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A linearization method for low catalytic activity enzyme kinetic analysis

Paolo Toti[a], Antonella Petri[b], Valerio Pelaia[a], Ahmed M. Osman[c], Moreno Paolini[d], Carlo Bauer[a,*]

[a]*Department of Physiology and Biochemistry, Biochemistry Unit, University of Pisa, via S. Zeno 51, I-56127 Pisa, Italy*
[b]*Centro Studio C.N.R. per le Macromolecole Stereordinate ed Otticamente Attive, Dipartimento di Chimica e Chimica Industriale, Università di Pisa, via Risorgimento 35, 56126 Pisa, Italy*
[c]*Institute of Inland Water Management and Waste-Water Treatement (RIZA), P.O. box 17, 8200 AA Lelystad, The Netherlands*
[d]*Department of Pharmacology, Biochemical Toxicology Unit, University of Bologna, via Irnerio 48, I-40126, Bologna, Italy*

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Abstract

A kinetic analysis was made and a linear plot based on the general rate equation derived by Laidler [Can. J. Chem. 33, 1614–1624] is proposed. This linearization method allows determining the kinetic parameters (K_m, k_{cat}) and $[E]_0$ for enzymes with low catalytic activity. The method was applied to chloroperoxidase from *Caldariomyces fumago* [EC 1.11.1.10], whose kinetic parameters K_m^{app}, k_{cat}^{app}, and $[E]_0$ with monochlorodimedone as substrate, were obtained by using the linearization plot and the V_{max} value (calculated by Eadie–Hofstee plot).

This plot could also be useful to the study of abenzyme kinetics provided the concentration of the latter is either higher or equal than K_m value.

Keywords: Linear plot; Kinetic parameters; Michaelis–Menten equation; Chloroperoxidase; Catalytic antibody

1. Introduction

In the kinetic studies of enzyme catalyzed reactions, the most widely used assumption is the Briggs and Haldane steady-state hypothesis [1], according to which the concentrations of enzyme intermediates are constant. Thus the rates of their changes are equal to zero and, the differential equations of the system reduce to a set of algebraic equations. These equations are then simplified by assuming that the initial substrate concentration is much higher than the initial enzyme concentration [2,3]. However, in enzyme systems, this condition is not always necessarily satisfied. Indeed, for enzymes of low turnover and high substrate affinity, it may be necessary to use relatively large concentration of enzyme compared to the substrate. In such a case, an appreciable fraction of the substrate can be bound to the enzyme and the concentration of the free substrate can be substantially different from initial substrate concentration [4,5]. The problems with evaluation of kinetic parameters of enzyme catalysis, in which the rates are slow and consequently the simplifying assumption $[E] \ll [S]$ is not valid, have been recognised a long time ago, but they have gained importance after the discovery of abenzymes [6,7] (antibody enzymes). The catalytic antibodies of high specificity can be prepared against any substrate and their specific activity is so low that abenzymes can be considered as enzymes with low catalytic activity. Many biological systems exhibit two or more enzyme–substrate intermediate steps [8–10], in which an initial fast transient phase is followed by a step with a low variation in reactant concentration. When the ratio of initial enzyme to substrate concentration is high, a redistribution of the substrate to the different intermediates takes place [8]. If the enzyme catalyzes the unique intermediate system [11,12], and the concentration of the free substrate is different from the

* Corresponding author. Tel.: +39 502213173; fax: +39 502213170.
E-mail address: bauer@dfb.unipi.it (C. Bauer).

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initial one, the Michaelis–Menten equation [13] in its normal form is not valid, and a different hyperbola describes v as a function of the total substrate concentration [14].

The aim of this paper is to show that this hyperbola can be linearized by a new method to obtain the enzyme fundamental constants (V_{max} and K_m), the total enzyme concentration and the catalytic constant. This linearization method was employed to determine the kinetic parameters of chloroperoxidase under conditions of low catalytic activity.

2. Experimental procedures

2.1. Materials

Chloroperoxidase from *Caldariomyces fumago* [EC 1.11.1.10] (11000 U/ml) and *tert*-butylhydroperoxide (70% v/v in water) were supplied by Fluka and were used without further purification. Monochlorodimedone was a Sigma Chemical product and was used without further purification. All other reagents used were of analytical grade.

2.2. Kinetic measurements

The assay mixture of chloroperoxidase consisted of: potassium chloride (0.55 mmol), *tert*-butylhydroperoxide (0.9 μmol), a fixed amount of chloroperoxidase (176 U), and various concentrations of monochlorodimedone ($(1\cdot10^{-7}|7.7\cdot10^{-6})$ in a total volume of 27.4 ml of potassium phosphate buffer (0.1 M, pH 2.75).

The rate of chloroperoxidase-catalyzed reaction was measured in a controlled quartz cell of 10 cm path-length at 25 °C by monitoring the decrease of absorbance at 278 nm due to the conversion of monochlorodimedone (MCD, $\varepsilon_{278}=1.22\cdot10^4$ M^{-1} cm^{-1}) to dichlorodimedone (DCD, $\varepsilon_{278}=1.6\cdot10^2$ M^{-1} cm^{-1}) [15]. 200 μl of the commercial enzyme was added to 2.5 ml of 0.1 M phosphate buffer, pH 6, after which 200 μl of this solution was taken and added to 0.1 M phosphate buffer pH 6 containing 20 mM KCl and 33 μM *tert*-butylhydroperoxide, tBuOOH (final volume 27.4 ml). At pH 6 the enzyme activity was found to be stable. All kinetic measurements were performed in triplicate.

Further, the UV spectrum of chloroperoxidase was recorded in the absence of the substrates, monochlorodimedone, and the oxidant, *tert*-butylhydroperoxide.

3. Theory

3.1. Steady-state equation of Laidler

A single-substrate system, assuming the absence of effects of activators and inhibitors, is represented by the Michaelis–Menten scheme [16,17] (see Scheme 1), where E, S, ES and P represent enzyme, substrate, enzyme–substrate complex and product, respectively. $[E]_0$ and $[S]_0$ indicate initial concentrations of enzyme and of substrate. From the Michaelis–Menten scheme of reaction, in 1955, Laidler [19] proposed the following general steady-state equation:

$$[ES]=\frac{k_2+k_{-1}+k_1\cdot\left([E]_0+[S]_0-[P]\right)\pm\left[k_2+k_{-1}+k_1\cdot\left([E]_0+[S]_0-[P]\right)\right]\cdot\sqrt{1-\dfrac{4\cdot k_1^2\cdot[E]_0\cdot\left([S]_0-[P]\right)}{\left[k_2+k_{-1}+k_1\cdot\left([E]_0+[S]_0-[E]\right)\right]^2}}}{2\cdot k_1} \quad (1)$$

where [ES] depends on the total substrate concentration. The rate equation which can be easily obtained from Laidler's formulation ($v=k_{+2}\cdot[ES]$) contains (besides the V_{max} and K_m values) the total enzyme concentration $[E]_0$ and the microscopic rate constant k_2 of the Michaelis–Menten reaction scheme. In the Michaelis–Menten equation, the rate constant is inseparably linked with the V_{max} expression ($V_{max}=k_2\cdot[E]_0=k_{cat}\cdot[E]_0$).

3.2. Linearization method of general steady-state equation

Because under the conditions described, the known linear plot are not applicable [20–23], for the estimation of V_{max}, K_m, k_2 and $[E]_0$ values in the general rate equation, we propose here a linearization method, which can be used to determine the enzyme constants.

$$\underset{[E]_0\text{-}[ES]}{E}+\underset{[S]_0\text{-}[ES]\text{-}[P]}{S}\underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}}\underset{[ES]}{ES}\xrightarrow{k_2}\underset{[E]_0\text{-}[ES]}{E}+\underset{[P]}{P}$$

Scheme 1. Michaelis-Menten scheme for one-substrate enzyme reaction; steady state concentrations of E, S, ES and P are indicated.

By using Laidler equation when relatively large concentration of enzyme is used, [S] can be expressed as a difference between the initial substrate concentration and the steady-state concentration of enzyme–substrate complex and the product concentration; but we assume (in the case of low catalytic enzyme activity) that at the beginning of the steady-state [P] is close to zero.

On algebraic manipulation Laidler equation can be rearranged in a general steady-state rate Eq. (2) [24]:

$$v = \frac{1}{2}\cdot k_2 \cdot \left\{ \left([S]_0 + [E]_0 + k_m\right) - \sqrt{\left([S]_0 + [E]_0 + K_m\right)^2 - 4\cdot[E]_0\cdot[S]_0} \right\} \tag{2}$$

since $k_2 \cdot [E]_0 = V_{max}$ and $k_2 \cdot [ES] = v$, we can write our linearization Eq. (3):

$$\frac{[S]_0}{v} = \frac{K_m}{V_{max} - v} + \frac{1}{k_2} \tag{3}$$

The linear plot of $[S]_0/v$ against $1/(V_{max}-v)$ is illustrated in Fig. 1. The ordinary Michaelis–Menten equation and the general rate Eq. (2) have the same asymptote V_{max} [4].

Substituting $v = V_{max}/2 = k_2 \cdot [E]_0/2$ and $[S]_0 = [S]_0^{hs}$ (where $[S]_0^{hs}$ is the half-saturation concentration) into Eq. (2) we get:

$$[S]_0^{hs} = K_m + \frac{1}{2}\cdot[E]_0 \tag{4}$$

Finally, by calculating $\delta v_0/\delta[S]_0$ and considering $[S]_0$ tending to zero, we obtain the equation of the tangent straight line to the general curve at the origin of axes:

$$v = \frac{V_{max}}{K_m + [E]_0}\cdot[S]_0 \tag{5}$$

The slope of this tangent can be compared with the slope of the ordinary Michaelis–Menten equation V_{max}/K_m; the intercept on the V_{max} horizontal line will be $K_m + [E]_0$ in agreement with that given by the graphical method of Dixon [4,5]. This graphical method of Dixon can be used not only to determine enzyme concentration but also the K_m value. This is done by suitably drawing straight lines from the origin of axes and determining the points of intersection with the V_{max} straight line. The accuracy of the method depends mainly on drawing the V_{max} line at the correct level, which must be determined by using excess substrate. Further, the constancy of the intervals giving the K_m value provides a check on its proper positioning.

3.3. Determining the V_{max}

Eq. (3) enables us to obtain separately k_2, K_m and $[E]_0$, provided that the V_{max} value is known. However, generally V_{max} is not known and correct positioning of the experimental abscissa points is required for its determination. This problem can be

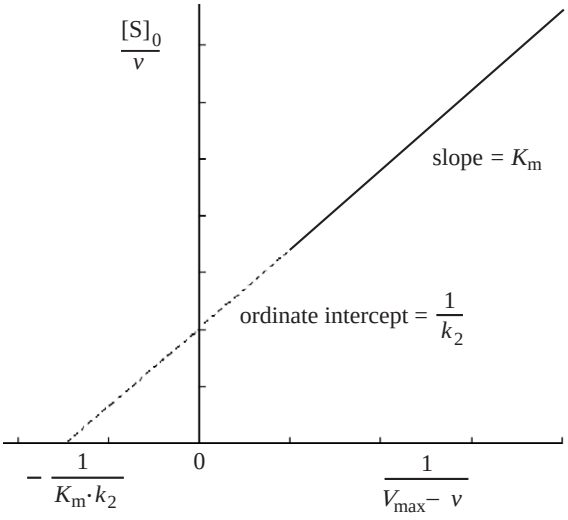


Fig. 1. Determination of enzyme constants (K_m, k_2 and $[E]_0$) by using Eq. (3).

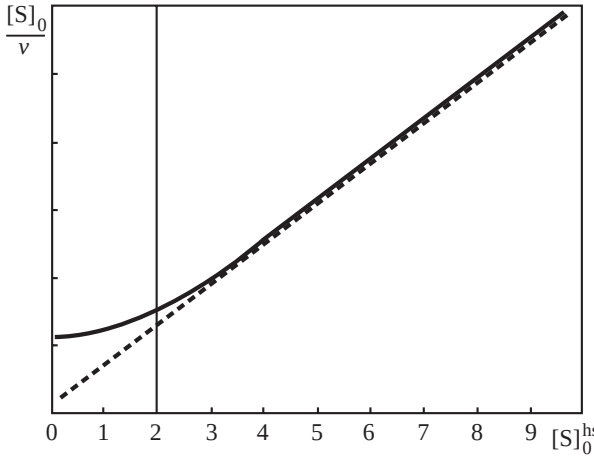


Fig. 2. Hanes plot of ordinary Michaelis–Menten (dashed line) and general (solid line) steady-state rate equation.

resolved graphically as proposed by Dixon [4,5]. A hypothetical V_{max} value can be extrapolated from the experimental points and the linearization plot can be drawn on this basis; if the points do not lie on a straight line, the V_{max} value can be adjusted until a straight line is obtained [4,5]. Using this approach one can determine the V_{max} value initially by using a linear classical plot (as Lineweaver-Burk or Hanes), in fact the ordinary Michaelis–Menten and general steady-state rate Eq. (2) share the same V_{max} value and the two curves coincide at a suitable high $[S]_0$ value; subsequently this V_{max} value is inserted into Eq. (3).

For a comparison, we have used Hanes plot of $[S]_0/v$ versus $[S]_0$, which, directly, gives the value of $1/V_{max}$ as straight line slope [20,21,25]. By using either general rate Eq. (2) or Michaelis–Menten formulation with a given set of parameters, we may obtain two series of data (v, $[S]_0$). If the condition $[E]_0 \ll K_m$ prevails, the general rate equation will become very close to Michaelis and Menten one [18]. If this condition does not prevail, for the Hanes plot, the two series of data give the same slope in the interpolation provided that the initial substrate concentration is greater than $2 \cdot [S]_0^{hs}$. For the general rate equation data, however, at low substrate concentration, the points diverge from a straight line as shown in Fig. 2.

To obtain a first evaluation of maximum velocity (which we call V'_{max}) all points (except these below saturation, if they cause a curvature as shown in Fig. 2) are interpolated. Next, the experimental value of marked initial substrate concentration ($[S]_0^{hs\prime}$) is determined. Subsequently, every point is connected with the next one by a line segment, and the segment that contains the point ($V'_{max}/2$, $[S]_0^{hs\prime}$) is determined. Once the $[S]_0^{hs\prime}$ value is found, the points with initial substrate concentration greater than $2 \cdot [S]_0^{hs\prime}$ can be plotted, by using Hanes equation, to obtain the correct V_{max} value (Fig. 3).

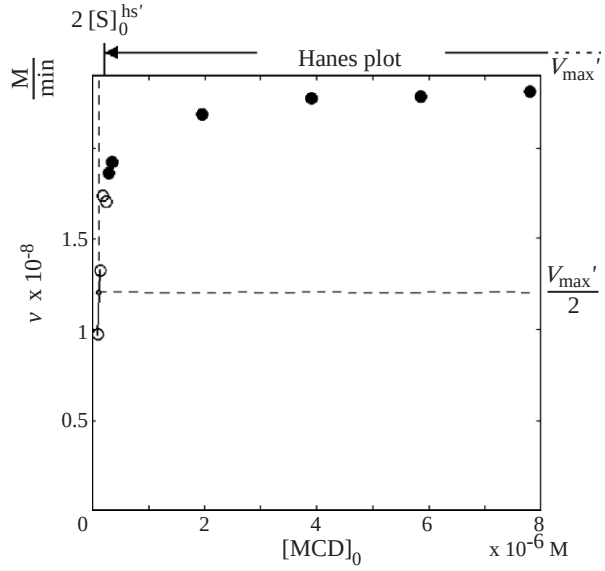


Fig. 3. Direct plot of the experimentally determined initial velocities of chloroperoxidase-catalyzed reaction and method for calculating $[S]_0^{hs\prime}$ value (solid points used in Hanes plot). Reaction mixture: potassium chloride (0.55 mmol), *tert*-butylhydroperoxide (0.9 μmol), chloroperoxidase (176 U), and various concentrations of monochlorodimedone ($1 \cdot 10^{-7}|7.7 \cdot 10^{-6}$) in a total volume of 27.4 ml of potassium phosphate buffer 0.1 M (pH 2.75).

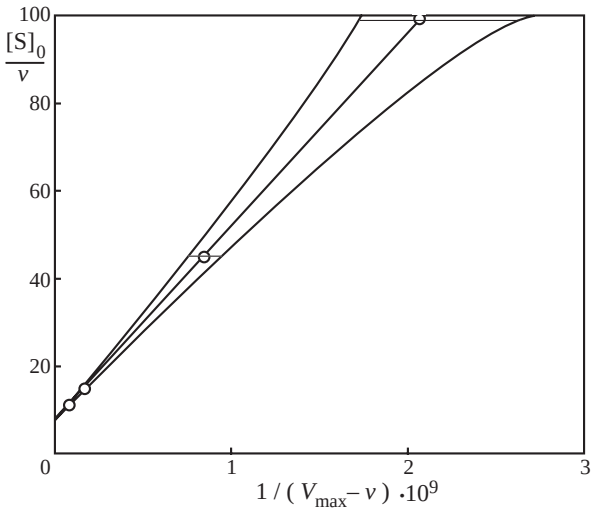


Fig. 4. Linearization of simulated data (v_0; $[S]_0$) obtained by using experimental kinetic parameters (see text: $[E]_0=1.56\cdot10^{-7}$ M, $K_m=4.51\cdot10^{-8}$ M, $k_2=1.49\cdot10^{-1}$ s^{-1} and $k_{-1}=10$ s^{-1}) and plot obtained with V_{max} value affected by a ±1% error.

3.4. Determination of the kinetics parameters and $[E]_0$

The V_{max} value (obtained as described above) can now be introduced in Eq. (3) and used for calculating the abscissa points corresponding to the experimentally measured velocities. In the Hanes linearization, the theoretical V_{max} limit becomes the experimental result, whereas for the linearization (3), V_{max} is a datum. At high substrate concentrations, and, therefore, with the rate approaching V_{max} value, the plot of $[S]_0/v$ versus $1/(V_{max}-v)$ should give a straight line whose slope and ordinate intercept are K_m and $1/k_2$, respectively. From these data, total enzyme concentration can be calculated as V_{max}/k_2, provided that $2\cdot[S]_0^{hs\prime}$ is closer to $[E]_0+2\cdot K_m$ than $2\cdot K_m$.

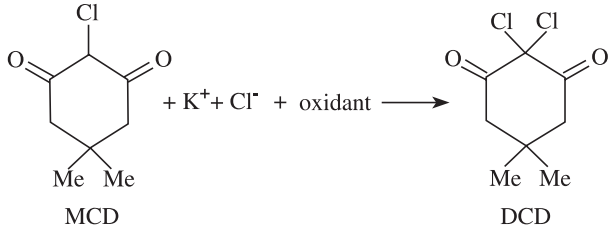
3.5. Effect on errors

The data transformation take place systematic errors; it is possible to classify the error types into three forms, on the determination of V_{max}, v and $[S]_0$ values. It is worthwhile noting that the abscissa errors due to the $1/(V_{max}-v)$ transformation is bigger than the ordinate errors due to $[S]_0/v$. The abscissa error effect is more important in the data points close to saturation, in fact in this situation v tends to V_{max}. The error effects on abscissa caused by bad determinations of V_{max}, v are shown in Fig. 4. This is the reason why we use, for the linearization, the data points far from saturation plateau.

4. Discussion

We have presented a linear plot based on Laidler's general steady state equation for enzyme kinetics [18] which could be used for the determination of kinetic constants for low catalytic activity enzyme at high enzyme concentration compared to the substrate. Under these conditions, the commonly used linear plots, based on the Briggs and Haldane [1] steady state equation, are not applicable due to the assumption that enzyme concentration should be much lower than the substrate concentration. The general steady state equation proposed by Laidler is not constrained by this assumption and hence the presented linear plot is suitable.

We have applied the presented linear plot to chloroperoxidase CPO from *C. fumago*, a heme enzyme, which participates in the production of the natural product caldariomycin [26]. Although the primary biological function of CPO is chlorination [26], this enzyme also catalyzes other oxidative reactions characteristic of other heme-based enzyme such as horseradish peroxidase, catalase and cytochrome P450 [27]. Using monochlorodimedone as substrate (Scheme 2) [15] and suitably reducing the concentration of the oxidant, tBuOOH, the turnover of CPO was lowered. In addition, with this oxidant the rate of catalase dismutation was reduced [28]. Thus, the values of



Scheme 2. Chloroperoxidase catalyzed oxidative chlorination of monochlorodimedone.

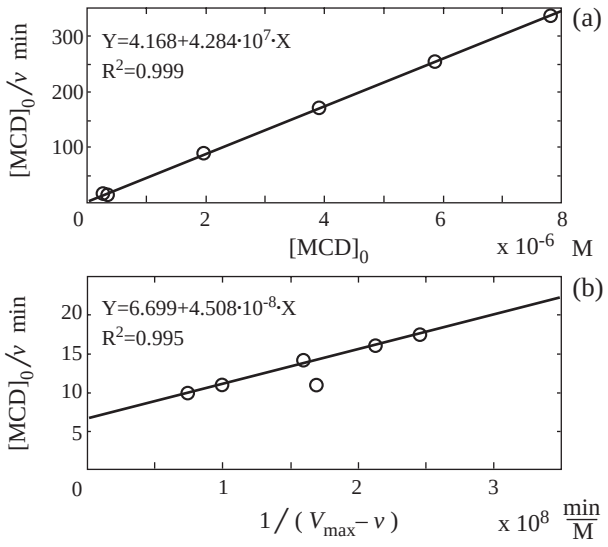


Fig. 5. Linearization of the experimental points: (a) by Hanes plot, (b) by using Eq. (3).

the fundamental enzyme constants K_m and k_{cat} (apparent K_m^{app} and k_{cat}^{app}) were found to be $4.51(\pm 0.19)\cdot 10^{-8}$ M and $1.49(\pm 0.07)\cdot 10^{-1}$ min^{-1}, respectively (Fig. 5b). Likewise, the enzyme concentration, $[E]_0$, was determined. The $[E]_0$ value was calculated to be $1.56(\pm 0.07)\cdot 10^{-7}$ M.

The three set of experimental data points were fitted into Laidler equation (assuming [P]=0) by non-linear regression with a average quality program (the MatLab metafile program "curvefit.m"), with initial estimates kinetics parameters obtained from the linearization plot, obtaining very similar values: K_m^{app} and k_{cat}^{app} values, $4.31(\pm 0.21)\cdot 10^{-8}$ M and $1.69(\pm 0.11)\cdot 10^{-1}$ min^{-1}, respectively, were determined as well as the $[E]_0$ value, $1.35(\pm 0.09)\cdot 10^{-7}$ M; it is worthwhile noting that, if the experimental data are in a limited number, the kinetics parameter values obtained by the non-linear fitting depend on the initial estimates used, whereas a linearization plot is a more objective method.

Although K_m^{app} and k_{cat}^{app} values could vary depending on the experimental conditions (e.g. concentration of the oxidant), the total enzyme concentration was a fixed value, representing the initial amount of enzyme added to the reaction mixture. By using the obtained $[E]_0$ value, the molar extinction coefficient ε of chloroperoxidase was obtained in the λ range 350–750 nm. This calculated spectrum was comparable with the experimentally determined one with the pure enzyme. For instance, at 400 nm, an extinction coefficient of $8.60(\pm 0.42)\cdot 10^4$ and $9.4(\pm 0.63)\cdot 10^4$, respectively for linear and non-linear fitting, were calculated, whereas the experimental value determined at this wavelength was $9.35(\pm 0.37)\cdot 10^4$. This indicated that the new method is appropriate for analysis of enzymes with low catalytic activity.

This method is also useful in the case where the ratio $[E]_0/K_m$ is bigger than 10. In these particular case the determination of the free substrate is very difficult; if $[S]_0 < 1/2[E]_0 + K_m$ (or $[S]_0^{hs}$), all substrate is complexed to enzyme, but in the case in which the substrate concentration is bigger than the half-saturation value the system expresses a V_{max}, and so information on the molar fraction falls down.

5. Conclusions

A kinetic analysis and a linearization method to obtain the kinetic parameters and $[E]_0$ for a low catalytic activity enzyme has been described. This linearization can be obtained from the general rate equation derived from Laidler steady-state equation that replaces the ordinary Michaelis–Menten one if relatively large concentrations of enzyme are used. This method was applied to determine the kinetic parameters of chloroperoxidase from *C. fumago* under the condition of low catalytic activity. The chloroperoxidase UV spectrum obtained from the calculated $[E]_0$ was quite similar to the experimental one supporting the validity of the proposed linearization plot. As seen in the previous section, a suitable set of starting zero point values is necessary for correct application of non-linear regression methods; so it is possible to use the values from linear fitting for this purpose.

This general method is expected to be applicable also to the abenzyme kinetics provided that their concentration is greater than K_m.

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