INHIBITORS

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INHIBITORS - DIFFERENT TYPES

INHIBITORS

REVERSIBLE

COMPETITIVE

UNCOMPETITIVE

NONCOMPETITIVE

IRREVERSIBLE

COVALENT

TIGHT BINDING

TRANSITION STATE ANALOGS

NONSPECIFIC

SPECIFIC

ACTIVE SITE REAGENTS

AFFINITY LABELS

PHOTOAFFINITY LABELS

SUICIDAL INACTIVATORS
Active site titration of trypsin

\[ \text{Trypsin} \rightarrow \text{NPGB} \rightarrow \text{Inhibitor} \]

FAST

SLOW

THREE DIFFERENT REVERSIBLE INHIBITORS

COMPETITIVE

UNCOMPETITIVE

NONCOMPETITIVE
### Competitive Inhibition

Both Substrate and Inhibitor compete for the same active site on the enzyme.

**Enzyme - inhibitor complex**

- **E + S → E-S Complex**
- **E-S Complex → E + P**
- **E + I → EI**

**Competitive Inhibitor**

- **E + S → ES → E + P**
- **E + I → EI**

**Malonate is a competitive inhibitor of succinate dehydrogenase reaction**

**Competitive Inhibition**

- **1/v**
- **1/S**

**Slope changes**
- **Intercept on Y-axis no change**

**At very high [S] conc**

- **There is no inhibition**

**Examples**

- **Succinate**
- **Fumarate**
- **Malonate**

**Chemical Structures**

- **Succinate**
- **Fumarate**
- **Malonate**
Uncompetitive Inhibition
Substrate binds to free enzyme.
Inhibitor binds only to ES complex.

Uncompetitive Inhibitor
S reacts with E and I reacts
Only with ES complex

Slope: No change
Intercept on Y-axis: Varies

Constant amount of ES is removed by I. Therefore, slope is constant
**Noncompetitive Inhibition**

- Substrate binds to E and EI complex.
- Inhibitor binds to E and ES complex.

Noncompetitive Inhibitor

- $E + S \rightarrow ES \rightarrow E + P$
- $I \quad I$
- $EI \leftrightarrow S \quad ESI$

**Both Slope and Intercept on Y-axis: Varies**

Noncompetitive Inhibition

- $S$ reacts with $E$ and EI complex.
- $I$ reacts with $E$ and ES complex.

Graph showing the relationship between $1/v$, $1/S$, and inhibitor concentration.
IRREVERSIBLE INHIBITORS ARE OF TWO TYPES

How to distinguish between reversible and irreversible inhibitors?

FOR REVERSIBLE INHIBITOR

FOR IRREVERSIBLE INHIBITOR
Tight Binding Inhibitors - Transition State Analogs

Transition State Analogs are Potent Enzyme Inhibitors

Compounds resembling the transition state of enzyme catalyzed reactions should be effective inhibitors of enzymes.

- Linus Pauling (1946)
Phosphonates are good analogs of phosphate esters

\[
\begin{align*}
\text{Phosphate ester} & : \quad R - O - P - O - R' \\
\text{Phosphonate} & : \quad R - O - \overset{\ominus}{P} - R' 
\end{align*}
\]

Aspartate transcarbamoylase and its transition state analog

\[
\begin{align*}
\text{Carbamoyl phosphate} & : \quad \overset{\ominus}{O}P\overset{\ominus}{O}NH\_2 \\
\text{Aspartic acid} & : \quad H\_2N - COOH \\
\text{Transition State} & : \quad \overset{\ominus}{O}P\overset{\ominus}{O}NH\_2 \\
\text{N-Carbamoylaspartate} & : \quad H\_2N - NH\_2 - COOH \\
\text{N-(phosphonacetyl)-L-aspartic acid} & : \quad \overset{\ominus}{O}P\overset{\ominus}{O}NH\_2 - COOH
\end{align*}
\]
ACONITASE REACTION

CITRATE  \xrightarrow{\text{CIS - ACONITATE}} \text{ISOCITRATE}

2-NITROISOCITRATE  \xrightarrow{\text{STABLE ANALOG}} \text{TRANSITION STATE}

Proline racemase reaction

D-Proline  \xrightarrow{\text{Planar Transition State}} \text{L-Proline}

Pyrrole -2-carboxylic acid  \xrightarrow{\Delta -1} \text{Pyrroline-2-carboxylic acid}

160 fold higher affinity than substrates
TRIOSE PHOSPHATE ISOMERASE REACTION

**Aldolase reaction**

\[ \text{Km} = 4 \times 10^{-4} \text{ M} \]

**Enediol intermediate**

\[ \text{K} = 1 \times 10^{-8} \text{ M} \]
Transition state analog - Adenosine deaminase

ADENOSINE

\[ \text{Km} = 3 \times 10^{-5} \text{ M} \]

TRANSITION STATE

\[ \text{Kf} = 3 \times 10^{13} \text{ M} \]

INOSINE

\[ \text{Kf} = 5 \times 10^{-6} \text{ M} \]

PURINE HYDRATE

DHYDRO PURINE COMPETITIVE INHIBITOR

2'-DEOXYCOFORMYCIN

Penicillin is a tight binding inhibitor of peptidyltransferase

Penicillin

D-ala D-ala Peptide
Penicillin inhibits transpeptidase reaction

Penicillin

\[
\text{peptidyl transferase} \quad \text{D-ala} \quad \text{D-ala} \quad \text{Peptide} \\
\downarrow \\
\text{peptidyl transferase} \quad + \quad \text{D-ala} \\
\downarrow \\
\text{penta gly peptide} \quad + \quad \text{HO} \\
\downarrow \\
\text{Crosslinked peptide}
\]

Pepstatin is a potent transition state analog of aspartyl proteases

(Hydroxyethylene group inside the peptide)

N-acetyl PEPSSTATIN - A MICROBIAL PEPTIDE
Methotrexate binds almost 1000 fold tighter than the substrate to dihydrofolate reductase.
OTHER TRANSITION STATE INHIBITORS

- LYSOZYME - LACTONE
- RIBONUCLEASE - URIDINE VANADATE
- ISOCITRATE LYASE - 3-NITRO PROPIONATE
- RIBULOSE BISPHOSPHATE CARBOXYLASE - CARBOXY ARABINITOL 1,5-BISPHOSPHATE
- GLUTAMINE SYNTHETASE - METHIONINE SULFOXIMINE
- GAMMA-GLUTAMYL CYSTEINYL SYNTHETASE - S-(n-BUTYL)-HOMOCYSTEINE SULFOXIMINE

THERE ARE TWO KINDS OF COVALENT INHIBITORS

SPECIFIC INHIBITOR

COVALENT INHIBITORS

NONSPECIFIC INHIBITOR
Nonspecific Covalent Inhibitors

They react invariably with any enzyme as long as a particular reactive amino acid is present at or near the active site. Generally the reaction leads to inactivation of the enzyme.

**Best examples are active site reagents.**

- Serine - organophosphorus compounds
- Histidine, cysteine and Lysine - Haloacetates, Halomethylketones
  - Histidine -diethylpyrocarbonate
  - Arginine - 1,2-diketocyclohexane
- Cysteine - Thiol reagents, pCMB, DTNB
- Metal ion containing enzymes - cyanide, azide, carbon monoxide
- Carbonyl groups - reduction, hydroxylamine
- Lysine - anhydrides
- Carboxyl groups - esterification.

Modification of arginine

**Reversible blocking of Arginine by cyclohexadione.**

Arginine is modified by 1,2-cyclohexadione (or phenylglyoxal). The vicinyl dihydroxy adduct can be stabilized by borate.
Carbonyl group modifications

- **Phenylhydrazone**
- **NH\textsubscript{2}OH** (Hydroxylamine)
- **Oxime**
- **NaBH\textsubscript{4}** (Sodium borohydride)
- **CHOH group**
- **Imine**

Cysteine modification (DTNB)

- **DITHIONITROBENZOIC ACID (DTNB)**

Addition of excess DTNB leads to quantitative derivatization of cysteine and stoichiometric liberation of the yellow colored aromatic nitrothiol.
A number of reagents react with cysteine. Ethyleneimine, NEM, iodoacetate (as well as iodoacetamide) and \( p \)-hydroxymercuribenzoate will modify cysteine as shown below.

Dithiothreitol reduces disulfides and iodoacetate blocks them.
Blocking of lysine with TNBS

TNBS (2,4,6-trinitrobenzenesulfonic acid) arylates lysines. The product can be quantified at 367 nm.

Modification of lysine

Lys: Carbonyl compound
Schiff's Base: Stable imine
Chymotrypsin inactivation by diisopropylfluorophosphate

Inactivation of glyceraldehyde-3-phosphate dehydrogenase by \( p \)-chloromercuribenzoate
Iodoacetamide and Iodoacetic acid react with SH, imidazole and NH₂

\[
\text{SH} + \text{ICH}_2\text{COOH} \rightarrow \text{SCH}_2\text{COOH}
\]

\[
\text{SH} + \text{ICH}_2\text{CONH}_2 \rightarrow \text{SCH}_2\text{CONH}_2
\]

SPECIFIC COVALENT INHIBITORS

AFFINITY LABELS

PHOTO AFFINITY LABELS

SUICIDAL INACTIVATOR
Affinity Label - TPCK

Top: Normal Chymotrypsin substrate
Bottom: \( p \)-toluenesulfonylphenylalanylchloromethylketone
Arrow indicates the site of cleavage

TRIOSE PHOSPHATE ISOMERASE
REACTION - AFFINITY LABELLING

GLYCOLALDEHYDE
3-PHOSPHATE
FORMATION
VIA ENEDIOXYL
INTERMEDIATE

ACTIVE SITE LABELING BY 3-BROMOACETOL PHOSPHATE
PHOTOAFFINITY LABEL

Attach a photoreactive group such as diazoacetyl group to a substrate analog. Allow the enzyme bind to the photoaffinity label. Shine light on the complex. The photolabile group is activated and a reactive intermediate is generated. The reactive intermediate rapidly inactivates the enzyme by binding to essential amino acids near near the active site.
PHOTOAFFINITY LABEL - HOW IT WORKS

Step 1. Photoaffinity label binds to the protein.
at a site directed by the substituent group (R).

Step 2. Light is shined on the complex to eliminate the photo labile group.

Step 3. The reactive group generated attaches itself to the nearly nucleophile killing the protein.

6E, 11Z-hexadecadienyl diazoacetate interaction with pheromone binding protein

Inactivated Enzyme

Enzyme
Mechanism of Suicidal Inactivation

Inactivation

Product formation

First report on suicidal inactivation - Bloch’s group

β-hydroxydecanoylthioester dehydrase

NAC = SCH₂CH₂NHCOCH₃

HIS - ENZYME
Criteria for suicidal inactivation

• **Inhibitor should be unreactive.**
• **It should become reactive only after activation by enzymatic action.**
• **Conversion of unreactive to reactive inhibitor should be due to specific catalytic function of the enzyme.**
• **Nonspecific interaction should be minimum.**
  (a) Nontarget enzyme reaction
  (b) Escape of reactive intermediate before reaction.

How to identify suicidal inactivators?

• **Best way to identify is the characterization of enzyme inhibitor complex by physico-chemical studies.**

• **But this could be difficult in some cases. So one needs some easy techniques to identify the suicidal inactivators.**
Kinetic experiments can identify suicidal inactivators

- The loss of enzyme activity should follow time dependent first order kinetics at fixed concentration of inhibitor.
- (If a reactive inhibitor comes out and then reacts with the enzyme, it will obviously follow second order kinetics. Moreover second addition of enzyme to this reaction mixture will cause an increased rate of inactivation due to accumulated inhibitor. Also addition of nucleophiles will reduce the rate of inactivation in this mode).

Kinetics of inactivation

- The rate of inactivation should follow Michaelis Menten type kinetics.
- Substrate (or competitive inhibitor) should protect the enzyme from inactivation.
Inactivation Kinetics

- Inactivation Should be irreversible. (Show by dialysis, gel filtration etc.,)
- Inactivator should be bound covalently to the enzyme (show with radiolabel or other techniques).
- Stoichiometry of binding should be one to one mole.
- Partitioning between catalysis and inactivation should be determined.

Chymotrypsin is an endopeptidase that cleaves proteins on the carboxyl side of aromatic amino acids (Phe, Tyr, Trp) and Leu.
Suicidal inactivation of chymotrypsin

Chymotrypsin Suicidal inactivation by 6- chloropyrane
Highly specific chymotrypsin inactivator

6-iodomethylene naphthyl tetrahydropyran-2-one (1.7 turnover per inactivation)

Stoichiometric titrant for chymotrypsin

Isatoic anhydride

Anthranilyl enzyme resistant to hydrolysis
**β- lactamase inhibitor - Sultamicillin**

Sultamicillin

- Amoxacillin - peptidyl transferase inhibitor
- Sulbactam - lactamase suicidal inactivator

**β- lactamase inhibitor - Sulbactam**

- Lactamase
- Tetrahedral intermediate
- Acyl enzyme adduct

- Acyl enzyme adduct
- Dead enzyme
β-lactamase inhibitor - Olivanate

Both natural and synthetic carbapenems containing pyrrolidine ring inactivate lactamase by the following mechanism.

Lactamase suicidal substrate

One mole of acetyl methylene penicillanic acid kills lactamase with IC₅₀ value of $1.4 \times 10^{-9}$ M.
Cephalosporinase inhibitor based on 3'-exo substituent on cephalosporin hydrolysis

Allyl sulfoxide functionality is uncovered in the acyl enzyme intermediate allowing 2,3-sigmatropic rearrangement

Sulfenate ester reacts and inactivates the enzyme

β-glycosidase inhibitor: β - D-galacto pyranosyl- p-nitrophenyltriazine in activated a β- glycosidase by adding on to the methionine at 500 position.
Inactivation of alanine racemase by o-acetylserine

Enzyme

Schiff's Base

Inactive enzyme

$X = \text{halide also could do the same inactivation.}$

Inactivation of pyridoxal phosphate containing enzymes-1

Enzyme

Schiff's Base

Inactive enzyme

Hydrolysis to pyruvate and further turnover

However, in three carbon systems, it is the PALP that gets inactivated and not the enzyme.
Formation of PALP adduct

Inactivation is caused by the reaction of product with PALP

Inactivation of D-amino acid oxidase by 1-chloro-1-nitroethane

Stable N5- acetylated reduced flavin
Thymidylate synthetase inactivators

5-nitro dUMP derivative

bicyclic triazinyl pyrimidine

Thymidylate synthetase inactivator

5-ethynyl-dUMP

Inactive enzyme
Reaction catalyzed by S-Adenosyl-L-homocysteinase

S-Adenosyl-L-homocysteine (SAH)

Adenosine

E-NAD E-NADH

H₂O

L-Homocysteine

Enzyme is locked up in the E-NADH form and is inactive. Accordingly, ara-adenosine is a suicidal inactivator, while adenosine is not an inactivator.
Inactivation by tight binding product

Cytochrome P 450
Side chain cleaving enzyme

Enzyme uses a cysteine thiol group to abstract the methyl group.
The resultant thioether enzyme is irreversibly inactivated.

DEATH OF A REPAIR ENZYME

Enzyme uses a cysteine thiol group to abstract the methyl group.
The resultant thioether enzyme is irreversibly inactivated.
Instead of using cysteine as the active site amino acid, use aspartic acid (or glutamic acid). Once the carboxyl group abstracts the methyl group, use hydrolytic reaction to regenerate the enzyme.

Inactivation of 3-hydroxyanthranilate dioxygenase
AZT INHIBITS DNA POLYMERASE BY
CHAIN TERMINATION MECHANISM

3'-azido-3'deoxythymidine (AZT)

AZT gets converted into
AZT triphosphate and is
incorporated into the
growing chain of DNA. But
it lacks the 3' hydroxyl to
allow further growth of the
chain. Hence chain
termination occurs.