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SUICIDE ENZYME INACTIVATORS AND OTHERS

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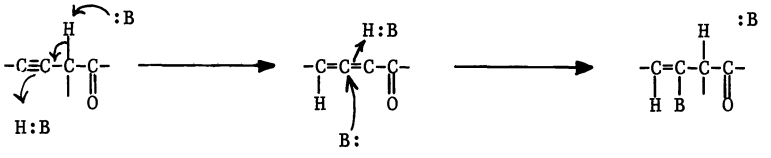
Abstract - Several enzyme inactivators are described which utilize the catalytic capability of the enzyme to inactivate the enzyme. They are relatively unreactive molecules which are activated at the enzyme-active site to reactive species which then form a covalent bond with a functional group at the active site. For example, some inactivators have the following structure: R-C≡C-CH_2-CO-R'. Through enzyme catalyzed proton abstraction these inactivators are converted to R-CH=C=CH-CO-R'. This conjugated allene then forms a bond with a functional group at the active site. Additional inactivators based on other chemical transformations are also described.

The work carried out by K. Bloch and collaborators about ten years ago, suggested the following approach to the inactivation of enzymes (1): An enzyme is presented with a substrate analogue which is chemically as unreactive as possible. This substrate analogue binds to the active site of the enzyme and the enzyme operates on this substrate analogue as it would on the normal substrate. The action of the enzyme results in the conversion of the substrate analogue to a reactive species. This reactive species then reacts with a catalytic group at the active site to form a covalent bond. This leads to enzyme inactivation. We called this type of inactivator suicide enzyme inactivator and shall use this designation throughout this discussion. Others have referred to it as mechanism-based inactivators, or k_{cat} inactivators (2). An important property of suicide inactivators is that they are produced at the active site and are not released into the solution. This property enhances their specificity and should make them useful in biological systems. They differ from the classical active site directed inactivators (3), which are intrinsically reactive molecules. I do not want to minimize the utility of active site directed inactivators. These inactivators have been enormously useful in the study of enzyme mechanisms.

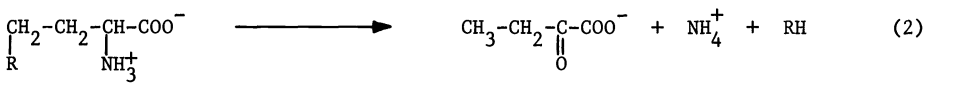
From the work of K. Bloch (1), the following principle of suicide inactivation emerged:



(1)

The target enzyme is an enzyme which in the normal catalytic process, abstracts a proton from the substrate, i.e., the catalytic process proceeds through a carbanion mechanism. The inactivator is an acetylenic analog of the substrate. When this inactivator binds to the active site, the enzyme abstracts a proton as it would in the normal catalytic process. Proton abstraction leads to the formation of an allene which is conjugated with a carbonyl group and hence subject to nucleophilic attack by a base (B) at the active site. Consequently, the enzyme becomes covalently labeled and is rendered catalytically inactive. Acetylenic suicide enzyme inactivators have probably been more widely used than any other class of suicide inactivators.

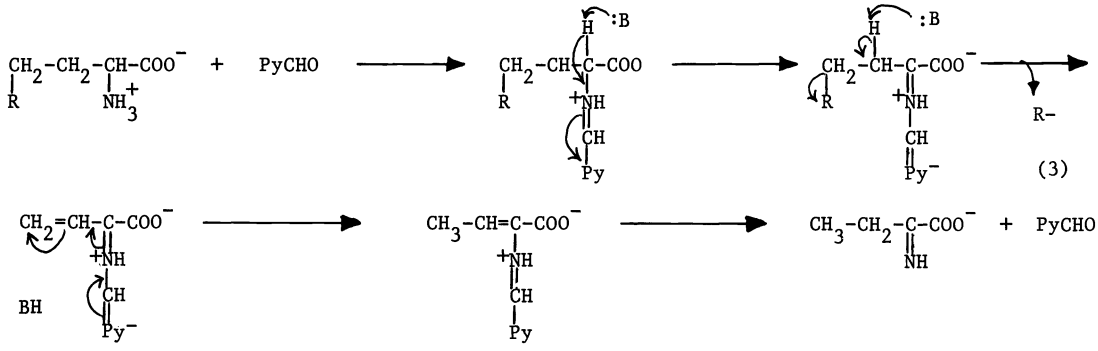
A case which has been extensively studied is the inactivation of γ-cystathionase (4), an enzyme which utilizes pyridoxal-phosphate. This enzyme catalyzes the following reaction:



(2)

R = -OH, $-S-CH_2-CHNH_3^+-COO^-$, -Cl

A minimal mechanism for this reaction is shown in Equation 3. For purposes of the present



(3)

PyCHO = enzyme bound pyridoxal-phosphate

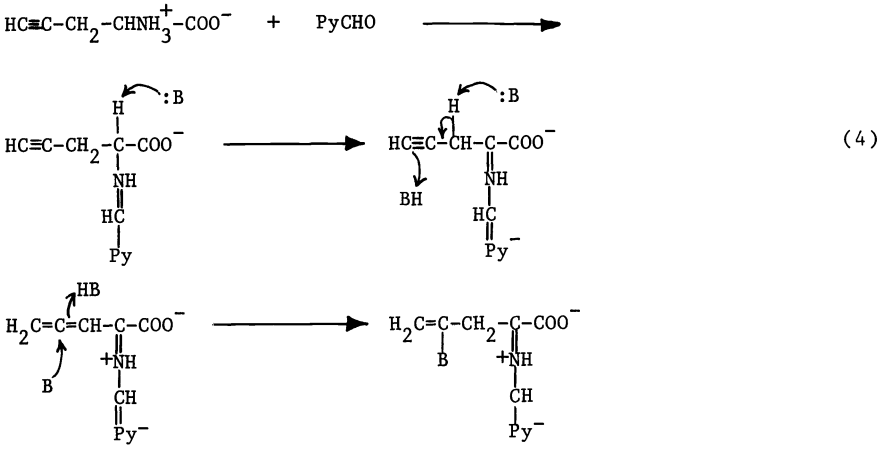
B = nucleophile(s) at the active site of the enzyme

discussion, the important aspects of this mechanism are: 1) The β-hydrogen of the substrate becomes activated as a result of imine formation at the α-carbon. 2) The enzyme can abstract a proton from the β-position. 3) The enzyme interacts with the γ-carbon of the substrate.

Propargyl glycine (I) is an effective inactivator of this enzyme. At 6.6×10^{-5} M $t_{1/2}$ inactivation ≈ 2 min.

I II

The analogous compound (II), containing a double bond, does not inactivate. The mechanism of inactivation involves the following reaction sequence (4). Inactivation occurs through the addition of a nucleophile at the active site to an allene generated from the acetylenic inactivator.



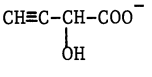
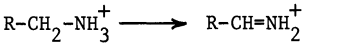
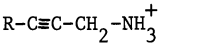
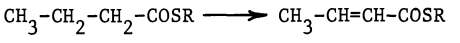
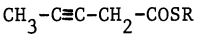
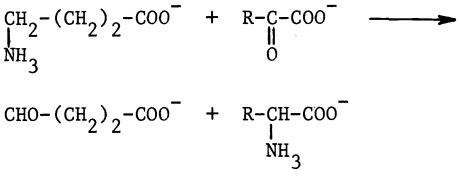
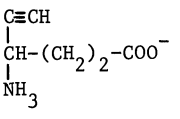
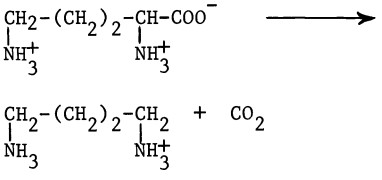
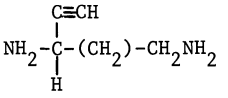
(4)

The inactivation mechanism closely parallels the catalytic mechanism. An imine is initially formed at the α-carbon. Next, abstraction of the β-hydrogen occurs which leads to formation of a conjugated allene, to which an active site nucleophile adds. The active site nucleophile which adds to the allene has, so far, not been completely identified. It is either a sulfhydryl group or the OH- of a tyrosine residue. Formation of the allene requires addition of a proton to the δ position of the inactivator. It is likely that the base which normally interacts with the γ-position of the substrate provides this proton. Thus the ability of the enzyme to interact with the γ-position of the substrate is important in bringing about the inactivation by the suicide inactivator.

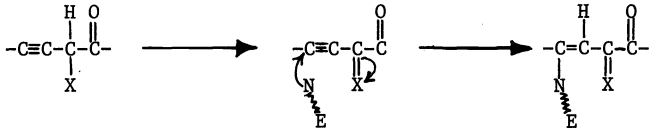
A large number of acetylenic inactivators have been reported. A few examples are shown in Table I.

Table I

Enzymes Inactivated by Acetylenic Inactivators

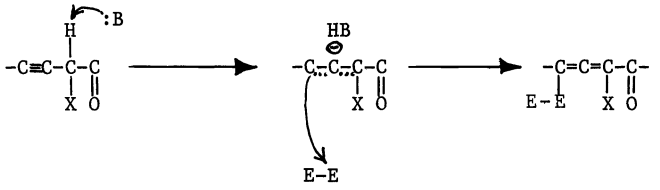
Enzyme and Reaction Catalyzed	Inactivator
1) Lactic Oxidase (flavoprotein) 	
2) Amine Oxidase (flavoprotein) 	
3) Butyryl-CoA Dehydrogenase (flavoprotein) 	
4) GABA Transaminase (Pyridoxal-P)[18] 	
5) Ornithine Decarboxylase[19] 	

It now appears very likely that allene formation is not involved in all cases of inactivation by acetylenic suicide inactivators. Some acetylenic inactivators probably operate through additional mechanisms represented by Equations 5 and 6.



(5)

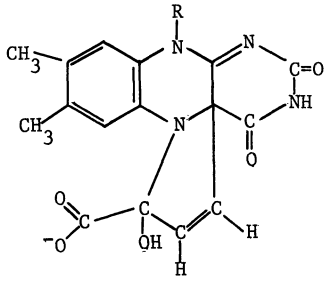
E⁓N = active site nucleophile



(6)

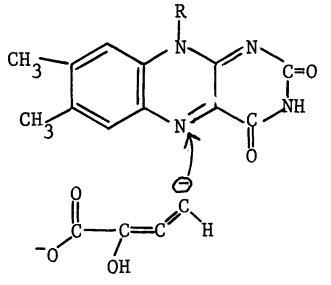
E-E = electrophile at the active site

In many reactions in which acetylenic inactivators are used, the carbon adjacent to the acetylenic group becomes oxidized. This results in the formation of a Michael acceptor which can react with a nucleophile (Equation 5). It has also been proposed that in some cases, an allenic carbanion is formed which adds to an electrophile at the active site (Equation 6). This mechanism has been proposed for the inactivation of lactic oxidase (Table I, Reaction 1). The inactivator, α-OH-butynoic acid does not react with a functional group at the active site; instead an adduct is formed with the flavin (I). It is obvious



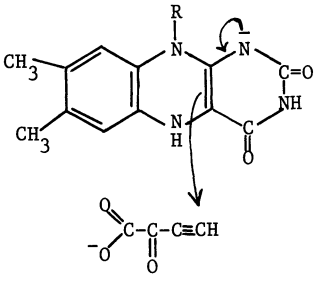
(I)

that this adduct is not formed through the mechanism of Equation 1. It has been proposed that this compound is formed through the addition of an inactivator derived allenic anion to the flavin (Equation 7).



(7)

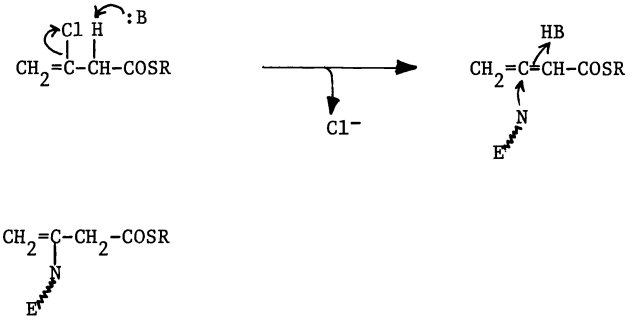
This allenic anion seems unlikely, although it might be stabilized at the active site of an enzyme. A more attractive mode of obtaining this adduct can be envisioned. Initially, the acetylenic inactivator is oxidized to a keto-acid and the flavin is reduced. The reduced flavin then reacts with the oxidized inactivator through a Michael addition as shown in Equation 8.



(8)

Inactivation of amine oxidase (Table 1, reaction 2) also leads to a flavin-inactivator adduct which cannot be formed through the mechanism shown in Equation 1. On the other hand, the inactivation of butyryl-CoA dehydrogenase (Table 1, reaction 3) most probably involves allene formation and subsequent addition of a nucleophile to the allene, analogous to the inactivation of γ-cystathionase.

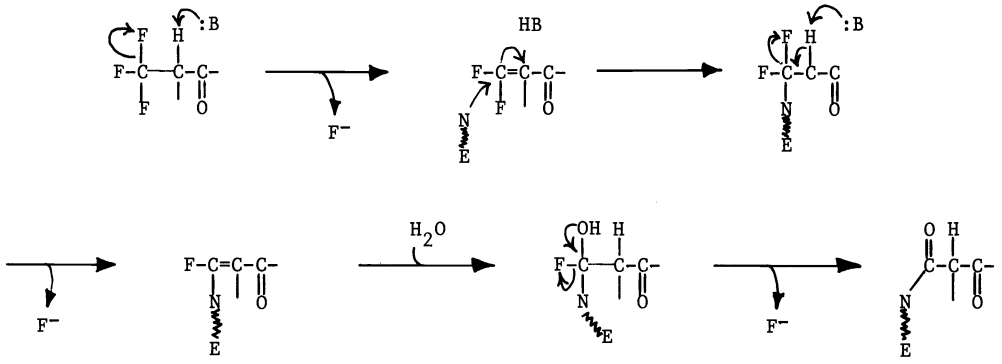
Butyryl-CoA dehydrogenase has also been inactivated through a variation of the approach just discussed. The pantotheine ester of 2-Cl-2-butenoic acid is an effective irreversible inactivator. A probable mechanism of inactivation is shown in Equation 9. The advantage of this



(9)

inactivator is that, unlike the acetylenic inactivators, protonation of the γ-position is not necessary and, therefore, the requirements for inactivation is less severe. This type of inactivator may work in cases where acetylenic inactivators fail. It is likely, although by no means certain, that several of the reactions shown in Table 1 proceed through the mechanism of Equation 5 (Michael addition) rather than through the mechanism of Equation 1.

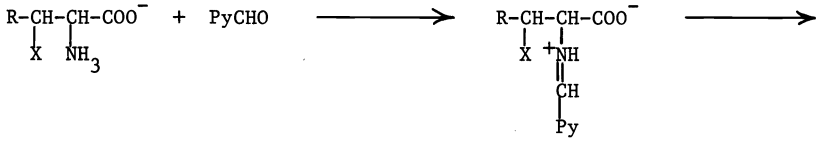
Another approach to suicide enzyme inactivation involves the use of compounds in which trifluoromethyl group is α to the carbanion (Equation 10).



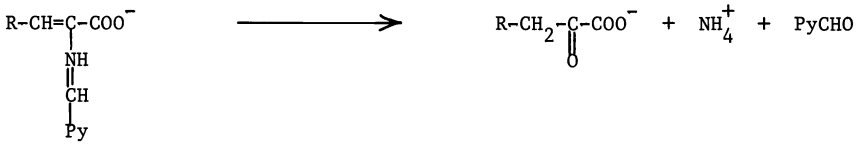
(10)

E⁓N = nucleophile at the active site

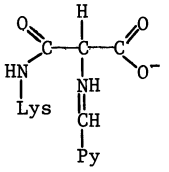
Other mechanisms for the loss of F^- can be envisioned, and in fact, the detailed step by which F^- is lost are unknown. It has, however, been established with model systems (5) and in an enzymic reaction (6) that acylation of a nucleophile occurs. Several pyridoxal-enzymes which catalyze β-elimination reactions are susceptible to inactivation by substrate analogues containing $-CF_3$. A minimal mechanism for β-elimination is shown in Equation 11.



(11)

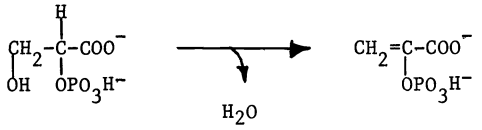


γ-cystathionase, an enzyme which has been discussed above, also catalyzes β-elimination reactions. The inactivation of this enzyme by trifluoroalanine occurs through the mechanism outlinedin Equation 10. Here, the ability of the enzyme to catalyze the abstraction of the α proton is basic to the mechanism of the inactivation reaction. Structure (II) represents the adduct formed at the active site. The amide carbon of this adduct is derived from the β-carbon of trifluoroalanine.



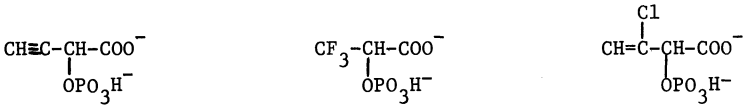
II

With the suicide inactivators discussed so far, one might expect to inactivate any enzyme which forms a carbanionic intermediate. Unfortunately, however, there are many examples where substrate analogues, which should "obviously" inactivate do not inactivate. Frequently, this is due to subtleties of the mechanism. I would like to illustrate this point with one example. Enolase catalyzes the elimination of H_2O from phosphoenol pyruvate (Equation 12). There is very compelling evidence that this reaction proceeds through an E-1cb mechanism (7). One of the pieces of evidence which supports the E-1cb mechanism is the observation that exchange of ^2H from phosphoenolpyruvate-α-d_1 proceeds more rapidly than elimination of H_2O.



(12)

We synthesized several potential suicide inactivators (Structure III, IV, V) for enolase (8).



III IV V

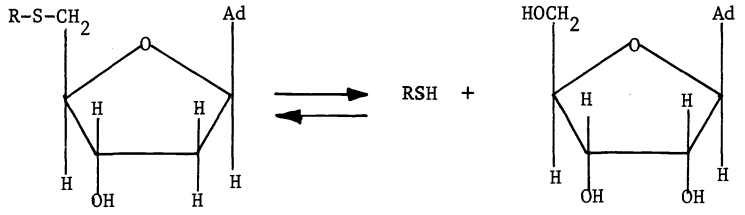
Compound III did not interact with the active site. The reason for its failure to bind is not clear. Compounds IV and V interacted well with the active site, i.e., they are good competitive inhibitors, but did not inactivate. Further investigations of the mechanism of action of enolase showed that the -OH group plays an important role in the abstraction of the substrate α-hydrogen (8). Table II shows the relative rate of the enzyme catalyzed exchange of the α-hydrogen with solvent tritons.

Table II

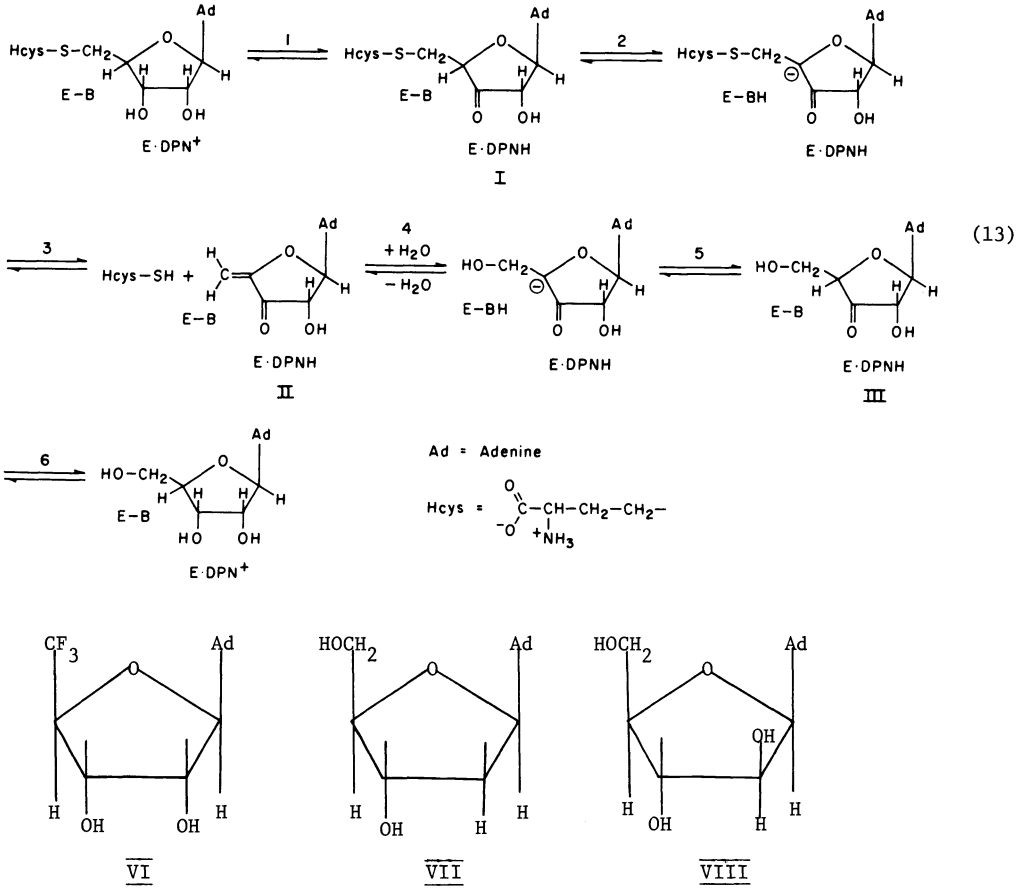
Substrate	Rate of α-Proton Exchange
$CH_2(OH)-CH(OPO_3H^-)-COO^-$	1
$CH_2(OPO_3H^-)-COO^-$	2×10^{-6}
$CH_3-CH(OPO_3H^-)-COO^-$	$< 10^{-6}$
$CH_3-CH_2-CH(OPO_3H^-)-COO^-$	$< 10^{-6}$

Exchange of the α-hydrogen of substrate analogues which do not have a β-OH group proceeds 10^6 fold slower than that of 2-phosphoglyceric acid. This explains the failure of suicide inactivators (IV, V) to inactivate, since the activation of these compounds is initiated by α-proton abstraction.

The next example of suicide inactivation which I will discuss may not involve covalent bond formation. S-adenosylhomocysteinase (SAHase) catalyzes the hydrolysis of S-adenosylhomocysteine (SAH) (Equation 12).

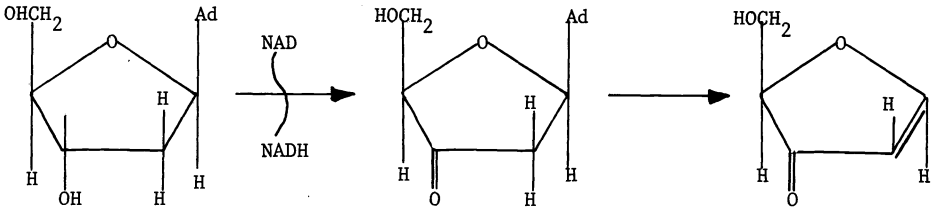


This reaction is quite unusual: In the reverse reaction, a mercaptan, or possibly a mercaptide ion, displaces an -OH group. How does the enzyme achieve this? The reaction sequence catalyzed by this enzyme is shown in Equation 13. The enzyme contains NAD which is extremely tightly bound. This tightly bound NAD serves as electron acceptor for oxidation of the 3'-position of the substrate to a keto group. The introduction of a keto group at C-3' facilitates the elimination of RSH which results in the formation of an α,β unsaturated ketone. H_2O finally adds to the α,β unsaturated ketone to produce adenosine. Through the temporary oxidation of C-3' of the substrate the displacement reaction is converted to an elimination and addition reaction. We tested C-5'-deoxytrifluoro adenosine (VI) as a suicide inactivator. This compound was inactive.



(13)

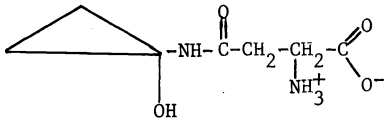
It was reported (10) that 2'-deoxyadenosine (VII) and ara-adenosine (VIII) irreversibly inactivate SAHase. It appeared to us that this inactivation could occur through the mechanism shown in Equation 14 (11).



According to this mechanism, 2'-deoxyadenosine binds at the active site and is oxidized at C-3'. This oxidation is also part of the normal catalytic process. 2'-deoxy-3'-keto-adenosine can eliminate adenine through a trans-elimination. We tentatively propose that adenosine, the normal substrate, does not undergo this elimination since a trans-elimination is not possible. Elimination of adenine produces a compound at the active site which can no longer reoxidize enzyme bound NADH. This fact alone would inactivate the enzyme. In addition, it is also possible that the sugar resulting from the elimination of adenine reacts with a nucleophile at the active site.

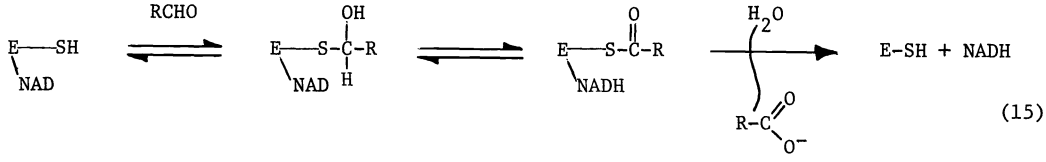
The mechanism proposed for the inactivation of SAHase is speculative, but there is now considerable supporting experimental evidence: 1) Addition of C^{14}-2-deoxyadenosine results in the formation of an enzyme-2'-deoxyadenosine complex which can be isolated by gel-filtration. 2) Addition of 2-deoxyadenosine to the enzyme results in the formation of enzyme bound NADH. The rate of NADH formation parallels the loss of enzyme activity. 3) When the enzyme inactivated with 2'-deoxyadenosine is denatured under mild conditions, no 2'-deoxyadenosine is recovered, but adenine has been isolated. 4) The inactivation was carried out with 2'[^{3}H], 2'-deoxyadenosine tritiated trans to adenine. No ^{3}H was found in the inactive complex but ^{3}H was found in the solvent.

The study of naturally occurring toxins could reveal new principles for the design of enzyme inactivators. We, therefore, investigated the mushroom toxin coprine (IX) (12)(13).



IX

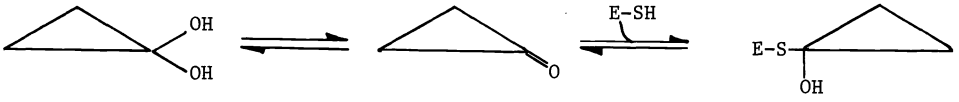
This compound causes severe illness when ingested together with alcohol. This suggested that coprine has Antabuse like effects, i.e., it inhibits aldehyde dehydrogenase and causes the accumulation of acetaldehyde. This was confirmed by administration of coprine and ethanol to mice. However, coprine has no effect on isolated aldehyde dehydrogenase. However, hydrolysis products of coprine, 1,1'-dihydroxycyclopropane or 1-NH_2, 1-OH cyclopropane inhibits aldehyde dehydrogenase in vivo and in vitro. All subsequent studies were done with 1,1-dihydroxycyclopropane (cyclopropanone hydrate) since we found that it was not possible to obtain samples of 1-NH_2, 1-OH cyclopropane which were not contaminated by chloroacetone. Before presenting the mechanism of inhibition of aldehyde dehydrogenase by cyclopropanone hydrate, the mechanism of action of yeast aldehyde dehydrogenase (Equation 15) needs to be considered. Yeast aldehyde dehydrogenase catalyzes the oxidation of aldehydes to the corresponding acids. NAD serves as an electron acceptor. The aldehyde reacts with an -SH group at the active site of the enzyme to form a thiohemiacetal. The thiohemiacetal is then oxidized



(15)

by enzyme bound NAD. The resulting thioester is hydrolyzed to give the final reaction product. The mechanism of inactivation by cyclopropanone hydrate is shown in Equation 16. It is proposed that cyclopropanone reacts with the -SH group at the active site to form an adduct, which results in enzyme inactivation. We do not know whether cyclopropanone formation is

enzyme catalyzed or whether cyclopropanone from solution reacts with the enzyme. As indicated the reaction is reversible, but the rate of dissociation of cyclopropanone hydrate from the enzyme is slow. In the presence of NAD the rate of dissociation of the enzyme-cyclopropanone adduct is further decreased. We were surprised to find that the rate of dissociation of NAD is also greatly decreased. When the enzyme is inactivated by cyclopropanone hydrate in the

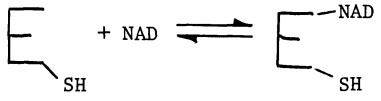
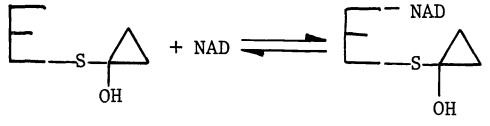
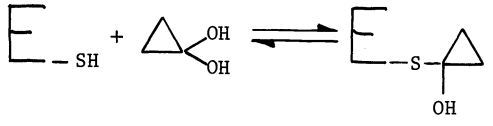
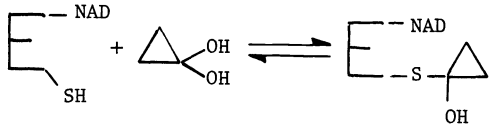


(16)

presence of NAD and then subjected to gel filtration, both NAD and cyclopropanone hydrate are associated with the protein fraction. When NAD alone is added to the enzyme, and the complex is subjected to gel filtration, NAD is completely separated from the protein. The tight binding of NAD to the enzyme is a unique property of the enzyme cyclopropanone hydrate by adduct. When the -SH group of the enzyme is modified with other reagents, such as iodoacetate, the binding of NAD to the enzyme is not enhanced. The dissociation constants for the various complexes as well as rate constants have been determined and are presented in Table III and Figure 1. The binding of cyclopropanone hydrate to enzyme is unexpectedly tight. Based on equilibrium constants for the reaction of hydrated aldehydes with mercaptans to form thiohemiacetals, one would expect $K_{diss} = 10^3$. The value determined is 10^2 fold higher. Furthermore, when both NAD and cyclopropanone hydrate are present, the binding of cyclopropanone hydrate and NAD is increased 10^4 fold. A consequence of this tight binding is the extremely slow rate of dissociation of NAD cyclopropanone hydrate from the enzyme. The reason for this tight binding is not certain. We have considered the following possibility. During intermediate stages in the catalytic process, it may be undesirable for intermediates to leave

Table III

Dissociation Constants for Reaction of Aldehyde Dehydrognease with NAD and Cyclopropanone Hydrate

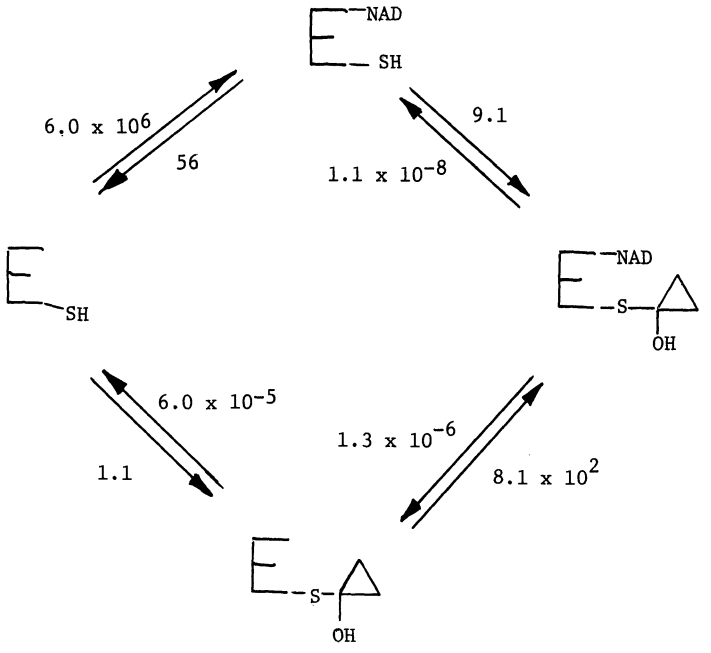
Reaction	K_{diss}
	7.5×10^{-5}
	1.6×10^{-9}
	5.5×10^{-5}
	1.2×10^{-9}

the active site and possibly for solvent to enter the active site. To achieve this, the enzyme converts to a "closed" form during certain stages of catalysis, i.e., a form in which the active site is insulated from the environment. The combination of enzyme and cyclopropanone hydrate leads to the formation of a structure which resembles a stage in catalysis in which the enzyme may be in the "closed" form. Normally oxidation would occur at this stage, but this cannot happen with cyclopropanone hydrate. Consequently, the enzyme remains locked in the closed form.

The principle involved here, i.e., the tight binding of cyclopropanone, may also be involved in other cases of enzyme inactivation. Several amine oxidases are inactivated by cyclopropylamine (14)(15). Bacterial alcohol dehydrogenase, an enzyme which contains these newly discovered cofactors methoxatin (16) and yeast alcohol oxidase, are inactivated by cyclopropanol (17). It is likely that these inactivators are oxidized to cyclopropylimine or cyclopropanone and that these compounds then react with a nucleophile at the active site of the enzyme.

Figure 1

Reaction of NAD and Cyclopropanone Hydrate with Aldehyde Dehydrogenase



unit time = min for all rate constants

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