Optimal duration of the preincubation phase in enzyme inhibition experiments

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Abstract

This report describes an algebraic formula to calculate the optimal duration of the pre-incubation phase in enzyme-inhibition experiments, based on the assumed range of expected values for the dissociation equilibrium constant of the enzyme–inhibitor complex and for the bimolecular association rate constant. Three typical experimental scenarios are treated, namely, (1) single-point primary screening at relatively high inhibitor concentrations; (2) dose-response secondary screening of relatively weakly bound inhibitors; (3) dose-response screening of tightly-bound inhibitors.

Key words: enzyme kinetics; inhibition; theory; mathematics; optimal design; tight-binding

1. Introduction

The experimental situation we are concerned with is a *bimolecular binding* experiment, in which reactants A and B are brought together in a homogeneous solution to form a noncovalent complex C. A typical example involves enzyme inhibition studies. The enzyme ("A") and the inhibitor ("B") are preincubated for a period of time sufficiently long to effectively establish full equilibrium. After the enzyme–inhibitor complex "C" is effectively fully formed a substrate is added to initiate the enzyme-catalyzed reaction.

Similar binding experiments are performed in other areas of biochemical and biophysical research, not necessarily involving enzymes and their inhibitors, perhaps involving protein–DNA interactions or pharmacological receptor–ligand binding instead. Again, the two respective reactants are brought together in a biochemical, biophysical, or pharmacological assay and allowed to equilibrate in order to investigate the strength of the binding interaction.

The main question addressed in this article is *how much time to allow* for nearly full equilibration, given the assumed values of the association and dissociation rate constants and also given a practically feasible range reagent concentrations. It is important that the preincubation time is neither too short nor too long for a number of practical reasons, such as avoiding possible chemical degradation of the enzyme upon prolonged exposure to the assay buffer.

In theory, full equilibrium can only be reached after infinitely long preincubation of reacting components. Thus, a practically useful question is what is the incubation time required to reach a sufficient degree of equilibration, for example 90, 95 or 99 percent. This reports presents a closed-form algebraic formula to achieve that particular goal.

2. Methods

2.1. Theory

2.1.1. General case

Let us assume that an inhibitor I and enzyme E associate reversibly to form a noncovalent molecular complex C. The bimolecular association rate constant is k_a and the corresponding first-order dissociation rate constant is k_d. It could be shown (see Appendix A for derivation) that under those assumptions the preincubation time need to achieve a particular degree of fractional equilibration is defined by Eqn (1).

$$t_f = \frac{1}{k_a \beta} \ln \frac{1 - f\,(\alpha - \beta)/(\alpha + \beta)}{1 - f} \tag{1}$$

$$\alpha \equiv [\mathrm{E}]_0 + [\mathrm{I}]_0 + K_d \tag{2}$$

$$\beta \equiv \sqrt{([\mathrm{E}]_0 + [\mathrm{I}]_0 + K_d)^2 - 4\,[\mathrm{E}]_0[\mathrm{I}]_0} \tag{3}$$

$$K_d = k_d/k_a \tag{4}$$

In Eqn (1), t_f is the preincubation time required to achieve a fractional approach to equilibrium measured by the parameter f. The allowable values are by definition between zero ($f = 0$, signifying that no enzyme–inhibitor binding occurred yet) and unity ($f = 1$, signifying full equilibration). For example, the value $f = 0.95$ represents the particular moment during the preincubation period when the enzyme–inhibitor complex is already 95% formed, in the sense that the concentration of the complex C is 95% of its equilibrium value. The auxiliary variables α and β (Eqns (2)–(3)) were introduced merely to simplify the display of Eqn (1); $K_d = k_d/k_a$ is the dissociation equilibrium constant. $[\mathrm{E}]_0$ and $[\mathrm{I}]_0$ are the initial (total, analytic) concentrations of the enzyme and the inhibitor.

2.1.2. Special case of primary screening

Primary high-throughput screening it conducted at a single (relatively high) concentration of the inhibitors, typically $[\mathrm{I}]_0 = 10\ \mu\mathrm{M}$, and at a single enzyme enzyme concentration that is as low as practicable. Essentially in all cases primary screening is conducted under the conditions where the enzyme concentration is very much lower than the inhibitor concentration, $[\mathrm{E}]_0 << [\mathrm{I}]_0$. Under such specialized experimental conditions $\alpha = \beta = [\mathrm{I}]_0 + K_\mathrm{d}$ and therefore Eqn (1) simplifies to Eqn (5). For example, the preincubation time to reach 95% equilibration during a primary screening assay can be calculated by using Eqn (6)

$$t_f = \frac{1}{k_\mathrm{a}\ ([\mathrm{I}]_0 + K_\mathrm{d})} \ln \frac{1}{1-f} \quad ;\ \text{if } [\mathrm{E}]_0 << [\mathrm{I}]_0 \quad (5)$$

$$t_{0.95} = \frac{3}{k_\mathrm{a}\ ([\mathrm{I}]_0 + K_\mathrm{d})} \quad (6)$$

Under the specialized primary screening conditions t_f is inversely proportional to the sum $[\mathrm{I}]_0 + K_\mathrm{d}$. Thus, the *maximum* expected equilibration time can be computed by setting K_d to a vanishingly small value, corresponding to an extremely tight binding inhibitor. The maximum necessary equilibration time (at the typical value $f = 0.95$) can then be computed simply as shown in Eqn (7).

$$t_{0.95}^{(\mathrm{max})} = \frac{3}{k_\mathrm{a}\ [\mathrm{I}]_0} \quad (7)$$

Thus, for example, if a primary inhibitor screening campaign is to be conducted at $[\mathrm{I}]_0 = 10^{-5}$ M, and if the slowest binder in the given inhibitor library can reasonably be expected to be characterized by the "on"-constant $k_\mathrm{a} = 10^3\ \mathrm{M}^{-1}\mathrm{s}^{-1}$, then the maximum necessary preincubation time is five minutes: $t_{0.95}^{(\mathrm{max})} = 3/(10^3 \times 10^{-5}) = 300$ sec = 5 min.

Note that at $[\mathrm{I}]_0 = 10\ \mu$M, this (at most) five-minute preincubation time applies to all conceivable enzyme inhibitors characterized by $k_\mathrm{a} \geq 10^3\ \mathrm{M}^{-1}\mathrm{s}^{-1}$, irrespective of their overall potency as measured by the dissociation equilibrium constant K_d. In fact, at $[\mathrm{I}]_0 = 10\ \mu$M, according to the kinetic analysis presented here, almost all compounds in any real-world inhibitor library will reach 95% equilibration in a much shorter incubation time typically measured in seconds rather than minutes or hours.

2.2. Heuristic simulations

All computations were performed by using the software DynaFit [1, 2]. An example simulation script, in this particular case a script used to generate *Figure 4*, is listed in Appendix B.

3. Results

3.1. Exponential approach to equilibrium

Figure 1 shows that the difference between preincubation time required to reach 90% and 95% equilibration is approximately the same as the difference between preincubation time required to reach 98% and 99% equilibration. In other words, the equilibration process slows down exponentially over time. In fact, by definition, full equilibrium ($f = 1$) could hypothetically be reached only at infinite incubation time.

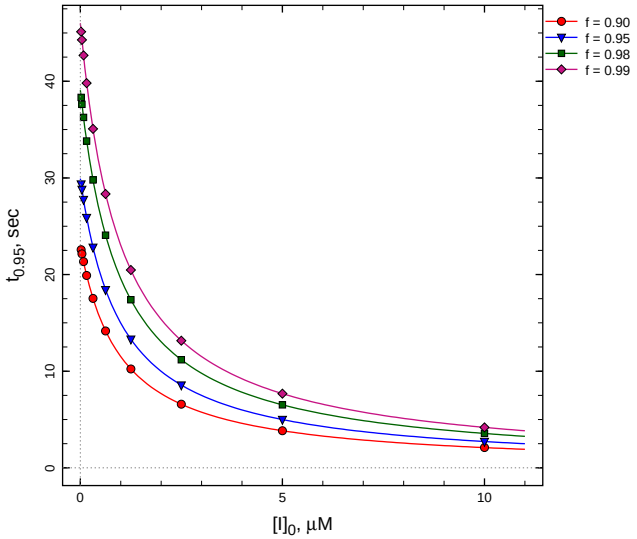

Figure 1: Simulated preincubation time required to reach, 90, 95, 98, or 99% equilibration at various concentrations of an inhibitor characterized by $K_\mathrm{d} = 1\ \mu$M and $k_\mathrm{a} = 10^5\ \mathrm{M}^{-1}\mathrm{s}^{-1}$. The enzyme concentration was $[\mathrm{E}]_0 = 1$ nM.

Given that all enzyme assays are characterized by a finite random experimental error, typically measured in single-digit percentage points, it should be sufficient to always compute the incubation time required to reach 95% equilibration, as opposed to insisting on 99% or higher equilibration extent. This value ($f = 0.95$) will be used through the rest of our kinetic analysis.

3.2. A primary screening scenario

A primary screen of a potentially large library of enzyme inhibitors often involves a single inhibitor concentration (typically 10 or 50 μM) assayed against the lowest feasible enzyme concentration, to economize the expenditure of materials. In this particular simulation study, we employed $[\mathrm{E}]_0 = 10$ pM and $[\mathrm{I}]_0 = 10\ \mu$M. The expected dissociation equilibrium constants for the putative inhibitor library ranged from 100 pM to 10 μM. *Figure 2* shows the calculated time required to reach 95% equilibration.

Figure 2 illustrates that at primary screening concentrations $[\mathrm{E}]_0 = 10$ pM and $[\mathrm{I}]_0 = 10\ \mu$M, no inhibitor regardless of its overall affinity and regardless of its bimolecular association kinetics ("fast" or "slow" binding, as long as $k_\mathrm{a} \geq 10^3\ \mathrm{M}^{-1}\mathrm{s}^{-1}$) takes longer than approximately five minutes to fully equilibrate. Note that most enzyme inhibitors reported in the literature actually associate much more rapidly than the "slowest" inhibitors considered in this study; the most typical value of associate rate constant appears to be in the range of $k_\mathrm{a} \approx 10^4 - 10^5\ \mathrm{M}^{-1}\mathrm{s}^{-1}$ [3]. See also Eqns (6)–(7) in the Methods section, which

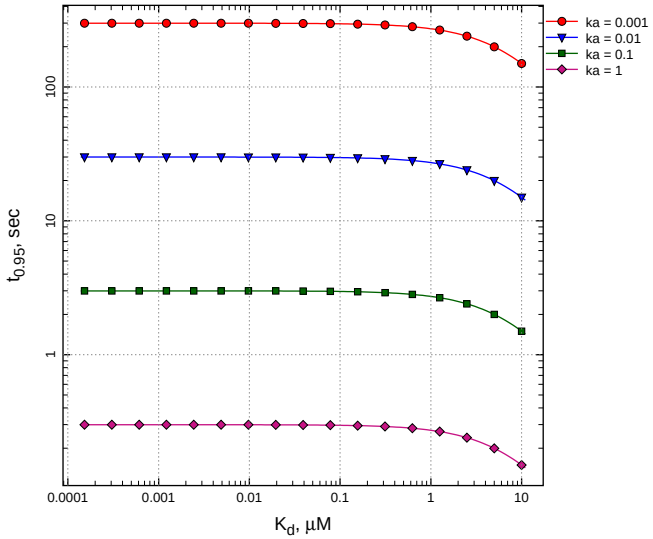

Figure 2: Simulated preincubation time required to reach 95% during a typical primary screen conducted at $[E]_0$ = 10 pM and $[I]_0$ = 10 μM. For details see text

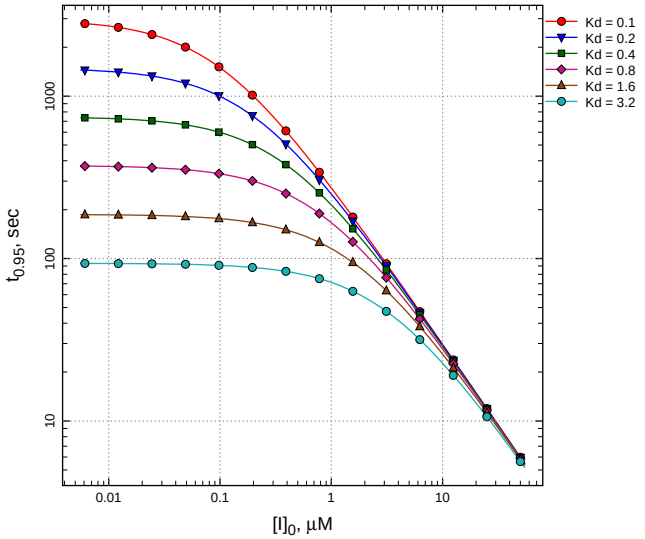

Figure 3: Simulated preincubation time required to reach 95% equilibration at various concentrations of low-affinity inhibitors. The assumed $k_a = 10^4$ M^{-1}s^{-1}. The dissociation equilibrium constant values in the figure Legend are in micromolar units. For details see text.

indeed predicts that at $[I]_0$ = 10 μM and $k_a \geq 10^3$ M^{-1}s^{-1} the maximum required preincubation time is exactly five minutes.

3.3. Dose-response screening of low-affinity inhibitors

Figure 3 displays the results of simulated 95% fractional equilibration time for a series of hypothetical low-affinity inhibitors, while assuming $k_a = 10^4$ M^{-1}s^{-1} and enzyme concentration $[E]_0$ = 1 nM. The inhibitor concentration is varied on the horizontal axis, as it would be in a typical dose-response (secondary) screening experiment.

Figure 3 illustrates that at inhibitor concentrations significantly lower than the dissociation equilibrium constant the incubation time required to reach 95% equilibration remains nearly constant. At inhibitor concentrations approaching K_d, the required incubation time starts to decrease gradually. Finally, at inhibitor concentrations significantly higher than K_d the required incubation time decreases rapidly, approximately following an exponential decay.

3.4. Dose-response screening of high-affinity inhibitors

Figure 4 shows the results of using Eqn (1) to calculating the preincubation time required for 95% equilibration (f = 0.95) of an inhibitor characterized by K_d is 0.01 nM and k_a = 10^6 M^{-1}s^{-1}. These particular numerical values of K_d and k_a would characterize a typical "fast on", "slow off", very high-affinity inhibitor.

Figure 4 illustrates probably the most important characteristic of high affinity inhibitors, namely, that the preincubation time to reach 95% equilibration is longest under the particular experimental conditions where $[E]_0 = [I]_0$. Note that appearance of local maxima on the simulated curves, approximately under the conditions where the enzyme and inhibitor concentrations are equal.

When the total concentration of the inhibitor is significantly lower the concentration of the enzyme (an experimental situation that does occur with some frequency in investigating the kinetics of "tight-binding" inhibitors) the time to reach 95% equilibration is approximately three or four times shorter than the the incubation time required at the point of equivalence.

4. Discussion

The general approach to deriving integrated rate laws similar to Eqn (1) is presented in a number of basic texts on chemical kinetics and ordinary differential equations (see for example [4, p. 56] [5, p. 22]). Boeker [6] previously derived a precursor of Eqn (1), and additionally also several related analogs of Eqn (1) relevant to enzyme kinetics. In this work we extended Boeker's earlier derivations by focusing specifically on the reaction time required to reach a certain extent of pre-equilibration in enzyme *inhibition* assays.

This topic is important for a number of practical reasons, especially because enzyme inhibition assays consume a large amount of material and human resources in the process of drug discovery. It is therefore imperative to always strive for optimal design of the enzyme inhibition experiments. Unnecessarily long preincubation time can lead to uncontrollable loss of enzyme activity due to prolonged exposure to the incubation buffer. On the other hand, insufficiently long preincubation time leads to inaccuracies in the determination of inhibition constants, because the enzyme–inhibitor equilibrium has not yet been established to a sufficient extent.

This work presents a simple algebraic formula, shown in Eqn (1), which can be used to estimate the enzyme–inhibitor

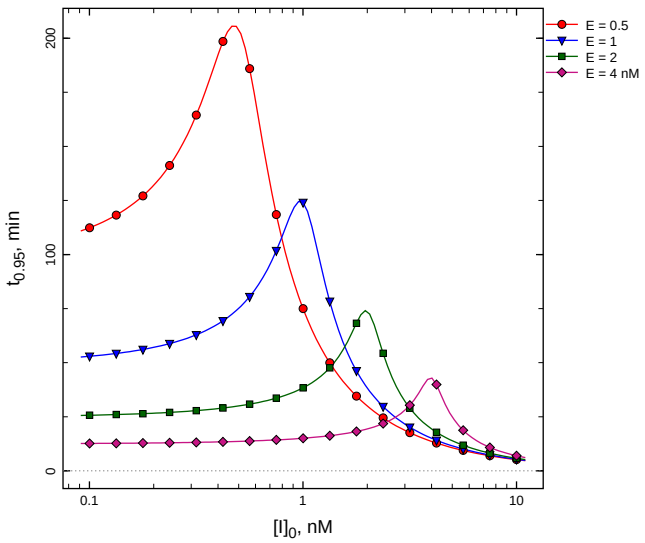

Figure 4: Simulated preincubation time required to reach 95% equilibration at various concentrations of the enzyme (legend) and the inhibitor (horizontal axis). The assumed bimolecular association rate constant is 10^6 $M^{-1}s^{-1}$ ("fast on") and the assumed affinity is K_d = 10 pM.

incubation time such that a desired degree of equilibration (e.g. 90, 95, of 99%) is reached at the end of the of the incubation interval. For the purposes of optimal experimental design, the application of the formula requires making realistic assumptions about the limiting values of of K_d and k_a.

An even simpler algebraic formula, shown in Eqn (6), can be utilized to decide on the necessary preincubation time in a primary (single-dose) screening campaign conducted at $[I]_0 >> [E]_0$. Under those particular conditions, deciding on the optimal duration of preincubation time involves only a single arbitrary assumption, namely, about the smallest plausible value of the association rate constant k_a. The results show any primary screening performed at 10 μM requires at most five minutes of preincubation while assuming that $k_a > 10^3$ $M^{-1}s^{-1}$. This in contrast to anecdotal evidence from various laboratories, in which primary inhibitor screening assays often include preincubation periods measured in hours, as opposed to minutes.

Eqn (1) can also be used to demonstrate that under secondary (dose-response) screening conditions, i.e. at relatively low inhibitor concentrations, preincubation times required to reach 95% approach to equilibrium can reach multiple hours if the enzyme concentration is insufficiently high.

Specifically, *Figure 4* illustrates that the overall the required incubation times are very much longer than the values expected for low-affinity inhibitors (see *Figure 3*). In addition, the required incubation time grows very substantially as the overall concentrations decrease. For example, as can be seen in *Figure 4*, at $[E]_0 = [I]_0 = 4.0$ nM the time to reach 95% equilibration is approximately 40 minutes (2400 sec). In contrast, at 0.5 nM concentrations the time to reach 95% equilibration is approximately 3.4 hours (12200 sec).

One possible lesson from this particular numerical experiment is that in order to shorten the preincubation time in assays of very high affinity ("slow off") inhibitors, the enzyme concentration should be as high as practically feasible. Previous theoretical and experimental studies [7, 8] demonstrated that it is possible to achieve reasonably good results with $[E]_0 = 10 \times K_d$ or even higher.

5. Summary and Conclusions

The newly derived algebraic Eqn (1) presented in this report can be utilized to optimize the preincubation time required for enzyme–inhibition studies. Given an approximate estimate of the bimolecular association rate constant k_a and the dissociation equilibrium constant K_d, and also given a feasible range of enzyme concentrations, it is possible to estimate the incubation time required for 95% equilibration.

Practical implications for optimal design of enzyme inhibition experiments are as follows. (1) In primary "percent inhibition" screening assays, using a single relatively high inhibitor concentration such as for example 10 μM, at most five minute preincubation is sufficient to achieve 95% equilibration. (2) In secondary dose-response screening assays, it is important to use the highest practically feasible enzyme concentration to avoid unnecessarily long preincubation times. (3) In the special case of extremely potent "tight binding" enzyme inhibitors, the optimal preincubation time should be based on the point of equivalence, where the concentrations of the enzyme and the inhibitor are equal.

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Appendix

A. Derivations

A.1. Reaction time course

The time-course of a bimolecular association $E + I \rightleftharpoons C$, characterized by the association rate constant k_a and the dissociation rate constant k_d, is described by the differential Eqn (A.1). Taking into account the mass-balance equations for the interacting components, Eqns (A.2)–(A.3), Eqn (A.1) can be solved analytically after separation of variables:

$$\frac{\mathrm{d[C]}}{\mathrm{d}t} = k_a[\mathrm{E}][\mathrm{I}] - k_d[\mathrm{C}] \tag{A.1}$$

$$[\mathrm{E}]_0 = [\mathrm{E}] + [\mathrm{C}] \tag{A.2}$$

$$[I]_0 = [I] + [\mathrm{C}] \tag{A.3}$$

$$\int_0^t \mathrm{d}t = \int_0^{[\mathrm{C}]} \frac{\mathrm{d[C]}}{k_a([\mathrm{E}]_0 - [\mathrm{C}])([\mathrm{I}]_0 - [\mathrm{C}]) - k_d[\mathrm{C}]} \tag{A.4}$$

Using the method of Laplace transforms, the solution of the integral Eqn (A.4) is obtained as shown in Eqn (A.5), where α and β are auxiliary variables defined in Eqns (2)–(3) above. An algebraic rearrangement yields [C] as a function of the reaction time, t, as shown in Eqn (A.6)

$$t = \frac{1}{k_a \beta} \ln \frac{1 - 2\,[\mathrm{C}]/(\alpha + \beta)}{1 - 2\,[\mathrm{C}]/(\alpha - \beta)} \tag{A.5}$$

$$[\mathrm{C}](t) = 2\,[\mathrm{E}]_0\,[\mathrm{I}]_0 \frac{1 - \exp(-k_a \beta\, t)}{\alpha + \beta - (\alpha - \beta)\,\exp(-k_a \beta\, t)} \tag{A.6}$$

A.2. Time required for fractional equilibration

Given the total (analytic) concentrations of components ($[\mathrm{E}]_0$ and $[\mathrm{I}]_0$), and also given a presumed value of the dissociation equilibrium constant K_d, the equilibrium concentration of the enzyme–inhibitor complex can be expressed as shown in Eqn (A.7).

$$[\mathrm{C}]_{eq} = \frac{\alpha - \beta}{2} \tag{A.7}$$

Let us define a particular concentration of the complex at the preincubation time t_f, such that $[\mathrm{C}]_f/[\mathrm{C}]_{eq} = f$, where f is by definition between zero and unity. The preincubation time t_f required to reach $[\mathrm{C}]_f$ can then be computed as shown in Eqn (A.9).

$$[\mathrm{C}]_f \equiv f\,[\mathrm{C}]_{eq} \quad , \quad 0 \le f < 1 \tag{A.8}$$

$$t_f \equiv \frac{1}{k_a \beta} \ln \frac{1 - 2\,[\mathrm{C}]_f/(\alpha + \beta)}{1 - 2\,[\mathrm{C}]_f/(\alpha - \beta)} = \frac{1}{k_a \beta} \ln \frac{1 - f\,(\alpha - \beta)/(\alpha + \beta)}{1 - f} \tag{A.9}$$

B. DynaFit simulation script

The following DynaFit [2] script was utilized to generate *Figure 4*. Note that units are nM and minutes. Thus, the notation `ka = 0.06` stands for $k_a = 0.06$ nM^{-1}min^{-1} = 10^6 M^{-1}s^{-1}. The values of `Kd` and all concentrations are in nanomoles per liter. The symbols `a` and `b` stand for α and β, respectively.

```
[task]
   task = simulate
   data = generic
[parameters]
   E, I, Kd, ka
[model]
   ka = 0.06
   Kd = 0.01
   f = 0.95
   a = E + I + Kd
   b = sqrt (a*a - 4*E*I)
   N = 1 - f*(a - b)/(a + b)
   D = 1 - f
   t95 = log(N/D)/(ka*b)
[data]
   variable I
   mesh logarithmic from 10 to 0.1 step 0.75
   directory ./project/TimeToEquil/data/simul
   plot logarithmic
   extension txt
   file d02 | param E = 0.5  | label E = 0.5
   file d03 | param E = 1    | label E = 1
   file d04 | param E = 2    | label E = 2
   file d05 | param E = 4    | label E = 4 nM
[output]
   directory ./project/TimeToEquil/output/simul
[settings]
{Output}
   XAxisLabel = [I]_0, nM
   YAxisLabel = t_{0.95}, min
[end]
```