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Kinetic Effects Due to Nonspecific Substrate-Inhibitor Interactions in Enzymatic Reactions

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ABSTRACT. Nonspecific protein binding is a commonly encountered problem with synthetic molecules designed as enzyme inhibitors. When the substrate for the enzymatic reaction is itself a protein, such nonspecific protein binding can also occur. Here, we demonstrate that this phenomenon can have a dramatic effect on the steady-state kinetic evaluation of such inhibitors. BIOCHEM PHARMACOL **55**;11:1785–1790, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. substrate-inhibitor interactions; protein binding; steady-state kinetics; protease; kinase; enzyme

In the design and synthesis of small molecule inhibitors of enzymes and receptors for therapeutic use, a commonly encountered problem is that of nonspecific binding of the molecules to proteins. Such binding events deplete the pool of free inhibitory molecules available for binding to and inhibiting the target enzyme. In most cases, such nonspecific protein binding manifests itself as an apparent diminution in inhibitory potency when cellular and *in vivo* assays, in which high levels of serum proteins are present, are compared with *in vitro* enzymatic assays of the same compounds. Such effects are also encountered in *in vitro* receptor-ligand binding assays, but here the relatively small protein content of the system and the typically large difference between the dissociation constants for nonspecific protein binding and specific receptor interactions make the effects easy to detect and correct for in the data analysis.

There is increasing interest in developing small molecule inhibitors of enzymes for which the substrate of the enzymatic reaction is itself a protein. Two contemporarily important examples of such systems are the proteinases and the protein kinases. In such systems, it is conceivable that the macromolecular substrate can act as a nonspecific sink for the inhibitor molecule, effectively depleting the concentration of free inhibitor in the reaction mixture. Indeed, in our own studies of protein kinase inhibitors, we have encountered situations in which nonspecific protein binding of the inhibitor to the substrate protein has perturbed our ability to correctly analyze inhibitor potency. The proprietary nature of this work precludes our presentation here of the experimental data on these inhibitors. However, this has stimulated us to consider what general effects such nonspecific substrate-inhibitor interactions might have on the steady-state kinetics of enzyme-catalyzed reactions. Here, we present a theoretical treatment for such events and demonstrate that they can have a profound effect on the experimental determination of inhibitor potency and mode of inhibitor interaction with the target enzyme. Similar treatments have been presented by Segel [1] for the situation in which the inhibitory species is actually the substrate-inhibitor binary complex, and by Morrison and Cleland [2, 3] for the interaction of inhibitory lanthanides with the cosubstrate Mg^{2+}-ATP complex for the enzyme hexokinase. To our knowledge, however, this is the first explicit treatment of the effects of nonspecific substrate-inhibitor interactions on the steady-state kinetics of enzyme-catalyzed reactions.

THEORY

We begin by defining nonspecific substrate-inhibitor interactions as the formation of a complex between the substrate and inhibitor molecules in which: 1) the substrate-inhibitor complex can still act as a substrate for the target enzyme (for simplicity we assume that the K_m for the substrate is unperturbed by inhibitor binding); and 2) the substrate-inhibitor complex is not itself inhibitory. In other words, the only effect of formation of the substrate-inhibitor complex is to diminish the concentration of free inhibitor that is available for binding to the target enzyme. To achieve true steady-state conditions, the target enzyme should be present at concentrations that are well below those of the substrate and inhibitor molecules (i.e. $[E] \ll [S]$ or $[I]$). For this reason we make the usual assumption that the formation of the ES and EI complexes do not affect significantly the concentrations of free substrate and inhibitor, respectively [4]. This is a reasonable assumption for

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many of the proteinases and protein kinases that one is likely to encounter. There are numerous examples in the literature where these enzymes are assayed in the range of 0.1 to 10 nM, while their proteinous substrates are present in micromolar to millimolar concentrations, conditions near or above the respective K_m for the substrates. In our own work, for example, we have assayed matrix metalloproteinases at low nanomolar concentrations with protein substrate for which the K_m values were 0.1 to >1.0 mM. In our most recent studies of mitogen-activated protein (MAP) kinase kinases, we have been able to assay activity at enzyme concentrations of 10 nM and protein substrate concentrations of ≥400 nM. Nevertheless, there may be cases where the substrate K_m is low enough to require working under conditions where [E] ≈ [S]. In such cases the equations to follow must be modified to reflect the depletion of free enzyme and substrate concentrations that attends formation of the enzyme–substrate complex. We do not deal with this less common situation explicitly here. The necessary corrections can be derived from consideration of the general effects of tight-binding substrates on enzyme reactions as discussed by several authors (see, for example, Refs. 5–7).

Under steady-state conditions, as defined above, the total concentration of inhibitor in the reaction mixture, $[I_T]$, is the sum of the free inhibitor concentration, $[I_F]$, the concentration of substrate-bound inhibitor, $[I_S]$, and the concentration of the enzyme-bound inhibitor, $[I_E]$. As discussed above, however, the low concentration of enzyme, relative to the concentrations of substrate and inhibitor, allow us to ignore the minor contribution of $[I_E]$ to the mass conservation equation. Thus:

$$[I_T] = [I_F] + [I_S] \tag{1}$$

or, by rearranging this mass conservation equation:

$$[I_F] = [I_T] - [I_S] \tag{2}$$

The concentration of substrate-bound inhibitor in such a system is a function of the total concentrations of inhibitor and substrate present in the reaction mixture, and of the dissociation constant, K_d^*, for the substrate–inhibitor complex (the term K_d^* is used here to denote the fact that we are dealing with an experimentally determined apparent dissociation constant, which may, in fact, result from the cumulative effects of multiple nonspecific binding events). There are a variety of biophysical methods by which the apparent K_d^* can be measured independently (see, for example, Ref. 8). In our own work, for example, we have often relied on preparation of a radiolabeled version of the inhibitor in conjunction with equilibrium dialysis or size exclusion chromatography for this purpose.

In the case where the substrate concentration in the reaction mixture is of the same order of magnitude as K_d^*, $[I_S]$ is related to $[I_T]$ by the following quadratic equation [9]:

$$[I_S] = \frac{([I_T] + [S] + K_d^*) - \sqrt{([I_T] + [S] + K_d^*)^2 - 4[S][I_T]}}{2} \tag{3}$$

Substituting this back into Eqn 2, we obtain:

$$[I_F] = [I_T] - \frac{([I_T] + [S] + K_d^*) - \sqrt{([I_T] + [S] + K_d^*)^2 - 4[S][I_T]}}{2} \tag{4}$$

In its most general form [4], that for the case of noncompetitive inhibition, the velocity of an enzymatic reaction in the presence of an inhibitor is described by the following equation:

$$v = \frac{V_{max}[S]}{K_m\left(1 + \frac{[I_F]}{K_i}\right) + [S]\left(1 + \frac{[I_F]}{\alpha K_i}\right)} \tag{5}$$

Eqn 5 describes the velocity equation for the case of a noncompetitive inhibitor, where the kinetic constants V_{max}, K_m, K_i, and αK_i have their usual meaning.

When the inhibitor under study also has non-specific binding affinity for the substrate molecule, the free inhibitor concentration must be corrected as define by Eqn 4. Combining this definition with Eqn 5, we obtain:

$$v = \frac{V_{max}[S]}{K_m\left(1 + \frac{[I_T] - \frac{([I_T] + [S] + K_d^*) - \sqrt{([I_T] + [S] + K_d^*)^2 - 4[S][I_T]}}{2}}{K_i}\right)} + \frac{V_{max}[S]}{[S]\left(1 + \frac{[I_T] - \frac{([I_T] + [S] + K_d^*) - \sqrt{([I_T] + [S] + K_d^*)^2 - 4[S][I_T]}}{2}}{\alpha K_i}\right)} \tag{6}$$

Eqn 6 provides an explicit description of the effects of nonspecific substrate–inhibitor interactions on the velocity of the enzymatic reaction in the presence of a noncompetitive inhibitor. The corresponding velocity equations for the cases of competitive and uncompetitive inhibition are given by Eqns 7 and 8, respectively.

$$v = \frac{V_{max}[S]}{K_m\left(1 + \frac{[I_T] - \frac{([I_T] + [S] + K_d^*) - \sqrt{([I_T] + [S] + K_d^*)^2 - 4[S][I_T]}}{2}}{K_i}\right) + [S]} \tag{7}$$

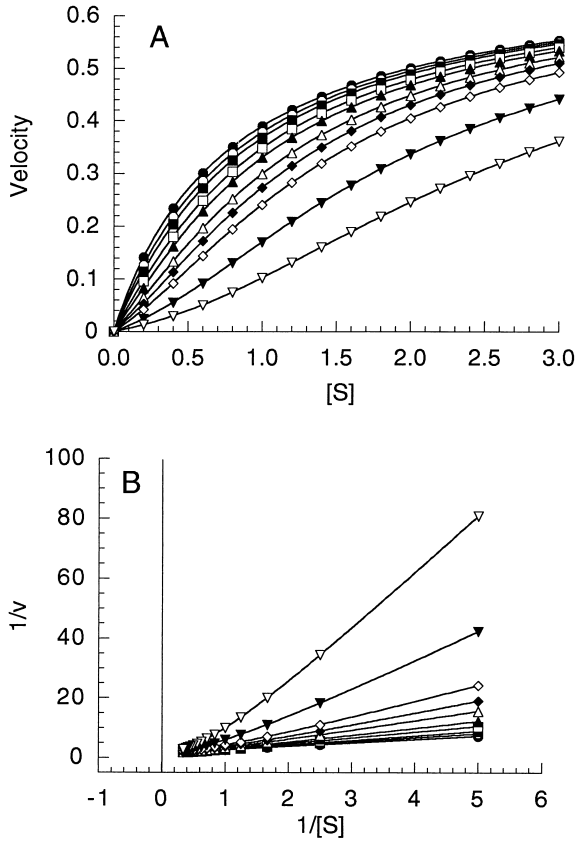

FIG. 1. Untransformed (A) and double-reciprocal (B) plots of the dependence of enzyme velocity on substrate concentration in the presence of various concentrations of a competitive inhibitor that also binds nonspecifically to the substrate. Steady-state parameters were as described in the text. The inhibitor concentrations used in this simulation were: 0 (●), 5 (○), 10 (■), 20 (□), 30 (▲), 50 (△), 70 (◆), 100 (◇), 200 (▼), and 400 (▽) nM.

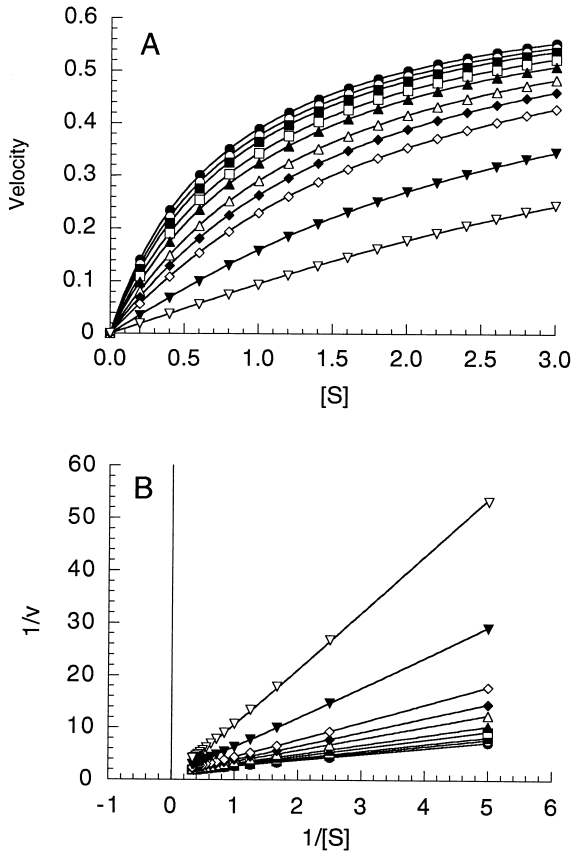

FIG. 2. Untransformed (A) and double-reciprocal (B) plots of the dependence of enzyme velocity on substrate concentration in the presence of various concentrations of a noncompetitive inhibitor that also binds nonspecifically to the substrate. Steady-state parameters and symbols are as for Fig. 1.

$$v = \frac{V_{max}[S]}{K_m + [S]\left(1 + \dfrac{[I_T] - \dfrac{([I_T] + [S] + K_d^*) - \sqrt{([I_T] + [S] + K_d^*)^2 - 4[S][I_T]}}{2}}{\alpha K_i}\right)} \quad (8)$$

EFFECTS OF SUBSTRATE–INHIBITOR INTERACTIONS ON STEADY-STATE KINETICS

The effects of nonspecific substrate-inhibitor interactions on the steady-state kinetics of an enzyme in the presence of various concentrations of an inhibitor are illustrated in Figs. 1–3 for the cases of competitive, noncompetitive, and uncompetitive inhibition, respectively. For each of these hypothetical cases, we have set the steady-state parameters as follows: $V_{max} = 0.7$ arbitrary velocity units; $K_m = 0.8$ μM; K_i or $\alpha K_i = 0.025$ μM; and $K_d^* = 0.5$ μM.

In the absence of inhibitor, the reaction velocity dependence on substrate concentration follows the expected square hyperbolic function described by the Michaelis–Menten equation:

$$v = \frac{V_{max}[S]}{K_m + [S]} \quad (9)$$

At low inhibitor concentrations, the data appear still to conform to equation 9; only minor deviations from a square hyperbolic function can be discerned here. As the inhibitor concentration increases, however, the deviations become striking. For competitive and noncompetitive inhibition (Figs. 1 and 2) the velocity dependence on substrate concentration takes on a sigmoidal character. This is the result of the lowering of free inhibitor concentration, hence enzyme inhibition, as the substrate concentration exceeds K_d^*. The velocity observed at higher substrate concentrations is therefore unexpectedly high, due to the combination of the general effect of substrate on the enzyme reaction and the diminution of the free inhibitor pool due to substrate binding. As the substrate concentration increases further, an increasingly large proportion of the inhibitor molecules are bound to substrate until no free inhibitor is left, and the reaction attains its uninhibited velocity. The effect is similar in the case of uncompetitive inhibition (Fig. 3), but here the curves take on a quasi-linear appearance at high substrate and high inhibitor concentrations.

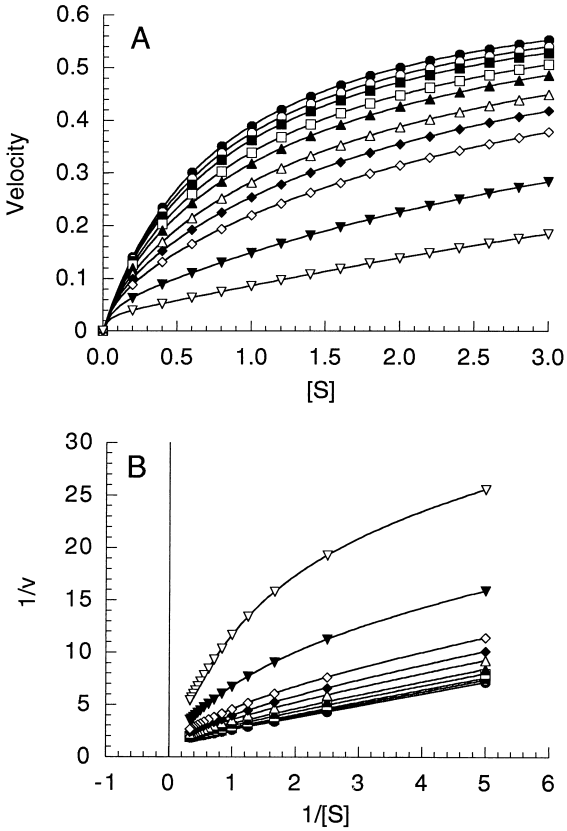

FIG. 3. Untransformed (A) and double-reciprocal (B) plots of the dependence of enzyme velocity on substrate concentration in the presence of various concentrations of an uncompetitive inhibitor that also binds nonspecifically to the substrate. Steady-state parameters and symbols are as for Fig. 1.

While the sigmoidicity observed in Fig. 1A is unique to the effects of nonspecific substrate–inhibitor interactions, it is not unexpected that high substrate concentrations can overcome the inhibitory effects of a competitive inhibitor [4]. Surprisingly, however, one observes a similar diminution of inhibition with increasing substrate concentrations for noncompetitive and uncompetitive inhibitors as well. For these types of inhibitors, one does not normally expect increasing substrate concentrations to diminish the effects of the inhibitor; again, here the diminution of inhibition with increasing substrate concentration is a result of the reduced concentration of free inhibitor brought about by nonspecific substrate–inhibitor complexation.

If the data in the top panels of Figs. 1–3 are plotted in double-reciprocal form, one obtains the plots illustrated in the lower panels of these figures. Rather than the linear relationship between 1/v and 1/[S] that is typically observed in steady-state enzyme kinetics [4], here the plots demonstrate significant curvature, as all of the data converge at infinite substrate concentration (i.e. as 1/[S] approaches zero). In the case of competitive inhibition, the data points display an up-turning curvature, whereas for uncompetitive inhibition the curvature is in the opposite direction. With the kinetic parameters chosen for this example, the curvature is less apparent in the case of noncompetitive inhibition. In this situation, one might be tempted to fit the experimental data to a series of linear functions that would appear to converge at the y-axis. This pattern is the classical signature for *competitive* inhibition. Hence, analysis of the experimental data by double-reciprocal plots alone could result in a mis-classification of the modality of inhibitor interactions with the enzyme. In contrast, however, our experience with competitive inhibitors that display nonspecific interactions with substrate indicates that both the sigmoidicity of the curves in the untransformed plots (Fig. 1A) and the curvature in the double-reciprocal plots (Fig. 1B) are immediately obvious from graphs of the experimental data (data not shown).

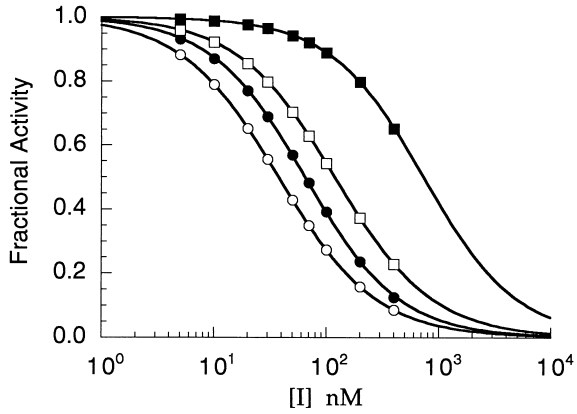

FIG. 4. Effect of substrate concentration on the concentration–response plot for a competitive inhibitor that also binds nonspecifically to the substrate. The open circles and squares represent the simulated data for a competitive inhibitor in the absence of substrate interactions, at substrate concentrations of 0.4 and 3.0 μM, respectively. The closed circles and squares are the corresponding data at 0.4 and 3.0 μM of substrate, respectively, when the substrate and inhibitor interact with a K_d^* value of 0.5 μM.

EFFECTS OF SUBSTRATE–INHIBITOR INTERACTIONS ON CONCENTRATION–RESPONSE PLOTS

A commonly employed means of evaluating the potency of multiple inhibitors of the same enzyme is to measure the diminution of initial velocity as a function of inhibitor concentration at a fixed concentration of substrate. The fractional velocity remaining is then graphed as a function of inhibitor concentration on a semilog scale, in what is commonly referred to as a concentration–response plot. The inhibitor concentration that reduces the initial velocity to half of the uninhibited velocity, referred to as the IC_{50}, is then taken as a relative measure of the inhibitor potency [4].

Figures 4–6 illustrate the effects of nonspecific substrate–inhibitor interactions on the concentration–response plots obtained for our hypothetical examples of competitive, noncompetitive, and uncompetitive inhibition, respectively. In all three cases, the net effect is the same, that is, a shifting of the apparent IC_{50} value to higher inhibitor

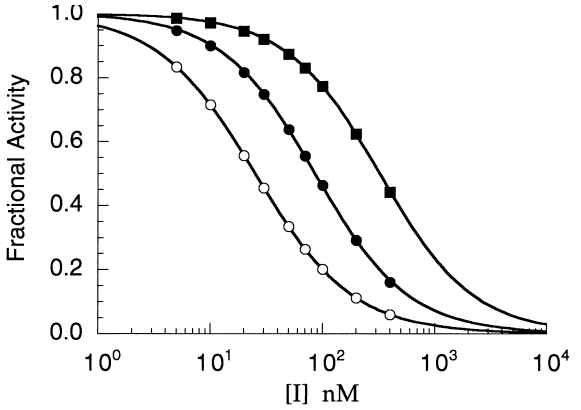

FIG. 5. Effect of substrate concentration on the concentration–response plot for a noncompetitive inhibitor that also binds nonspecifically to the substrate. The open circles represent the simulated data at any substrate concentration when the inhibitor does not interact with substrate. The closed circles and squares are the corresponding data at 0.4 and 3.0 μM of substrate, respectively, when the substrate and inhibitor interact with a K_d^* value of 0.5 μM.

concentrations. As one would expect, the perturbation of the IC_{50} value is greatest at the highest substrate concentration used for the analysis.

For all three types of inhibitors considered here, Cheng and Prusoff [10] have derived equations that relate the measured IC_{50} value to the inhibitor dissociation constant, K_i. Using these relationships, one can derive an estimate of the K_i value for each inhibitor type from the IC_{50} value, knowledge of the substrate concentration used in the experiment, and the K_m of the substrate. We have performed such calculations for the concentration–response plots illustrated in Figs. 4–6, and have summarized the results in Table 1. As can be seen from this table, even at low substrate concentrations there is a significant error in

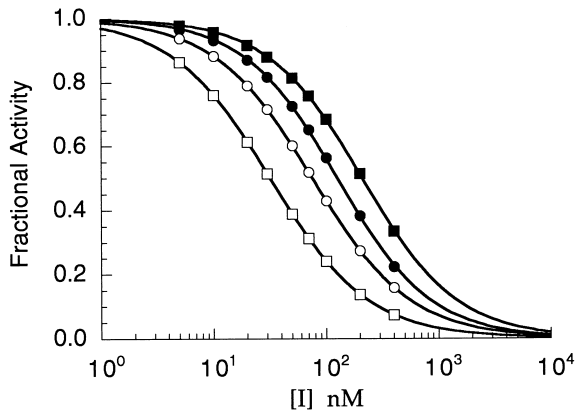

FIG. 6. Effect of substrate concentration on the concentration–response plot for an uncompetitive inhibitor that also binds nonspecifically to the substrate. The open circles and squares represent the simulated data for the inhibitor in the absence of substrate interactions, at substrate concentrations of 0.4 and 3.0 μM, respectively. The closed circles and squares are the corresponding data at 0.4 and 3.0 μM of substrate, respectively, when the substrate and inhibitor interact with a K_d^* value of 0.5 μM.

TABLE 1. Perturbation of the apparent K_i (or αK_i) values obtained from concentration–response plots for competitive, noncompetitive, and uncompetitive inhibitors displaying nonspecific interactions with substrate

Inhibitor type	$[S]/K_m$	Apparent K_i or αK_i (nM)*	% Error from true K_i†
Competitive	0.50	43	72
Competitive	3.75	155	520
Noncompetitive	0.50	85	240
Noncompetitive	3.75	322	1188
Uncompetitive	0.50	42	68
Uncompetitive	3.75	155	560

*The true K_i value in each case is 25 nM.

†Percent error is calculated as 100 multiplied by the difference between the true and calculated K_i, divided by the true K_i [11].

the calculated K_i values for all three inhibitor types, resulting from the influence of nonspecific substrate–inhibitor interactions. As expected, this error is magnified as the substrate concentration is increased.

CONCLUSIONS

In this brief report, we have demonstrated that nonspecific substrate–inhibitor interactions can have a profound effect on the analysis of the steady-state kinetics of enzyme-catalyzed reactions. One's abilities to diagnose the modality of inhibitor interactions with the target enzyme (i.e. competitive, noncompetitive, or uncompetitive) and to quantify inhibitor potency are dramatically compromised by the occurrence of such nonspecific binding interactions between the inhibitor and the substrate.

The types of deviations in steady-state kinetics demonstrated here can result from multiple causes other than nonspecific substrate–inhibitor interactions. For example, similar effects can be manifested as a result of tight-binding and/or time-dependent inhibitors of the enzyme. For tight-binding inhibitors, where the inhibitor K_i is similar in magnitude to the concentration of enzyme in the assay, the depletion of free inhibitor concentration due to formation of the enzyme–inhibitor complex is no longer insignificant. Hence, one observes deviations from classical steady-state kinetics in the presence of such inhibitors. With time-dependent inhibitors, the onset of inhibition is slow on the time scale of enzyme turnover. Because of this, perturbations in the measured values of initial velocities can occur if care is not taken to account for such effects. These and other causes of deviation from classical steady-state kinetics have been reviewed recently in Ref. 4. Thus, some caution must be exercised in assuming that nonspecific substrate–inhibitor interactions are the cause of experimentally observed deviations in the kinetic behavior of an enzyme. Care should be taken to consider, and discount on the basis of experimental evidence, other causes for the observed deviations.

For enzymes that act upon small molecular weight substrates we expect that the occurrence of nonspecific

substrate–inhibitor interactions will be extremely rare. In the case of enzymes that act upon macromolecular substrates (i.e. proteins), however, the possibility of these interactions is increased greatly. We expect that examples of such interactions will be encountered as researchers continue to target enzymes, such as proteinases and kinases, for inhibition by small molecules. As stated in the introduction, in fact, we have ourselves encountered examples of such effects in our studies of inhibitors of protein kinases.

The theoretical treatment described here provides a quantitative means of assessing the effects of such interactions on the steady-state kinetics of enzymatic reactions. Experimental data of the type illustrated in Figs. 1A, 2A, and 3A can be fit directly to Eqn 6 with both the values of K_d^* and the inhibitor constant(s) simultaneously estimated as fitting parameters. Alternatively, when nonspecific substrate–inhibitor interactions are suspected, the researcher can rely on independent biophysical methods to verify the existence of such interactions, and to determine the value of K_d^*. As mentioned in the text, a variety of methods are well established for this purpose, including techniques such as equilibrium dialysis, size-exclusion chromatography, and various spectroscopic methods. With the value of K_d^* thus established, one can readily apply Eqn 6 to a set of experimentally measured velocities at varying substrate and inhibitor concentrations (i.e. as in Figs. 1A, 2A, and 3A) to determine correctly the values of the inhibitor constant(s). Hence, the use of these methods in data analysis can provide a means of assessing the true affinity of such inhibitors for their target enzymes. In addition, this treatment may prove useful in understanding and segregating the structure–activity relationships for enzyme inhibition and nonspecific protein (i.e. substrate) binding by small molecule inhibitors.

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