Slow-onset Enzyme Inhibition and Inactivation

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Abstract

Interactions between modifiers and enzymes can either occur rapidly, on the time scale of diffusion-controlled reactions, or they can be slow processes observable on the steady-state time scale. Slow interactions in hysteretic enzymes serve to dampen cellular responses to rapid changes in metabolite concentration as part of regulatory mechanisms. Naturally occurring inhibitors of several enzymes, such as the macromolecular proteinaceous inhibitors of peptidases, may act slowly when forming complexes with their targets. To allow physiologically meaningful rates of enzyme inhibition, the modifier concentration is kept at high levels in nature but problems arise when these levels drop for some reason. The slow-onset inhibitory behavior of enzyme modifiers used as drugs may represent a handicap if their concentration at the target site is insufficient and/or the kinetic constants are inadequate to warrant pharmacologically meaningful rates of enzyme inhibition. A truthful knowledge of mechanisms and kinetic constants of such systems is mandatory for making predictions on the efficiency of the modifiers *in vivo*.

Introduction

Slow interactions in enzymology gained popularity after Frieden coined the term *hysteretic enzymes* for "... those enzymes which respond slowly (in terms of some kinetic characteristic) to a rapid change in ligand, either substrate or modifier, concentration" [1].

Frieden also derived an integrated rate equation, see equation (1) below, which relates the increase of product concentration with time. This equation was later shown to apply to a vast group of enzymatic mechanisms, whether or not enzyme hysteresis was involved, and the necessary mathematical background for inhibitors was further developed by Cha [2 – 4] and Morrison *et al*. [5 – 7].

Reversible enzyme inhibitors have been classified by Morrison in four categories (Table 1) depending on the rate of formation of their complexes with enzymes [5]. There are many instances in which the binding step of modifiers to enzymes occurs rapidly, whereas the sluggishness of the process is due to events other than the formation of the first complex. For this reason, the more general expression *slow-onset inhibition* will be used in place of slow-binding inhibition. The term 'slow' is vague as the time taken by these reactions varies from seconds to hours. However, it is generally agreed that slow-onset inhibition represents transient kinetics observed on the steady-state time scale and that the slowness of inhibition is understood in terms relative to the catalytic step, which is usually a faster process. There is no clear demarcation between the slow and fast categories in Table 1. For classical inhibitors the condition $[I] \approx [I]_t$ (the subscript means total concentration) could be trusted if it were not for the relative magnitudes of $[E]_t$, $[I]_t$ and K_i, which are not considered in Morrison's classification. In fact, a classical inhibitor can manifests tight-binding properties if $[E]_t$ is comparable in magnitude to K_i. On the other hand, typical high-affinity inhibitors show their efficiency already for $[I]_t$ $[E]_t$ at low enzyme concentrations to meet the condition $[I]_t$ $[E]_t$ K_i. Then, the relationships for the tight-binding and the slow, tight-binding classes of inhibitors in Table 1are better appreciated with the modification $[I]_t$ $[E]_t$ and K_i in place of $[I]_t$ $[E]_t$.

Table 1. Classification of the reversible enzyme inhibitors according to Morrison [5]. The original relationship between the total enzyme and inhibitor concentrations for the tight-binding and the slow, tight-binding cases, $[I]_t \approx [E]_t$, is changed here into $[I]_t \approx [E]_t$ and K_i.

Type of inhibition	Relationship between $[E]_t$ and $[I]_t$	Rate of formation of the inhibited complex
Classical	$[I]_t >> [E]_t$	fast
Tight-binding	$[I]_t \approx [E]_t$ and K_i	fast
Slow-binding	$[I]_t >> [E]_t$	slow
Slow, tight-binding	$[I]_t \approx [E]_t$ and K_i	slow

Irreversible enzyme inhibition, more appropriately called inactivation, is typically a slow process under *in vitro* conditions and can be treated analogously to slow-onset inhibition. The term *modifier* describes in general inhibition, inactivation and activation. This chapter is dedicated to slow-onset inhibition and inactivation, while slow-onset activation will not be

treated. Steady-state and pre-steady-state equations for slow-onset activation have been derived by Hijazi and Laidler [8], and an overview of hysteretic, allosteric activators has been published by Neet *et al*. [9].

General Aspects of Slow-onset Inhibition

There are several reasons why an inhibitor acts 'slowly', including (1) the existence of an intermediate whose structure recalls that of the transition state; (2) the inhibitor equilibrates rapidly between two or more forms, one of which makes up only a small proportion of all forms and interacts with the enzyme; (3) binding of the modifier to the enzyme to form an intermediate adsorption complex is a fast process, which is followed by a slow structural rearrangement to a second inhibitory complex; (4) only a rare form of the enzyme, which equilibrates between conformers, interacts with the modifier; (5) more trivially, it may be impossible to achieve sufficiently high modifier concentrations so that the rate of the second-order association reaction with enzyme is slow. The information supplied by the analysis of the transient phase in slow-onset inhibition experiments is superior to that gained from steady-state data because the exponential approach to steady-state can be used for calculating at least some individual rate constants for the inhibitory steps. The mechanism of inhibition and kinetic constants can be extracted from data either using integrated rate equations, when available, or by numerical integration.

For some mechanisms, integrated rate equations can be derived under restrictive assumptions but this is not always possible. The assumptions require $[S] \approx [S]_t$ and $[I] \approx [I]_t$, which means $[S]_t$, $[I]_t >> [E]_t$, i.e. experiments must be properly designed to avoid excessive substrate turnover (say $\leq 10\%$) and to circumvent tight-binding between enzyme and inhibitor. In the presence of a slow-onset inhibitor, an enzyme-catalyzed reaction in which substrate is transformed into product (P) can be described by Frieden's equation mentioned above, which reads

$$[P] = v_s t + \frac{v_z - v_s}{\lambda}\left(1 - e^{-\lambda t}\right) + d, \tag{1}$$

where v_s and v_z are the velocities at steady-state and at time zero, respectively, and λ is the frequency constant of the exponential phase with reciprocal time as dimension. The parameter d, for displacement, was added to the original equation to account for any non-zero value of [P] or background of measured signal proportional to it at time zero. If an integrated rate equation exists and experiments can be set up to fulfill the assumptions, the equation can be fitted to progress curves by non-linear regression. In several cases, unambiguous diagnosis of the mechanism is possible by extracting the information contained in the expressions of the parameters v_s, v_z and λ. These are functions of inhibitor and substrate

concentrations for a given mechanism and allow the calculation of rate constants. The graphical representation of slow-onset inhibition according to equation (1) is shown in Figure 1.

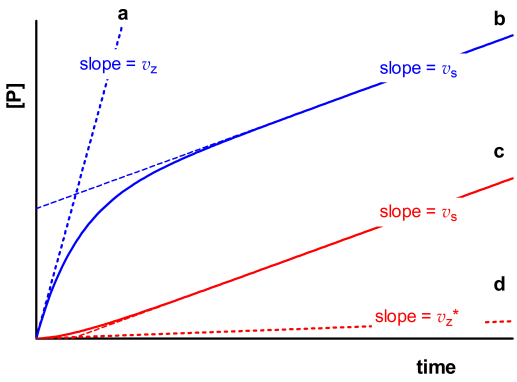
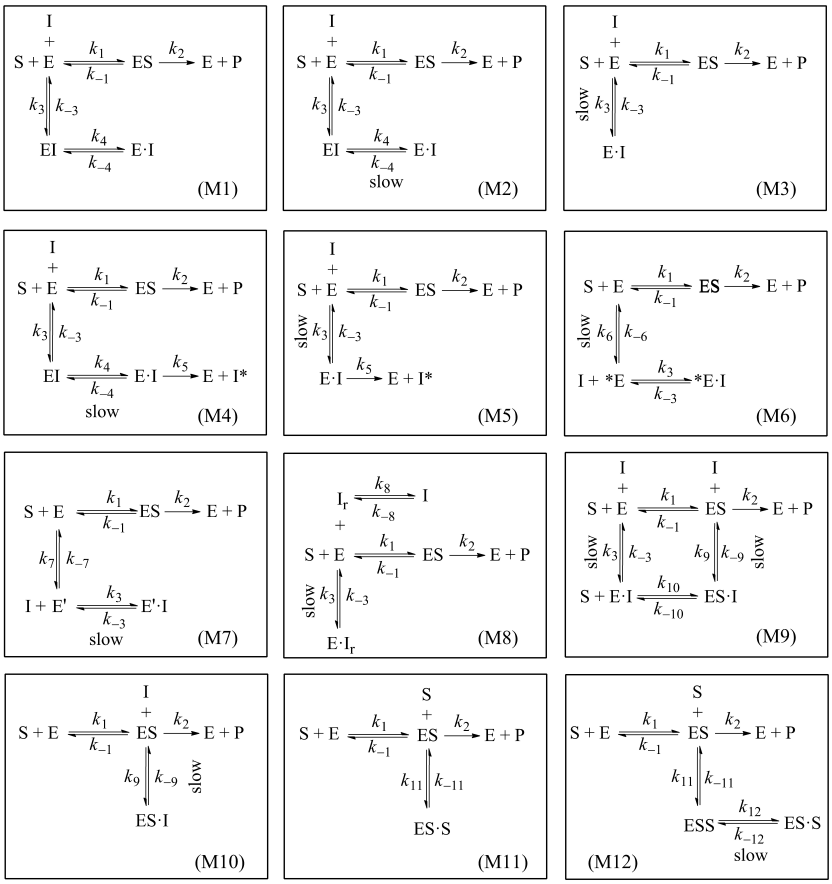


Figure 1. Progress curves for a generic slow-onset inhibition mechanism. The curve in (b) is obtained when substrate and inhibitor are mixed and reaction is started by adding enzyme. Curve (c) represents a reaction started by adding substrate to enzyme and inhibitor that had been preincubated for a sufficiently long tome to allow complex formation. The dashed line (a) is the tangent at time zero for curve (b) and dashed line (d) is the corresponding tangent for curve (c).

All mechanisms discussed below are drawn as the simplest one-substrate/one-product reaction for purely practical reasons with the implicit agreement that the same mechanisms can be applied to multi-reactant enzymes. For instance, in the case of a two-substrate/two-product enzymatic reaction, one substrate is kept at a constant concentration (not shown in the scheme) while the concentrations of the second substrate and inhibitor are varied. The measurements are then repeated exchanging the roles of the varied substrate.

The most frequent mechanism of slow-onset inhibition, both among naturally occurring and synthetic inhibitors, is of the linear competitive type. A general form is shown in Scheme 1, panel M1[1], in which no assumptions are made about the relative rates of equilibration of the two inhibitory steps. For this mechanism, a progress curve consisting of a double exponential followed by a linear steady-state release of product is expected but the derivation of an integrated rate equation is bound to restrictions, of which the absence of tight-binding between enzyme and inhibitor is the most important. However, it is right M1 in Scheme 1 that is often characterized by tight-binding.

[1] Reaction mechanisms, labeled Mx, where x is a progressive number, are grouped in this chapter as composite schemes to allow easier finding and cross-referencing



Scheme 1. Mechanisms giving rise to slow-onset inhibition. The labels M1 through M12 are introduced for facilitating cross-referencing, e. g. M3 is read 'mechanism 3'. E = enzyme, S = substrate, P = product, I = inhibitor. The steps labeled *slow* indicate qualitatively their relative rate of equilibration with respect to the other steps, which are assumed to be much faster. EI represents an adsorptive complex, while E●I, *E●I, E'●I, E●I_r, ES●I and ES●S denote reversible, non-covalent complexes. The numbers identifying kinetic constants of similar paths in diverse mechanisms are the same.

The first approach for analyzing the general mechanism M1 consists in fitting the generic equations (2) and (3) to progress curves and in evaluating which equation produces the best fit.

$$Y = A_1\left(1 - e^{-\lambda_1 t}\right) + kt + d \tag{2}$$

$$Y = A_1\left(1 - e^{-\lambda_1 t}\right) + A_2\left(1 - e^{-\lambda_2 t}\right) + kt + d \tag{3}$$

Y is a signal proportional to product concentration, A_1 and A_2 represent amplitudes of the exponential phases, λ_1 and λ_2 are frequency constants, k is the slope of the straight line following the exponentials and d the value of Y at $t = 0$. If equation (3) fits data better than equation (2), any further use of equation (1) for non-linear regression analysis is discouraged. Instead, numerical integration is the method of choice in this case. However, even if the restrictions mentioned above can be avoided, this powerful approach may fail to extract the information from progress curves if λ_1 and λ_2 are *coupled*. That is to say, if both λ_1 and λ_2 contain the rate constants of the forward and reverse inhibitory reactions, the two steps of M1 cannot be separated from one another because they are 'mixed together'. Without analyzing in full the complexity of this system, such problems arise when the values of k_{-3} and k_4 are similar. Simulations of progress curves for this general slow-onset inhibition mechanism with various combinations of rate constants, reveals that the distinction between single exponential and double exponential reaction profiles is often very subtle and can be disclosed only after accurate statistical analysis. Fitting equations (1) or (2) generates progress curves that, by sight, appear nicely superimposed to data, though with worsening of the fit in dependence on inhibitor concentration if a single exponential is fit where a double exponential would better do the job. Introducing noise in the artificial data to simulate experimental error renders this distinction even more difficult and sometimes impossible. Yet, the most significant detail is that the kinetic constants calculated by fitting a single exponential equation to a biexponential progress curve can deviate considerably from the true values even if the fitted curve, as judged by inspection, appears to be almost perfect. It is difficult to ascertain from hundreds of reports in the literature whether the most appropriate model was fit to data because the overwhelming majority of published slow-onset inhibition cases were directly addressed to two variants of the general mechanism M1, namely M2 and M3 in Scheme 1, which can be analyzed with the monoexponential equation (1). These mechanisms are discussed in the next section.

A suggested approach to data analysis for the general mechanism M1 by numerical integration is shown in Figure 2. To appreciate the efficiency of the method, artificial data were simulated and random scatted was added to mimic experimental error. Two sets of fake data were produced, one for reactions started by adding enzyme (panel a) and another for reactions started with substrate (panel b). The idea is that data set (a) should represent more closely the association process, while data set (b) should give more information on the dissociation of the EI and E●I complexes. The set of differential equations for M1 was then globally fitted to all data by a combination of numerical integration and non-linear regression using KinTek software [10, 11]. When the kinetic constants k_3, k_{-3}, k_4 and k_{-4} were allowed to float freely during the global fit, it was impossible to obtain a unique solution; the values depended very much from the initial guesses and diverged considerably from the theoretical values used to generate the fake data. Data set (b) was then excluded from global fitting and only set (a) was analyzed in a first iteration until stable values of k_3 and k_{-3} were obtained. After constraining k_3 and k_{-3} to vary in a constant ratio, global fit was performed with data sets (a) and (b), which provided four kinetic constants very close to their

theoretical values. Although the satisfactory overlap between data and best fits in Figure 2 is not yet a guarantee that the fits truly represent the system, running the FitSpace explorer [10] confirmed that all parameters were well constrained by data.

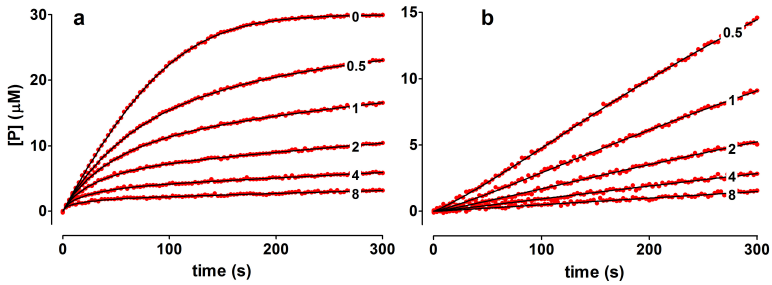


Figure 2. Analysis of progress curves for the general slow-onset inhibition mechanism (a) in Scheme 1. (a) Progress curves for reaction started by adding enzyme to a mixture of substrate and inhibitor. (b) Reactions started by adding substrate to enzyme and inhibitor preicubated for 300 s to allow complete equilibration of the EI and E●I complexes. The curves were simulated with the following constants and concentrations: $k_1 = 100\,\mu M^{-1}\ s^{-1}$, $k_{-1} = 1990\,s^{-1}$, $k_2 = 10\,s^{-1}$, $k_3 = 0.1\,\mu M^{-1}\ s^{-1}$, $k_{-3} = 0.05\,s^{-1}$, $k_4 = 0.04\,s^{-1}$, $k_{-4} = 0.005\,s^{-1}$, $[E]_t = 0.05\,\mu M$, $[S]_t = 30\,\mu M$, $[I]_t$ (μM, the numbers shown inthe two panels), $K_m = (k_{-1} + k_2)/k_1 = 20\,\mu M$. Random scatter was added to simulate experimental error (red traces). The solid lines represent best fits obtained by combining numerical integration and non-linear regression using KinTek software [10, 11].

A critical inspection of Figure 2 shows that it had been impossible to analyze the progress curves with equation (1). In fact, the available substrate (30 μM) is used up to a great extent during reaction and, at least for the lowest inhibitor concentrations, a proportion of the inhibitor is bound to the enzyme, which cannot be neglected. The assumptions $[S] \approx [S]_t$ and $[I] \approx [I]_t$ are thus violated in at least some progress curves. As shown by trace '0' in panel (a), also the reaction with substrate alone can be included in the global fit, which gives further support to the quality of the analysis. The success in using curves with high substrate turnover for data analysis depends on product inhibition and this possibility can be considered in the model. It is also important to remark that in the progress curves containing two exponential phases, the fastest of these processes may be completed within a short time. Such experiments should be performed with a rapid-mixing device like the stopped-flow to minimize loss of essential data at the very beginning of the reaction. Particularly useful in this case is a logarithmic acquisition time to intensify data collection in the early phases of the reaction [12]. Using conventional photometry there is high risk to missing part or all of the first exponential, thus hampering proper analysis of the mechanism. Not shown here is another useful option, the inclusion in the global fit of progress curves obtained at fixed inhibitor concentration and variable substrate concentrations. This is useful to assess the character of the inhibition, i.e. for discriminating between competitive, mixed or

uncompetitive inhibition. Published papers on 'linear competitive', slow-onset inhibition deserve as a remark that the competitive nature of inhibition was not always ascertained and analysis was performed by taking M2 or M3 in Scheme 1 for granted.

Specific Cases of Slow-onset Inhibition

For all mechanisms discussed in this section, the integrated rate equation and the parameters therein presume the absence of inhibitor depletion after binding to the enzyme. This can experimentally be avoided if $[E]_t$ can be maintained at least one order of magnitude lower than $[I]_t$. Mechanisms M2 to M12 in Scheme 1 have in common the same integrated rate equation (1) but in some cases characteristic combinations of the expressions for v_s, v_z and λ. M2 and M3 can be distinguished from one another because λ depends linearly on [I] for M3 and hyperbolically for M2, while v_z depends hyperbolically on [I] in mechanism M2 while it is independent of [I] in M3 [equation groups (4) and (5)]. The kinetic constants for M2 can be calculated by non-linear regression analysis of λ versus [I], with k_{-4} representing the value of λ extrapolated for $[I] = 0$, and $(k_4 + k_{-4})$ as the asymptote of λ for $[I] \rightarrow \infty$. From the expression of v_z and v_s and the known value of [S] and K_m, K_i and the overall inhibition constant K_i* can be extracted. Analogously for M3, k_{-3} is calculated as the intercept and k_3 from the slope of a plot of λ versus [I].

$$\upsilon_Z = \frac{V[\mathrm{S}]}{K_m\left(\frac{1+[\mathrm{I}]}{K_i}\right)+[\mathrm{S}]}; \qquad \upsilon_S = \frac{V[\mathrm{S}]}{K_m\left(\frac{1+[\mathrm{I}]}{K_i^*}\right)+[\mathrm{S}]}; \qquad \text{(M2)}$$

$$\lambda = k_{-4} + \frac{k_4[\mathrm{I}]}{K_i\left(\frac{1+[\mathrm{S}]}{K_m}\right)+[\mathrm{I}]}; \quad K_i = \frac{k_{-3}}{k_3}; K_i^* = K_i\left(\frac{k_{-4}}{k_4+k_{-4}}\right) \tag{4}$$

$$\upsilon_Z = \upsilon_0 = \frac{V[\mathrm{S}]}{K_m+[\mathrm{S}]}; \quad \upsilon_S = \frac{V[\mathrm{S}]}{K_m\left(1+\frac{[\mathrm{I}]}{K_i}\right)+[\mathrm{S}]}; \qquad \text{(M3)}$$

$$\lambda = k_{-3} + \frac{k_3[\mathrm{I}]}{1+\frac{[\mathrm{S}]}{K_m}}; \qquad K_i = \frac{k_{-3}}{k_3} \tag{5}$$

M4 and M5 represent 'inhibitors' that are in reality substrates. At first sight they can be misidentified as true inhibitors because, for a given extent of time, progress curves are the same as those shown in Figure 1b. The expressions of v_s, v_z and λ are given in the equation groups (6) and (7), from which it is seen that discrimination between M2 versus M4 and M3 versus M5 is impossible using the dependence of v_z, v_s and λ upon [I] because the shapes of these plots are the same. However, if the reaction is started by adding substrate (curve c in

Figure 1), the slope of the linear part of the curve is independent of the preincubation time between enzyme and inhibitor for M2 and M3 while this increases with increasing preincubation time for M4 and M5. This because formation of I* reduces the amount of I that can react with the enzyme to form inhibited complex(es) thus favoring substrate turnover. For integrating the rate equations of M4 and M5 the additional assumption $[I^*] << [I]_t$ is necessary, meaning that equation groups (6) and (7) are valid only if $[I]_t$ is sufficiently large and the measuring time sufficient short to satisfy this condition. The kinetic constants for M4 and M5 can be calculated likewise their respective counterparts M2 and M3, with the difference that only the sums ($k_{-4} + k_5$) can be calculated for M4 and ($k_{-3} + k_5$) for M5, not the individual values. These represent the net 'off' constants of the E●I complex. Examples of M4 and M5 can be found among polypeptides and proteins that bind to peptidases. Depending on the conditions, they can behave as inhibitors or can be cleaved as substrates as seen in the interaction between the thyroglobulin type-1 domain and the cysteine peptidase cathepsin L [13], which occurs according to mechanism M4. Although progress curves were shown without fitting to a particular equation, the slow-onset inhibitory interaction between Factor Xa and the leech-derived inhibitor antistasin appears to be a further example of mechanism M4 [14].

$$\upsilon_z = \frac{V[\mathrm{S}]}{K_m\left(1+\frac{[\mathrm{I}]}{K_i}\right)+[\mathrm{S}]}; \qquad \upsilon_s = \frac{V[\mathrm{S}]}{K_m\left\{1+\frac{[\mathrm{I}]}{K^*_{i,temp}}\right\}+[\mathrm{S}]}; \qquad \text{(M4)}$$

$$\lambda = k_{-4} + k_5 + \frac{k_4[\mathrm{I}]}{K_i\left(1+\frac{[\mathrm{S}]}{K_m}\right)+[\mathrm{I}]}; \quad K_i = \frac{k_{-3}}{k_3}; K^*_{i,temp} = K_i\frac{k_{-4}+k_5}{k_4+k_{-4}+k_5} \qquad (6)$$

$$\upsilon_z = \upsilon_0 = \frac{V[\mathrm{S}]}{K_m+[\mathrm{S}]}; \qquad \upsilon_s = \frac{V[\mathrm{S}]}{K_m\left(1+\frac{[\mathrm{I}]}{K^*_{i,temp}}\right)+[\mathrm{S}]}; \qquad \text{(M5)}$$

$$\lambda = k_{-3} + k_5 + \frac{k_3[\mathrm{I}]}{1+\frac{[\mathrm{S}]}{K_m}}; \qquad K_{i,temp} = \frac{k_{-3}+k_5}{k_3} \qquad (7)$$

The two particular cases of slow-onset inhibition M6 and M7 can be observed if an enzyme exists in equilibrium between different conformations and only one of these binds the inhibitor. The two mechanisms differ for the relative positions of the slow and fast steps. In M6 equilibration between two enzyme forms occurs slowly and only the state labeled *E reacts with the inhibitor. Although inhibitor binding may occur at the rate of diffusion, the overall inhibition process is slow because of the limited availability of the *E-form (the inhibitor 'must wait' for it). This mechanism it is likely to occur with enzymes that exist in distinct conformational states. M6 can be distinguished for the characteristic dependence of λ on [I], which is a concave-up hyperbola (λ decreasing for increasing [I]) as shown in the

equation group (8). While this allows the determination of k_6 and k_{-6}, it must be noted that M6 is a pretty complex mechanism because, depending on the equilibrium between E and *E, progress curves can show a lag both in the presence and in the absence of the inhibitor and v_z may or may not depend on [I] [6, 15].

In M7 the enzyme isomerizes in a fast process but inhibition proceeds slowly because the inhibitor can react only with a rare form E'. Since λ for M7 is a linear function of [I], the mechanism cannot be distinguished from M3 on the basis of this parameter, and also v_z and v_s are the same as for M3. In this case other sources of information, such as knowledge of enzyme structure from crystallography or different spectroscopic properties of E and E', must be invoked.

$$\lambda = \frac{k_6}{1+\frac{[S]}{K_m}} + \frac{k_{-6}}{1+\frac{[I]}{K_i}}; \quad K_i = \frac{k_{-3}}{k_3} \qquad \text{(M6)} \tag{8}$$

$$\lambda = k_{-3}\,\frac{1+\frac{[S]}{K_m}+\frac{[I]}{K_i}}{1+\frac{[S]}{K_m}}; \qquad v_z \text{ and } v_s \text{ as in M3} \qquad \text{(M7)}$$

$$K_m = \frac{(k_{-1}+k_2)(k_7+k_{-7})}{k_1 k_7}; \quad K_i = \frac{k_{-3}(k_7+k_{-7})}{k_3 k_7} \tag{9}$$

M8 is analogous to M7 but in this case slow-onset inhibition is due to equilibration of the inhibitor between different molecular forms of which only a rare species reacts with the enzyme. A well-documented case of M8 is the inhibition of cathepsin B by the peptide aldehyde leupeptin [16, 17]. The slow-onset inhibitory behavior of this system, originally attributed to a hysteretic effect on the part of the enzyme [16], was later demonstrated by NMR to be due to equilibration of leupeptin in aqueous solution between three forms: a cyclic carbinolamine (42%), a leupeptin hydrate (56%) and the free aldehyde (2%) [17]. Only the free aldehyde behaves as inhibitor, which represents 2% of the mixture at any concentration. As shown in equation group (10), the dependence of λ on [I] for M8 is linear as it is for M3 in equation group (5). Hence, the two mechanisms cannot be distinguished from one another by kinetic measurements. The effective concentration of the inhibitor must be measured by an independent method that allows calculation of the equilibrium constant K_r. Since $K_r << 1$, the effective inhibitor concentration is reduced by the factor K_r, which explains slow-onset inhibition.

$$\lambda = k_{-3} + \frac{k_3 K_r [I]}{1+\frac{[S]}{K_m}}; \qquad K_r = \frac{[I_r]}{[I]} = \frac{k_{-8}}{k_8} \ll 1 \qquad \text{(M8)} \tag{10}$$

A neglected aspect is the possible occurrence of mixed inhibition (M9) besides the widely reported competitive inhibition. Equations for this system are shown in the equation group (11), which show that λ is a linear function of [I], v_z is independent of [I] and v_s is hyperbolically dependent on [I]. Although these patterns are indistinguishable from those of M3, the dependence of λ on [S] is different for M3 and M9. Thus, a set of progress curves at fixed inhibitor concentration and variable [S] will show the identity of the mechanisms. The systematic measurement of the substrate dependence of progress curves for slow-onset inhibition is anyhow highly recommended for both diagnostic and computational purposes.

$$\upsilon_z = \frac{V[\mathrm{S}]}{K_m+[\mathrm{S}]}; \qquad \upsilon_z = \frac{V\frac{[\mathrm{S}]}{K_m}}{1+\frac{[\mathrm{I}]}{K_i}+\frac{[\mathrm{S}]}{K_m}\left(1+\frac{[\mathrm{I}]}{\alpha K_i}\right)}; \quad (\mathrm{M9})$$

$$\lambda = \frac{k_{-3}+\frac{k_{-9}k_{10}}{k_{-10}}[\mathrm{S}]}{1+\frac{k_{10}}{k_{-10}}[\mathrm{S}]} + \frac{k_3+k_9\frac{[\mathrm{S}]}{K_m}}{1+\frac{[\mathrm{S}]}{K_m}}[\mathrm{I}]; \quad K_i = \frac{k_{-3}}{k_3}; \quad \alpha K_i = \frac{k_{-9}}{k_9} \tag{11}$$

M10 represents slow-onset uncompetitive inhibition, which was shown to occur in enzymatic reactions involving two substrates and two products. Inhibition of the enoylreductase FabI from *Escherichia coli* by triclosan belongs to this type [18] although, for technical reasons, the authors could not use progress curves for analyzing slow-onset inhibition. Equation group (12) lists the expressions that apply to M10.

$$\upsilon_z = \frac{V[\mathrm{S}]}{K_m+[\mathrm{S}]}; \qquad \upsilon_s = \frac{V[\mathrm{S}]}{K_m+[\mathrm{S}]\left(1+\frac{[\mathrm{I}]}{\alpha K_i}\right)}; \qquad (\mathrm{M10})$$

$$\lambda = k_{-9} + \frac{k_9[\mathrm{I}]}{1+\frac{K_m}{[\mathrm{S}]}}; \qquad \alpha K_i = \frac{k_{-9}}{k_9} \tag{12}$$

The dependence of λ on [I] is linear as it is for M3 but the dependence of λ on [S] can discriminate between the two cases because λ decreases or increases with increasing [S] for M3 or M10, respectively. These diagnostic properties and the way kinetic constants can be extracted are displayed in Figure 3.

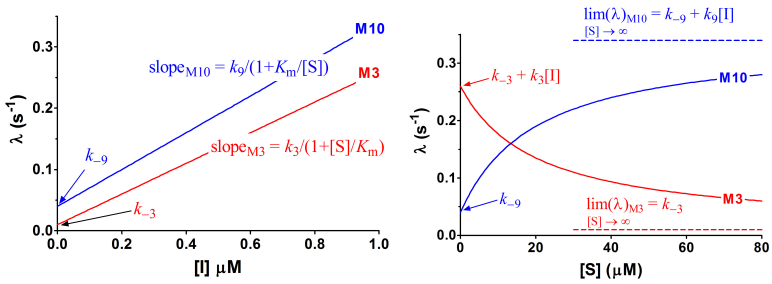


Figure 3. Dependence of the frequency constant λ on substrate and inhibitor concentration for mechanisms M3 and M10. The characteristic intersection points on the ordinate in both panels, as well as the asymptotes for [S] →∞ in the right panel (dashed lines) allow calculation of kinetic constants. Curves were simulated with the following parameters: $k_3 = 0.5\,\mu M^{-1}\ s^{-1}$, $k_{-3} = 0.01\ s^{-1}$, $k_9 = 0.6\,\mu M^{-1}\ s^{-1}$, $k_{-9} = 0.04\ s^{-1}$, $K_m = 20\,\mu M$. The fixed [S] in the left panel was 20 μM, and the fixed [I] in the right panel was 0.5 μM.

Inhibition by excess substrate, as shown in M11, is formally equivalent to uncompetitive inhibition. Classically, substrate inhibition has been treated as a fast process. However, it is possible that binding of substrate molecules to a site different from the catalytic center exhibit the properties of slow-onset inhibition, if nothing else because the secondary binding may have lesser affinity than that at the catalytic center. This means that the secondary binding may be slow because saturation of this process requires very high substrate concentrations and, while progressively increasing [S] in an experiment, a threshold is reached for which substrate begins to appreciably bind to it but this concentration is still too low to allow the path S + ES → ES●S to be sufficiently fast. M12 is a degenerated form of M11 in the same way as M3 is a degenerated form of M2. M12 was documented for the enzyme cathepsin K, which owns an exsosite to which ligands, including the substrate itself, can bind [19]. The expressions of the parameters in equation (1) for M11 and M12 are given in the equation groups (13) and (14), respectively.

$$v_z = \frac{V[\mathrm{S}]}{K_m + [\mathrm{S}]}; \qquad v_s = \frac{V[\mathrm{S}]}{K_m + [\mathrm{S}]\left(1 + \frac{[\mathrm{S}]}{K_{si}}\right)} \qquad \text{(M11)}$$

$$\lambda = k_{-11} + \frac{k_{11}[\mathrm{S}]}{1 + \frac{K_m}{[\mathrm{S}]}}; \qquad K_{si} = \frac{k_{-11}}{k_{11}} \qquad (13)$$

$$v_z = \frac{V[\mathrm{S}]}{K_m + [\mathrm{S}]\left(1 + \frac{[\mathrm{S}]}{K_{si}}\right)}; \qquad v_s = \frac{V[\mathrm{S}]}{K_m + [\mathrm{S}]\left(1 + \frac{[\mathrm{S}]}{K^*_{si}}\right)} \qquad \text{(M12)}$$

$$\lambda = k_{-12} + \frac{k_{12}[\mathrm{S}]}{K_{si}\left(1 + \frac{K_m}{[\mathrm{S}]}\right) + [\mathrm{S}]}; \quad K_{si} = \frac{k_{-11}}{k_{11}}; \ K^*_{si} = K_{si}\frac{k_{-12}}{k_{-12} + k_{12}} \qquad (14)$$

Other mechanisms for slow-onset inhibition besides those listed in Scheme 1 are not shown here. These are likely to occur in practice but not all of them can be unambiguously identified if not supported by adequate and sufficiently precise data.

The tight-binding condition was not treated in this section devoted to the use of analytical solutions of differential equations. Indeed, tight-binding represents just an experimental issue, not an intrinsic property of the mechanisms. Furthermore, an analytical, integrated rate equation that takes into account inhibitor depletion can be derived for M3 but not for M2 [3], while for the other mechanisms in Scheme 1 this point was not explicitly addressed in the literature. Although this derivation is possible for some systems, the necessary assumptions are too restrictive to be useful in practical situations. Alternatively, numerical integration methods can be used in place of non-linear regression. For this purpose, a system of dedicated differential equations can easily be written for each one of the mechanisms in Scheme 1 and any other mechanism of this type. This labor is even superfluous when using modern software [10, 11]. The issue is that several models must be applied until the best one is found but this may be a tedious and time-consuming approach. As far as the pre-steady-state of progress curves consist of a single exponential, i.e. as long as equation (2) fits data better than equation (3), the identification of the appropriate model for numerical integration is possible by preliminary analysis with the diagnostic criteria outlined above using analytical solutions. Analyzing the parameter dependence on [I] and [S] will give hints as to which mechanism comes closer to that described by the experiment, no matter if too much substrate is turned over during the observation time and if plotting parameters against $[I]_t$ instead of [I] will produce bias in the values of the kinetic constants. These can be used later as initial guesses for refinement by numerical integration once the appropriate model has been identified.

Enzyme Inactivation

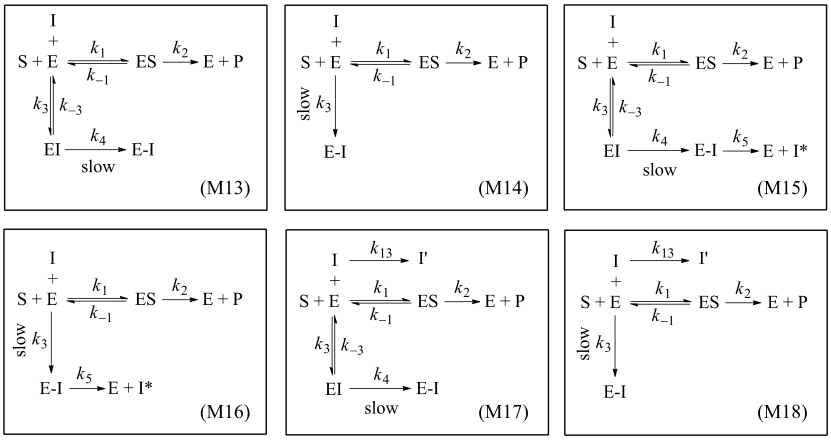
Modifiers can react with elements of the catalytic center of enzymes forming covalent bonds that result in enzyme inactivation. Common mechanisms observed experimentally for this category of modifiers are listed in Scheme 2 (M13 – M18), where the symbol E-I distinguishes at a glance inactivation from reversible inhibition. Typically, E-I covalent bonds are formed slowly and reactions can require minutes to hours to proceed to completeness depending on the characteristic constants of the system and reactant concentrations. For M13 and M14 the integrated rate equation is (15) and the parameters are given in the equation groups (16) and (17):

$$[\mathrm{P}] = \frac{v_z}{\lambda}\left(1 - e^{-\lambda t}\right) + d, \tag{15}$$

$$v_z = \frac{V[\mathrm{S}]}{K_m\left(1+\frac{[\mathrm{I}]}{K_i}\right)+[\mathrm{S}]}; \quad \lambda = \frac{k_4[\mathrm{I}]}{K_i\left(1+\frac{[\mathrm{S}]}{K_m}\right)+[\mathrm{I}]}; \quad K_i = \frac{k_{-3}}{k_3}. \qquad \text{(M13)} \tag{16}$$

$$v_z = v_0 = \frac{V[\mathrm{S}]}{K_m+[\mathrm{S}]}; \quad \lambda = \frac{k_3[\mathrm{I}]}{1+\frac{[\mathrm{S}]}{K_m}} \qquad \text{(M14)} \tag{17}$$

Equation (15) differs from (1) for the absence of the term v_s because k_{-4} and k_{-3} equal zero in M13 and M14, respectively. The diagnosis of these mechanisms is generally easy because a steady-state is absent in the progress curves (the single exponent levels off to a line parallel to the time axis for increasing time) and because plots of λ versus [I] intersect the ordinate at zero after any correction for the term d in equation (15). In general however, this last criterion should not be taken as a demonstration of irreversibility because small values of k_{-4} and k_{-3} in mechanisms M2, M3 and others, particularly in presence of experimental scatter, can render impossible the distinction of a small value of the ordinate intercept from zero.



Scheme 2. Mechanisms of enzyme inactivation. Labeling as M13 – M18 continues the list of Scheme 1 and numbering of kinetic constants reproduces similar paths for the reversible counterparts in Scheme 1. E = enzyme, S = substrate, P = product, I = inactivator. E – I denotes a covalent bond between enzyme and inactivator.

Equation (15) is invalid for the temporary inactivation mechanisms M15 and M16 because the recycled free enzyme can again combine with substrate and inactivator. It is assumed that the enzymatically transformed inactivator, I*, has lost any affinity for the enzyme. The appropriate equation is (18), which contains the term v_∞ for the velocity 'at the end' of the exponential phase, i.e. $t = \infty$ in $e^{-\lambda t}$ [20]:

$$[\mathrm{P}] = v_\infty t + \frac{v_z - v_\infty}{\lambda}\left(1 - e^{-\lambda t}\right) + d. \tag{18}$$

The velocity term v_∞ resembles v_s in equation (1) and progress curves have the same shape as in Figure 1b, which give the impression of a reversible mechanism. Without knowledge of the temporary character of the inactivation from chemical or other information, an impulsive diagnosis of such progress curves would suggest either M2 or M3 as candidate mechanisms. This is evidenced from the expressions of λ in equation groups (19) and (20), which are formally identical to (4) and (5), respectively.

$$v_z = \frac{V[\mathrm{S}]}{K_m\left(1+\frac{[\mathrm{I}]}{K_i}\right)+[\mathrm{S}]}; \qquad v_\infty = \frac{V[\mathrm{S}]}{K_m\left\{1+\frac{[\mathrm{I}]}{K_i}\left(1+\frac{k_4}{k_5}\right)\right\}+[\mathrm{S}]} \qquad \text{(M15)}$$

$$\lambda = k_5 + \frac{k_4[\mathrm{I}]}{K_i\left(1+\frac{[\mathrm{S}]}{K_m}\right)+[\mathrm{I}]}; \qquad K_i = \frac{k_{-3}}{k_3} \tag{19}$$

$$v_z = v_0 = \frac{V[\mathrm{S}]}{K_m+[\mathrm{S}]}; \quad v_\infty = \frac{V[\mathrm{S}]}{K_m\left(1+\frac{k_3}{k_5}[\mathrm{I}]\right)+[\mathrm{S}]} \qquad \text{(M16)}$$

$$\lambda = k_5 + \frac{k_3[\mathrm{I}]}{1+\frac{[\mathrm{S}]}{K_m}} \tag{20}$$

Mechanisms M15 and M16 are different from mechanism-based (suicide) inhibition, which is not treated here. A way for discriminating kinetically M15/M16 from M2/M3 is to run experiments with enzyme and inactivator preincubated for various times, preferentially with low inactivator concentrations, and to start reactions by adding substrate. Provided the enzyme is stable during the measuring time, mechanisms M15 and M16 will show pre-incubation time-dependent regain of enzyme activity until reaching the rate v_0 after complete transformation of all added inactivator to the inert species I*. This cannot happen with mechanisms M2 and M3 but still the possibility of having to do with the reversible temporary mechanisms M4 and M5 cannot be ruled out, and here we have reached an end point in which enzyme kinetics must seek help from other methods for analyzing the fate of the inactivator after having been in contact with the enzyme. Examples are known from many published studies, such as a large screening of phosphadecalin derivatives as inactivators of acetylcholinesterase [20, 21], in which kinetic measurements were supported by NMR spectroscopy (see [20] and references therein).

The last two inactivation mechanisms in Scheme 2, M17 and M18, apply to unstable inactivators, to which a meticulous theoretical work has been dedicated [22]. The instability of some compounds in aqueous solution is often due to hydrolysis that leads to chemically transformed, inert species (I' in Scheme 2), which may represent a limitation for their practical use. Progress curves for M17 and M18 resemble those for reversible inhibition or temporary inactivation, i.e. they consist of an exponential burst followed by a linear

increase of product release as depicted in Figure 1b. An integrated rate equation cannot be derived for these cases but Topham [22] provided the following analytical approximation for M18 based on Maclaurin expansion:

$$[\mathrm{P}] = \frac{v_z}{k_{13}} e^{-A[\mathrm{I}]_t/k_{13}} \left\{ k_{13}t + \sum_{i=1}^{\infty} \left(\frac{A[\mathrm{I}]_t}{k_{13}}\right)^i \frac{\left[1-\left(e^{-k_{13}t}\right)^i\right]}{i \times i!} \right\} \quad \text{(M18)} \qquad (21)$$

$$A = \frac{k_3 K_m}{K_m + [\mathrm{S}]}$$

A useful property of this treatment is that equation (21) applies to competitive, uncompetitive and mixed inactivation by an unstable modifier. Moreover, the same equation is suitable for analyzing the effects of unstable activators. Every mechanism owns a dedicated expression of the apparent second-order rate constant A in equation (21) [22]. These and other mechanisms are not discussed in this chapter.

For M18, the rate constants k_3 and k_{13} can be calculated by non-linear regression fit of equation (21) to data with appropriate adjustment of the number of terms (i) in the Maclaurin expansion. The choice of the necessary terms depends on the value of k_{13}. For instance, with $k_{13} = 0.005\ \mathrm{s}^{-1}$ the third term is sufficient, whereas with $k_{13} = 0.001\ \mathrm{s}^{-1}$ expansion must be performed to the 10th term [20]. The complex analytical solution for M17, also provided by Topham [22], may overwhelm the endurance of end users less experienced in enzyme kinetics, in which case numerical integration can be managed easier.

The Physiological and Pharmacological Significance of Slow-onset Enzyme-modifier Interactions

Mechanisms M2 – M18 have in common a slow monoexponential phase for either inhibition or inactivation, for which the half-time can be calculated as $t_{1/2} = \ln 2/\lambda$ [23], where λ is the frequency constant in equations (4) – (20). After seven half-times the exponential phase for enzyme-modifier association is more than 99% complete and a *delay time for inhibition/inactivation* can be defined as DTI = (7 × 0.693)/λ or

$$\mathrm{DTI} \approx 5/\lambda. \qquad (22)$$

This is the time needed by both naturally occurring and exogenous modifiers used as drugs for neutralizing unwanted enzyme activities. Knowing the inhibition or inactivation mechanism, the related kinetic constants, the substrate concentration and K_m, λ can be calculated and then introduced in (22) for computing the DTI. From the kinetic parameters, the extent of substrate turnover during the DTI can be calculated and this information can be used to estimate the required modifier concentration at the target site for hindering unwanted effects in due time [24]. Even knowing an approximate value of DTI can be of great help because

the successful use of enzyme modifiers as drugs depends on modifier bioavailability, concentration at the target site and mechanism of inhibition/inactivation. Specifically, if depends linearly on [I], DTI can be made as short as the modifier concentration can be increased because DTI→ 0 for [I] →∞. However, if the dependence of λ on [I] is hyperbolic, DTI cannot be shortened below a given threshold because DTI will level off to a plateau even increasing [I] to infinity. Thus, for a modifier operating with mechanism M2, DTI→ $5/(k_{-4} + k_4)$ for [I] →∞ and the success in the practical use of this modifier will depend on the values of k_{-4} and k_4.

These simple considerations emphasize that every effort put in determining mechanisms of action and kinetic parameters for slow-onset enzyme modification as accurately as possible is not an academic exercise. On the contrary, this knowledge is indispensable for predicting the physiological and pharmacological significance of the modifiers and can help in the chemical design of new drugs.

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