Chemistry and Enzymology of k_{cat} Inhibitors

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Irreversible enzyme inhibitors possessing latent reactive groupings which are specifically unmasked by the action of the target enzyme are extraordinarily specific. In this article a general approach to the design of such irreversible enzyme inhibitors is discussed. These inhibitors are so constructed that they require chemical activation by the target enzyme. Upon activation, a chemical reaction ensues between the inhibitor and enzyme resulting in the irreversible inhibition of the latter. Thus, the enzyme by its specific mode of action catalyzes its own inactivation.

We may begin with the usual model for an enzymatic process:

$$S + E \underset{K_s}{\rightleftarrows} ES \xrightarrow{k_{cat}} E + P$$

where S is the substrate, E is the enzyme, K_s is a dissociation constant for the binding of the substrate to the enzyme, and k_{cat} is a catalytic constant that reflects the probability that substrate molecules, once bound, will be converted to the product P. Although k_{cat} is easily measured, K_s is not. Kinetic measurements, of course, usually afford k_{cat} (V_{max}, the maximum velocity) and K_m, the Michaelis constant. The term K_m may or may not be equal to K_s. Nevertheless, we choose to describe the enzymatic process in terms of k_{cat} and K_s because these terms have definite meaning in terms of the mechanism of the reaction and can serve as a useful basis for the classification of inhibitors.

The first type of irreversible inhibitor that might be called a "K_s inhibitor" is exemplified by affinity labeling agents (*1*). These reagents are constructed by attaching a chemically reactive functional group to a moiety that resembles the substrate. Selectivity of action is based on the much higher concentration of the noncovalent complex between reagent and active site than of complexes between the reagent and other reactive groups. Thus, this selectivity is based on a physical mechanism rather than on a chemical one and, in practice, is solely dependent on the magnitude of the dissociation constant (K_s) for the reagent (*2*). Unfortunately, these reagents are still rarely selective enough to be very useful in multicomponent systems, let alone in vivo. The lack of overall selectivity of these agents is a consequence of the strong possibility of reaction with other biomolecules before a fruitful encounter occurs with the target enzyme. These reagents could, in fact, only be made sufficiently selective to be of general pharmacological interest if they could be designed with K_s's perhaps in the nanomolar range. However, even when the three-dimensional structure of an enzyme has been known, it has not yet been possible to formulate the kind of molecule that would bind with such a low K_s to most enzymes (*3*).

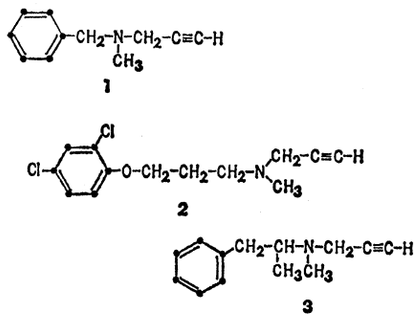
Since the specificity of enzyme action resides primarily in the k_{cat} term (*4*), one would do much better to focus on this term rather than on K_s when contemplating the design of a specific and irreversible inhibitor. This other class of inhibitors can be designated as "k_{cat} inhibitors" because their mechanism (or mechanisms) of action depends on catalytic conversion. These k_{cat} inhibitors have the potential for far greater specificity than the K_s inhibitors and furthermore are easier to design since much more is known about enzyme mechanism than about the forces that govern binding. Specifically, k_{cat} inhibitors can be constructed to possess latent reactive groupings that are selectively activated by the target enzyme at its active site. The reactive grouping, once generated, then engages in a chemical reaction with the enzyme leading to its irreversible inhibition. Since inhibitors of this type are not rendered active until acted upon by the target enzyme, they cannot react with other biomolecules, and therein lies the basis of their specificity. These inhibitors, in addition to obviously utilizing the K_s terms, take advantage of the major factor (k_{cat}) upon which enzyme specificity is based. We shall now turn to examples of k_{cat} inhibitors that exist in the literature.

The paradigm of this kind of inhibitor is to be found in studies on the irreversible inhibition of β-hydroxydecanoyl thioester dehydrase by $\Delta^{(3,4)}$ decynoyl *N*-acetyl cysteamine (NAC) (*5*). This enzyme, which is required for unsaturated fatty acid synthesis in *Escherichia coli*, catalyzes the reversible interconversion of hydroxydecanoyl thioesters with their α,β-*trans* and β,γ-*cis* counterparts (scheme 1).

The k_{cat} inhibitor of this enzyme, $\Delta^{(3,4)}$-decynoyl-NAC, is treated as a substrate by the enzyme. However, unlike the β,γ-olefinic substrate, the acetylene is converted into the highly reactive conjugated allene, which immediately alkylates an active site histidine residue (*6*) (scheme 2).

Since the enzyme can be totally inactivated by a stoichiometric amount of this acetylene, a single turnover is all that is required for irreversible inhibition to occur. The high degree of specificity of this inhibitor in multicomponent systems and in vivo was demonstrated in the experiments of Kass and Bloch (*7*). These experiments showed that β-hydroxydecanoyl thioester dehydrase was absolutely required for unsaturated fatty acid synthesis in *E. coli*.

Flavin-linked monoamine oxidases are also irreversibly inhibited by molecules that contain an acetylenic moiety (*8*). Compounds such as pargyline (**1**), chlorgyline (**2**), and deprenyl (**3**) are irreversible inhibitors of the enzyme, and in certain instances they have been used clinically (*9*).



In order for the inhibition to occur the acetylenic moiety must be positioned β

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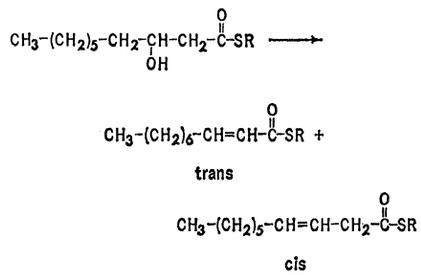
to the amino group and as a result it is covalently attached to the flavin. Furthermore, we have found that *cis*-3-haloallylamines are irreversible inhibitors of this enzyme (*10*). As would be expected, the primary acetylenic amine propargyl amine also irreversibly inactivates the enzyme. All of these results can be understood in terms of a plausible mechanism for reactions cocatalyzed by flavin (*11*) (scheme 3). When the R group is in an acetylene or vinyl halide, the conversions shown in scheme 4 can occur.

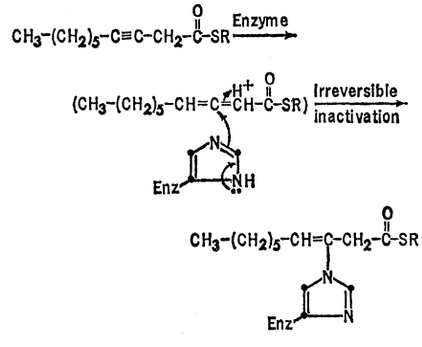
In both cases, the proton on the α carbon is labilized by the enzyme, but instead of the electrons flowing toward the flavin, they are subverted, with resultant isomerization of the molecules. Thus, the generation of highly reactive allenes and allylic halides is achieved from their unreactive counterparts. That flavin-linked amine oxidases can serve as isomerases under certain conditions is already known from the demonstration that β-chloroalanine can be converted into pyruvic acid by D-amino acid oxidase (*12*).

Other flavin-linked enzymes are also susceptible to irreversible inhibition by acetylenes. Abeles and co-workers have demonstrated that flavin-linked lactate dehydrogenase is irreversibly inhibited by 2-hydroxy-3-butynoic acid (*13*). In this case also, the inhibitor reacts with the flavin cofactor. The great specificity of this inhibitor was used to advantage in studies by Abeles *et al.*, which were designed to determine the physiological role of membrane-bound lactate dehydrogenase in *E. coli* (*14*). In addition, the monoamine and diamine oxidases in the plasma that are not linked to flavins suffer irreversible inhibition in the presence of the acetylenic propargylamine (*15*). These enzymes require two cofactors for their activity; the first has been positively identified as cupric ion and the second might be a pyridoxal derivative (*16*). This second cofactor is capable of forming a Schiff base with the primary amine substrates and, as is expected, the enzyme is inhibited by standard carbonyl reagents such as hydrazine (*17*). The conversion shown in scheme 5 could mediate the actual irreversible inhibition step. This interpretation is consistent with the observation that propargylamine has no effect on the enzymes when the cupric ion is absent (*15*). Therefore, the acetylene is not inhibitory in and of itself, but must be enzymatically converted into the active substance.

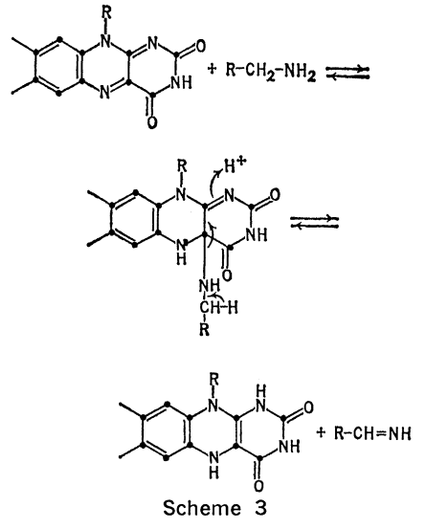
Enzymes that use the coenzyme pyridoxal phosphate are also quite susceptible to k_{cat} inhibitors. In 1949, Wood and Gunsalus showed that bacterial threonine dehydratase was irreversibly inhibited by serine (*18*). The mechanism of action of this enzyme requires abstraction of the proton on the α carbon of the amino acid with concomitant elimination of H_2O to form the aminoacrylate intermediate (scheme 6). When serine is the substrate, the highly reactive aminoacrylate that is generated apparently engages in a Michael reaction with an active-site amino acid residue, resulting in the inactivation of the enzyme (*19*). It is reasonable to suspect that the steric hindrance of the methyl group normally prevents this reaction from occurring when threonine is the substrate. It would be interesting to know how the cell normally compartmentalizes its threonine dehydratase with respect to serine to prevent this mode of inhibition from occurring. Along similar lines, L-serine sulfate has been shown to be an irreversible inhibitor of soluble aspartate aminotransferase (*20*), and β-chloroalanine irreversibly inhibits both this enzyme (*21*) and L-aspartate-β-decarboxylase (*22*). The mechanisms of action of these enzymes involve the initial cleavage of the bond between the α carbon and the hydrogen. Once



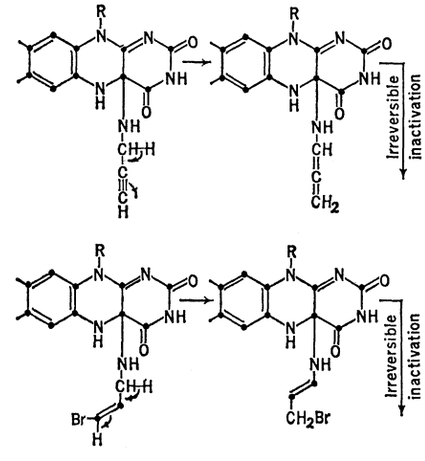
Scheme 1



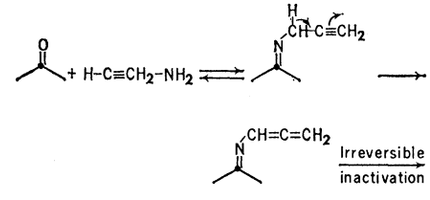
Scheme 2



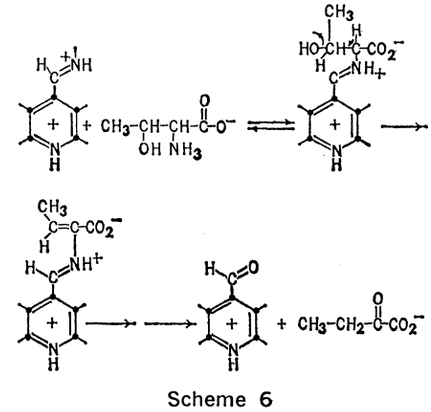
Scheme 3



Scheme 4



Scheme 5

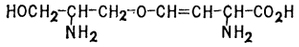


Scheme 6

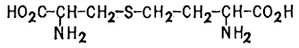
this occurs, with the inhibitors as substrates, the reaction sequences depicted in scheme 7 can intervene.

The subsequent reaction of either of these molecules with critical amino acid residues results in the inactivation of the enzyme. These observations would lead one to expect that β,γ-unsaturated amino acids ought be potent irreversible enzyme inhibitors since their enzymatic conversions would lead to similar highly reactive intermediates (scheme 8).

Several β,γ-unsaturated amino acids have been described (*23*). Physiological studies have been reported for two of them and, indeed, the results are exceedingly interesting. Rhizobitoxine [2-amino-4-(2-amino-3-hydroxypropoxy)-*trans*-but-3-enoic acid] is an irreversible inhibitor of β-cystanthionase (*24*) from bacteria and plants. The compound shows strong antimetabolic activity, as expected, and resembles cystathionine, the natural substrate for the enzyme.

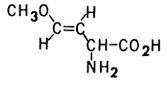


Rhizobitoxine



Cystathionine

This pyridoxal-linked enzyme degrades cystathionine by the mechanism shown in scheme 9. I propose that rhizobitoxine is an irreversible inhibitor of this enzyme by virtue of the fact that it can be enzymatically converted into the reactive intermediates shown in scheme 9. In this instance a chemical reaction ensues between the rhizobitoxine and the pyridoxal phosphate cofactor. The second compound of this type is L-2-amino-4-methoxy-*trans*-3-butenoic acid (AMB) (*25*).



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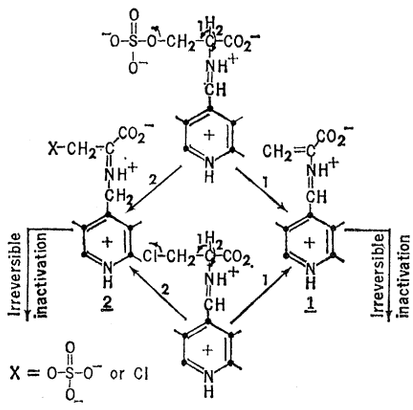
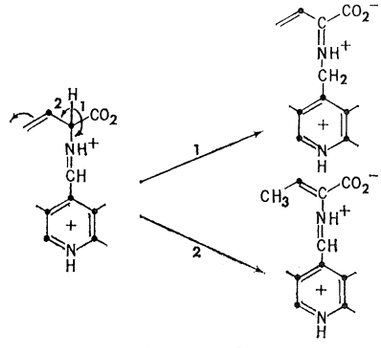
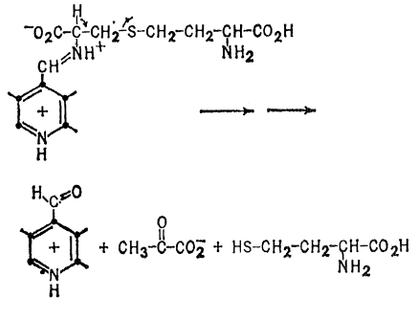
This compound has been shown to exhibit strong antimetabolic activity against bacteria. I recently found that it is an irreversible inhibitor of the pyridoxal phosphate-linked aspartate aminotransferase (*26*). Irreversible inhibition only occurs when the vitamin B_6 cofactor is in the aldehyde form, suggesting that Schiff base formation between the AMB and cofactor precedes the inhibition step. A mechanism similar to the ones described above can account for this (scheme 10). Other pyridoxal-containing amino acid metabolizing enzymes may also be affected by this inhibitor. It is interesting that the antibacterial effects of AMB can be overcome by the addition of D- and L-alanine, L-2-aminobutanoic acid, D-glutamic acid, and D-aspartic acid. In addition, my colleagues and I have found that the parent β,γ-unsaturated amino acid 2-amino-3-butenoic acid is also an irreversible inhibitor of aspartate aminotransferase, as would be expected from the mechanisms shown above.



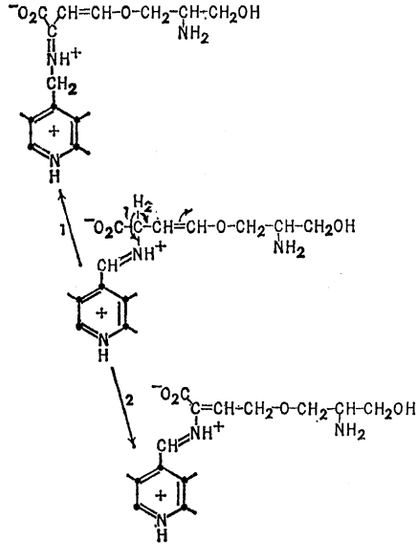
Scheme 7



Scheme 8



a

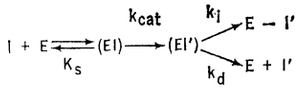


b

Scheme 9

We would expect that, in general, β,γ-unsaturated amino acids will be found to be potent irreversible inhibitors of amino acid metabolizing enzymes that are linked to pyridoxal phosphate. Furthermore, we would expect that other natural toxins in addition to rhizobitoxine and AMB, will also be found to operate as k_{cat} inhibitors.

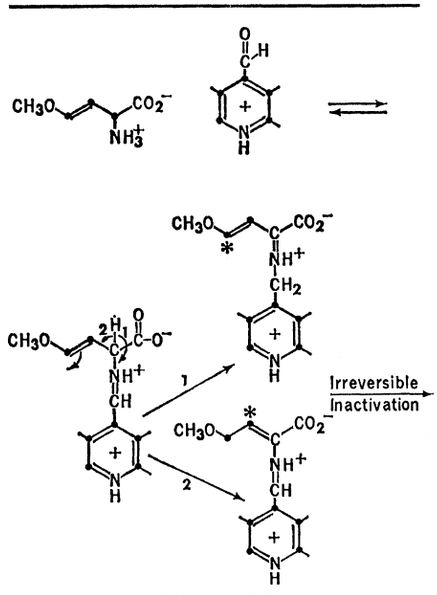
A pattern emerges from the studies summarized above. Three factors are crucial to the successful design of these inhibitors. In the first instance, the enzyme must convert a chemically unreactive molecule to a reactive one. This is a necessary, but not a sufficient condition. Second, the reactive molecule must be generated within bonding distance of a crucial active site residue capable of engaging in a chemical reaction with it. Even when these two conditions are met, inhibition might still not result: once formed, the reactive molecule can follow two routes. If I is the proinhibitor, and I′ is the inhibitor, the following scheme can be set up, where k_i is the rate of covalent bond formation between the enzyme and inhibitor and k_d is the rate of dissociation of the enzyme inhibitor complex. It is useful to define the ratio of k_i/k_d as the inhibitory constant K_{inh}.



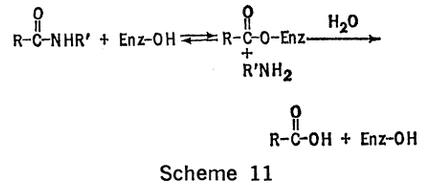
The more effective the inhibitor, the larger the magnitude of this constant; with normal substrates, of course, $K_{inh} = 0$. Experimentally, the easiest way to set limits on its value with inhibitors would be to measure the percentage of enzyme inactivated when it is challenged with a stoichiometric amount of inhibitor. With β-hydroxydecanoyl thioester dehydrase it appears that K_{inh} is greater than 100 since the inhibitor inactivates the enzyme stoichiometrically. In general, however, one would expect the k_d to be rather larger than k_i simply because the rates of enzyme-substrate dissociation are usually rather larger than rates of bond breaking and bond making. This means that, even when a reactive molecule with the right

chemistry is generated at the active site, its rapid dissociation may preclude the possibility of direct chemical reaction with an active site residue. Therefore, with the k_{cat} inhibitors reported here, one might expect that there must have been an unusually small k_d or an unusually large k_i to make K_{inh} observable. Since very reactive molecules such as carbenes or free radicals were not generated in any of these cases, k_d would be expected to be small. This is precisely the case. With the enzymes that require cofactors this follows directly from the fact that the reactive molecule once generated is held at the active site by covalent bonds to the cofactor. For example, in the pyridoxal phosphate linked enzymes, the reactive derivatives are held there by Schiff base formation to the pyridoxal moiety so that sufficient time is allowed for a reaction with the enzyme. In the case of β-hydroxydecanoyl thioester dehydrase—the single enzyme that acts without a cofactor, where this mode of inhibition has been found—the rate of enzyme-substrate dissociation has been observed to be unusually slow and comparable to the rates of the chemical reactions within the complex (*27*). Thus, in this instance, the allenic inhibitor is held at the active site by secondary forces long enough for reaction with a histidine residue. Hence, the third condition which must be met in the design of these inhibitors is that k_d must be decreased or k_i increased. The former seems to be the case that obtains in the examples cited here.

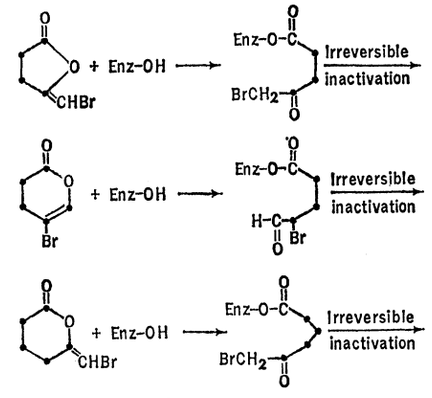
In apposition to these examples, it is of interest to consider a case where a reactive molecule is generated at the active site but where there is nothing to prevent its rapid dissociation. Yeast alcohol dehydrogenase can be irreversibly inhibited by the reactive Michael acceptor acrolein (*28*). This inactivation appears to be a consequence of a reaction of acrolein with an active site sulfhydryl group. Acrolein is generated by the enzyme when it oxidizes allyl alcohol (*28*), and during this process the enzyme slowly becomes irreversibly inhibited, but this inhibition only becomes noticeable after the enzyme produces all of the acrolein it can produce in the presence of limited amounts of oxidizing agent [nicotinamide adenine dinucleotide, (NAD)] (*29*). This means that no direct inactivation by acrolein occurs, but that the enzyme is generating its own affinity labeling agent. This is understandable when one considers the fact that the actual oxidation step with NAD involves direct hydride transfer between the substrate and the cofactor with no covalent bonds holding the putative inhibitor to the active site. Thus, the acrolein, once formed, can quickly diffuse into solution without engaging in a direct reaction with the enzyme.



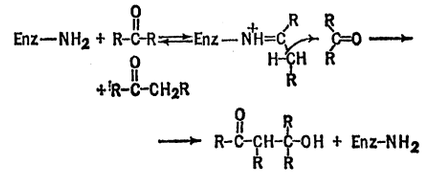
Scheme 10



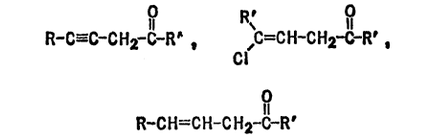
Scheme 11



Scheme 12



Scheme 13



Scheme 14

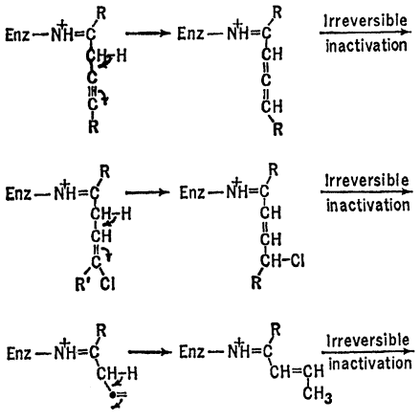
The question of how one should, in general, design k_{cat} inhibitors for classes of enzymes other than those reported here is of interest. From the arguments developed previously, we would expect that these inhibitors would be easier to design for enzymes whose mechanisms involve covalent catalysis simply because this ensures sufficient time for the chemical reaction. The number of enzymes that use covalent catalysis is by no means small. Certainly, many enzymes that require a cofactor use this mechanism, but in addition many that do not require cofactors also use it (*30*). Some of the chemistry that might prove useful as starting points for the design of k_{cat} inhibitors for different classes of enzymes whose mechanisms involve covalent catalysis is outlined below.

Proteases

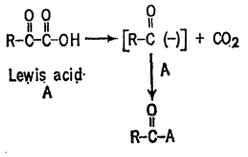
The mechanisms of many proteolytic and esterolytic enzymes are known to proceed through acyl-enzyme intermediates (scheme 11). (*31*). The serine proteases like chymotrypsin and trypsin are representatives of many proteases which utilize this mechanism. Since these enzymes are irreversibly inhibited by chloro- and bromoketones and since they can hydrolyze lactones, one would expect halolactones of the kind shown in scheme 12 to be k_{cat} inhibitors of these enzymes. In each instance, a chemically unreactive vinyl bromide would be converted to the highly reactive bromoketone, which is held at the active site by the *O*-acyl bond. Of course, the bromolactone moiety would have to be attached to specificity determinants to ensure efficient binding to the various proteases—for example, guanidine-benzoate group for trypsin (*32*).

Aldolases

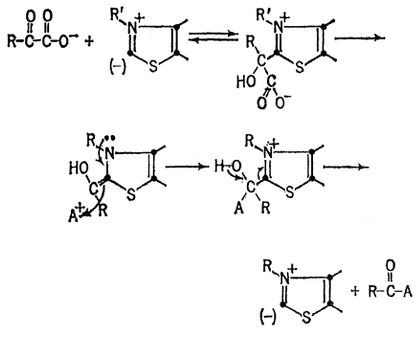
Class 1 aldolases catalyze aldol and reverse aldol condensations through the agency of a Schiff base formed by condensation of a substrate keto group and the ϵ-amino group of an active site lysine (scheme 13) (*33*). Thus, compounds of the kind shown in scheme 14 might very well be ef-



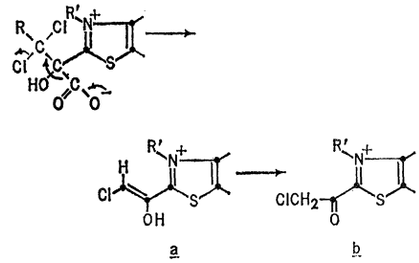
Scheme 15



Scheme 16



Scheme 17



Scheme 18

fective k_{cat} inhibitors of these enzymes. In these cases, the labilization of the C-H bond at the α position would result in an isomerization reaction which yields highly reactive products as shown in scheme 15.

Thiamine Cocatalyzed Reactions

Enzymes which use thiamine as a cofactor generally catalyze the kinds of reactions shown in scheme 16. The enzymatic mechanism involves the sequence of events shown in scheme 17 (*34*). α-Dichloropyruvate derivatives might be ideal k_{cat} inhibitors of these enzymes. These compounds themselves are not reactive since they are unable to undergo first-order nucleophilic substitution reactions (S_N1) or second-order (S_N2) reactions. However, they can undergo elimination reactions which would render them highly reactive during a thiamine cocatalyzed reaction. With these compounds, the elimination route would be possible so that the highly reactive molecules shown in scheme 18 might be generated. The terminal carbon in *a* (scheme 18) would be highly reactive as a Michael acceptor, and in *b* the reactive chloroketone moiety has been generated. Both *a* and *b* would be capable of reacting with a whole host of active site nucleophiles.

The chemistry outlined here is not meant to be exhaustive, but merely suggestive of the approaches that should lead to the fruitful design of the k_{cat} inhibitors.

Specific and irreversible inhibitors of the kind discussed here should have important uses for defining the physiological roles of enzymes in higher organisms. The mutant technique, which proved to be so valuable in delineating their physiological roles in bacteria, is clearly impractical here. One is left with the possibility of analyzing mutants which randomly arise, as in molecular diseases. It would be far better to be able specifically and irreversibly to block an enzyme of interest and then observe the physiological response. It should be mentioned that irreversible inhibitors are crucial to this task. Simple competitive inhibitors are only likely to be effective when applied to rate-limiting enzymes in a sequence of reactions and are also not very useful when the physiological concentration of the natural substrate is high, as, for example, with glucose. Finally, it would be expected that k_{cat} inhibitors might serve as a rational basis for the design of therapeutically useful agents.

References and Notes

1. B. R. Baker, *Design of Active-Site-Directed Irreversible Enzyme Inhibitors* (Wiley, New York, 1967).
2. The chemical makeup of the active-site region cannot be known until an active-site residue is specifically labeled, until the protein is degraded, and until the sequence of amino acid residues of the labeled region is determined. Therefore, when designing an affinity labeling agent for an enzyme, the usual guideline is rough isosterism with the substrate. When there is little or no knowledge of the types of amino acid residues at the active site, it is impossible to rationally choose the chemically reactive functionality. Usually, a functionality of fairly broad reactivity, such as a bromoketone, is chosen. Photoactivatable moieties have been used to obtain even broader chemical reactivity [Y. Stefanovsky and F. H. Westheimer, *Proc. Natl. Acad. Sci. U.S.A.* **70**, 1132 (1973)].
3. An important advance in the solution of this problem has been made with the advent of "transition state" inhibitors. Exceptionally low k_i's have been observed with many of these inhibitors. See, for example, G. E. Lienhard, *Science* **180**, 144 (1973).
4. It is clear that enzymes owe part of their selectivity to the binding term (k_s). However, it is also well recognized that many molecules will bind to the active site, but only a few will actually serve as substrates. Indeed, hypotheses such as that of nonproductive binding, induced fit, and strain have all been proposed in part to account for observations of this kind [W. P. Jencks, *Catalysis in Chemistry and Enzymology* (McGraw-Hill, New York, 1969), pp. 289–291].
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