Steady-state enzyme kinetics

Matthew D. Lloyd
(University of Bath, UK)

Steady-state enzyme kinetics is a cornerstone technique of biochemistry and related sciences since it allows the characterization and quantification of enzyme behaviour. Enzyme kinetics is widely used to investigate the physiological role of enzymes, determine the effects of mutations and characterize enzyme inhibitors. Well-known examples of enzyme inhibitors used to treat diseases include anti-infectives (e.g., penicillin, clavulanic acid and HIV protease inhibitors); anti-inflammatories (e.g., aspirin and ibuprofen); cholesterol-lowering statins; tyrosine kinase inhibitors used to treat cancer; and Viagra. Commonly, new disease treatments are discovered by using enzyme kinetics to identify the few active compounds residing within a large compound collection ('high-throughput screening'). The subject of enzyme kinetics is typically introduced to first-year undergraduates with a mathematical description of behaviour. This Beginners Guide will give a brief overview of experimental enzyme kinetics and the characterization of enzyme inhibitors. Colorimetric assays using a microtitre plate will be considered, although most principles also apply to other assays.

Preliminary considerations

Steady-state kinetics measures rates at the beginning of the reaction time course ('initial rates') because this is when inhibitor effects are most easily seen, and complications are minimized. If possible, a continuous assay should be used with a coloured reaction product, as this allows the most accurate rate measurements. The reaction rate in change in absorbance per minute ($\Delta A\ min^{-1}$) can be converted into, e.g., nmol min^{-1} using the Beer–Lambert law (equation 1). If there is a choice of assays, the one where the product has the longest wavelength (as this will minimize background noise) and the product which is the most intensely coloured (has the largest extinction coefficient ε) should be used. Using the longest reporter wavelength also reduces assay interference by the inhibitor:

$$A = \varepsilon c l \quad (1)$$

Product concentration (c in, e.g., molar, M) can be calculated from the absorbance at a particular wavelength (A) if the extinction coefficient (ε in, e.g., $M^{-1}\ cm^{-1}$) and pathlength (l in cm) are known.

Special mention should be made of assays which follow 4-nitrophenol release from a substrate at 405 nm. 4-Nitrophenoxide (deprotonated form) is very yellow (ε_{405} = 18.1 $mM^{-1}\ cm^{-1}$) whilst 4-nitrophenol (protonated form) is almost colourless (ε_{405} = 0.2 $mM^{-1}\ cm^{-1}$). The pK_a of 4-nitrophenol is 7.14, so at pH 7.5 only ~70% of product exists as 4-nitrophenoxide. A stopped assay where the reaction is quenched with, e.g., 0.1 M NaOH aq. at several time points is needed to ensure full colour development and the correct measurement of rates.

An amount of enzyme which gives a signal that can be easily distinguished from background noise needs to be used. Detergent is often included in assays to prevent inhibitor aggregation (which is a significant source of 'false-positive' results in inhibitor studies). If more than one assay is available, then Z' (equation 2) can be used to decide which assay performs the best. A Z' value >0.5 and ideally >0.7 is required. Once assay conditions have been finalized, the K_m value for the substrate should be determined. Substrate concentrations at or close to the K_m value are often used in further experiments:

$$Z' = 1 - \frac{3SD\ (positive\ control) + 3SD\ (negative\ control)}{signal\ from\ positive\ control - signal\ from\ negative\ control} \quad (2)$$

Equation 2 calculates the Z' value, which can range between 0 and 1 (with 1 being a 'perfect' assay). SD is the standard deviation.

A final consideration is how to measure rates. Absorbance is measured over the required time course using a spectrophotometer or a plate reader, the latter allowing the reading of several samples at the same time. The simplest method of determining rates (i.e., $\Delta A\ min^{-1}$) is to plot absorbance vs time at low substrate conversions ('initial rates'). This method is convenient but considerably under-estimates rates. An alternative is to fit absorbance readings over time to an integrated rate equation which gives better estimates and corrects for substrate depletion. An online program exists to derive rates from experimental data (see Olp *et al.*, 2020) which also allows them to be corrected for the time between starting the reaction and the first measurement.

Dose–response curves and measurement of IC_{50} values

The first step in determining inhibitor activity is often to determine a dose–response curve. Enzyme is incubated with varying concentrations of inhibitor, e.g., for 10 minutes. Most inhibitors will bind to the enzyme in <1

Summary Box

- Behaviour of Michaelis-Menten enzymes is described by the kinetic parameters V_{max} (the maximum rate of reaction under the specified conditions) and K_m (substrate concentration at which the observed rate is half that of V_{max}).
- Potency of inhibition can be determined using dose–response curves to measure the IC_{50} (the concentration of inhibitor which reduces enzyme activity to half that in its absence).
- Rapid dilution experiments can be used to assess inhibition reversibility.
- Reversible enzyme inhibitors can be competitive (K_m increases), non-competitive (V_{max} decreases), mixed competitive (K_m increases, V_{max} decreases) or uncompetitive (K_m and V_{max} are decreased to the same extent). Inhibitor potency is quantified using K_i (the concentration of inhibitor changing the observed K_m and/or V_{max} by twofold).
- Saturating irreversible inhibitors can be characterized using $k_{inactivation}$ (the maximum rate of inactivation) and K_I (inhibitor concentration where inactivation is half its maximum rate).

second but binding of inhibitor can be much slower than this. Many inhibitors have limited solubility in buffer and readily precipitate, and so concentrated inhibitor stocks should be prepared in DMSO and mixed with a larger volume of enzyme stock. At the end of the pre-incubation period, the sample is split into repeats, substrate solution added and rates measured.

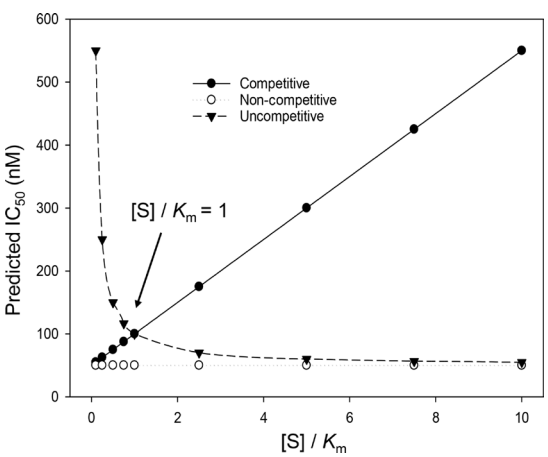
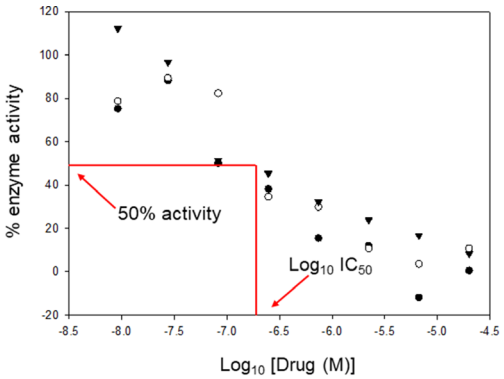


Figure 1. Predicted IC_{50} values for various inhibitor types at various substrate concentrations, assuming K_i = 50 nM. The predicted IC_{50} value for mixed inhibitors depends on the proportion of competitive and non-competitive components of inhibition.



Parameter	Value	StdErr	CV(%)	Dependencies
min	-4.958e+0	1.114e+1	2.247e+2	0.7238928
max	8.746e+1	1.905e+1	2.179e+1	0.8522084
$logIC_{50}$	-6.702e+0	2.856e-1	4.261e+0	0.7510544
Hillslope	-9.174e-1	5.506e-1	6.002e+1	0.7970488

Figure 2. An example dose–response curve for a well-behaved inhibitor.

A typical upper inhibitor concentration in the assay is 100 μM, although this may need to be increased for weakly binding inhibitors such as those identified from fragment screens. A 1 in 3 dilution series is used, which for 11 concentrations spans a range of 100 μM to 1.69 nM. Two inhibitors can be measured in duplicate in each run, with the 12th positions occupied by positive and negative controls. A substrate concentration equal to the K_m value is often used as this enables the effects of different inhibitors to be most easily observed (Figure 1).

The IC_{50} value is determined by plotting the reaction rate against Log_{10} molar drug concentrations, followed by fitting of the curve (Figure 2). For a well-behaving inhibitor, the enzymatic rates at the highest and lowest concentrations should be close to the negative and positive controls, respectively, and the slope in the middle part of the curve should be ~–1. Failure to behave in this way needs to be thoroughly investigated, as it means that there are problems with the inhibitor which may render it unusable (see Copeland for further details). Three independent determinations of IC_{50} values for each inhibitor are usually made. Log_{10} molar inhibitor concentrations are generally used in dose-response curves, which means that results should be quoted as $-\log_{10}$ IC_{50} (usually denoted as pIC_{50}) ± standard error of the mean. An important point is that the determined pIC_{50} value will depend on the substrate concentrationused in the assay (Figure 1).

The possibility of tight-binding inhibition should be considered with potent inhibitors (those with low IC_{50} values). This occurs when the ratio of available drug to active enzyme is less than ~20:1 and often manifests itself by unusual dose–response curves. A detailed discussion is beyond the scope of this article and readers should consult the textbook by Copeland for further details.

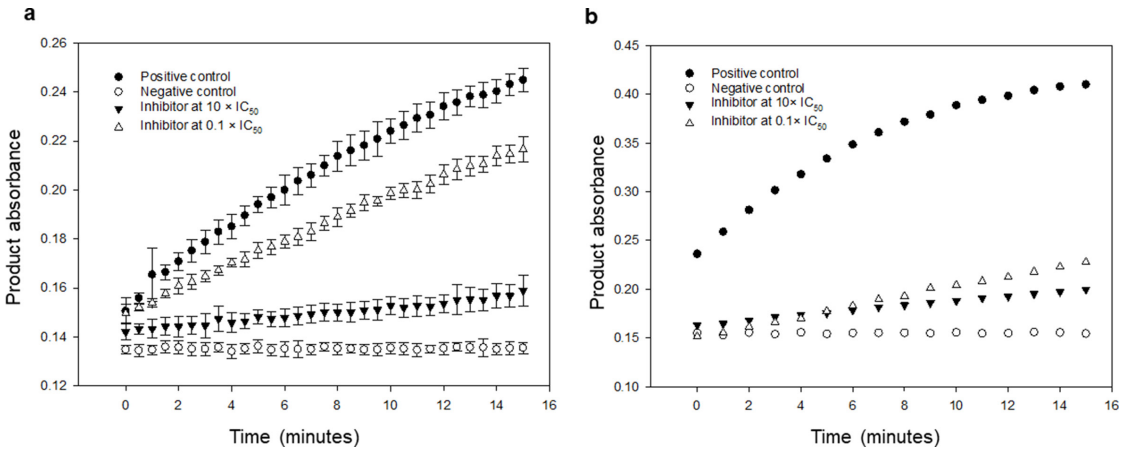


Figure 3. A rapid dilution experiment with (a) a rapidly reversible inhibitor and (b) an irreversible inhibitor showing the differences in substrate conversion at high (10× IC_{50}) and low (0.1× IC_{50}) inhibitor concentrations.

Determining if the inhibitor is reversible

Proper characterization of an inhibitor requires a determination of whether it binds reversibly to the enzyme or not. A convenient method to determine this uses a rapid dilution experiment (Figure 3). In this experiment enzyme is incubated with inhibitor at 10× IC_{50} value, which should reduce reaction rates to ~10% of that in the positive control. In a second experiment, concentrated enzyme (e.g., 100× assay concentration) is incubated with inhibitor at 10× IC_{50}. Immediately before assaying, the enzyme/inhibitor mixture is diluted such that enzyme is at the same concentration as in the positive control; this also dilutes the inhibitor (by 100× in this example, to 0.1× IC_{50} value). For a rapidly reversible inhibitor this results in restoration of activity to ~90% of that in the no-inhibitor positive control (Figure 3a). In contrast, little difference in rates is observed between the first and second experiments for an irreversible inhibitor (Figure 3b). Irreversible inhibition can arise when inhibitor binds covalently to the enzyme or when inhibitor binds to the enzyme non-covalently but the inhibitor is released slowly.

Determination of K_i values

K_i values are related to the thermodynamic strength of reversible inhibitor binding and are used to compare potencies between different reversible inhibitors. The most common method to determine K_i values is to measure enzymatic activity in the presence of variable substrate concentrations at several fixed inhibitor concentrations. A wide range of substrate concentrations should be used, e.g., 8 equally spaced concentrations between 0.1× and 10× K_m, which gives rates between 9% and 91% of V_{max}. Inhibitor concentrations to be used in this experiment ([I]) can be conveniently calculated using equation 3. Inhibitor concentrations should be chosen to give rates of 75%, 50% and 25% (v_i) of the uninhibited reaction (v_0) (so v_0/v_i will be 1.33, 2 and 4, respectively):

$$[I] = IC_{50}\,[(v_0/v_i)\,-1]^{1/h} \qquad (3)$$

Equation 3 is used for determining inhibitor concentrations for steady-state kinetic analysis. The IC_{50} value is that determined when the substrate concentration in the assay is equal to its K_m value, and h is the slope from the dose–response curve (the 'Hill coefficient').

The obtained data should be fitted to all types of inhibition (competitive, non-competitive, mixed and uncompetitive) for both dead-end (full) and partial inhibition modes (dead-end inhibition means that no enzyme activity remains at saturating inhibitor concentrations, whilst significant activity is present for a partial inhibitor under the same conditions). Competitive inhibitors will increase K_m with no change in V_{max}; non-competitive inhibitors decrease V_{max} without changing K_m; mixed competitive (a.k.a. mixed) inhibitors will have both effects; and uncompetitive inhibitors will decrease both K_m and V_{max} to the same extent. More than one viable solution will usually fit the data, and so the problem becomes how to tell which is the correct answer. This is done by inspection of various plots (Figure 4) and the use of statistical calculations.

Several plots can be used to analyse the data, including the Direct Linear plot (Figure 4a), the Direct ('Michaelis–Menten') plot (Figure 4b) and various linear transformations of the Michaelis–Menten equation (e.g., Figure 4c and d). Each plot has advantages and disadvantages and so all of them should be used in this analysis. In addition, a residuals plot showing the difference between calculated and observed rates vs

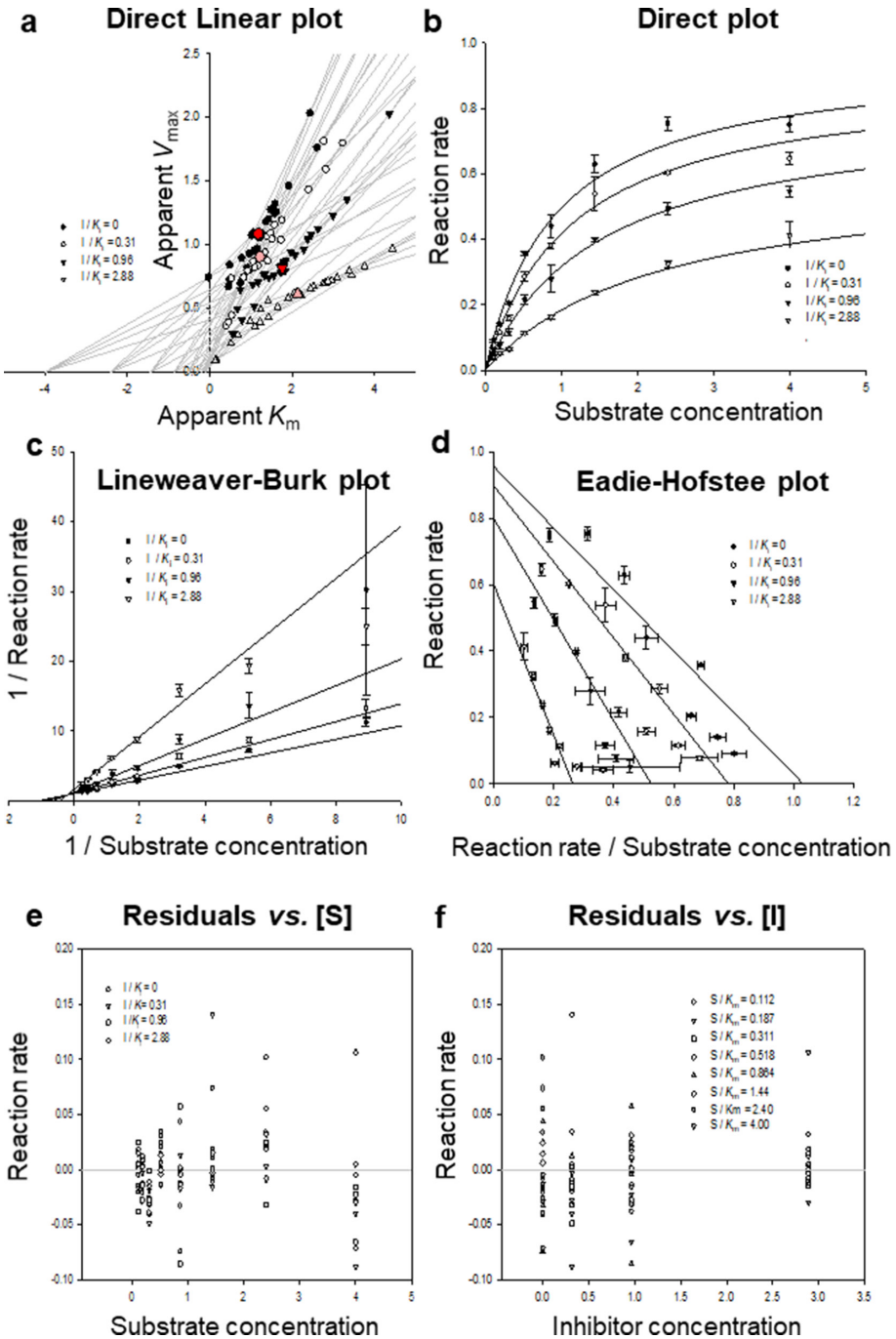


Figure 4. An illustration of kinetic plots for a mixed competitive inhibitor. Note that substrate and inhibitor concentrations are relative to K_m and K_i values, respectively, and rates are relative to V_{max}. S and I are substrate and inhibitor, respectively.

substrate and inhibitor concentrations (Figure 4e and f) should be produced, as this allows systematic deviations from predicted behaviour to be readily identified. The following steps usually result in the identification of the correct type of inhibition:

1. Examine the Direct Linear plot (Figure 4a) and observe the general trends for K_m and V_{max} values with increasing inhibitor concentrations.
2. Look at the statistical ranking of the different types of inhibition and check which are consistent with the trends observed in (1).
3. Look at the calculated kinetic parameters for the most likely solutions. Reject those where K_m and K_i values are much larger or smaller than experimental substrate or inhibitor concentrations, or where V_{max} is much larger or smaller than the observed rates.

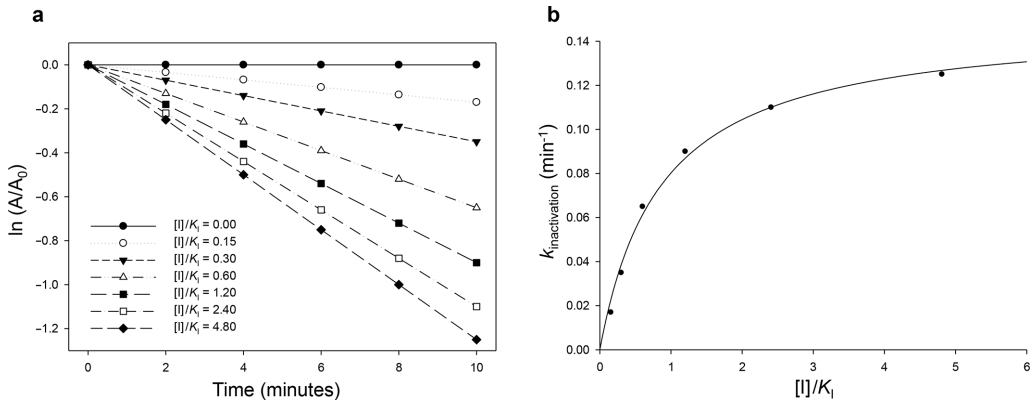


Figure 5. Behaviour of a saturating irreversible inhibitor. (**a**) Plot of the natural logarithm of fractional remaining activity vs time to determine the rate constant for enzyme inactivation at each inhibitor concentration. (**b**) Plot of inactivation rate constants vs inhibitor concentration to determine the maximum rate of inactivation ($k_{inactivation}$) and K_I value (inhibitor concentration at which inactivation is half its maximum rate).

Reject any solutions with very large errors in any of the kinetic parameters.

4. Examine the Direct plot (Figure 4b), Eadie–Hofstee plot (Figure 4d) and residuals plots (Figure 4e and f) for the remaining solutions and look for systematic deviations. Examine the Lineweaver–Burk plot (Figure 4c) for points near the *Y*-axis departing from a straight line (substrate inhibition). The presence of significant deviations suggests the solution is not correct or more complex behaviour is being observed.
5. Apply Occam's razor (which says that the simpler solution is usually correct). Partial inhibition (mixed inhibition in this case) usually gives a statistically 'better' fit to the data, but this solution should be rejected if it makes little difference to K_m, V_{max} or K_i values and/or their associated errors.
6. The commonly observed types of inhibition are competitive and mixed competitive. Uncompetitive inhibition is rare but is occasionally observed. Non-competitive inhibition generally arises with multi-substrate enzymes, and the inhibitor will usually only behave in this way with one of the substrates. If non-competitive inhibition is observed, consider whether the inhibitor could be irreversible (see next section) or has aggregated (adding 0.1% detergent to the assay should diminish or abolish inhibition).

Irreversible inhibitors

The fundamental characteristic of irreversible inhibitors is that inhibition increases upon increasing the length of time that the enzyme is exposed to inhibitor. There are fundamentally two different types of irreversible inhibitor, those where inactivation rates saturate at higher inhibitor concentrations and those which do not. The former behaviour (Figure 5) usually indicates inhibitor binding to a defined pocket in the enzyme (often the active site), whilst the latter usually indicates binding to several non-specific sites. The latter behaviour is typically observed with non-specific protein modification reagents.

When characterizing an irreversible inhibition, concentrated enzyme is pre-incubated with several different concentrations of inhibitor and activity determined after different pre-incubation times. For most irreversible inhibitors, enzymatic activity decays with first-order kinetics at each fixed concentration. Plotting the natural logarithm (ln) of activity at the pre-incubation time point/activity at pre-incubation time = 0 vs time for each inhibitor concentration yields a series of straight lines (Figure 5a), with the slope corresponding to the first-order rate constant. Saturating inhibitors will show behaviour such as that displayed in Figure 5b when inactivation rates are plotted against inhibitor concentration. This allows determination of $k_{inactivation}$ (the maximum rate of inactivation) and K_I (the inhibitor concentration at which the rate of inactivation is half its maximum value; not to be confused with K_i the reversible inhibitor constant). The effectiveness of different irreversible inhibitors can be compared using $k_{inactivation}/K_I$ values.

Conclusions

Enzyme inhibitors are the single largest class of small-molecule drugs used in modern human and veterinary medicine and are highly successful. The development of new enzyme inhibitors for the treatment of many different diseases is being actively pursued. Enzyme kinetics will remain a key technique for the foreseeable future, for characterization of inhibitor properties, the optimization of drug potency and inhibitor identification by high-throughput screening and related techniques. ■

Further reading

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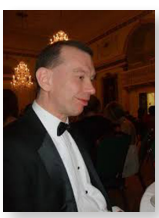

Matthew Lloyd is Senior Lecturer in the Department of Pharmacy & Pharmacology at the University of Bath and researches enzyme inhibitors as treatments for diseases such as cancer, using enzyme kinetics and chemical biology approaches. He is also a 4th Degree Black Belt (International Instructor) in the Ch'ang-Hon ('ITF') style of Taekwon-Do. Email: M.D.Lloyd@bath.ac.uk; Twitter: @DrMatthewLloyd