First-order Kinetics in the Study of Enzymes: Applications to the Reporter Substrate Method and to the Estimation of k_{cat}/K_m.

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The authors wish to dedicate this paper to the memory of the late Michel Rinné (1941-2009) whose contribution in making the data analysis program very user-friendly was invaluable.

Abstract.

Background: in enzyme kinetic studies, it is often necessary to determine pseudo first-order rate constants.

Objective: we wish to describe a simple, rapid and efficient method for doing so.

Method: a very simple program is presented.

Results: this program is applied to the study of inactivation phenomena and to the determination of k_{cat}/K_m.

Conclusion: the described program is particularly useful when applied to the reporter substrate method.

Keywords: Reporter substrate, enzyme inactivation, β-lactamases, k_{cat}/K_m, avibactam, imipenem, nitrocefin, ceftazidime.

1. Introduction.

IC_{50} values are often measured to estimate the efficiency of an enzyme inhibitor or inactivator (I). Although they might represent a preliminary approach, they do not supply information about the exact mechanism of the inhibition/inactivation phenomenon. Whenever possible, it is much more useful to monitor the time-course of substrate disappearance (or product formation). In the following analysis, we will consider that, in the absence of inhibitor/inactivator, substrate disappearance remains linear or quasi-linear (reference sample) and that the initial inhibitor/inactivator concentration is much larger than the total concentration of the enzyme (E_0). If the inactivator itself is a poor substrate (Appendix, models 4, 5 and 7, see example 2) it is assumed that its concentration remains constant throughout the whole time-course: $(I)_t = I_0 = (I)$. Under these conditions, the addition of an inhibitor with which the $E + I \rightleftharpoons EI$ equilibrium is established within the mixing dead-time of the experimental method (i.e. 5-10 s for manual mixing but 5-10 ms or less if stopped-flow equipment is utilized) is expected to yield a line exhibiting a lower absolute slope than that of the reference sample. This inhibition situation is discussed in many biochemistry or enzymology textbooks [1, 2] and will not be further considered here.

If the studied compound acts as an inactivator or a slow-binding inhibitor, the rate of the enzymatic reaction will progressively decrease in time to reach complete inactivation or a steady-state in which the enzyme is only partially inactivated or inhibited.

2. Materials and methods.

Avibactam was obtained from Pfizer. Ceftazidime was purchased from Fresenius Kabi (Belgium) and nitrocefin from Oxoid. The *Enterobacter cloacae* P99 β-lactamase was produced and purified as described [3]. The purification of the OXA-427 β-lactamase [4] will be described elsewhere.

Reaction time-courses were monitored with the help of Speccord 50 Plus end Specord 200 (Analytikjena) spectrophotometers.

The method for analysing the kinetic data is described below (see “practical aspects).

3. Theoretical aspects.

3.1. Complete inactivation

If the rate of the enzymatic reaction is zero or negligible at the end of the time-course (this translates into a horizontal line in the (S) *vs* t curve), the decrease in this rate is given by a simple equation

$$v_t = v_0 e^{-k_a t} \quad (1)$$

where v_t is the rate at time t

or $\ln v_t = \ln v_0 - k_a t$ (2)

and the slope of the line ln v_t *vs* t yields the value of k_a.

3.2. Non negligible rate at the steady-state

This translates into a line of non-negligible slope (v_{ss}) at the end of the experiment [5, 6]. The decrease in the rate is then characterized by Eq 3 and 4

$$v_t - v_{ss} = (v_0 - v_{ss}) e^{-k_a t} \quad (3)$$

or

$$ln(v_t - v_{ss}) = ln(v_0 - v_{ss}) - k_a t \quad (4)$$

The value of k_a is obtained by plotting ln(v_t-v_{ss}) *vs* t.

In both cases, complete or incomplete inactivation, k_a is a pseudo-first-order rate constant that depends upon the concentration of I according to equations and parameters characteristic of the exact mechanism of the reaction between E and I (see Appendix).

3.3. Protection by the substrate

If the reporter substrate concentration is not << its K_m value, its presence somehow protects the enzyme against inactivation [3, 6, 7]. The measured k_a value thus decreases with increasing reporter substrate concentrations.(see the Appendix for complete equations). If the reporter substrate concentration is well below its K_m value, no correction is necessary.

3.4. Measurement of V/K_m

Equation 1 also characterises the rate of an enzymatic reaction when its time time-course is monitored with an initial substrate concentration << K_m, so that the reaction obeys first-order kinetics

$$(S) = S_o e^{-k_a t}$$

and $k_a = \frac{kcat}{K_m} E_o$ = V/Km (5)

where E_o is the total enzyme concentration.

In this case, $v_t = \frac{d(P)}{dt} = -\frac{d(S)}{dt} = k_a S_o e^{-k_a t} = v_o e^{-k_a t}$ (6)

and, in consequence the slope of the line ln v_t *vs* t also yields the value of k_a from which the k_{cat}/K_m value can be deduced if E_0 is known.

4. Practical aspects. Examples

We have developed a simple program [8] that allows a rapid determination of the k_a values, especially in the cases of spectrophotometric or spectrofluorimetric measurements. The (S) *vs* t [or (P) *vs* t] curve is first divided in about 50 points. Each point thus represents the average of several individual measurements, which decreases the reading errors. For instance for a 3-min run with a measurement every 0.5 s, each point represents the average of 7 individual readings. Moreover, the total number of points can be modified. It is most often decreased so that each point represents the average of a larger number of individual readings. In the following examples, we will utilise reaction time-courses monitored by UV or visible spectrophotometry (absorbance measurements, A). The rate at time t is calculated as $\left|\frac{A_t - A_{t+i}}{i}\right|$ where i is the time interval between 2 points. When the final rate is 0 or close to 0, the last values are rejected because the relative error on $|A_t - A_{t+i}|$ becomes too large (see below). Similarly, when a steady-state is reached, the points that are too close to the steady-state should also be rejected. The steady-state rate (v_{ss}) is computed by linear regression using the last time-points. It is thus important to make sure that the steady-state has really been reached.

4.1. Example 1: Complete inactivation (examples can be found in refs 9-12) **.**

Inactivation of active-site serine β-lactamases by avibactam obeys model 7 [9], but with the exception of KPC-2, the k_3 value is zero. In addition, in the example presented here (the class D OXA-427 enzyme), the k_{-2} value is so low (< 2 x $10^{-5}s^{-1}$) that it can be neglected so that the inactivation, observed over a period of a few minutes occurs according to model 2 and allows to determine k_2/K.

Figure 1 presents the progressive inactivation of OXA-427 by 44 µM avibactam in the presence of 83 µM ceftazidime as a reporter substrate (S, K_m = 68 µM for the detailed experimental conditions, see the legend of the figure). The hydrolysis of ceftazidime results in a decrease of the absorbance at 260 nm. 358 readings were recorded with a time interval of 0.5 s. In the analysis of the data, the number of points was reduced to 14 so that each point represents the average of 24 readings. The logarithmic plot was drawn with the first 10 points, yielding a k_a value of 1.59 x 10^{-2} s^{-1} with a relative error of 10%. The experiment was repeated twice and the average of the 3 results was (1.6 ± 0.07) x 10^{-2} s^{-1}.

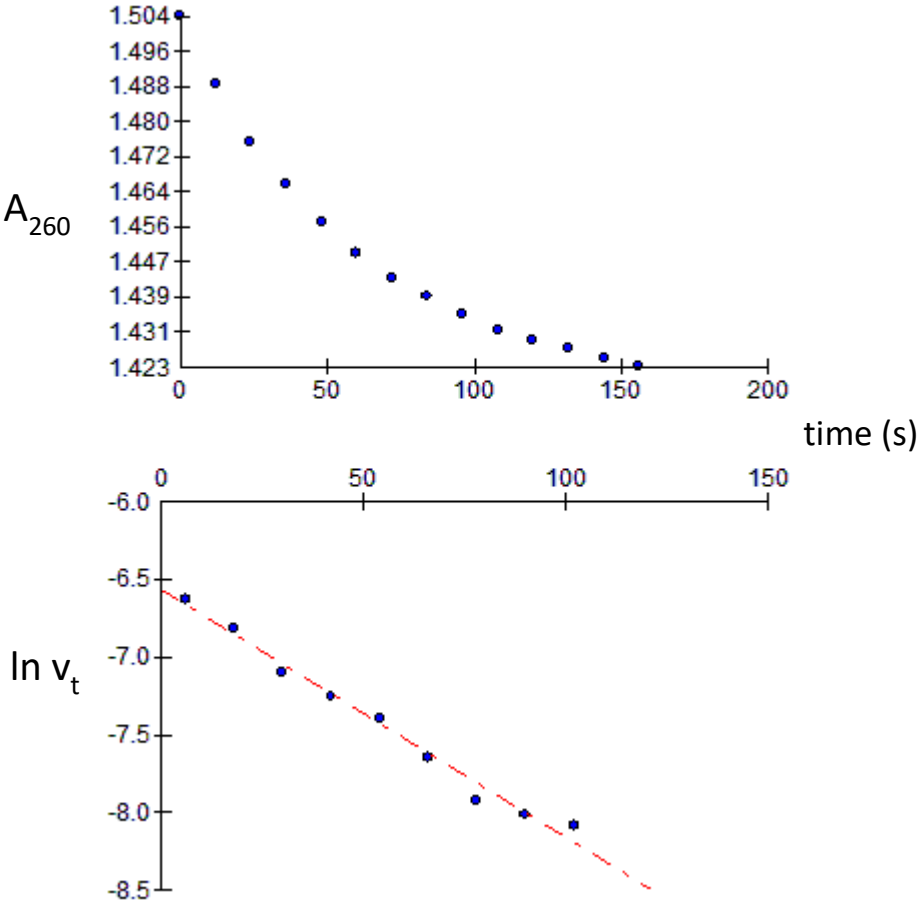

Figure 1.

Panel A: Monitoring of A_{260} *vs* t upon hydrolysis of 83 µM ceftazidime by the OXA-427 β-lactamase in the presence of 44 µM avibactam at 30°C. The total volume was 455 µL in 100 mM sodium phosphate, pH 6.2 and the enzyme concentration 0.18 µM. The mixing dead-time was about 10 s.

Panel B: ln v_t *vs* t, computed with the first 10 points of Panel A.

Figure 2 shows a similar experiment with 33 µM avibactam. Here, each point corresponds to the average of 21 readings and a k_a value of 1.25 x 10^{-2} s^{-1} is deduced with a relative error of 7%. As above, the average of 3 experiments yielded (1.26 ± 0.06) x 10^{-2} s^{-1}.

In all cases, it was verified that, after about 8 min, the inactivation was complete (residual activity < 0.5%).

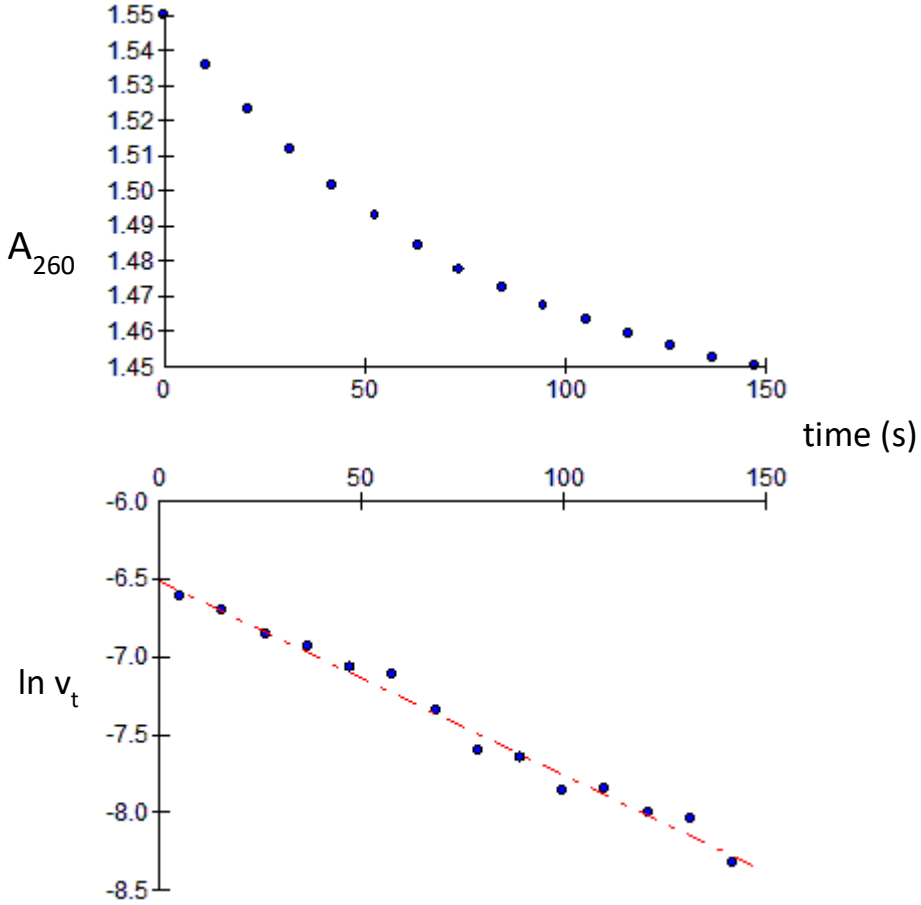

Figure 2.

Panel A: Monitoring of A_{260} *vs* t upon hydrolysis of 83 µM ceftazidime by the OXA-427 β-lactamase in the presence of 33 µM avibactam at 30°C. The total volume was 450 µL in 100 mM sodium phosphate, pH 6.2 and the enzyme concentration 0.18 µM. The mixing dead-time was about 10 s.

Panel B: ln v_t *vs* t, computed with the 15 points of Panel A.

Since the k_a values remain proportional to the avibactam concentration within the limits of experimental errors, it can be concluded that the K' value is significantly larger than 44 µM and

a k_2/K' value of 370 ± 40 $M^{-1}s^{-1}$ is calculated. After correction for the protection by the reporter substrate, a k2/K value of 820 ± 80 $M^{-1}s^{-1}$ is obtained. Note that, in this simple case,

$$\frac{k_2}{K} = \frac{k_2}{K'} \quad \frac{K_m+(S)}{K_m} \qquad (7)$$

4.2. Example 2: Incomplete inactivation

Imipenem is a very poor substrate of the class C *Enterobacter cloacae* P99 β-lactamase [13]. The k_{cat} value, corresponding to k_3 (model 7 with $k_{-2} = 0$) is 3×10^{-3} s^{-1} which results in the accumulation of EI*, the acylenzyme. In this case, the reporter substrate was 100 µM nitrocefin ($k_{cat} = 780$ s^{-1}, $K_m = 25$ µM) in 45 mM sodium phosphate, pH 7.5 and the absorbance increase was monitored at 485 nm.

Figure 3 shows the results recorded with 2.06 µM imipenem. 553 readings were recorded over a period of 110 s with a time interval of 0.2 s. In the analysis of the data, the number of points was reduced to 25 so that each point represents the average of 22 readings. The steady-state rate (v_{ss}) was derived from points 21-25 and the logarithmic plot drawn with points 1-11 yielding a $(k_a)_{obs}$ value of 5.58×10^{-2} s^{-1} with a relative error of 7%.

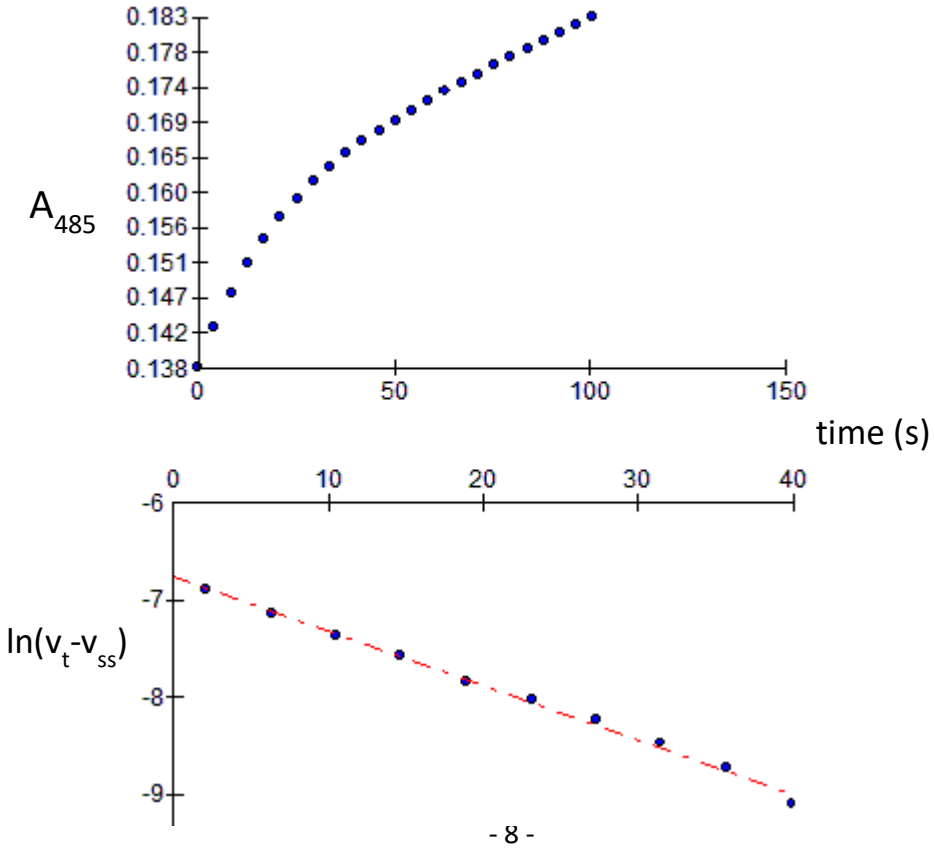

Figure 3.

Panel A: Monitoring of A_{485} *vs* t when 0.2 nM P99 β-lactamase was incubated in the presence of 100 µM nitrocefin and 2.06 µM imipenem at 30°C. The total volume was 490 µL. The mixing dead-time was about 10 s.

Panel B: ln v_t *vs* t, computed with the first 11 points of Panel A.

Four repeats of the experiment yielded a k_a value of (5.26 ± 0.55) x 10^{-2} s^{-1}. Since (I) << K' [13], equation A9 simplifies to $k_2(I)/K' = k_a - k_3$ = (4.96 ± 0.55) x 10^{-2} s^{-1} and k_2/K' = 24000 ±2600 $M^{-1}s^{-1}$ which yields a k_2/K value of 120000 ±13000 $M^{-1}s^{-1}$ after correction for the protection by nitrocefin (K_m = 25µM). This is in fair agreement with the result of Galleni *et al* (60000 ± 400 $M^{-1}s^{-1}$) if one takes account of the slightly different experimental conditions (pH 7.5 here *vs* 8.2 in ref 13). From these results, a K_i value for Imipenem $\left(=\frac{k_3 K}{k_2}\right)$ of 21 ± 2 nM was derived. However, a K_i value of 41 ± 8 nM could also be obtained from the residual activity at the steady-state (8.9 ± 1.6 %) in excellent agreement with that reported by Galleni *et al* (40 nM). The difference between the two K_i values obtained in the present paper might be explained by the fact that the 20 nM value was obtained by assuming a k_3 value of 3 x $10^{-3}s^{-1}$ [13] that might be somewhat higher at pH 7.5.

4.3.Example 3: Measurement of k_{cat}/K_m

In the presence of 40 mM $NaHCO_3$ (in 100 mM sodium phosphate, final pH 6.65, the k_{cat} and K_m values for the hydrolysis of ceftazidime by the OXA-27 beta-lactamase (at 30°C) are respectively 8.1 ± 1.1s^{-1} and 98 ± 13 µM. In the example presented in figure 4, the hydrolysis of 5 µM ceftazidime by 0.31 µM enzyme was monitored at 30°C over a period of 2 min (241 readings). The total absorbance variation was – 0.049 absorbance units. In the presented analysis, the number of points was reduced to 20, each point thus representing the average of 12 readings. The deduced k_a value was 2.56 x 10^{-2} s^{-1} with a relative error of 5.5%. The experiment was repeated 3 times yielding an average k_a value of (2.55 ± 0.25) x 10^{-2} s^{-1}. The error is thus larger than that deduced from the analysis of a single curve. This is probably due to the error in the pipetting of the added enzyme sample (5 µl). From these data, a k_{cat}/K_m value of (82300 ± 8000) $M^{-1}s^{-1}$ is obtained that is in excellent agreement with that deduced from the individual k_{cat} and K_m values [85000 ± 13000 $M^{-1}s^{-1}$] with a significantly lower error.

It should be noted that an important advantage of the present method over the direct analysis of the (S) *vs* t curve is that there is no need to obtain a reliable final value of the absorbance that might make it necessary to monitor the reaction for a longer period of time and that is not always very accurate. Indeed, one can show that an error of 0.005 absorbance units on this final value can translate into a very large error on the k_a value. For instance, with a final absorbance value of 0.110, the V/K_m value is 2.59 x $10^{-2}s^{-1}$ while it is 2.02 x 10^{-2} s^{-1} with a final absorbance value of 0.105.

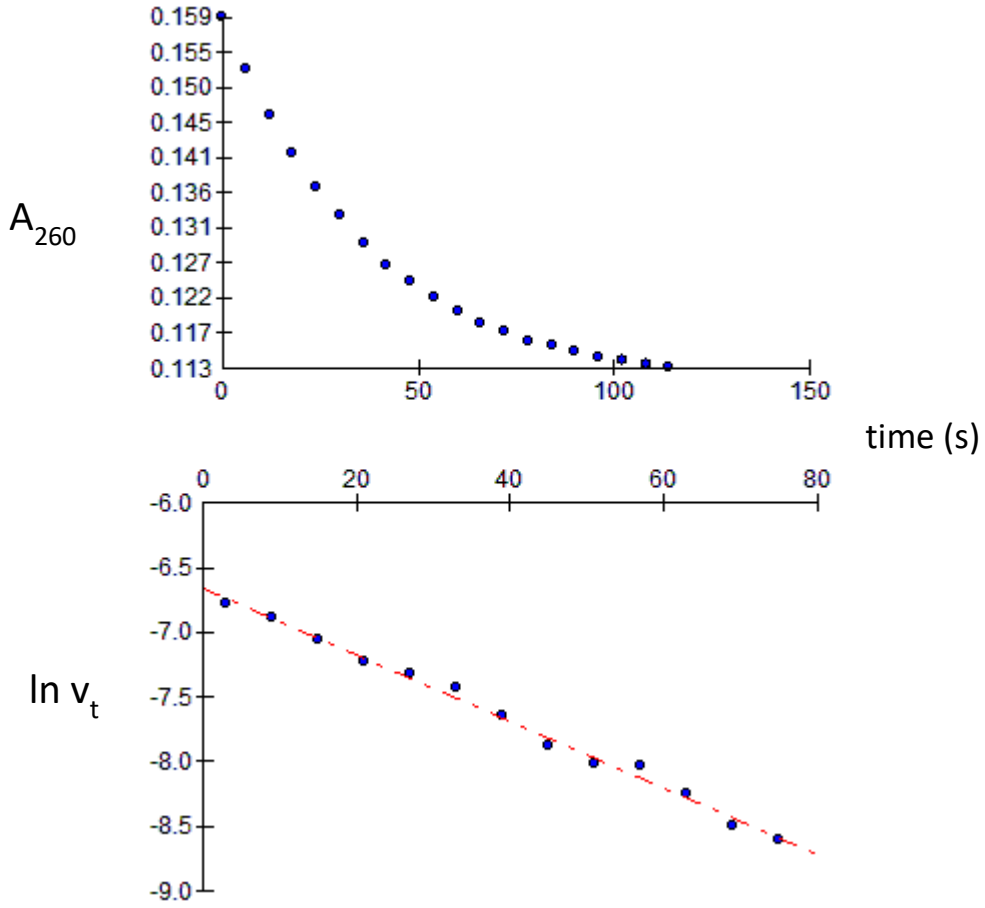

Figure 4.

Panel A: Monitoring of A_{260} *vs* t upon hydrolysis of 5 µM ceftazidime by 0.31 µM OXA-427 β-lactamase at 30°C. The total volume was 425 µL. The mixing dead-time was about 10 s.

Panel B: ln v_t *vs* t, computed with the first 14 points of Panel A.

5. Discussion and conclusions.

The method that is described here allows an easy and rapid determination of the first-order rate constants that characterise the inactivation of an enzyme or the rate of the enzymatic reaction when the substrate concentration is well below the K_m value. Good estimates of these constants can be obtained by repeating measurements. Due to the relatively short time needed to obtain good time-courses, this can be easily done. In the case of inactivation experiments, a prior determination of the IC50 value is helpful in choosing the range of inactivator concentrations to be utilized.

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Conflict of interest.

The authors declare no conflict of interest.

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Appendix – Values of the k_a for different models.

The following equations can be found in various textbooks or articles [14, 15 and other articles cited in the text] but we thought that it was useful to summarise the most common possibilities.

1) Models resulting in complete inactivation

Model 1

$E + I \overset{k_1}{\rightarrow} EI^*$ where EI* is generally a covalent adduct

$$k_a = k_1(I) \quad \frac{K_m}{K_m+(S)} \qquad \text{(A1)}$$

Model 2

$$E + I \overset{K}{\rightleftharpoons} EI \overset{k_2}{\rightarrow} EI^*$$

EI is usually a non-covalent complex and K its dissociation constant. EI* is most often a covalent adduct.

$$k_a = \frac{k_2(I)}{K\prime+(I)} \quad \text{where } K' = K\frac{K_m+(S)}{K_m} \qquad \text{(A2)}$$

If (I) << K', k_a is directly proportional to (I) [= k_2(I)/K') as in model 1.

Note that if the first step is not in rapid equilibrium, (K = k_{-1}/k_1 where k_{-1} is not much larger than k_2), the decrease of enzyme activity *vs* t is the sum of two exponentials and this more complex situation is not analysed here.

2) Models resulting in a steady-state or an equilibrium

Model 3

$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EI$$ (slow-binding inhibitors)

$$k_a = k_{-1} + k_1(I)\ \frac{K_m}{K_m+(S)} \qquad \text{(A3)}$$

Manual mixing methods (dead-time: 5-10 s) allow the measurement of k_a values up to 0.1 s^{-1}. Utilisation of stopped-flow equipment (mixing dead-time: 5-10 ms or less) allows to measure k_a values up to 100 s^{-1} or even more.

Model 4

$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EI \overset{k_2}{\rightarrow} E + X$$

$$k_a = k_{-1} + k_2 + k_1(I)\ \frac{K_m}{K_m+(S)} \qquad \text{(A4)}$$

In this case, I behaves as a poor substrate. If it is a good substrate, stopped-flow methods can be used but if the reaction is too rapid, even stopped-flow methods might not yield adequate results.

The individual value of k_2 can be obtained by measuring the maximum rate of X formation with saturating concentration of I ($V = k_{cat}E_0$ and $k_2 = k_{cat}$) in the sole presence of I (no reporter substrate).

Model 5

$$E + I \overset{k_1}{\rightarrow} EI \overset{k_2}{\rightarrow} E + X \quad or \quad E + I \overset{k_1}{\rightarrow} EI^* \overset{k_2}{\rightarrow} E + X$$

$$k_a = k_2 + k_1(I)\ \frac{K_m}{K_m+(S)} \qquad \text{(A5)}$$

Similar to model 4 but k_{-1} is negligible when compared to k_2.

Model 6

$$E + I \overset{K}{\rightleftharpoons} EI \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} EI^*$$

$$k_a = k_{-2} + \frac{k_2(I)}{K\prime+(I)} \quad \text{where} \qquad K' = K\frac{K_m+(S)}{K_m} \qquad \text{(A6)}$$

In the absence of reporter substrate, the proportion of active enzyme at equilibrium is

$$\frac{(E)}{E_o} = \frac{k_{-2}K}{k_{-2}K+(I)\ (k_{-2}+k_2)} \qquad \text{(A7)}$$

If (I) << K this simplifies to: $\frac{(E)}{E_o} = \frac{K_{eq}}{(I)+K_{eq}}$

The global equilibrium constant: $K_{eq} = \frac{(E)(I)}{(EI*)}\ is\ \frac{Kk_{-2}}{k_2}$ (A8)

Model 7

$$E + I \overset{K}{\rightleftharpoons} EI \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} EI^* \overset{k_3}{\rightarrow} E + X$$

$$k_a = k_{-2} + k_3 + \frac{k_2(I)}{K\prime+(I)} \qquad \text{(A9)}$$

Again, the individual values of k_{-2} and k_3 can be obtained by measuring the k_{cat} value for the I → X reaction in the absence of reporter substrate:

$$k_{cat} = \frac{k_2k_3}{k_{-2}+k_2+k_3} \qquad \text{(A10)}$$