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COMMENTARY

MEANINGS OF K_i FOR CONVENTIONAL AND ALTERNATE-SUBSTRATE INHIBITORS

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Inhibition constants (K_i) are commonly reported to express the ability of a competitive inhibitor to decrease the velocity of an enzyme-catalyzed reaction. Unfortunately, there are frequent misinterpretations of the meanings of these constants. For example, the K_i value has been considered to be equal to the concentration of inhibitor (I) that produces 50 per cent inhibition of the reaction velocity. This is obviously incorrect because the per cent inhibition will vary with the concentration of the competing substrate. The K_i value has also been interpreted to be the dissociation constant (K_I) of the inhibitor from the EI complex. This is correct in only a few cases. The relationship between K_i and K_I can depend upon: (1) the reaction mechanism of the enzyme (i.e. the binding sequence for the substrates) (2) which substrate is being varied (3) the concentration(s) of the non-varied substrate(s), and (4) whether the inhibitor possesses any alternative substrate activity.

The following commentary provides the rationale and simple equations required to convert the variable constant, K_i, into the true constant, K_I. It also points out how studies with a competitive inhibitor can reveal reaction mechanisms, and explains how the K_m for an alternative substrate can be determined from simple competitive inhibition studies. Some of the equations and rules presented here also appear in Refs. 1–4.

Inhibitors devoid of substrate activity (dead-end inhibitors)

Determination of K_i *values.* Kinetic data are usually presented as double reciprocal plots of the velocity (v) versus the concentration of substrate (S) in the absence and presence of inhibitor (primary

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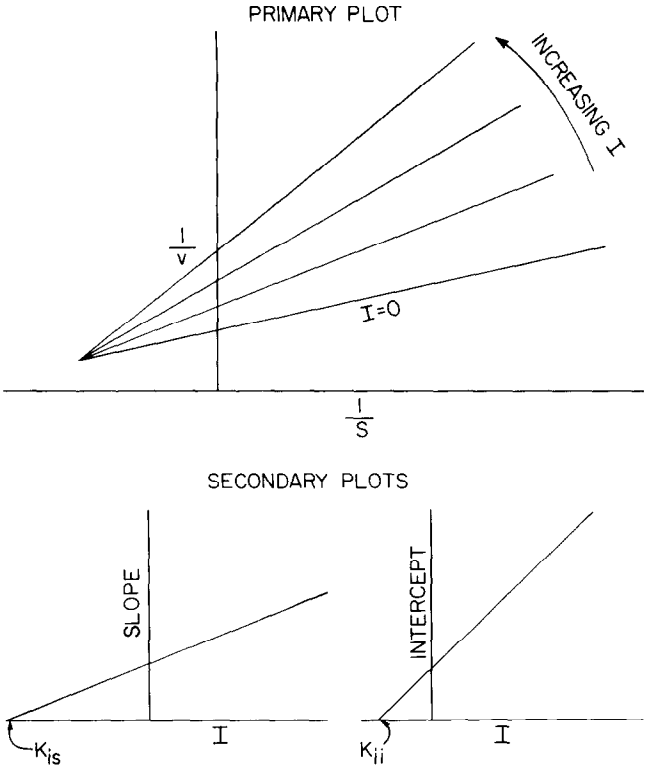


Fig. 1. Graphical presentations of kinetic data.

Table 1. General Rule 1*: To distinguish the type of inhibition

For a plot of $1/v$ vs. $1/S$:
A dead-end inhibitor affects
(1) The $1/v$ axis intercept,
if I and the varied S combine with different enzyme forms and if saturation with the varied S cannot overcome the inhibition.
(2) The slope,
if (a) I and the varied S combine mutually exclusively with the same enzyme form(s), or (b) I binds before (upstream from) the varied S with an enzyme form that is reversibly connected to the form combining with the varied S (i.e. the varied S must add after I with no irreversible steps between the times of addition of both ligands).

* From Ref. 2.

graph of Fig. 1). This graph is preferred because most investigators are able to readily recognize the different patterns of inhibition when the data are displayed in this manner. It should be noted that the data are not accruately analyzed directly from such graphs, but rather from statistical analysis of least squares fits of the data to hyperbolas [5, 6]. Computer programs have been written to facilitate the calculations [7, 8].

The presence of a dead-end inhibitor (one that does not participate in the chemical reaction) may cause an increase in the slope and/or the intercept of a double reciprocal plot. Inhibitors that only increase the slope produce competitive inhibition and generate a family of lines that intersect on the $1/v$ axis (see Fig. 2A). A secondary plot of the slope versus the concentration of the inhibitor will reveal the $K_{i(\text{slope})}$ or K_{is} as shown in Fig. 1. Uncompetitive inhibitors only affect the intercepts and generate a family of parallel lines. Replots of intercepts (Fig. 1) produce a $K_{i(\text{intercept})}$ or K_{ii}. When an inhibitor increases both the slope and the intercept as in the primary plot of Fig. 1, the inhibition is noncompetitive and the lines intersect to the left of the $1/v$ axis. Noncompetitive inhibition yields both a K_{is} and a K_{ii}. These constants may or may not have equal values.

Rules for predicting patterns of inhibition and interpreting the inhibition constants. The rules for predicting the patterns of inhibition for a dead-end inhibitor are presented in Table 1. These rules are similar to those for product inhibitors [9] with the additional requirement of the upstream binding (part 2a) of the inhibitor relative to the varied substrate. Since, unlike product inhibitors, dead-end inhibitors cannot reverse a reaction sequence, they will not affect the slope if they bind after the varied substrate [2].

By definition, an irreversible step occurs when a product is released from the enzyme. This is because all velocity measurements are taken during the initial part of the reaction when the concentration of product formed is too low to permit any significant recombination with the enzyme. Saturation with a substrate will also produce an irreversible step.

The procedure for relating K_i to the dissociation constant (K_I) utilizes the rules shown in Table 2. To begin, it is most useful to make a linear diagram indicating the various enzyme forms and the sequence of addition of each substrate and the inhibitor. Next, refer to Table 2 to determine

Table 2. General Rule 2: To establish the relationship between K_i and K_I

If the inhibitor affects	then the inhibition constant is:	and, to relate K_i to K_I, consider the concentration of the varied S to be:
(a) Slope	K_{is}	Zero
(b) Intercept	K_{ii}	Infinite

Table 3. Definition of substrate constants for sequential mechanisms

Constant	Extrapolated concentration of cosubstrate	Meaning of constant: Ordered — Steady-state	Random — Rapid-equilibrium	Random — Steady-state
K_A	Zero	K_d of A from EA*	K_d of A from EA	†
K_B	Zero	Does not exist	K_d of B from EB	†
$K_{m(A)}$	Infinite	True K_m for A	K_d of A from EAB‡	True K_m of A
$K_{m(B)}$	Infinite	True K_m for B	K_d of B from EAB‡	True K_m of B

* K_d = dissociation constant.

† Steady-state random mechanisms occur when the rate of dissociation of the substrate(s) is slower than the V_{max}. This may cause a distortion of the initial velocity patterns with resultant K_A or K_B values that are not equal to the dissociation constants of A or B from the enzyme. In the case of hexokinase [12] the rate of dissociation of glucose is slower than V_{max} producing a K_{ATP} that is considerably lower than the dissociation constant for ATP. Both substrates of phosphofructokinase dissociate at rates slower than the V_{max} and both K_A and K_B appear lower than their respective dissociation constants [11].

‡ In this case, the true K_m is also the dissociation constant.

whether the concentration of the varied substrate should be extrapolated to zero or to infinity. Finally, make the extrapolation and note on the diagram if there are any non-varied substrates remaining that will affect the binding of the inhibitor. Also note the enzyme form to which this non-varied substrate binds and the proper kinetic constant (see Table 3) that describes the association.

For most cases where the extrapolation results in the retention of only one enzyme-inhibitor species, K_i will be equal to K_I multiplied by $[1 + X]$. If the influence of the non-varied substrate is to impede the binding of the inhibitor, "X" will be equal to the ratio of the concentration of the substrate to its proper kinetic constant. If the non-varied substrate facilitates the binding of the inhibitor, "X" will be the inverse of this ratio. If the extrapolation from Table 2 reveals that there are no influential non-varied substrates remaining, "X" will equal zero and the K_i will directly equal the K_I.

Single substrate reactions. A competitive inhibitor and the substrate of a single substrate reaction will always bind to the same form of the enzyme. The resulting slope effects may be replotted to calculate the K_{is}. Rule 2 of Table 2 states that the factors required to relate the K_{is} to the K_I are revealed by considering the concentration of the substrate to be zero. Since there are no cosubstrates, in the absence of the substrate, there is nothing left to affect the binding of the inhibitor to the enzyme. Therefore, for all single substrate reactions, the K_{is} of a competitive inhibitor is equivalent to its dissociation constant (Equation 1.0).

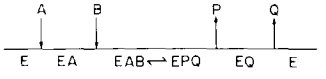
$$K_{is} = K_I \qquad (1.0)$$

Two substrate reactions. It is more common for enzymes to catalyze reactions involving two substrates rather than just one. The additional substrate may confer some complexity on the relationships between K_i and K_I. The nature of this relationship is dependent upon the mechanism by which the substrates bind to the enzyme. Basically, there are two ways for substrates to do this. The *sequential mechanism* requires that all substrates be simultaneously bound to the enzyme before any product is formed. The second mechanism requires that the first substrate bind, "donate" a chemical group to the enzyme, and depart. The next substrate then binds, accepts that chemical group, and it too departs. The enzyme thus fluctuates or ping-pongs between the unmodified and the chemically modified forms and hence the name *ping-pong mechanism*.

(a) *Sequential mechanisms.* The sequential mechanism is referred to as *ordered* if the substrates bind in a compulsory step-wise fashion. That is, substrate A binds first, and then substrate B will bind to the EA complex to form the ternary EAB complex. The mechanism is *random* if either A or B binds first. Sequential mechanisms are further divided into *rapid-equilibrium* and *steady-state* subclasses. Detailed explanations of these subclasses can be found in Refs. 10 and 11. The following discussion will exclude the uncommon rapid-equilibrium ordered mechanism.

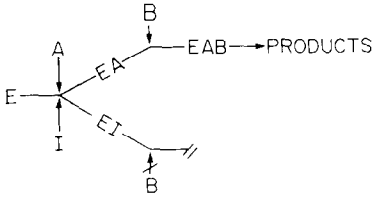
There are a number of constants that can be obtained from the sequential mechanisms. These constants are defined in Table 3*. The first two constants are called K_A and K_B and in most cases represent dissociation constants of substrate A or substrate B from their respective complex with enzyme. K_A and K_B are the constants that exist when the concentration of the cosubstrates are extrapolated to zero. The $K_{m(A)}$ and $K_{m(B)}$ constants are the true Michaelis constants and exist when the concentration of the cosubstrates are extrapolated to infinity. For the rapid-equilibrium random mechanism, these constants are also the dissociation constants of A or B from the ternary EAB complex.

(i) *Ordered sequential mechanisms.* The ordered sequence is shown in Diagram 1.0. P and Q are the products of the reaction.



D1.0

Case 1.1 (Diagram 1.1)—The inhibitor, I, competes with A for binding to free enzyme, E, but



D1.1

cannot promote the binding of B. Therefore, there is no formation of an EIB complex. The less common case where EIB is formed will be discussed below (for the most part, inhibitors do not usually mimic substrate A well enough to convert the enzyme into a form that will accept substrate B). Consider the inhibition, with A as the varied substrate. Since A and I both bind to enzyme form E, there will be a slope effect, and only a slope effect. The inhibition will be competitive and a K_{is} can be obtained. The rule from Table 2 states to extrapolate to zero A. Without A, B cannot bind. So with no influence of B on the K_{is}, the K_{is} is equal to the dissociation constant (K_I) (Equation 1.1a).

$$K_{is} = K_I \qquad (1.1a)$$

This relationship is true for any inhibitor that is competitive with the first substrate of an ordered sequential mechanism regardless of the concentration of the second (non-varied) substrate.

When B is the varied substrate (A not saturating), the results will be different. Since B and I bind to different forms, and saturation by B would not nullify the inhibition, there is an intercept effect. As I binds to an enzyme form that lies upstream to B (that is, before) and is not separated from it by any irreversible steps, there will also be a slope effect. The inhibition will therefore be noncompetitive and both a K_{is} and a K_{ii} can be determined. The factors relating the K_{is} to the K_I are determined by considering the

* The convention used here is different from Cleland's which denotes the dissociation constants from the binary complexes as K_{ia} and K_{ib} and the true K_m's as K_a and K_b.

concentration of B to be zero (from Table 2). It can be seen from Diagram 1.1 that with B absent substrate A will still impede the binding of I. Furthermore, the enzyme form to which A binds is E and the kinetic constant which describes this association (from Table 3) is K_A. Therefore, as shown in Equation 1.1b, K_{is} is equal to K_I multiplied by $[1 + A/K_A]$ and its value will increase as the concentration of A is increased relative to its dissociation constant (K_A). The true K_I may be calculated from this equation.

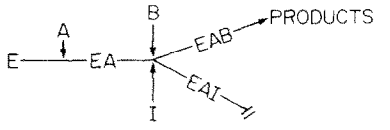
$$K_{is} = K_I(1+A/K_A) \qquad (1.1b)$$

The K_{ii} is related to the K_I by imagining the concentration of B to be infinite (Table 2). This will convert all EA to the EAB complex. The constant that describes the association of A with the enzyme under these conditions is $K_{m(A)}$ (Table 3). Thus, it is the ratio of A to its true K_m that is included in the equation to calculate K_I from K_{ii} (Equation 1.1c).

$$K_{ii} = K_I\,(1+A/K_{m(A)}) \qquad (1.1c)$$

The dissociation constant of an inhibitor competitive with the first substrate of an ordered mechanism can therefore be determined either directly from the K_{is} of the competitive inhibition or indirectly from either the K_{is} or the K_{ii} of the noncompetitive inhibition versus the second substrate. To be consistent with this mechanism, all three values must be equal. Figure 2 shows an example of this type of inhibition where GMP reductase is inhibited by XMP (xanthosine 5'-monophosphate) [13]. The first substrate of this ordered sequence is GMP. The second substrate is NADPH. As seen in Fig. 2A, XMP is competitive versus GMP, the first substrate, and, in 2C, is noncompetitive versus NADPH, the second substrate. The three determinations of the dissociation constant in this situation produced very similar values. Another interesting feature of this type of inhibition is that since I and A compete for binding, the effect of increasing I would be virtually identical to the effect of decreasing A. Therefore, the increase of I in Fig. 2C causes the family of lines to pivot at a locus that is exactly identical to the locus at which the family of lines pivots in Fig. 2B when A is decreased in the absence of any inhibitor. Therefore, both the vertical and horizontal coordinates of the common point of intersection of the lines in Fig. 2B are the same as those of the lines in Fig. 2C.

Case 1.2 (Diagram 1.2)—I competes with B for binding to EA. Of course this will produce competitive inhibition versus B. The extrapolation to zero B shows that the binding of the inhibitor will be influenced by the binding of substrate A to form



D1.2

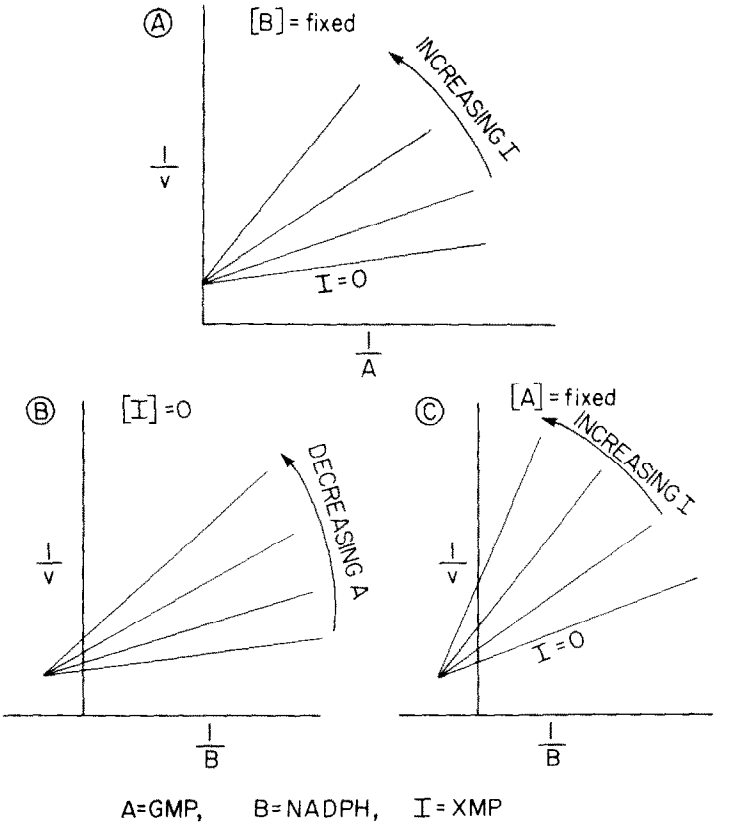


Fig. 2. Inhibition of GMP reductase (schematically reproduced from Ref. 13).

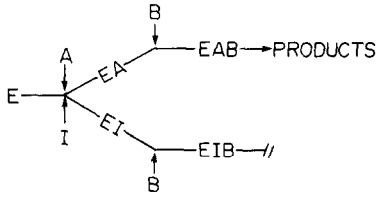
E. Since I binds to the EA complex, its binding is dependent upon the prior binding of A (i.e. A facilitates the binding of I). Therefore, K_{is} is equal to K_I multiplied by $[1 + K_A/A]$, and the K_{is} will decrease as the concentration of A increases (Equation 1.2a).

$$K_{is} = K_I (1 + K_A/A) \qquad (1.2a)$$

An examination of the inhibition versus A shows that A and I combine with different forms of the enzyme and saturation by A does not overcome the inhibition, but actually enhances it. Thus, there is an intercept effect. There is no slope effect, however, because the inhibitor binds after or downstream from A (when A is extrapolated to zero, there is no EA for I to combine with). The inhibition is therefore uncompetitive. To determine the factors that relate K_{ii} to K_I, consider the concentration of A to be infinite. Therefore, it is the true K_m for B and not the dissociation constant of B that enters into the calculation (Equation 1.2b).

$$K_{ii} = K_I (1 + B/K_{m(B)}) \qquad (1.2b)$$

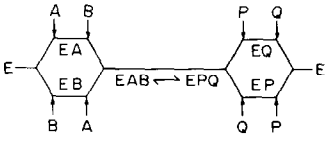
Case 1.3 (Diagram 1.3)—I competes with A for binding to E and EIB is formed. This case is similar to Case 1.1 except that I mimics A well enough to promote the binding of B. Formation of an EIB



D1.3

complex is readily detectable because the inhibitor will induce substrate-inhibition when B is the varied substrate. As mentioned above, this case is uncommon and will not be discussed in detail here. A good discussion and example of this type of inhibition can be found in Ref. 14 which describes the inhibition of thymidylate synthetase by bromodeoxyUMP.

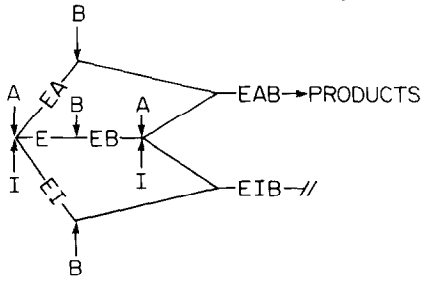
(ii) *Random sequential mechanisms*. The random binding mechanism shown in Diagram 2.0 illustrates



D2.0

that either substrate A or B can bind first in the process of forming the EAB complex.

Case 2.1 (Diagram 2.1)—I competes with A for binding to either E or the EB complex. In this



D2.1

situation, there are two dissociation constants. K_I describes the affinity of I towards E and K_I^* describes the affinity of I towards the EB complex. Thus, the K_{is} from the competitive inhibition versus A is a composite of both of these dissociation constants. At low B, E will be available to I and K_I will dominate. At saturating B, E will be forced into the EB complex and K_I^* will dominate. At in-between (real life) concentrations of B, K_{is} is represented by Equation 2.1a, which contains two unknowns and thus requires that K_{is} be measured at several different concentrations of B to determine both K_I and K_I^*. The situation is simplified by examining the inhibition versus B at non-saturating A. This results in non-competitive inhibition and produces a K_{is} and a K_{ii}. It can be seen in Equation 2.1b that the K_I is discernible from the K_{is} (B extrapolated to zero) and in Equation 2.1c that the K_I^* is obtained from the K_{ii} (B extrapolated to infinity).

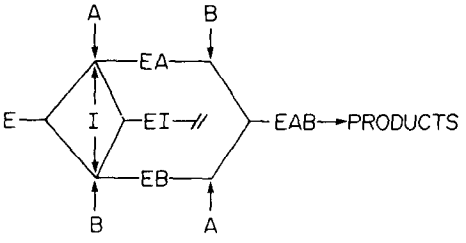
$$K_{is} = \frac{K_I (1 + B/K_B)}{[1 + (K_I/K_I^*)(B/K_B)]} \qquad (2.1a)$$

$$K_{is} = K_I (1 + A/K_A) \qquad (2.1b)$$

$$K_{ii} = K_I^* (1 + A/K_{m(A)}) \qquad (2.1c)$$

Since random mechanisms are symmetrical, a competitive inhibitor of B is analyzed by substituting B for A in Equations 2.1b and 2.1c. An example of this type of analysis can be found in Ref. 15.

Case 2.2 (Diagram 2.2)—I competes with A and



D2.2

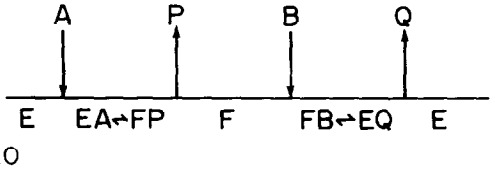
B for binding to E. When I is large enough to cover the binding sites for both A and B, it will produce competitive inhibition with respect to either substrate.

Equations 2.2a (A is varied) and 2.2b (B is varied) show that the K_{is} is affected by the concentration of non-varied substrate relative to its dissociation constant. This type of inhibition is exemplified in Ref. 16.

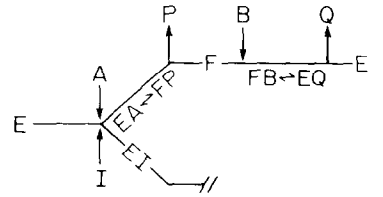
$$K_{is} = K_I (1 + B/K_B) \qquad (2.2a)$$

$$K_{is} = K_I (1 + A/K_A) \qquad (2.2b)$$

(b) *Ping-pong mechanism*. Diagram 3.0 illustrates



D3.0



3.1

the typical ping-pong mechanism. Here F is the modified and E the unmodified form of the enzyme.

Case 3.1 (Diagram 3.1)—I competes with A for binding to E. The K_{is} from this inhibition is equal to the K_I because at zero A, there is no form F available for B to bind (Equation 3.1a).

$$K_{is} = K_I \tag{3.1a}$$

An examination of the inhibition versus B reveals that I and B bind to different forms of the enzyme and saturation by B will not overcome the inhibition. An intercept effect is therefore created. However, although I binds upstream from B, there is no slope effect because the binding of I and B is separated by the release of P which is an irreversible step. So, the inhibition is uncompetitive. At infinite B, it is the $K_{m(A)}$ that relates K_{ii} to K_I (Equation 3.1b).

$$K_{ii} = K_I (1 + A/K_{m(A)}) \tag{3.1b}$$

Case 3.2: I competes with B for binding to F. The second half of the reaction sequence has the same properties as the first half. Thus, a competitive inhibitor with respect to B will be treated identically to a competitive inhibitor of A. That is, the K_{is} from the competitive inhibition is equal to the K_I (Equation 3.2a) and the K_{ii} from the uncompetitive inhibition versus A is related to the K_I by the true $K_{m(B)}$ term as shown in Equation 3.2b. The inhibition of transcarboxylase [17] provides an example of inhibitors of this type.

$$K_{is} = K_I \tag{3.2a}$$

$$K_{ii} = K_I (1 + B/K_{m(B)}) \tag{3.2b}$$

Use of inhibitors to determine reaction mechanisms. It becomes obvious that to relate K_{is} or K_{ii} to K_I, the reaction mechanism must first be known. However, on the brighter side, the patterns of inhibition themselves may reveal the mechanism and thereby create a self-sufficient system of analysis. Fromm [3, 18] has pointed out that dead-end inhibitors produce inhibition patterns that are unique for each reaction mechanism and, therefore, can be used as powerful diagnostic tools. For example, as described by Fromm and above, a competitive inhibitor of substrate A produces noncompetitive inhibition with B and a competitive inhibitor of B produces uncompetitive inhibition with A in an ordered sequential mechanism (Cases 1.1 and 1.2). The patterns are competitive, noncompetitive and competitive, noncompetitive for similar experiments with a small inhibitor and a random mechanism (Case 2.1). A large inhibitor produces competitive inhibition with both A and B of the random mechanism (Case 2.2). Finally, the patterns are competitive, uncompetitive and competitive, uncompetitive with the ping-pong mechanism (Cases 3.1 and 3.2). Thus, either two or four inhibition experiments may establish patterns that uniquely define the reaction mechanism.

Alternate-substrate inhibitors

Alternative substrates are compounds which can replace the normal substrate of an enzyme-catalyzed reaction. The nomenclature is modified for these compounds. As depicted in Equations 4.1 and 4.2, the alternative substrate S^* replaces S and produces the alternate product P^* with a maximal velocity of V^*. C is the common cosubstrate and Q the common product. When S^* is used as an inhibitor, it is critical to measure only the unique product produced from S. This product is circled in Equation 4.1. If the formation of Q, or the sum of P and P^* is measured, the velocity will be a combination of the two reactions and non-linear double reciprocal plots may result. When the ratio of V/V^* is greater than about 15, the velocity of the reaction with S^* is not a significant contribution, and S^* can be considered a dead-end inhibitor and will give the patterns already discussed above.

$$S + C \overset{V}{\longrightarrow} P + Q \tag{4.1}$$

$$S^* + C \overset{V^*}{\longrightarrow} P^* + Q \tag{4.2}$$

Patterns of inhibition. Table 4 states that S^* will produce competitive inhibition versus S and noncompetitive inhibition versus C. This is true for any mechanism regardless of the binding order of S and C. There is no uncompetitive inhibition with alternate-substrate inhibitors of significant V^*. The observation of uncompetitive inhibition means that V^* is negligible and S^* is behaving as a dead-end inhibitor.

Kinetic constants of S^*. The K_{is} from the competitive inhibition of S by S^* is equivalent to the apparent K_m for S^* at the fixed concentrations of the cosubstrate(s) (Equation 4.3).

$$K_{is} = K'_{m(S^*)} \tag{4.3}$$

The use of alternative substrates as competitive inhibitors is a very efficient way to conserve enzyme when determining apparent K_m values for alternative substrates with low V^* values (e.g. Ref. 13). Alternative substrates may also be studied as inhibitors to ascertain their true constants.

If V^* approaches zero, alternate-substrate inhibitors are analyzed in the same manner as dead-end

Table 4. Alternate-substrate inhibitors*

When an alternative substrate (S^*) is used as an alternate-substrate inhibitor, it will produce competitive inhibition versus the substrate it replaces (S) and noncompetitive inhibition versus the cosubstrates, provided that the velocity contribution from S^* is not measured.

*From Ref. 2.

inhibitors and dissociation constants are obtained. If V^* is significant, the alternative substrate's K_{S^*} (concentration of C extrapolated to zero) and/or its true Michaelis constant, $K_{m(S^*)}$ (C extrapolated to infinite), may be calculated by the following equations.

Ordered sequential mechanisms. Because S^* diverts the flux of the reaction, the double reciprocal plots may not be linear. For this reason, discussions of this mechanism are not within the scope of this manuscript, but can be found in Refs. 2 and 4.

Random sequential mechanisms. An alternate-substrate inhibitor replacing either substrate will produce noncompetitive inhibition versus the substrate it does not replace. The K_{S^*} is obtained from the K_{is} as shown in Equation 4.4. The $K_{m(S^*)}$ is obtained from the K_{ii} (Equation 4.5). The meanings of K_{S^*} and $K_{m(S^*)}$ are described in Table 3.

$$K_{is} = K_{S^*}\,(1 + S/K_S) \tag{4.4}$$

$$K_{ii} = K_{m(S^*)}\,(1 + S/K_{m(S)}) \tag{4.5}$$

Ping-pong mechanisms. The true K_m for S^* can be obtained from the K_{ii} of the noncompetitive inhibition versus the cosubstrate. The relationship is shown in Equation 4.6.

$$K_{ii} = K_{m(S^*)}\,(1 + S/K_{m(S)}) \tag{4.6}$$

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