis-Menten experimental data for the conversion of sucrose into glucose and fructose at various initial sucrose concentrations ($s_0$). Lines: Model fitting results. (B) Degree of substrate-product conversion ($C_P/s_0$) vs time for different $s_0$. (C) Residual fitting error (%) for each $s_0$ in relation to time. (D) Contour plot of SSE$_{\text{min}}$/SSE (sum of squares error) and $r^2$ (correlation coefficient) at distinct values of the kinetic constants defined by Equation (1), that is, $k_1+$ and $k_1-$. This type of plot shows the location (values ~1) of the rate constant combinations that best fit the data for $s_0 = 0.333$ M in panel A. (E) Contour plot of SSE$_{\text{min}}$/SSE and $r^2$ at distinct values of $k_2-$ and $k_2$. (F) Contour plot of SSE$_{\text{min}}$/SSE and $r^2$ at distinct values of $k_2$ and initial enzyme concentration ($e_0$).
internal looping of \(i, j, k,\) and \(l\) (Figure 1 and Appendix S1). Any of these statistical parameters could be used to obtain the accurate fit and the confidence contour surface.\(^{31,39}\) Since the parameters for the kinetic constants and initial enzyme concentration change from \(n_i\) to \(n_f\) divided into \(n\) intervals according to the values of \(i, j, k,\) and \(l,\) for \(n = 20\) (as used here) the total number of possible rate constants and initial enzyme concentration combinations will be given by \(20^4\) possibilities. Thus, by calculating for instance the SSE and \(r^2\) for each rate constant and enzyme combination, it is possible to find the combination that provides the best SSE and \(r^2\) results, allowing for the plot of the final fitting together with the experimental results. If the fit is not satisfactory, the initial (\(n_i\)) and/or final (\(n_f\)) value must be changed. In this case, the solution is obtained by maximization of \(r^2\) or \(\text{SSE}_{\text{min}}/\text{SSE}\) which furnishes the kinetic parameters corresponding to the best fit. If a value \(\geq 0.95\) for \(r^2\) is used instead of the maximum value, then the solution would correspond to a region with a relatively large range of possible kinetic values.

More flexible plots can be shown to the range of these values for each rate and initial enzyme concentration, Figure 2D to F. In this regard, Johnson et al\(^{35,40}\) incorporated an important step by proposing surface and contour plots based on the maximization of \(\text{SSE}_{\text{min}}/\text{SSE}\). This situation is illustrated by the contour plots obtained for the combinations of rate constants indicated in Figure 2D to F for 0.333 M sucrose (substrate). Thus, for \(n = 20\), the starting values were \(n_i = 2 \times 10^{-2}\) and \(n_f = 6 \times 10^{-2}\) for \(k_{1+}\), \(n_i = 6 \times 10^{-3}\) and \(n_f = 9 \times 10^{-3}\) for \(k_{1-}\) and \(n_i = 1 \times 10^{-2}\) and \(n_f = 5 \times 10^{-2}\) for \(k_2\) and \(n_i = 5 \times 10^{-4}\) and \(n_f = 1.5 \times 10^{-3}\) for \(e_o\). These contour plots was complemented to highlight the contribution of the parameter \(\text{SSE}_{\text{min}}/\text{SSE}\) of Johnson et al\(^{35,40}\) by applying additionally the \(r^2\) parameter. In Figure 2D to F, the color lines indicate values from \(\text{SSE}_{\text{min}}/\text{SSE}\) and the contour levels specified by the tags refer to the \(r^2\) region. In this case, the difference between \(r^2\) and \(\text{SSE}_{\text{min}}/\text{SSE}\) was particularly clear. Both parameters showed a good fit for values close to 1. However, as shown in Figure 2D to F, a change in \(r^2\) from 0.999 to 0.95, usually still considered as an exceptionally good fit, led to a 10-fold decrease in \(\text{SSE}_{\text{min}}/\text{SSE}\). These findings indicate that \(r^2\) provides a macro view of the optimized region of adjustment, whereas \(\text{SSE}_{\text{min}}/\text{SSE}\) provides details regarding on low \(r^2\) variations.

Based on Figure 2D to F that involves \(20^4\) combinations of rate constants, we concluded that for an estimated enzyme concentration of \(e_o = 9.7368 \times 10^{-4}\) M and a substrate (sucrose) concentration of 0.333 M, the combination of \(k_{1+} = 0.0516\) (M.s\(^{-1}\)), \(k_{1-} = 0.0074\) (s\(^{-1}\)) and \(k_2 = 0.0332\) (s\(^{-1}\)) provides the fit shown in Figure 2A (black dashed line and circles, with % residual errors shown in Figure 2C). This fit yielded \(r^2 = 0.9994, \text{SSE}_{\text{min}}/\text{SSE} = 1\) and a residual error <1% (Figure 2C).

If the approach proposed by Johnson et al,\(^{30}\) that it is based only on \(\text{SSE}_{\text{min}}/\text{SSE}\) is used, the results may not reflect the best fit condition because \(r^2\) does not necessarily reach suitable values. The present proposal does not have such a limitation since it considers the \(r^2\) values additionally. After this initial adjustment, the results obtained for the rate constants are used as starting values for the second adjustment according to the equations shown in Figure 1 for \(d > 1\).

In this case, when the search range (Sr) is equal to 2, the search will look for rate constants in a range two times higher and lower than the solution previously obtained. This allows the results of each experimental condition to be linked, and permits the assessment of progressive changes in the rate constants. Figure 2A shows the results for the fit obtained by direct integration of the rate equations (fourth-order Runge-Kutta method) after applying such approach successively. As shown by this figure and the product: substrate ratio (Figure 2B), this method provides a very good fit of all the original Michaelis-Menten results and Figure 2C shows that the residual plot of the fit for the data was <6%. This program\(^{40}\) was run in a desktop computer within a 2.5-GHz Intel processor that generated the contour plots shown in Figure 2D to F in 2 to 3 minutes using well-constrained initial parameters.

The method described here can handle SEs or deviations, which is included in the computational process. In this case, the approach can be applied based on the average experimental results and the values for SE or deviation. As mentioned before, the results were obtained directly from numerical integration of the kinetic equations (Equations (6)-(8)) using a fourth-order Runge-Kutta method, with no need to use equations such as (12) to (14) to calculate the rate constants. The values obtained for each rate constant at distinct initial substrate concentrations plotted as shown in Figure 3A to C were dependent on substrate concentration. Thus, an increase in \(s_o\) from 0.0052 to 0.333 M decreases \(k_{1+}\) from 0.5346 to 0.0516 (Ms\(^{-1}\)) and \(k_2\) oscillates from 0.0319, 0.0638, and 0.0332 second\(^{-1}\), stabilizing in this last value, whereas \(k_{1-}\) increases from 0.0012-0.0011 to 0.0074 second\(^{-1}\). This profile of responses reflected the unbalanced stoichiometry of each component in the mixture and fluctuations in the medium that affected enzyme mobility and its ability to effectively bind substrate. To characterize the dependence of the kinetic constants on substrate concentration we considered the possible occurrence of enzyme species with distinct states of affinity. Such a condition is analogous to systems involving the heterogeneity of tobacco mosaic virus observing in pressure-induced dissociation\(^{26,41}\) and drying processes that promote heterogeneous structures over time.\(^{27,28}\) In these investigations, the distinct structures were correlated to different fractions populating each experimental condition. Formally, the equations in these reports that are equivalent to those described here are equations 11 to 13 in Reference 26, equations 2 to 5 in Reference 27 and equations 2 to 6 in Reference 28.
Intrinsic results obtained from the fit procedure for the rate constants and for $K_M$ results as a function of $s_o$. (A) Symbols: intrinsic results obtained from the fit procedure for the rate constant $k_{1+}$ vs $s_o$. Lines: Nonlinear fitting done using the equation $k_i = \sum_{a=1}^{4}(f_{ia}k_{ia})$ for each rate constant so that $\sum_{a=1}^{4}(f_{ia})=1$, according to previous reports.\(^{26-28}\) (B) Symbols: intrinsic results obtained from the fit procedure for the rate constant $k_{1-}$ versus $s_o$. (C) Symbols: intrinsic results obtained from the fit procedure for the rate constant $k_2$ vs $s_o$. The solid lines in (B) and (C) were calculated using the equation given in (A). (D) Symbols: $K_M$ data obtained directly from the relationship in Equation (9). Continuous line: curve obtained by applying the equation in (A) to the $K_M$ results.

A nonlinear fit (Figure 3A-C) allowed adjustment of the kinetic constants to assess their values at any substrate concentration and permitted two- or three-dimensional plots for several kinetic parameters (Figures 4-10). Based on these results, $K_M$ was calculated directly using the rate constants that changed with values for $s_o$, such that an increase in substrate concentration increased the $K_M$ values, for example, the $K_M$ increased from 62 to 786.7 mM up to $s_o = 0.333$ M (Figure 3D). Calculation of the $K_M$ based on steady-state conditions by using the $V_M$ and $s_o$ plot reported by Johnson et al.\(^{39}\) yielded a $K_M = 16.7$ mM and $V_M = 0.76$ mM/min. The use of a Lineweaver-Burk plot in the present work yielded $K_M = 15.5$ mM and $V_M = 0.638$ mM/min. The differences in the $K_M$ values calculated from Lineweaver-Burk plot indicated that this constant had a complex behavior. For short reaction times the values of $K_M$ approximated those calculated from Lineweaver-Burk plot. The significant increase in $K_M$ with $s_o$ concentration (Figure 3D) may reflect a conformational change in the enzyme under these conditions. Such a change may be related to conformational drift described in the literature to explain the
Figure 4  Relation of product formation ($C_P = x_2$), initial substrate concentration ($s_o$) and time (A) Surface plot of the results obtained for product formation ($C_P = x_2$) vs time and initial substrate concentration ($s_o$). (B) Level diagrams obtained from cuts of the surface data for $s_o$ vs time at distinct product concentrations. (C) Level diagrams obtained from cuts of the surface data for product formation vs $s_o$ at different times. (D) Level diagrams obtained from cuts of the surface data for product formation vs time at distinct $s_o$ concentrations.

loss of protein-protein affinity during the dissociation of oligomers by pressure or low temperature.\textsuperscript{42,43} At high substrate concentrations the enzyme may show lower activity because of an increase in $K_M$ as observed here.

To evaluate the relevance of these results, we examined product formation ($C_P$) as a function of the initial substrate concentration and time (Figure 4A). The respective surface cuts are shown in Figure 4B to D. As already explained, when the fitting process was done automatically, the time results for the extent of reaction were calculated for each substrate condition ($x$). Analysis of Figure 4C shows for the first time that product formation ($C_P$) increased with substrate concentration up to an initial substrate concentration ($s_o$) of $\sim 0.15$ M. Figure 4D shows that the entire hyperbolic nature of these parameters can be predicted using the proposed model so that it is not necessary to exclude the nonlinear part of the experimental results, in contrast to the steady-state hypothesis. The velocities of product formation ($v_2(t)$) can be determined directly using Equation (8) under the same conditions as those of Figure 5. These results agree with previous
FIGURE 5  Relation of velocity of product formation ($v_2$), initial substrate concentration ($s_0$) and time. (A) Surface plot of the results obtained for the velocity of product formation ($v_2$) vs time and initial substrate concentration ($s_0$). (B) Level diagrams obtained from cuts of the surface data for $s_0$ vs time at distinct velocities of product formation. (C) Level diagrams obtained from cuts of the surface data for the velocity of product formation versus $s_0$ at different times. (D) Level diagrams obtained from cuts of the surface data for the velocity of product formation vs time at distinct $s_0$ concentrations.

Figure 6A shows $1/v$ as a function of $1/s_0$, the Lineweaver-Burk plot, and time, and Figure 6B to D shows the respective surface cuts. As shown in classic double reciprocal plots (Figure 6C and inset), the behavior was nonlinear. Recent studies also showed nonlinear behavior on Lineweaver-Burk plot for horseradish peroxidase. Such results were attributed to enzyme conformational changes associated to change in the apparent rate constants.
The assumption of a maximum constant velocity of reaction was not applicable in many conditions. This can be explained by a substrate induction of enzyme conformational drift. Moreover, the inhibitory effect of substrate on enzyme activity probably contributed to the shape of this curve. For $1/s_o > 20$ (Figure 6C inset) and short reaction times, the approach described here using a Lineweaver-Burk plot furnished $K_M = 15.5$ mM, while the $K_M$ calculated directly from the constants that generate this plot was 120 mM for $1/s_o = 20$. This discrepancy indicates that the calculation of $K_M$ requires careful consideration since an increase in substrate concentration can result in significant deviations of enzymatic activity from ideality, which usually is assumed in the steady-state conditions.

Additional aspects explored in this model included the influence of substrate depletion (Figure 7), free enzyme concentration (Figure 8) and complex concentration (Figure 9). The curves for complex formation shown in Figure 9D indicate that as the initial substrate concentration increased so did amount of complex formed ($\sim 2.8 \times 10^{-4}$ at $s_o = 0.333$ M, with $e_o = 9.7368 \times 10^{-4}$), but even in this condition the free enzyme concentration was relatively high, Figure 8D, ($\sim 6.9368 \times 10^{-4}$), corresponding to only 28.7% of the ES saturation if one considers the total amount of enzyme in solution. These findings indicate that when undertaking a Lineweaver-Burk analysis, the reliability of this method is compromised since the enzyme is not predominantly in the complex state. Experimental procedures to detect different enzyme species and their properties should confirm these data but require special methods, such as fast kinetic and spectroscopic.
measurements with fluorescent probes. Such analyses can also be done using the present approach by loading and fitting directly the complex or turnover data, as described by Kumar et al.

A very relevant feature of the proposed approach is the possibility of establishing connections between kinetics and thermodynamics. Such kind of correlation was recently described for metabolic pathway analysis using the flux of intermediary components in response to enzyme concentration perturbations. In the present work we proposed a direct way to obtain the energy parameters. Thus, as already mentioned, the rate constants in Figure 3 were developed for the Michaelis-Menten data with respect to the initial substrate concentration $s_o$. When adjustments are made for distinct temperatures such as $k$ vs $T$, these data can be promptly fitted by the Arrhenius equation, which provides the activation energy of each step. The observed change in the rate constants with respect to the initial substrate concentration $s_o$ pointed to a conformational change in the enzyme in the presence of ligands. In their initial publication, Michaelis and Menten also
FIGURE 8  Relation of free enzyme concentration ($C_E$), time and initial substrate concentration ($s_0$). (A) Surface plot of the results obtained for the free enzyme concentration ($C_E$) vs time and initial substrate concentration ($s_0$). (B) Level diagrams obtained from cuts of the surface data for $s_0$ vs time at distinct free enzyme concentrations. (C) Level diagrams obtained from cuts of the surface data for the free enzyme concentration vs $s_0$ at different times. (D) Level diagrams obtained from cuts of the surface data for the free enzyme concentration vs time at distinct $s_0$ concentrations.

expected an influence of substrate (sugar) mass action on the activity of invertase. This change in enzyme conformation can be related to a physical quantity referred to here as “conformational drift parameter” $\delta$. An analogous phenomenon was previously described by Weber$^{42}$ as the “conformational drift of protein subunits” to explain the decrease in the free energy of association of monomers after protein dissociation by pressure or low temperature. The quantification of $\delta$, which modulates the energy of ligand association/dissociation, involves a relationship between the rate constant and the initial substrate concentration, as expressed by Equations (15) to (17).

$$k_{1+}(s_0) = k_{1+}(s_{0i})s_0^{\delta_{+}},$$

(15)

$$k_{1-}(s_0) = k_{1-}(s_{0i})s_0^{\delta_{-}},$$

(16)
$k_2(s_0) = k_2(s_{0i})s_0^{\delta_2}$, \hspace{1cm} (17)

where $k(s_o)$ is the rate constant at any $s_o$, $s_{0i}$ is the reference substrate concentration, considered here as 0.333 M and $\delta$ is the conformational drift parameter that controls the strength of mass action of $s_0$ at each rate constant. The parameter $\delta$ in these equations is obtained by fitting results from Figure 3 and is therefore not necessarily constant. Consequently Equations (15) to (17) cannot be used to calculate $k$ as a function of $s_o$, but serve to calculate $\delta$ from $k$ and $s_o$. Another point to be noted in these equations is that these parameters are obtained with respect to an initial substrate concentration of reference $s_{0i}$. Under these conditions, when $s_o = s_{0i}$, $\delta$ should be zero in all reactions (see Figure 10B).
FIGURE 10  Potential energies of transition $G_{1+}$, $G_{1-}$ and $G_2$, energy change of transition ($\Delta G$), conformational drift parameters of $s_0$ mass action ($\delta_{1+}$, $\delta_{1-}$, and $\delta_2$) and conformational drift parameter change of transition ($\Delta \delta$) as a function of initial substrate concentration ($s_o$). (A) Potential energies of transition $G_{1+}$, $G_{1-}$ and $G_2$ obtained from the theoretical results for $k_{1+}$, $k_{1-}$, and $k_2$, respectively, using Equations (18) to (20). (B) Conformational drift parameters of $s_0$ mass action ($\delta_{1+}$, $\delta_{1-}$, and $\delta_2$) obtained from the theoretical results for $G_{1+}$, $G_{1-}$ and $G_2$, respectively, using Equations (21) to (23). (C) Energy change of transition ($\Delta G_{E \rightarrow S \rightarrow ES}$, $\Delta G_{ES \rightarrow E + P}$ and $\Delta G_{S \rightarrow P}$), respectively, using Equations (24) to (26). (D) Change in the conformational drift parameter ($\Delta \delta_{E \rightarrow S \rightarrow ES}$, $\Delta \delta_{ES \rightarrow E + P}$ and $\Delta \delta_{S \rightarrow P}$) according to the Equations (24) and (25). The results shown in panels C and D were obtained using Equations (24) to (26).

To relate the kinetic constants to the corresponding Gibbs free energy, the natural logarithmic is applied to both sides of Equations (15), (16) and (17) followed by multiplication by $-RT$ (where R is the gas constant and T the temperature), which yields

\begin{align*}
G_{1+}(s_0) &= G_{1+}(s_{0i}) - \delta_{1+}RT\ln(s_0), \quad (18) \\
G_{1-}(s_0) &= G_{1-}(s_{0i}) - \delta_{1-}RT\ln(s_0), \quad (19) \\
G_2(s_0) &= G_2(s_{0i}) - \delta_2RT\ln(s_0), \quad (20)
\end{align*}
where $G(s)$ is the potential energy of transition between the states whose directions are indicated by the subscripts, with subscript “1+” referring to the transitions from $E + S$ to $ES$, “1−” to the reverse process and “2” to the transition of complex $ES$ into $P + E$.

Rewriting Equations (18), (19) and (20) we obtain:

$$\delta_{1+} = \frac{G_{1+}(s_0) - G_{1+}(s_0)}{-RT\ln(s_0)}.$$  \hspace{1cm} (21)

$$\delta_{1-} = \frac{G_{1-}(s_0) - G_{1-}(s_0)}{-RT\ln(s_0)}.$$  \hspace{1cm} (22)

$$\delta_{2} = \frac{G_{2}(s_0) - G_{2}(s_0)}{-RT\ln(s_0)}.$$  \hspace{1cm} (23)

From these relationships, the resulting constants $k_{1-}$, $k_{1+}$, and $k_{2}$ (Figure 3) can be related to the respective $G_{1-}$, $G_{1+}$, and $G_{2}$ (Figure 10A). Since these potential energies are related to the $s_0$ concentrations, they are relative with respect to this parameter at each transition $G$. Hence, an increase in $s_0$ from 0.052 to 0.333 M also increases the potential energy of enzyme/substrate binding ($G_{1+}$), while the opposite is observed for the transition from complex to enzyme and product $G_{1-}$. The complex dissociation into enzyme and product remains approximately constant ($G_{2}$) (Figure 10A); In these cases, it is possible to observe a minimum and maximum points between 0.015 and $s_0$ (0.333 M). The relative conformational drift parameter $\delta$ for $s_0$ mass action can be obtained using Equations (21) to (23) (Figure 10B). Based on the results for $\delta_{1+}$ and $\delta_{1-}$ we conclude that these parameters reflect the density of substrate surrounding each species. For positive values of $\delta_{1+}$, $\delta_{1-}$, and $\delta_{2}$, an increase in $s_0$ will increase the potential energy, while the opposite will be observed for negative values of $\delta$. This in turn indicates that states with a greater ability to capture substrate are preferentially formed to “buffer” the solution with respect to $s_0$. Although this analysis is based on constant values of $\delta$, Figure 10B also shows that a decrease in $\delta_{1-}$ from 0.6 to 0 implies a reduction in the potential energy $G_{1-}$ from $\sim$16.6 to $\sim$12.2 kJ; such a change alters the species balance and makes the complex more unstable in solution. The differences of complex stability can be measured by calculating the net potential energy and the net conformational drift change. This difference can be expressed in relation to all these parameters at any $s_0$ concentration, as follows:

$$G_{E+S\rightarrow ES} = G_{1-} - G_{1+} \quad \text{and} \quad \delta_{E+S\rightarrow ES} = \delta_{1-} - \delta_{1+}.$$  \hspace{1cm} (24)

$$G_{ES\rightarrow P} = G_{2} - G_{1-} \quad \text{and} \quad \delta_{ES\rightarrow P} = \delta_{2} - \delta_{1-}.$$  \hspace{1cm} (25)

$$G_{S\rightarrow P} = G_{2} - G_{1+} \quad \text{and} \quad \delta_{S\rightarrow P} = \delta_{2} - \delta_{1+}.$$  \hspace{1cm} (26)

The net results for species formation shown in Figure 10C,D can be attained as follows. Equation (24) and Figure 10C show that the energy of complex dissolution $G_{E+S\rightarrow ES}$ corresponds to 15.0 kJ/mol at $s_0 = 0.052$ M, and it decreases to 5.0 kJ/mol with the increasing of $s_0 = 0.333$ M, indicating that at higher initial substrate concentration the complex is less stable. In contrast, high complex occurrence at low $s_0$ should in turn be associated with low substrate-enzyme dissociation seen as a potential state of $G_{ES\rightarrow P} = -8.03$ kJ/mol. Thus, as $s_0$ increases the concentration of complex in solution reduces and this effect can be enhanced by an increase in ($G_{ES\rightarrow P} \rightarrow -3.7$ kJ/mol) potential, which increases the complex dissociation in this direction.

In a similar manner, the conversion of substrate to product involves an initial energy change ($G_{S\rightarrow P} = 6.98$ kJ/mol) at $s_0 = 0.052$ M, and decreases to values close to $G_{S\rightarrow P} = 1.09$ kJ/mol at $s_0 = 0.333$ M. The irregular shapes observed in Figure 10C may reflect a conformational change in the enzyme in the presence of different substrate concentrations. We concluded that these fluctuations involve a change in the conformational drift parameter $\Delta \delta$, showed in Figure 10D. Hence, depending on the $s_0$ concentration, these steps can express different intensity in a cooperative/antagonistic effect on enzyme activity.

Finally, when these analyses are done with respect to pH (pH = $-0.4242\ln([H^+])$), Equations (21) to (26) will express the stoichiometry of proton $\text{H}^+$ release or binding during ES complex formation and dissociation (a phenomenon usually referred to in hemoglobin studies as the Bohr effect). The influence of other parameters, such as temperature, pressure and effectors, can be analyzed in a similar manner. The procedure described here can be readily adapted to investigate
other processes such as fermentation, cell division, cooperative binding of ligands, protein folding and others. In these cases, the proposed model can be tested by using the concept of extent of reactions and numerical integration in a manner similar to that described here.

4 | CONCLUSION

The analysis of enzyme kinetics is frequently complicated by the need for algorithm construction, the number of parameters involved, the time required for processing and the poor user friendliness of the various programs/softwares currently available. In this report, we have described an approach for classic enzyme kinetics analysis based on a program that allows detailed examination of various kinetic parameters. Our results show that this method provides additional information beyond what can be obtained by more traditional approaches. The algorithm is easily implemented and, with appropriate adjustments, may be applied to processes such as fermentation, cell growth, protein aggregation, protein folding and others. This method should also be useful in optimizing industrial processes through its ability to provide more details of each chemical process and greater predictability when intervening in physical and chemical parameters. Compared with previous approaches, the present proposal allows a complete characterization of any kinetic process free of restrictions such as quasi steady-state approximations, the use of the Lambert function and nonlinear exponential approximations that are usually present in current procedures. With appropriate adjustments, the methodology proposed here should be applicable to studies involving allosteric proteins, cooperativity in binding and folding, inactivation processes and any problem that can be associated to kinetic equations.

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The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS
Carlos Bonafe: Conceptualization; formal analysis; funding acquisition; validation; writing-original draft; writing-review and editing. Daniel Lima Neto: Formal analysis; validation; writing-review and editing. Cesar Aguirre: Formal analysis; validation; writing-review and editing. Silvio Melo: Conceptualization; methodology; writing-original draft. Wallisson Lima: Formal analysis; writing-review and editing. Jose Bispo: Conceptualization; data curation; formal analysis; funding acquisition; validation; writing-original draft; writing-review and editing.

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