Significance of Slow-Binding Enzyme Inhibition and Its Relationship to Reaction-Intermediate Analogues

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Received January 19, 1988 (Revised Manuscript Received June 1, 1988)

In the quest for new compounds with commercial utility in agriculture and medicine, many potent, reversible inhibitors of enzymes have been obtained, by accident or design. A number of the more potent of these chemically inert reagents have a potency comparable to that of irreversible, chemically reactive inhibitors, and yet retain a high degree of selectivity. Because of their potential for potency and selectivity, chemically stable enzyme inhibitors have received increasing attention in the design of drugs and agrochemicals. However, the inhibition kinetics of potent, reversible enzyme inhibitors is frequently complex, with time-dependent inhibition commonly observed.

A great deal of confusion has been associated with the time-dependent inhibition of enzymes by reversible inhibitors. Although time-dependent inhibition is expected for irreversible inhibitors such as group-specific reagents, affinity labels, or suicide substrates, the time-dependent nature of these inhibitors is attributed to a slow chemical modification of the enzyme. Due in large part to the intrinsic time-dependent nature of these chemically reactive inhibitors, slow inhibition has come to be associated with covalent bond formation between enzyme and inhibitor. However, in only a relatively few of the cases of inhibitors that act by chemically modifying an enzyme has the time-dependent step been rigorously established as covalent bond formation.

As an aid in identifying examples of time-dependent, reversible enzyme inhibition, Prof. John F. Morrison coined the term "slow-binding inhibition".[1] This term has an operational definition in that if the potency of an inhibitor changes on a time scale that is readily followed by methods commonly employed to assay enzymes, i.e., longer than a few seconds, that inhibitor is slow binding. As the nature of the time dependency does not enter into the definition, a large number of time-dependent phenomena, each of which may have an intrinsically different explanation, are lumped together under the title "slow-binding inhibition".

Recently, it has become increasingly obvious that slow-binding inhibition is commonly associated with analogues of intermediates of enzymic reactions.[2,3] These analogues are frequently exceptionally potent inhibitors, and as avidly bound ligands it is not surprising that they have exceptionally slow rates of dissociation. However, a large number of these inhibitors also have rather slow rates of association. It is this latter feature of reaction-intermediate analogues, slow association rates, that has most puzzled enzymologists. The range of kinetic constants encountered for slow-binding inhibitors is illustrated in Table I. The dissociation rate constants range from relatively rapid (0.21 s^{-1} for an inhibitor of adenosine deaminase)[9] to virtually irreversible (half-time for release of 1.4 years for an inhibitor of ribulosebisphosphate carboxylase);[29] association rate constants range from nearly diffusion limited (2.7×10^7 M^{-1} s^{-1} for an inhibitor of HMG-CoA reductase)[23] to exceptionally sluggish (0.23 M^{-1} s^{-1} for acetylene inhibition of hydrogenase).[24] The thermodynamic constants, which reflect the ratio of dissociation and association rates, span an equally wide range from weak binding (1.5×10^{-4} M for acetylene and hydrogenase)[24] to exceptionally avid (1.9×10^{-13} M for 2-carboxyarabinitol 1,5-bisphosphate (2CABP) and ribulosebisphosphate carboxylase).[29] The common feature of the slow-binding inhibitors listed in Table I is a slow rate of dissociation (somewhat less than 1 s^{-1}, such that the approach to steady state can be followed

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Table I
Kinetic Constants of Selected Slow-Binding Inhibitors

enzyme	inhibitor	association: assoc rate, M^{-1} s^{-1}	association: max rate,[a] min^{-1}	dissociation: s^{-1}	dissociation: half-time	K_i*	ref
acetolactate synthase	sulfometuron methyl	1500	0.15	1.2×10^{-4}	1.6 h	82 nM	4–6
acetolactate synthase	imazaquin	12	0.6	2.4×10^{-4}	0.8 h	20 μM	7
aconitase	nitro analogue of isocitrate	1.5×10^{6}	6.6	1.0×10^{-3}	11 min	680 pM	8
adenosine deaminase	deoxycoformycin	2×10^{6}	NS	5×10^{-6}	38 h	2.5 pM	9
adenosine deaminase	coformycin	9×10^{5}	NS	2×10^{-4}	0.96 h	220 pM	9
adenosine deaminase	DHMPR	7.5×10^{4}	NS	0.21	3.3 s	2.8 μM	9
adenosine deaminase	EHNA	2.9×10^{6}	36	0.005	2.3 min	1.7 nM	9
adenylate deaminase	deoxycoformycin	1.3×10^{4}	NS	4.6×10^{-3}	2.5 min	360 nM	9
adenylate deaminase	coformycin	9000	NS	1.8×10^{-4}	1.1 h	20 nM	9
adenylate deaminase	deoxycoformycin 5′-phosphate	9×10^{4}	NS	9×10^{-5}	2.1 h	1 nM	9
adenylate deaminase	coformycin 5′-phosphate	1×10^{5}	NS	5×10^{-6}	38 h	55 pM	9
adenylate deaminase	DHMPR 5′-phosphate	4×10^{4}	NS	0.1	6.9 s	2.5 μM	9
alanine racemase[b]	1-aminoethyl phosphonate	7	9.6	3.2×10^{-7}	25 days	1.25 μM	10, 11
1-aminocyclopropane carboxylate deaminase	1-aminocyclopropyl phosphonate	100	NS	8.5×10^{-4}	13.5 min	5.8 μM	12
aeromonas aminopeptidase	amastatin	2.1×10^{4}	NS	5.5×10^{-6}	35 h	260 pM	13
cytosolic leucine aminopeptidase	amastatin	4600	NS	1.4×10^{-4}	1.4 h	30 nM	13
microsomal aminopeptidase	amastatin	1280	NS	6.6×10^{-5}	2.9 h	52 nM	13
angiotensin converting enzyme	captopril	1.19×10^{6}	3.4	4×10^{-4}	29 min	330 pM	14
chymotrypsin	Me-succinyl-Ala-Ala-Pro-*boro*-Phe	5.9×10^{6}	1.2	9.6×10^{-4}	12 min	160 pM	15
chymotrypsin	chymostatin	3.6×10^{5}	ND	3.2×10^{-4}	36 min	390 pM	16
chymotrypsin	difluoro ketone peptide (7)	4.7×10^{5}	ND	6.7×10^{-3}	1.7 min	14 nM	17
cytidine deaminase	phosphinamide analogue of cytidine	8300	NS	7.8×10^{-6}	25 h	900 pM	18
elastase	acetyl-Ala-Ala-Pro-*ambo*-Ala-CF_3	290	ND	1.25×10^{-4}	1.5 h	430 nM	19
elastase	Z-Val-Pro-Val-CF_3	2.5×10^{4}	NS	4×10^{-5}	4.8 h	1.6 nM	20
enolase	phosphonoacetohydroxamate	5.33×10^{5}	NS	1.22×10^{-4}	1.6 h	230 pM (15 pM)[c]	21
glutamine synthetase	L-methionine sulfoximine *N*-phosphate	1×10^{6}	ND	2×10^{-13} -2×10^{-14}	10^{5} -10^{6} year	2×10^{-19} -2×10^{-20} M	22
HMG-CoA reductase	compactin	2.7×10^{7}	NS	6.5×10^{-3}	1.8 min	240 pM	23
hydrogenase	acetylene	0.23	3.8	3.5×10^{-5}	5.5 h	150 μM	24
isocitrate lyase	3-nitropropionate	1070	16	$<1.8 \times 10^{-5}$	>10 h	<17 nM	25
orotidine 5′-phosphate decarboxylase	BMP	2.5×10^{6}	ND	2.2×10^{-5}	8.7 h	8.8 pM	26
protocatechuate 3,4-dioxygenase	2-hydroxyisonicotinate *N*-oxide	640	120	6.45×10^{-5}	3.0 h	100 nM	27
protocatechuate 3,4-dioxygenase	6-hydroxyisonicotinate *N*-oxide	240	1.0	1.96×10^{-4}	0.98 h	1.6 μM	27
pyruvate dehydrogenase	thiamine thiazolone pyrophosphate	9500	NS	4.7×10^{-6}	40 h	500 pM	28
ribulosebisphosphate carboxylase	2CABP	7.8×10^{4}	2.2	1.5×10^{-8}	530 days	190 fM	29
ribulosebisphosphate carboxylase	4CABP	1.6×10^{5}	1.8	4.4×10^{-6}	1.82 days	28 pM	29
xanthine oxidase	alloxanthine	5400	0.37	1.8×10^{-4}	1 h	35 nM	30

[a] ND = not determined; NS = no saturation observed in the rate of tight complex formation. [b] The reported values are not internally consistent. Association and dissociation rate constants define a 46 nM K_i*, vs the 1.25 μM value reported. An initial inhibition constant of 0.8 mM and a maximal rate of tight complex formation of 9.6 min^{-1} give an association rate of 200 M^{-1} s^{-1}, further lowering the calculated K_i* to 1.6 nM. The higher affinity for the inhibitor calculated from these values would explain the incomplete reversal of enzyme inhibition on dilution of the enzyme–inhibitor complex observed (i.e., equilibrium was achieved). [c] The value in parentheses is the dissociation constant for the fully ionized, hydroxamate form of the inhibitor.

under the conditions commonly employed to assay enzymes). It is the intent of this review to examine those factors that determine the kinetic and thermodynamic constants of reversible inhibitors, and what consequences these parameters have on the potential utility of an enzyme inhibitor as a drug, agrochemical, or pesticide.

Thermodynamics: How Tight Can an Inhibitor Be Expected To Bind? By a simple extension of Eyring transition-state theory, the affinity of enzymes for transition-state structures (the most tightly bound reaction intermediates) can be estimated.[31-38] The assumption made in this approximation is that the transition state of a reaction will decompose at the same rate, kT/h (6×10^{12} s^{-1}), irrespective of whether it is free in solution or bound by an enzyme. With this assumption, and in the absence of other steps limiting the rate of the enzymic reaction (such as product release), the ratio of the rate of the enzymic reaction to the rate of the uncatalyzed reaction is equal to the ratio of the enzyme's dissociation constants for substrate and transition state. From consideration of the rate enhancements normally achieved by enzymes (10^{8}–10^{14}-fold)[34] and the dissociation constants typical of most low molecular weight substrates (10^{-3}–10^{-6} M), a

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"typical" range of 10^{-11}–10^{-20} M might be expected for the dissociation constants of transition-state structures for their enzymes. A good transition-state analogue could reasonably be expected to have a dissociation constant in this range. However, transition states are the most tightly bound and shortest lived (half-time of 0.1 ps) intermediates of the enzymic reaction. What might be expected for analogues of other enzyme-bound intermediates? By arguments similar to those used to estimate the enzyme's affinity for transition states, it can be shown that the enzyme will bind any intermediate of the reaction less tightly than a transition state to the extent it enhances the rate of its conversion to transition states before or after the intermediate in the reaction path.[3] Thus, the more stable an intermediate of the enzymic reaction is, the less tightly bound by the enzyme should it, or its analogue, be.

For analogues with affinities comparable to those of transition-state structures, intracellular concentrations favor complex formation. The minimum concentration of anything packaged in the volume of a eukaryotic or prokaryotic cell (a single molecule) is approximately 10^{-12} and 10^{-9} M, respectively. Thus, at stoichiometric concentrations of enzyme and a good transition-state analogue in vivo (likely to be several thousand-fold higher than the minimum concentrations since several thousand molecules of enzyme are likely to be present), virtually complete inhibition of the enzyme should be achieved, based on thermodynamic considerations alone.

Dissociation Rates: How Fast Can Inhibition Be Reversed? The primary advantage offered by irreversible inhibitors such as affinity labels or suicide substrates in designing pharmaceuticals or agrochemicals is potency. Irreversible inhibition can, in principle, give the minimum effective dose: one molecule of inhibitor for each molecule of enzyme to be inhibited. If the rate of release of a reversible inhibitor is exceptionally slow, then the advantage of an irreversible inhibitor can be gained without the need for chemical reactivity. For transition states of enzyme-catalyzed reactions, the estimated dissociation constants place upper limits on the dissociation rates of these intermediates. Since the dissociation constants are defined by the ratio of dissociation and association rate constants, and the association rates cannot exceed the diffusion limit, an upper limit for the dissociation rates of transition states is between 10^{-2} and 10^{-11} s^{-1}, or half-times for release between about 1 min and 2000 years. Since the transition state has a half-life of about 10^{-13} s in its conversion to product, these rates of dissociation are sufficiently slow to ensure that the transition state never leaves the enzyme. For a good transition-state analogue with an affinity comparable to that of the transition state, a slow rate of release is expected.

Several of the inhibitors listed in Table I have dissociation constants and rates of release comparable to those expected for transition-state analogues. Methionine sulfoximine *N*-phosphate is thought to mimic the transient tetrahedral species formed upon the attack of ammonia on γ-glutamyl phosphate (Figure 1). This inhibitor binds so tightly to glutamine synthetase that it can be released only by reversible pH denaturation of the complex. Extrapolation of this reversal rate from low pH (about 4.5) to neutrality gives an estimated

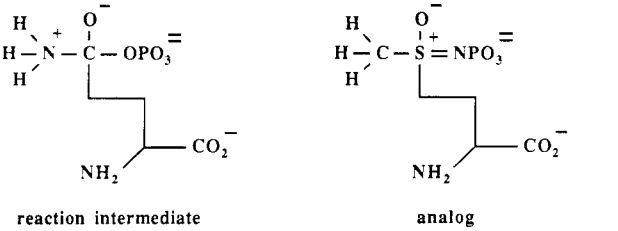

Figure 1. Reaction intermediate and analogue of the glutamine synthetase reaction.

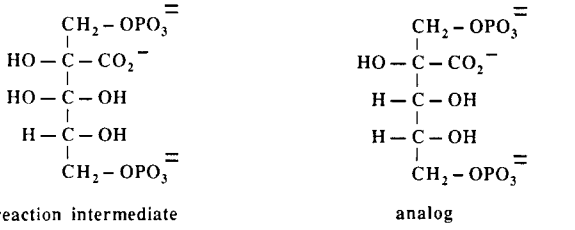

Figure 2. Reaction intermediate and analogue of the ribulosebisphosphate carboxylase reaction.

half-time for release of the inhibitor of 10^5–10^6 years.[22] Given the method by which this value was determined (the pH dependency is fourth-order in hydrogen ion concentration), it is not a reliable estimate, although the rate of inhibitor release is certainly exceptionally slow. 2CABP is an analogue of the 6-carbon intermediate of the ribulosebisphosphate carboxylase reaction, 2-carboxy-3-oxoarabinitol 1,5-bisphosphate (presumably with its carbonyl at C-3 hydrated) (Figure 2). The rate of release of this inhibitor has been determined by dual isotope exchange (^{3}H) inhibitor in the enzyme–inhibitor complex and ^{14}C-labeled exogenous inhibitor) to be 1.4 years.[29] This value together with the association rate determined by inactivation kinetics (7.8×10^4 M^{-1} s^{-1}) defines a dissociation constant of 1.9×10^{-13} M (190 fM). These extraordinary inhibitors lend credence to the estimated affinities and slow rates of dissociation expected for transition states. Inhibitors for the enzymes adenosine deaminase,[9] adenylate deaminase,[9] alanine racemase,[10,11] aminopeptidase,[13] cytidine deaminase,[18] and pyruvate dehydrogenase[28] have reported half-times for release in excess of a day. Although all of these inhibitors are reversible (in the sense that what is released by the enzyme is the same as what was initially bound), their rates of release are so exceptionally slow that the distinction between these inhibitors and truly irreversible inhibitors (such as affinity labels or suicide substrates), in a practical sense, is lost.

One of the consequences of blocking an enzyme on a major metabolic pathway can be a buildup of the substrate for that enzyme, leading to reversal of the inhibition.[39,40] However, when the rate of inhibitor release is sufficiently slow that the desired biological effect can be achieved and maintained for a sufficient duration, the compensating effects of metabolic blockade no longer need be considered. Certainly this would be true for many of the inhibitors listed in Table I.

Although transition-state analogues have been considered for purposes of establishing a reasonable range that might be expected for the release rates of reversible

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inhibitors, it is questionable whether many of the examples listed in Table I truly mimic transition-state structures. In the case of the exceptionally potent inhibitor of ribulosebisphosphate carboxylase, the intermediate that it resembles is sufficiently stable to allow its trapping after denaturation of the enzyme.[41] The intermediate in solution decomposes with a half-life of approximately an hour, substantially longer than the 0.1-ps half-life expected for a transition-state structure.[42] It would appear that exceptional avidity and extremely slow rates of release can be obtained for analogues of intermediates lower in energy and longer lived than transition states. If an intermediate of an enzymic reaction is labile with respect to reactions other than that catalyzed by the enzyme, then its release by the enzyme could prove detrimental. There would be selective pressure on such an enzyme, in an evolutionary sense, to maintain a slow rate of release for such an intermediate.

Association Rates: Why Are They Frequently So Slow for Reaction-Intermediate Analogues? Intermediates of the enzymic reaction other than transition states must be bound less tightly to the extent the enzyme catalyzes their reaction rates. However, where release of an intermediate by the enzyme can result in a detrimental side reaction, there will be selective pressure on the enzyme to maintain a slow rate of release for that intermediate to ensure it remains enzyme bound. The enzyme can satisfy the need to bind such an intermediate less tightly than transition states by lowering its association rate. For an enzyme-bound intermediate that is never released from the enzyme during the reaction, the association rate is without kinetic consequences. There will be evolutionary pressure on enzymes to pay the smallest price possible in dealing with metastable reaction intermediates, and it is likely that many enzymes will be constructed such that both the association and dissociation rates for these intermediates will be rather slow. During the evolution of an enzyme, slow association rates may be selected for enzyme-bound reaction intermediates that are more stable than transition-state structures. To the extent an analogue resembles a transition-state structure of an enzymic reaction it should be bound exceptionally tightly, and to the extent an analogue resembles a metastable reaction intermediate of the reaction it is reasonable to expect that both association and dissociation rates will be slow for the inhibitor, with only moderate affinity for enzyme (between that of substrate and transition state). The relative affinities for substrate, product, intermediate, and transition states, derived from a comparison of reaction coordinate diagrams for enzyme-catalyzed and noncatalyzed reactions, are illustrated in Figure 3.

A slow rate of association is not desirable in the design of drugs or agrochemicals, as it reduces the likelihood that the desired biologic effect will be obtained in vivo. At a concentration of 10 pM (1×10^{-11} M) methionine sulfoximine *N*-phosphate and 1 pM glutamine synthetase, complete inhibition would be obtained at equilibrium. However, at this concentration, with the association rate of the inhibitor (1×10^{6} M^{-1} s^{-1}) reported in Table I, glutamine synthetase would have a half-life in excess of 19 h. At a 1000-fold higher concentration of inhibitor (10 nM), the half-life of the enzyme would decrease to about 1 min, a rate far more likely to achieve inhibition of a target enzyme in vivo. Of the inhibitors listed in Table I, methionine sulfoximine *N*-phosphate has one of the faster association rates. The slower inhibitors would require far higher concentrations to be effective than those expected by consideration of their dissociation constants alone.

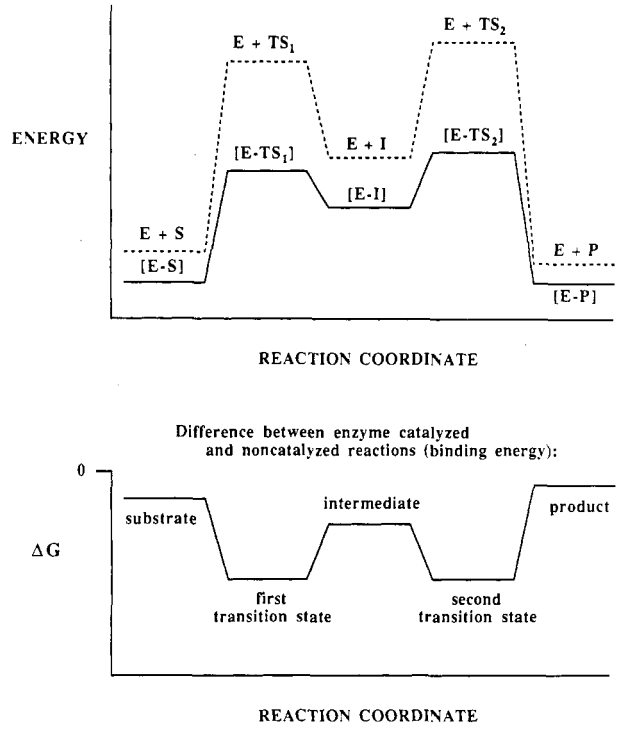

Figure 3. Reaction coordinate diagrams for an enzyme-catalyzed reaction (solid line) and the corresponding noncatalyzed reaction (dashed line) are illustrated in the upper profiles. Enzyme, substrate, the first transition state, a reaction intermediate, a second transition state, and product are designated by E, S, TS_1, I, TS_2, and P, respectively. The complexes of enzyme with reactants are indicated by enclosing the hyphenated partners in brackets. The binding energy (ΔG) for reactants, the difference between the enzymic and nonenzymic profiles, is illustrated in the lower profile.

Not only do many of the inhibitors listed in Table I have slow rates of association, but they also exhibit maximum rates of slowly reversible complex formation. A number of kinetic mechanisms (mechanisms 1–4, Scheme I) have been considered in characterizing slow-binding inhibition (in these mechanisms and in mechanisms 5 and 6, E, I, EI, E*, I*, and EI* are enzyme, inhibitor, rapidly reversible enzyme–inhibitor complex, a rare, slowly reversible enzyme form, a rare, slowly reversible inhibitor form, and slowly reversible enzyme–inhibitor complex, respectively).[1,19,43-45] Mechanism 1 is the simplest with direct formation of the slowly reversible complex (EI*). This type of binding mechanism would not have a maximum rate of association, nor would there be any inhibition at early times after mixing enzyme and inhibitor that could not be accounted for by the time-dependent formation of EI*. Mechanisms 2–4 differ in that they can account

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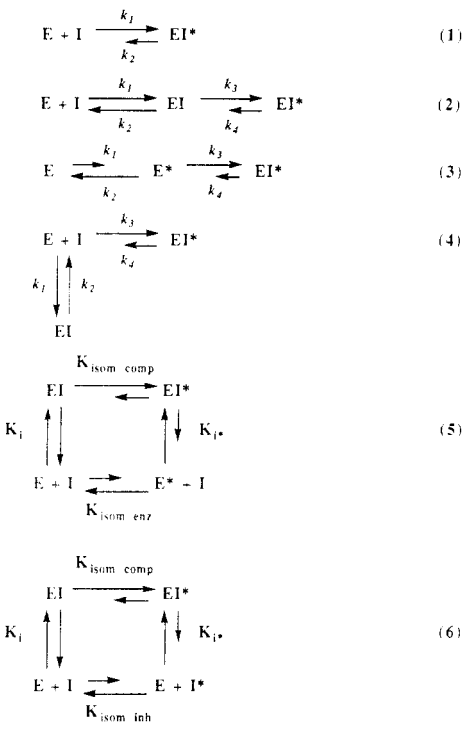
Scheme I



$$K_i = \frac{[E]\,[I]}{[EI]} \qquad K_{isom\ comp} = \frac{[EI]}{[EI^*]} \qquad K_i^{**} = \frac{[E]\,[I]}{[EI^*]}$$

Enzyme isomerization:

$$K_{i^*} = \frac{[E^*]\,[I]}{[EI^*]} \qquad K_{isom\ enz} = \frac{[E]}{[E^*]} \qquad K_i^{*} = \frac{[E + E^*]\,[I]}{[EI + EI^*]}$$

$$K_i^{*} = K_{i^*}\frac{(1 + K_{isom\ enz})}{(1 + K_{isom\ comp})}$$

$$K_i^{**} = K_iK_{isom\ comp} = K_{i^*}K_{isom\ enz} = K_i^{*}K_{isom\ enz}\frac{(1 + K_{isom\ comp})}{(1 + K_{isom\ enz})} \approx K_i^{*}$$

Inhibitor isomerization:

$$K_{i^*} = \frac{[E]\,[I^*]}{[EI^*]} \qquad K_{isom\ inh} = \frac{[I]}{[I^*]} \qquad K_i^{*} = \frac{[E]\,[I + I^*]}{[EI + EI^*]}$$

$$K_i^{*} = K_{i^*}\frac{(1 + K_{isom\ inh})}{(1 + K_{isom\ comp})}$$

$$K_i^{**} = K_iK_{isom\ comp} = K_{i^*}K_{isom\ inh} = K_i^{*}K_{isom\ inh}\frac{(1 + K_{isom\ comp})}{(1 + K_{isom\ inh})} \approx K_i^{*}$$

for a saturable rate of tight complex formation. Mechanisms 2 and 4 differ from mechanism 3 in that they can also account for rapidly reversible, initial inhibition (EI), as well as slowly reversible inhibition. Detailed accounts of the kinetics of these slow-binding inhibition schemes have been described.[1-3,43-45] The saturable, biphasic inhibition mechanisms all have a maximum rate of tight binding and a concentration of inhibitor that gives a half-maximal rate of inhibition (comparable to a Michaelis constant, or K_i for mechanisms 2 and 4). The ratio of these two kinetic constants (maximum rate/K_i) is equal to the rate constant for tight complex formation at low inhibitor concentration.

All of these mechanisms can be derived from two more general slow-binding inhibition mechanisms (mechanisms 5 and 6). The distinction between these two mechanisms is whether the slow step involves a reversible alteration of the enzyme ($K_{isom\ enz}$) or inhibitor ($K_{isom\ inh}$). For the enzyme this "isomerization" could involve reorientation of the protein (conformational change), water structure at the active site, or a change in oligomeric state. For the inhibitor the change could be hydration/dehydration of a carbonyl, change in ionization state, or a conformational change. These alterations could take place in the enzyme–inhibitor complex ($K_{isom\ comp}$) or in solution ($K_{isom\ enz}$ and $K_{isom\ inh}$). It does not matter from the standpoint of inhibition kinetics, however, whether the slow step occurs in the enzyme or the inhibitor. In most cases, equivalent behavior can be accounted for by either model. The experimental constants are the initial inhibition constant (K_i), the final, steady-state inhibition constant (K_i^*), and the limiting rate constants for tight complex (EI*) formation at low and high inhibitor concentrations. By making various rate constants in mechanism 5 equal to zero or very fast (combining steps), one can obtain each of the mechanisms 1–4. Slow changes in protein structures are known to occur,[46] and the best documented slow transitions in relatively small molecules are those for crown ether–cation interactions (dissociation rates with half-lives of 3 months and association rates as slow as 2.75×10^{-4} M^{-1} s^{-1}).[47] In both cases, the slow transitions are likely to involve energetically unfavorable transitions along the way to a net change that is favored.

Advantages of Reaction-Intermediate Analogues in the Design of New Pharmaceuticals or Agrochemicals. There are several attractive features of reaction-intermediate analogues in designing new enzyme inhibitors, such as (1) the potential to achieve virtually irreversible inhibition without the need for chemical reactivity (and an appropriately positioned enzymic nucleophile), (2) a simple de novo design strategy that does not require detailed structural information about the enzyme's active site, and (3) the likelihood that good mimics of a common intermediate will prove equally effective against several different, mechanistically related enzymes (and still maintain selectivity between these enzymes to the extent that their substrates are structurally different). In designing a new type of reaction-intermediate analogue, one can employ a rather simple strategy, which is best described as heteroatom or radical replacement.[3] Starting with a labile intermediate or transition-state structure that is likely to be on the reaction pathway of a given enzyme, a single atom or group of atoms (radical) can be substituted in the structure that would maintain the geometry and electronic nature of the intermediate to the extent possible, yet give a stable structure. Many examples of inhibitors that conform to this approach exist (reaction-intermediate analogues can be categorized by the type of substitution made, e.g., boron for carbon, phosphorus for carbon, hydrogen for hydroxyl, carbon or hydrogen for nitrogen, sulfur for carbon, vanadium for phosphorus, carbon or nitrogen for phosphorus, nitrogen for carbon, oxygen for carbon, and nitrogen for oxygen),[3] many of which are not only po-

(46) Glick, D. M. *Nature (London)* **1986**, *320*, 22.
(47) Rebek, J., Jr.; Luis, S. V.; Marshall, L. R. *J. Am. Chem. Soc.* **1986**, *108*, 5011.

tent inhibitors but exhibit slow-binding inhibition. An inhibitor designed by this approach that is potent and/or slow-binding provides evidence that the intermediate considered is on the enzymic reaction pathway. However, not every potent or slow-binding inhibitor is a reaction-intermediate analogue.

While a credible case can be made that most of the inhibitors listed in Table I possess their kinetic properties by virtue of their structural similarity to intermediates of the enzymic reactions inhibited, there are several notable exceptions. It is hard to imagine, for example, what intermediate of the hydrogenase reaction that acetylene might resemble.[24] After analyzing the inhibition of HMG-CoA reductase by compactin, Nakamura and Abeles concluded that a large portion of the inhibitor bound to a part of the enzyme close to, but outside of the enzyme's active site.[23] The herbicides that inhibit acetolactate synthase, sulfometuron methyl and imazaquin, seem to do so by binding to an evolutionary vestige of a quinone binding site that is no longer functional in this enzyme.[7] All of these latter three examples are relatively potent inhibitors that are also slow binding. The latter two examples (compactin and sulfometuron methyl) suggest other strategies for obtaining potent enzyme inhibitors. As evidenced by these inhibitors, additional binding energy can be gained by protein–ligand interactions outside of the enzyme's active site. Unfortunately, where any additional favorable interactions may be gained is difficult to predict in the absence of a high-resolution protein crystal structure. In cases where such structures have been obtained for the enzyme of interest, improvements in existing inhibitors or de novo design can be pursued with reference to the crystal structure alone.[3,48] Potent inhibitors obtained by such methods, will undoubtedly often exhibit slow-binding inhibition.

Concluding Remarks

There is reason to believe that slow rates of association will be a common feature of analogues of enzymic reaction intermediates that are more stable than transition states. This is borne out by the number of reaction-intermediate analogues that exhibit slow association rates. In any case, slow-binding inhibition (a phenomenon resulting from a slow rate of dissociation) will be encountered with any sufficiently potent, reversible inhibitor ($<10^{-9}$ M, for inhibitors with diffusion-limited association rates; this will occur with substantially less avid inhibitors, if their rates of association are slow). From the standpoint of inhibitor design for agricultural or pharmaceutical applications, a slow rate of dissociation is desirable, as it may be expected to enhance an inhibitor's effectiveness, while a slow rate of association is not, as it delays the time required for inhibition at a given concentration in vivo. Balancing the kinetic properties of an inhibitor between the Scylla of too fast a dissociation rate and the Charybdis of too slow an association rate is one of the current challenges facing those trying to design enzyme inhibitors with commercial utility via this route.

(48) Goodford, P. J. *J. Med. Chem.* **1984**, *27*, 557.