Enzyme Mechanisms

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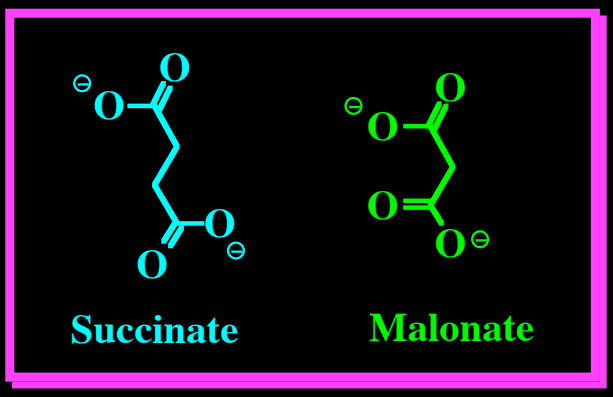
Enzymes are highly specific

- **Enzymes distinguish between homologues (succinate and malonate).**
- **Enzymes acting on D-amino acids will not touch L-amino acids at all (Chiral specific).**
- **Enzymes will distinguish between even prochiral centers (Prochiral specific).**
- **Even nonspecific enzymes show certain specific requirement in their substrate structure.**

Enzymes are highly specific

1. Enzymes distinguish between homologues.

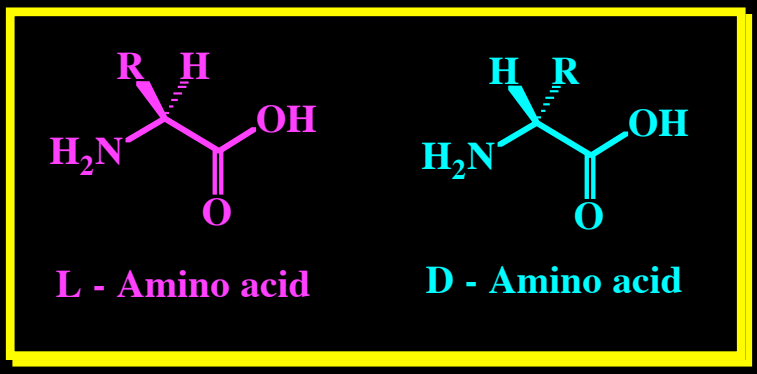
For succinate Dehydrogenase, succinate is the substrate, the lower homologue, malonate is an inhibitor.



Enzymes are highly specific

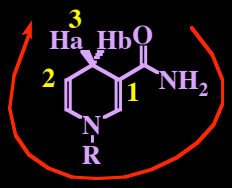
2. Enzymes attacking D-amino acid will not touch L-amino acid (Chiral specific).

D - amino acid oxidase will not oxidize L-amino acid at all.



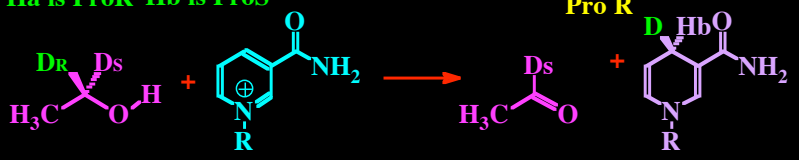
Enzymes are highly specific

3. Enzymes will distinguish between even prochiral centers (Prochiral specific).



Alcohol dehydrogenase transfers specifically Pro R hydrgen from ethanol to A side of NAD (to form Pro R in NADH)

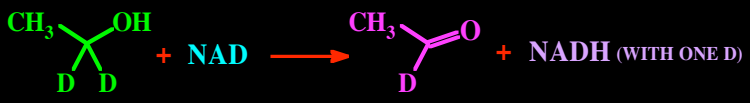
Ha is ProR Hb is ProS



Ethanol NAD Acetaldehyde NADH

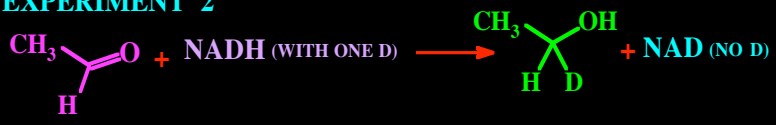
Alcohol dehydrogenase - Details of proton transfer mechanism

EXPERIMENT 1



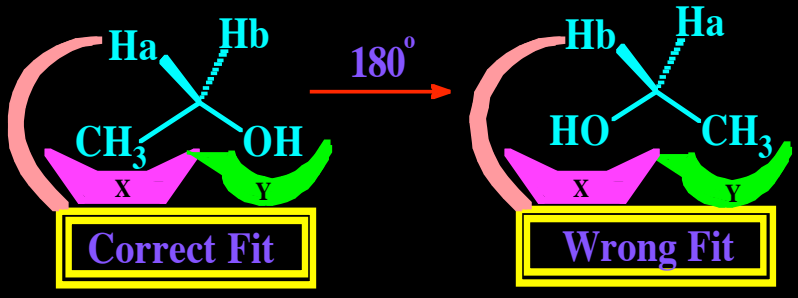
No Deuterium is lost in the solvent. Thus, D transfer is direct.

EXPERIMENT 2



All deuterium is transfered from NADH to alcohol. Therefore, the reaction seems to be stereospecific.

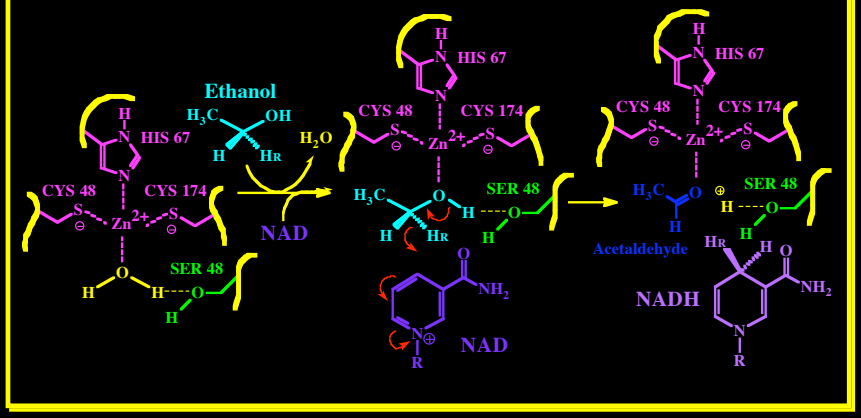
Two point attachment is enough to explain prochiral reactions



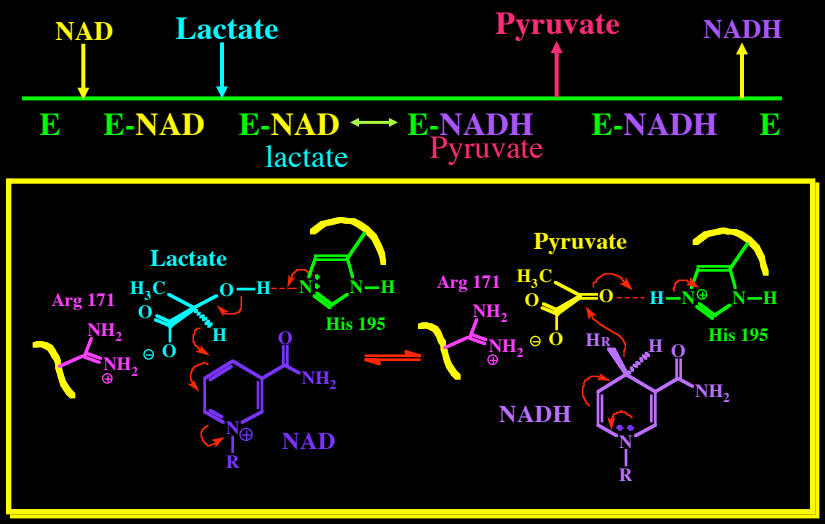
If methyl group binds to the site X and hydroxyl group to site Y then the enzyme could only pick up Ha proton. If we rotate the substrate by 180 degrees the correct binding sites would not be available.

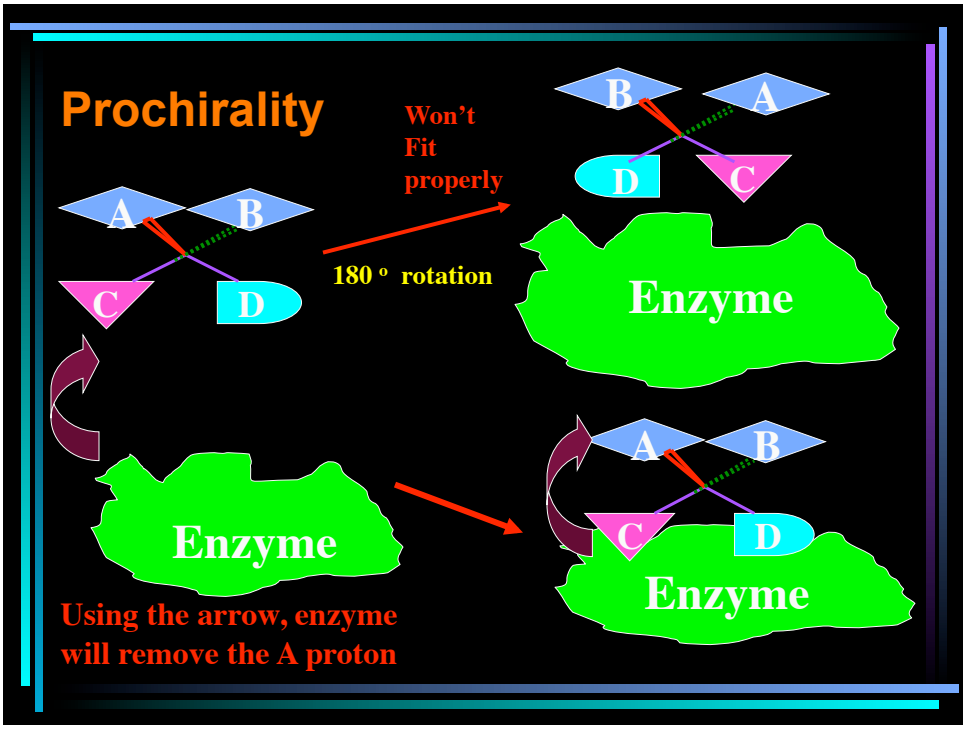
Alcohol Dehydrogenase Reaction

Pro R group is added and/or removed from both substrates



Lactate dehydrogenase also removes hydride from Pro R position of NADH (A side specific)





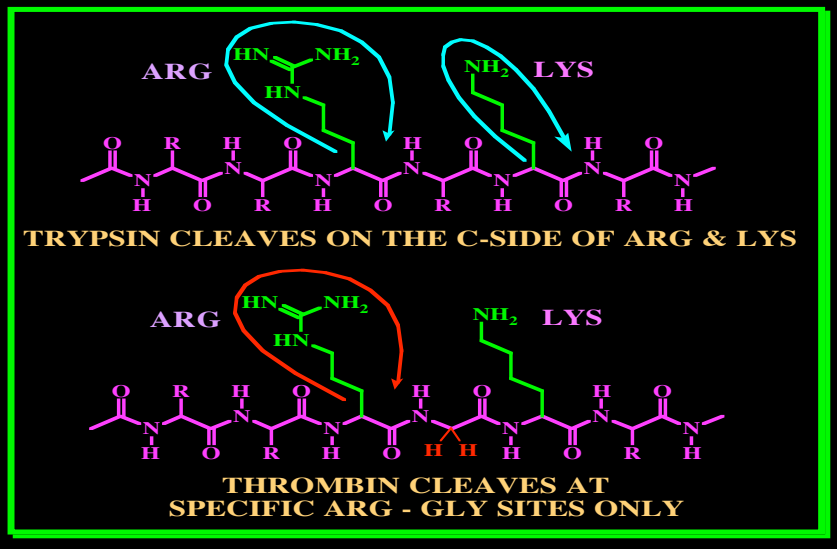
Enzymes are highly specific

4. Even nonspecific enzymes show certain specific requirement in their substrate structure.

Trypsin is a non specific protease. But even trypsin shows specificity in its requirement for having basic amino acid at the cleavage site.

While highly analogous enzyme thrombin, specifically will cleave only the Arg - Gly bond and none other.

Enzyme specificity - Trypsin versus thrombin

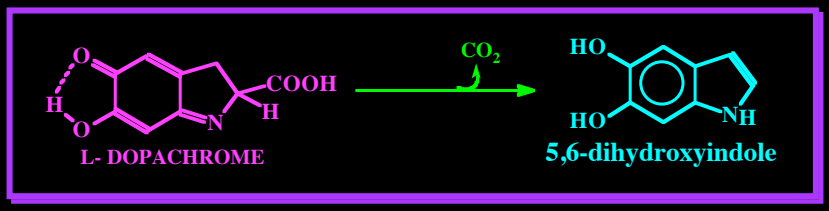


Enzymes are highly specific

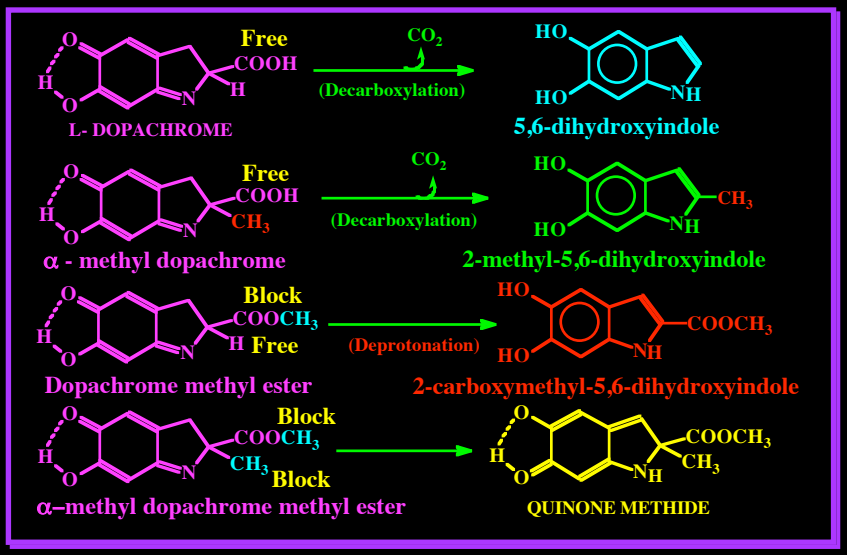
4. Even nonspecific enzymes show certain specific requirement in their substrate structure.

The unusual case with Insect dopachrome isomerase.

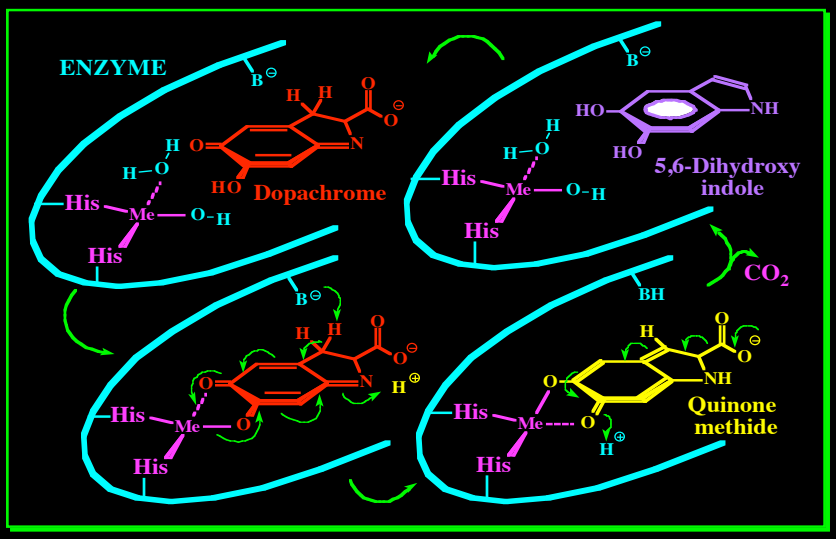
In 1990, we isolated an enzyme from insect blood that catalyzes the following reaction. While investigating the mechanism of action of this enzyme, it became clear that it is not a decarboxylase but an isomerase.



Reactions catalyzed by Insect dopachrome isomease



Proposed mechanism for insect dopachrome isomerase (decarboxylase) reaction

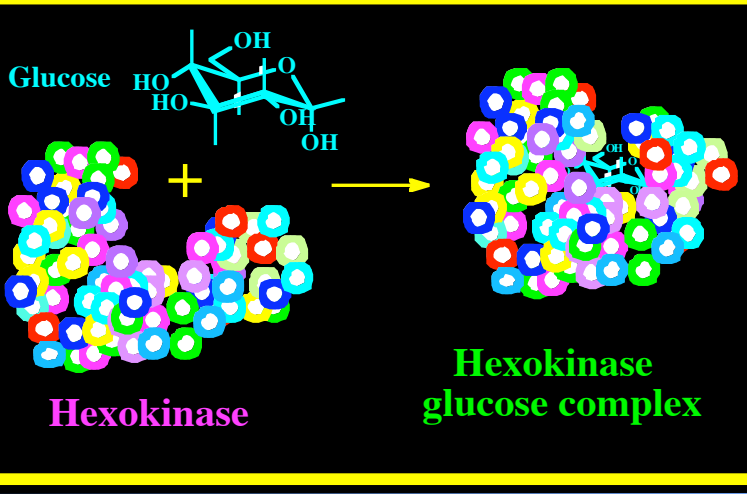


Enzymes perform these reactions by forming a tight E-S complex, which reduces the free rotation of the substrates and forces them to go to the transition state for better binding.

Enzymes are highly flexible and exhibit drastic conformational changes while performing the catalysis.

Koshland's Induced Fit Theory

Substrate binding causes conformational changes on the enzyme.



The superior power of enzymes over chemical catalysts is ascribed to the following factors

- Proximity & strain effects
- Electrostatic effects
- Acid-Base catalysis
- Covalent catalysis

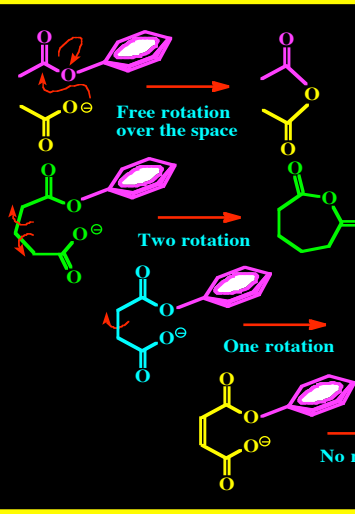
1. Proximity effect

- For an enzymatic reaction to occur, substrate must come in close proximity to the active site groups of the enzyme.
- Substrate should be properly oriented at the active site of the enzyme.
- Following the above positioning, the conformational changes on ES complex should cause sufficient strain to bring the ES complex to E-Transition state complex.

Proximity effects

- Reducing the freedom of motion of the substrate at the active site will increase the rate of the reaction.

Proximity effects - Proof



If the rate of this anhydride formation is taken as one the relative rates for the following reaction are

1×10^3

2×10^5

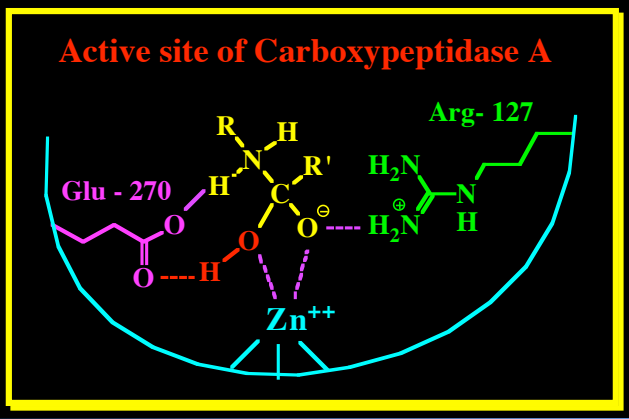
1×10^7

2. Electrostatic effects

- To exhibit proper binding, the charges on the substrate (and the transition state) are appropriately neutralized by oppositely charged groups on the enzyme. Thus the enzyme keeps certain charged groups at or near the active site even if the active site is well buried inside the protein.
- (Recall the general rule in protein structure which dictates that all charged amino acids should be present on the periphery of the protein to interact with the aqueous environment and the hydrophobic amino acids should be kept inside. Active site is the exception to this rule)

Electrostatic effect - example

Active site of Carboxypeptidase A
contains an acidic group (Glu 270) and a basic group (Arg 127). They are involved in stabilization of the transition state of the reaction.



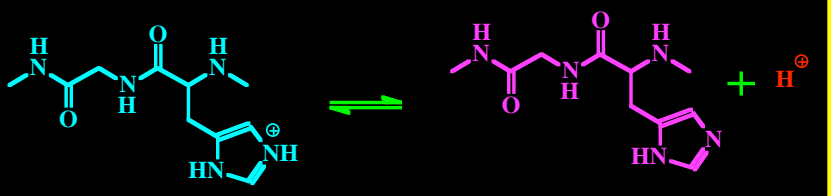
3. Acid - Base catalysis

- Enzymes do not use specific acid and specific bases for catalysis (like: H^+ or OH^-)
- Enzymes have reactive side chains at the active site to function as proton donors or acceptors for a reaction.
- These groups are called general acids and general bases. (general acid is a group that donates a proton and general base is a group that accepts a proton).

Imidazole group of Histidine

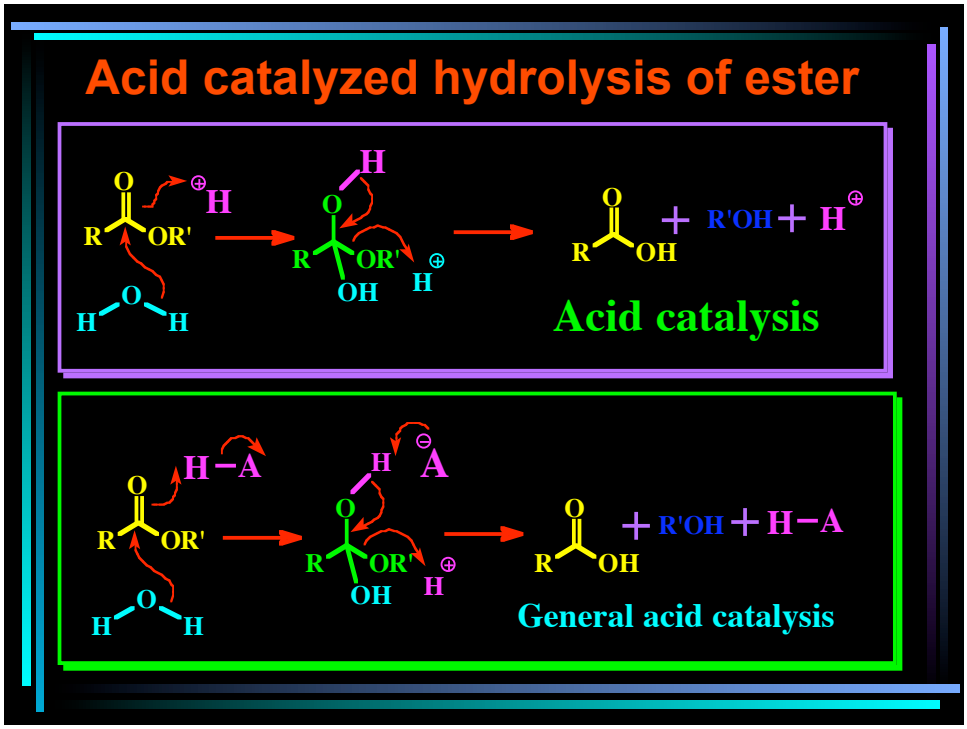
- The imidazole group of histidine often participates in several enzyme reactions at neutral pH, because it has a pK_a value of 6.
- It readily ionizes at the physiological pH value.
- The protonated form of imidazole serves as the general acid and its conjugate base is the general base.

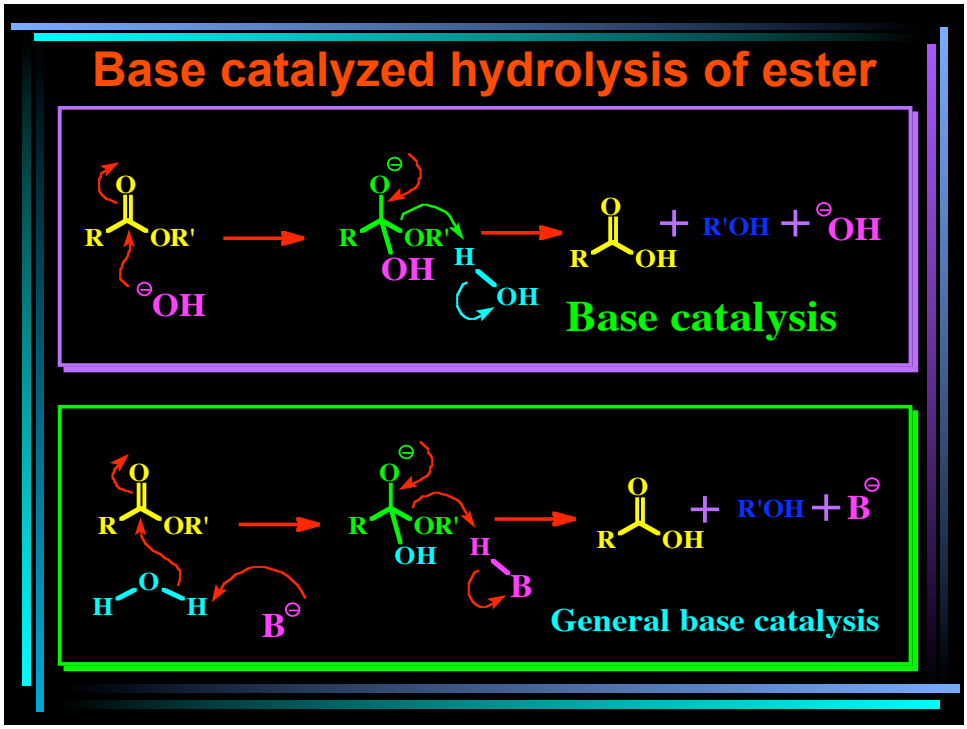
An example of a general acid - general base in enzymes



(General acid) (general base)

(Imidazole group of histidine)


Acid catalyzed hydrolysis of ester
Acid catalysis
General acid catalysis

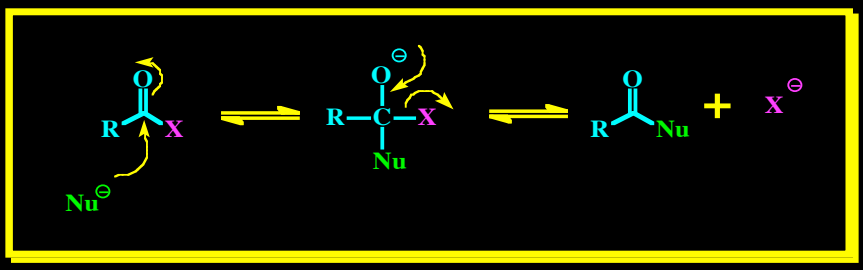

Base catalyzed hydrolysis of ester
Base catalysis
General base catalysis

4. Covalent catalysis

- Often enzymes use one of their side chains to form an unstable covalent bond with the substrate.
- This enzyme partial substrate intermediate is further degraded in a secondary reaction completing the catalytic cycle.

Nulceophilic Reactions

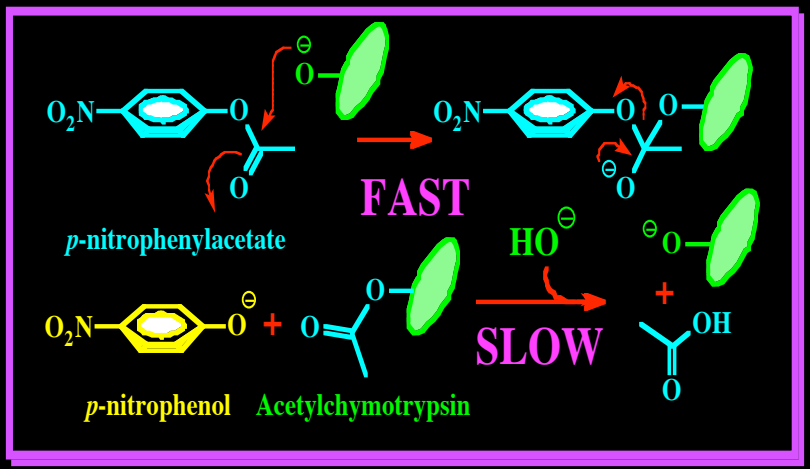
- **Reactions occurring with ionic compounds involve two species. An electron rich molecule called nucleophile, attacks an electron deficient center (called an electrophilic center). The nucleophile invariably has a negative charge (or an unshared electron pair). The following is an example of an electrophilic reaction.**



Nucleophiles participate in covalent catalysis - examples

- Hydroxyl group of Serine - chymotrypsin
- Thiol group of Cysteine - Papain
- Imidazole group of Histidine -
- Carboxyl groups of aspartic and glutamic acids -

Chymotrypsin - example of a covalent catalysis

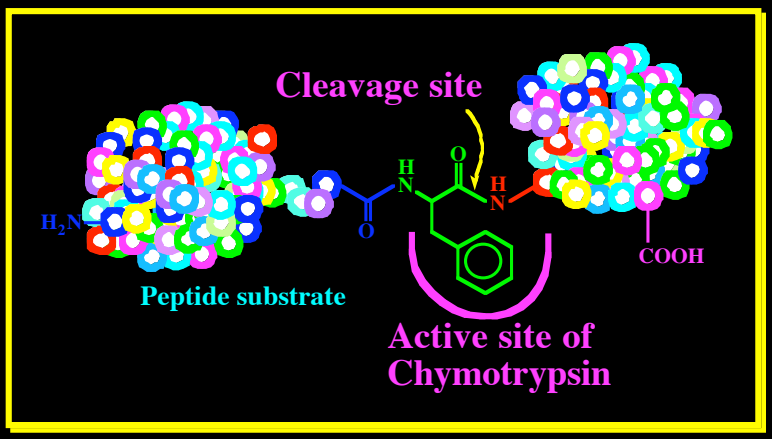


Enzyme mechanism
Case 1.
Chymotrypsin

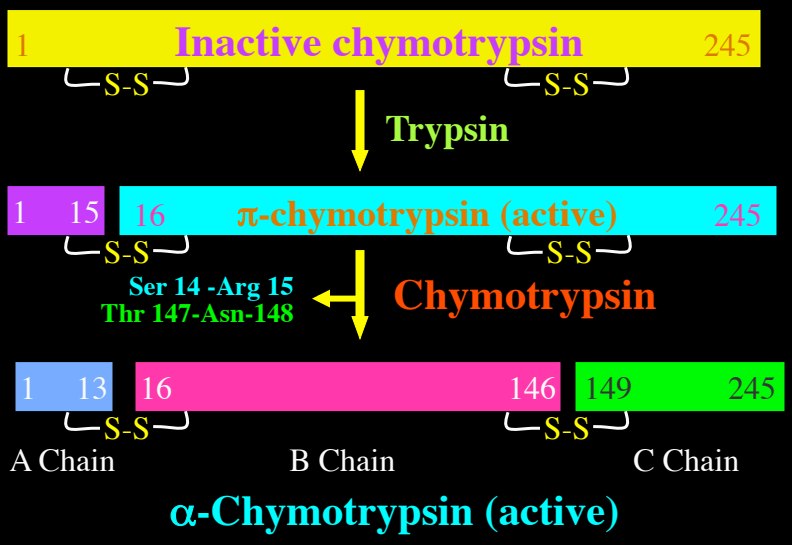
General Properties of Chymotrypsin

- It is a serine protease; M.Wt 25 kd; Three chains interconnected with disulfide bridges.
- It is an endopeptidase with specificity towards Phe, Tyr, Trp (and to some extend Met).
- It will also hydrolyze small peptides and esters as long as they possess the above structural requirements.
- The 3 - D structure down to 2 - Å resolution is available.
- It is an ellipsoid protein; Dimentions: 51 x 40 x 40 Å
- It has a lot of antiparallel β - sheets with very little α - helix.

Chymotrypsin cleaves peptides on the C - side of Phe, Tyr and Trp

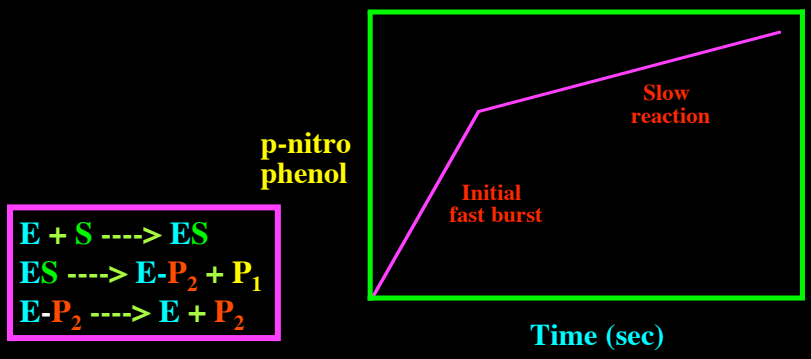


Activation mechanism of chymotrypsin



Chymotrypsin shows biphasic kinetics.

During hydrolysis of *p*-nitrophenylacetate, chymotrypsin shows a fast initial burst followed by a slow reaction. It turns out that the enzyme hydrolyzes *p*-nitrophenylacetate to *p*-nitorphenol and hold the acetate. The hydrolysis of enzyme bound acetate is a slow reaction limiting the rate of the entire reaction accounting for the initial biphasic kinetic behavior.

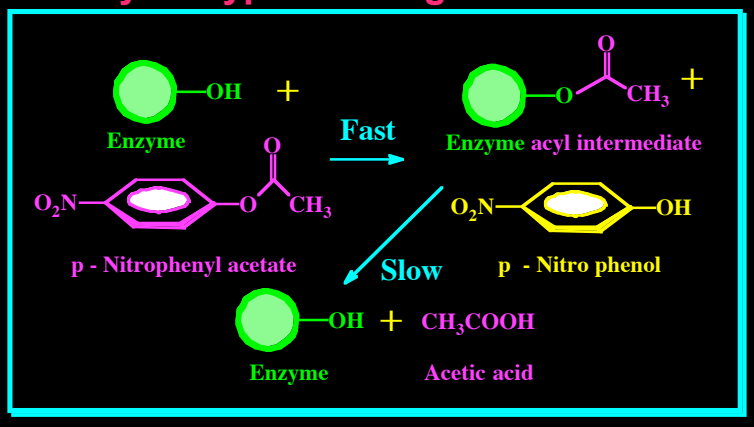


$E + S \longrightarrow ES$

$ES \longrightarrow E\text{-}P_2 + P_1$

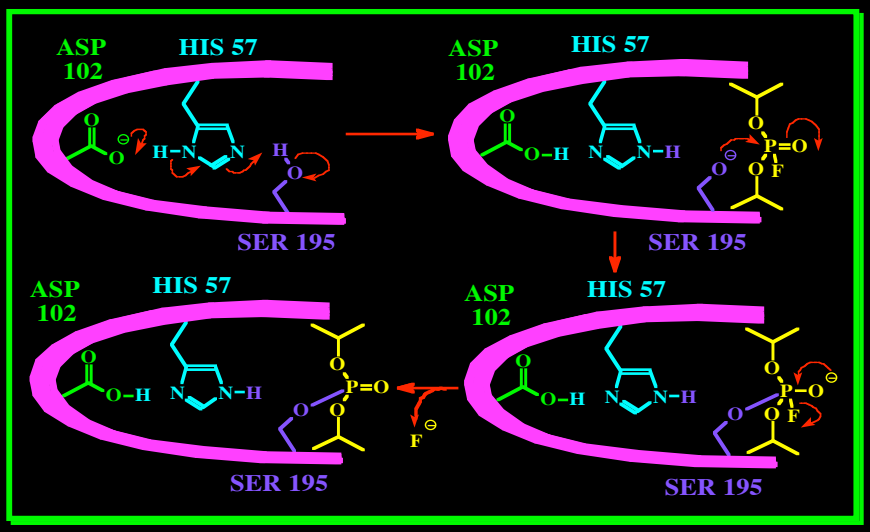
$E\text{-}P_2 \longrightarrow E + P_2$

Part of substrate gets bound to chymotrypsin during the reaction.

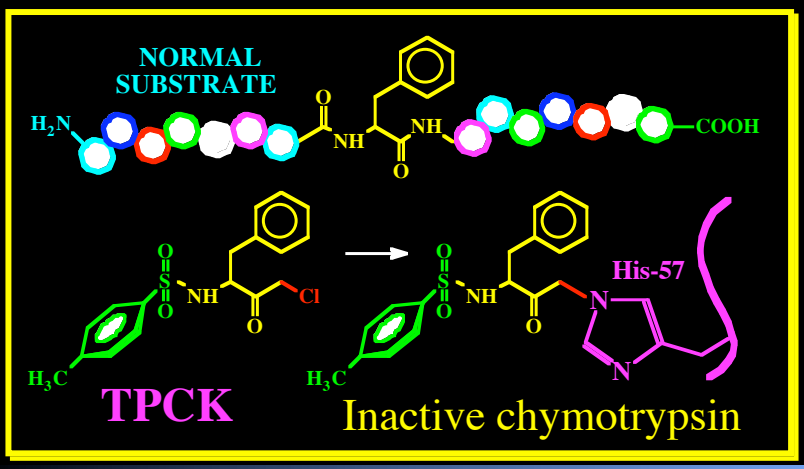


The residue binding to acyl group was identified to be Ser 195. Although the enzyme has a total of 28 Ser residues, only Ser 195 gets specifically acylated indicating a role in catalysis.

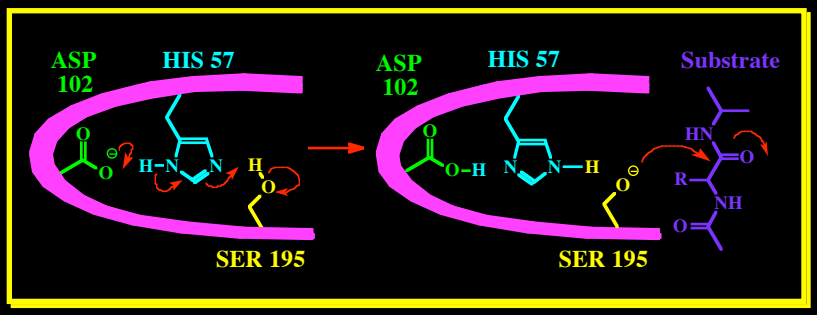
Diisopropylfluorophosphate acylates Ser 195 and inactivates chymotrypsin.



The affinity label, p-toluenesulfonyl phenylalanyl chloromethyl ketone (TPCK) specifically labels His 57 residue of chymotyrpsin. Therefore, His 57 is involved in the catalytic process of the enzyme.

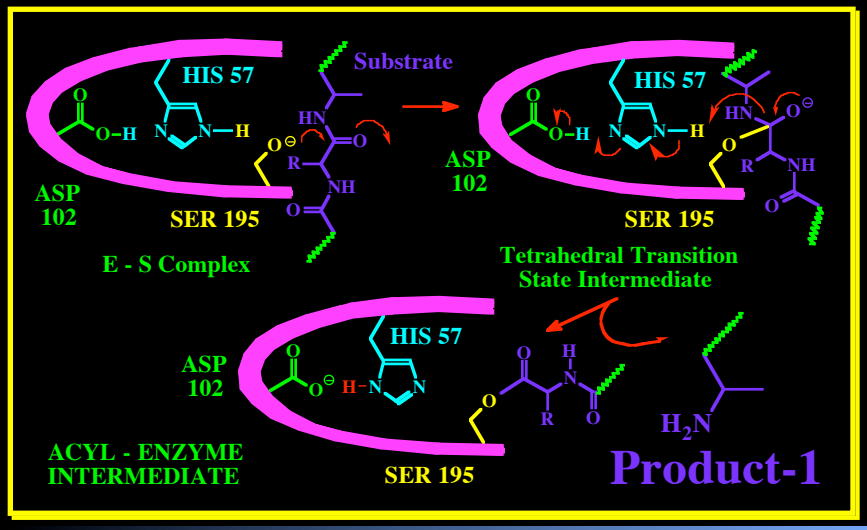


Catalytic triad at the active site of chymotrypsin

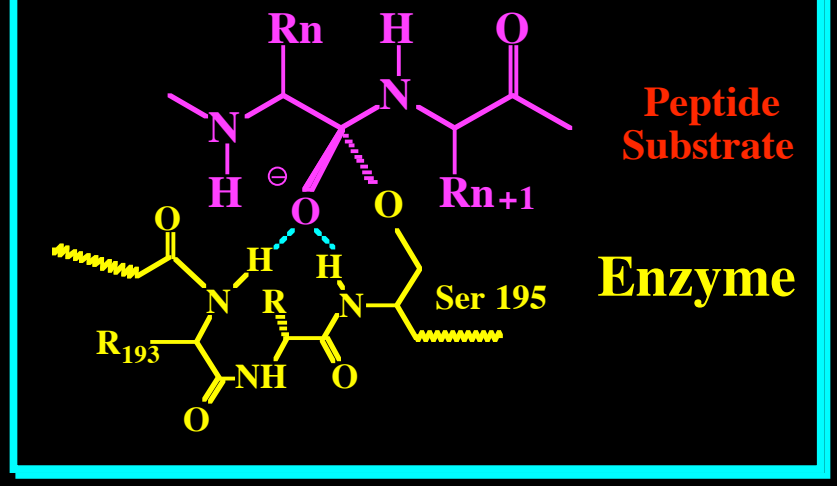


X - ray crystallographic studies reveal that a buried aspartyl group is in close proximity to His 57 and Ser 195. The ionized carboxyl group through His 57 activates the Ser to become $-CH_2O^-$, which becomes a powerful nucleophile for catalysis.

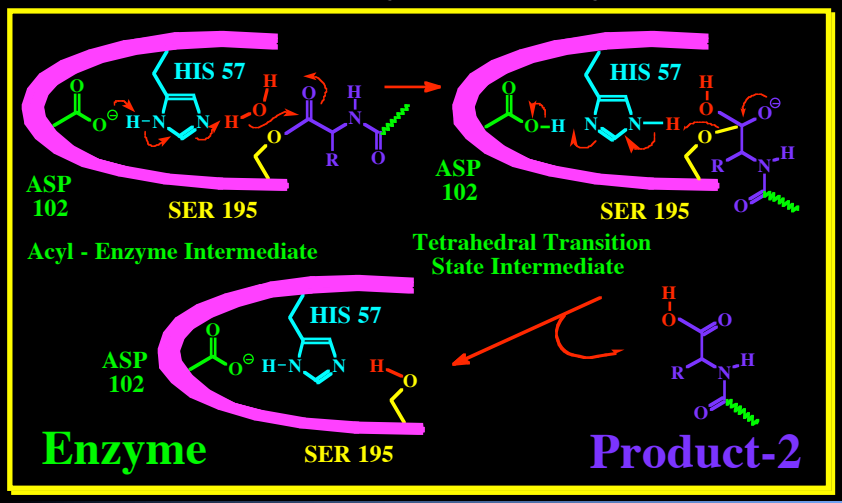
Ser OH serves as the nucleophile and attacks the peptide bond. This results in the formation of unstable tetrahedral transition state intermediate. The transition state breaks down to give acyl enzyme intermediate (Covalent catalysis) and product 1 which is the amine portion of the peptide.



Enzyme stabilizes the tetrahedral intermediate formed during acylation reaction by hydrogen bonding



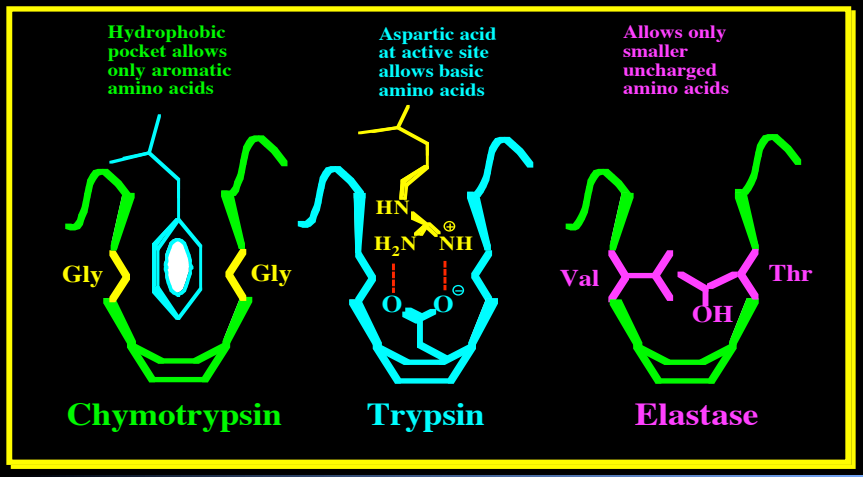
A water molecule is introduced into the active site in the second half of the cycle. Histidine serves as the general base. General base catalysis causes the formation of another unstable tetrahedral transition state intermediate. This intermediate breaks down to give acid portion of the peptide and liberates the free enzyme for the next cycle of reaction.



Chymotrypsin, Trypsin and Elastase

- They show 40% of the amino acids are identical.
- Interior 60% of the amino acids are identical.
- Their 3-D structure is very similar.
- All three have Asp - His - Ser Triad at the active site.
- All are inactivated by diisopropylfluorophosphate.
- They have same catalytic mechanisms.
- Oxyanion hole stabilizes tetrahedral intermediate.
- Sequence around active site serine in all is Gly-Asp-Ser-Gly-Gly-Pro.
- **But markedly different in substrate specificity.**

Chymotrypsin, trypsin and elastase are variation of the same theme.

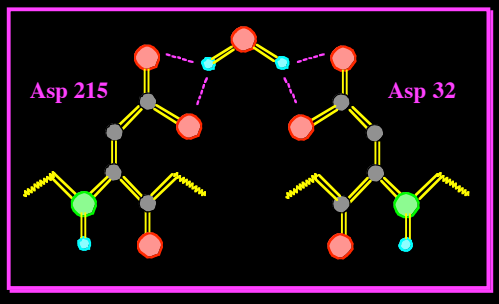


Classification of proteases

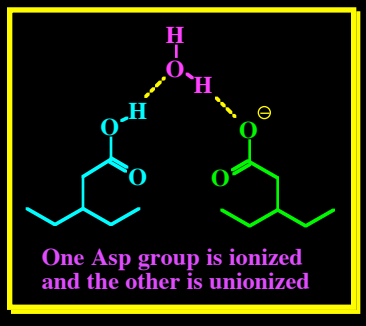
Protease type	Example	Active site	Active site amino acids
Serine protease	Chymotrypsin	Ser - 195	Ser - 195; Asp 102; – His 57
Aspartyl protease	Pepsin	Aspartic acids	Asp – 32; Asp – 215
Thiol protease	Papain	Cys – 25	Cys – 25; His – 159
Metallo protease	Carboxy-peptidase A	Zinc ion	Arg – 127; Glu - 270

Aspartyl Protease - Pepsin

Active site of pepsin is made up of two aspartyl side chains flanked by a water molecule.

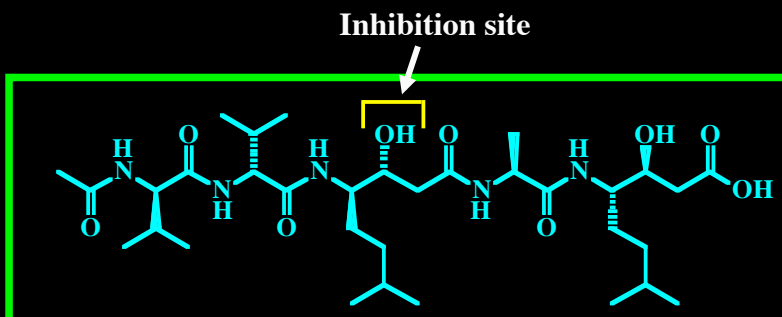


This makes the Asp group very acidic.



One Asp group is ionized and the other is unionized

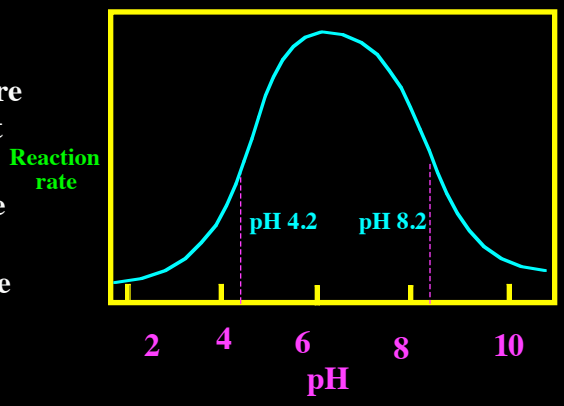
Pepstatin is a transition state inhibitor of pepsin



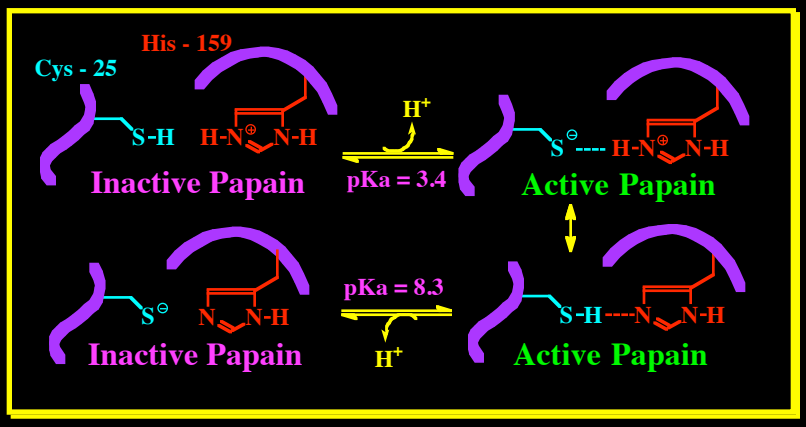
N-acetyl PEPSTATIN - A MICROBIAL PEPTIDE

pH optimum of papain

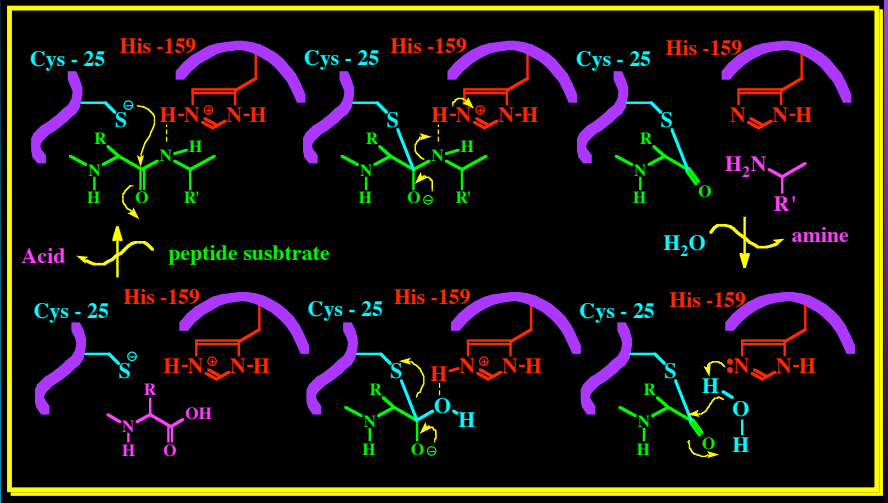
The inflection points of the rate versus pH profile gives the nature of ionizable groups at the active site.
In this case, 4.2 is due to SH of Cys -25 and 8.2 is due to imidazole of His 159.



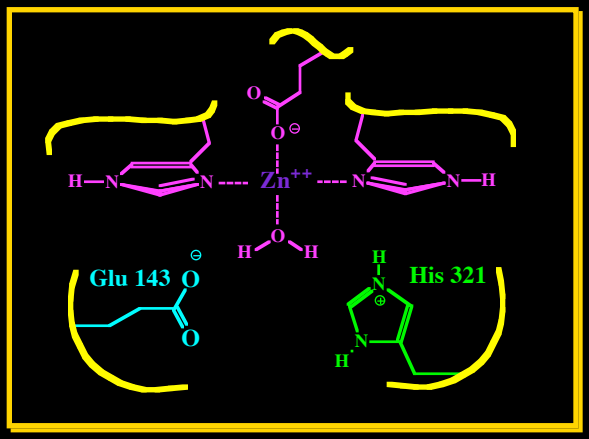
Active site of Papain has Cys and His



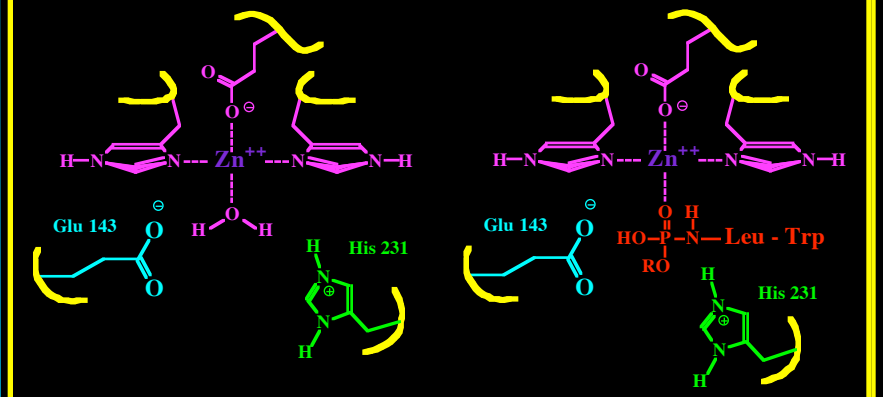
Mechanism of Action of Papain



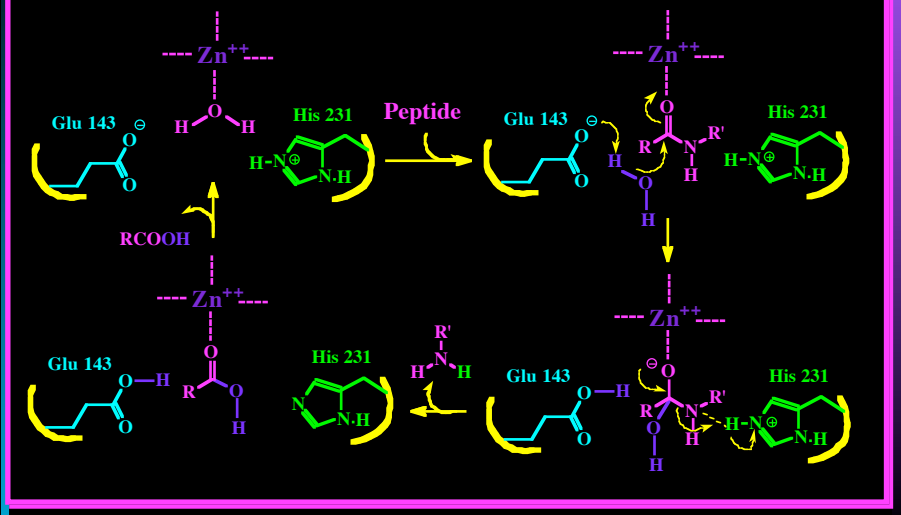
Active site of a metallo protease - Thermolysin



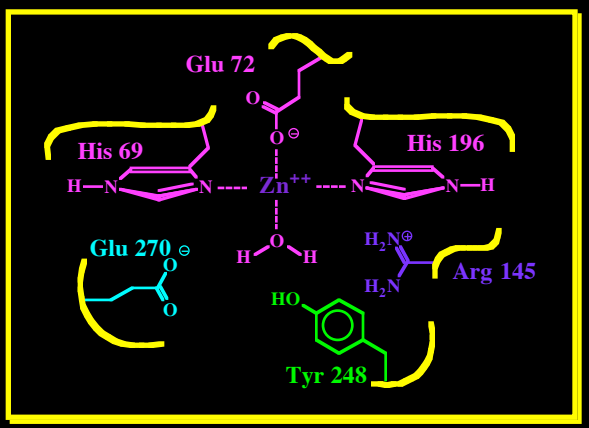
Thermolysin and its inhibitor

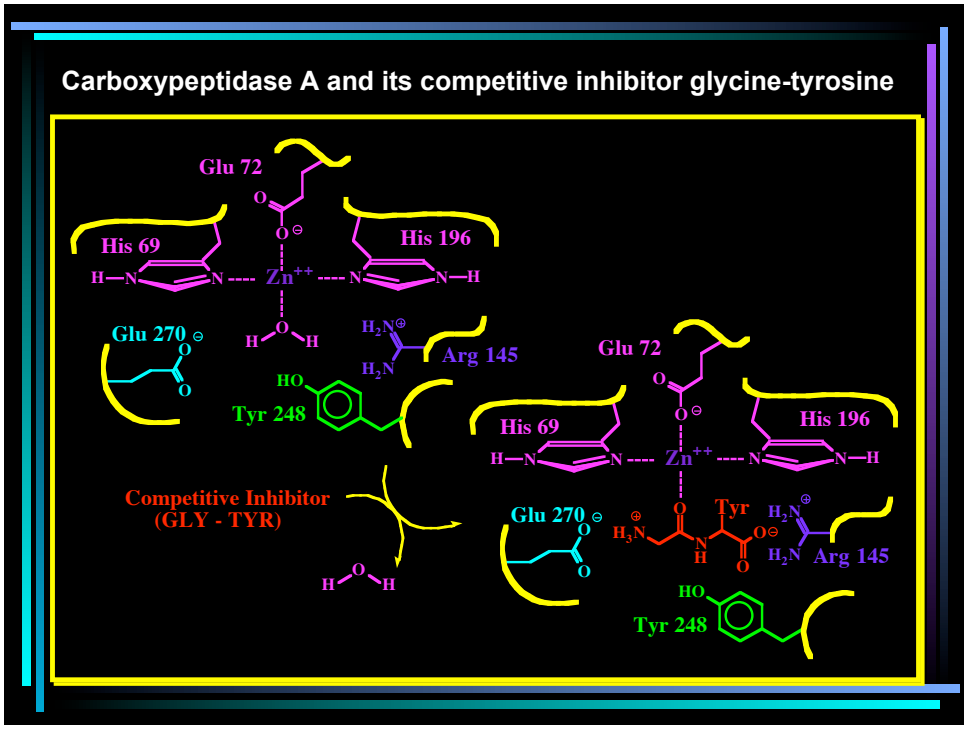


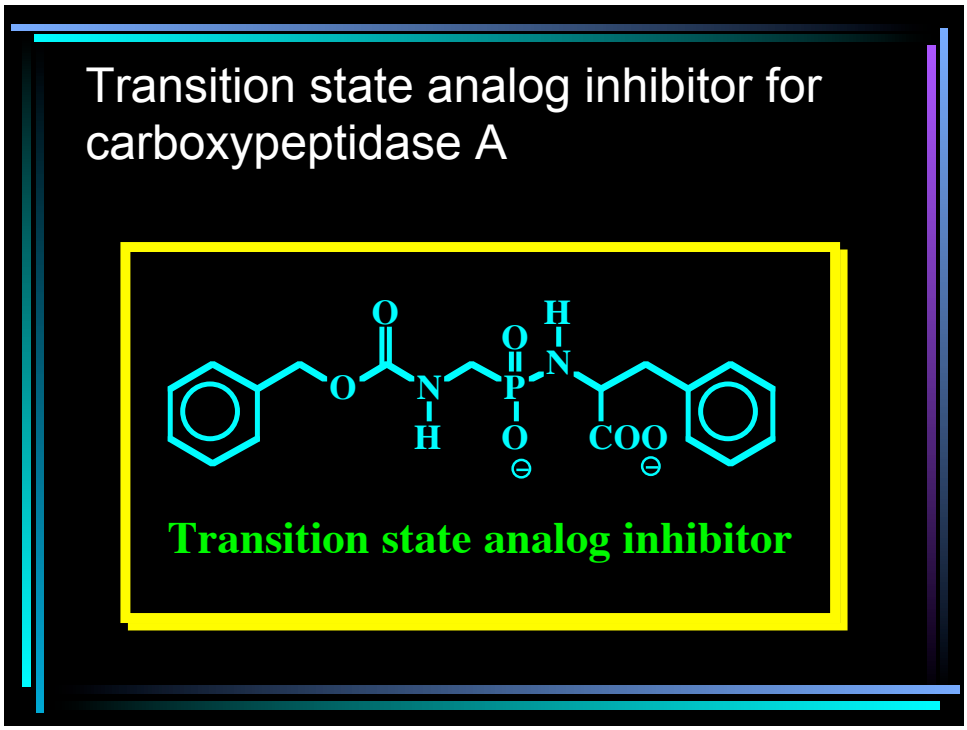
Mechanism of action of thermolysin



Active site of carboxypeptidase A



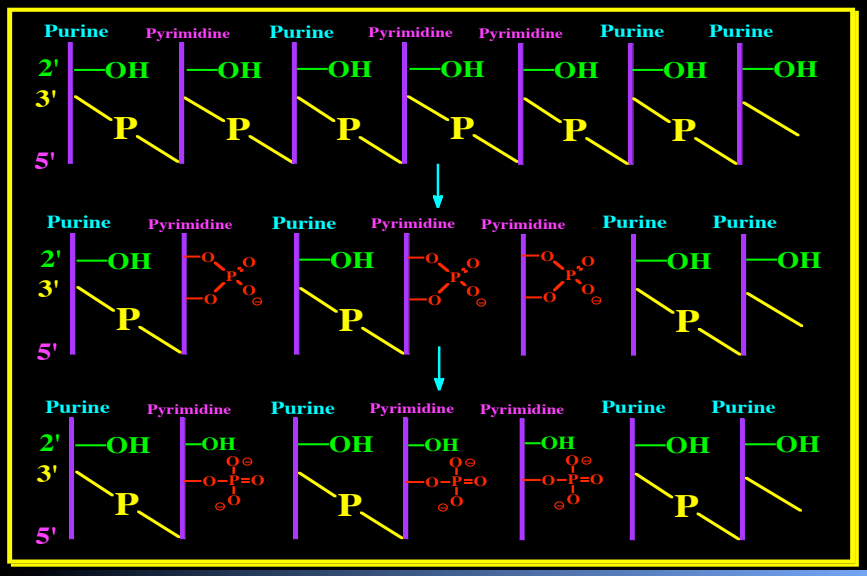

Carboxypeptidase A and its competitive inhibitor glycine-tyrosine
Glu 72
His 69
His 196
Zn++
Glu 270
Arg 145
Tyr 248
Competitive Inhibitor
(GLY - TYR)
Glu 72
His 69
His 196
Zn++
Glu 270
Tyr
Arg 145
Tyr 248


Transition state analog inhibitor for carboxypeptidase A
Transition state analog inhibitor

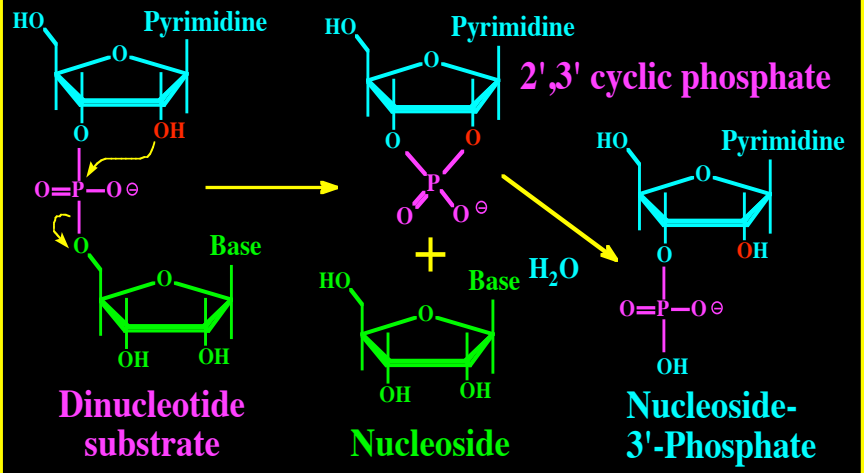
Enzyme mechanism
Case 2. Pancreatic Ribonuclease A

- 124 amino acid - single polypeptide chain containing enzyme.
- Hydrolyzes RNA molecules.
- Tightly packed. Interior has hydrophobic amino acids. Mainly β sheets.
- Has four disulfide derivatives.

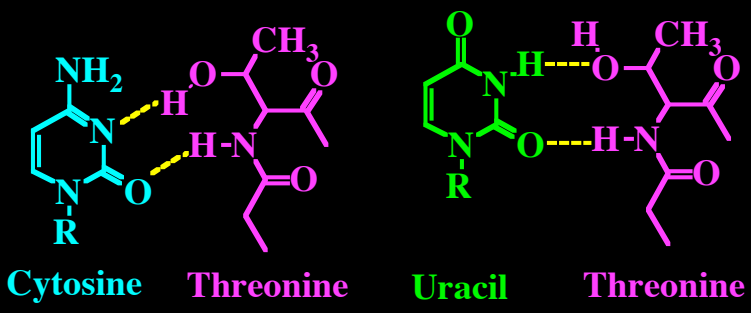
Reaction catalyzed by RNAse



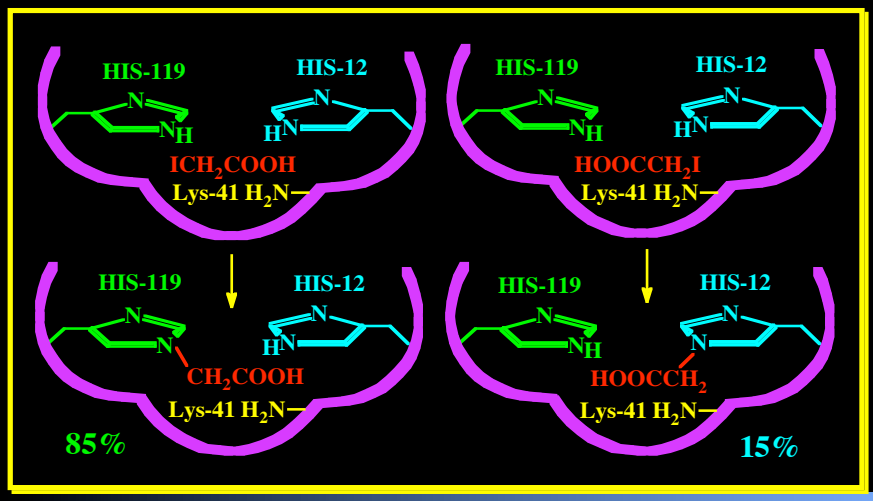
Reaction catalyzed by RNAse A



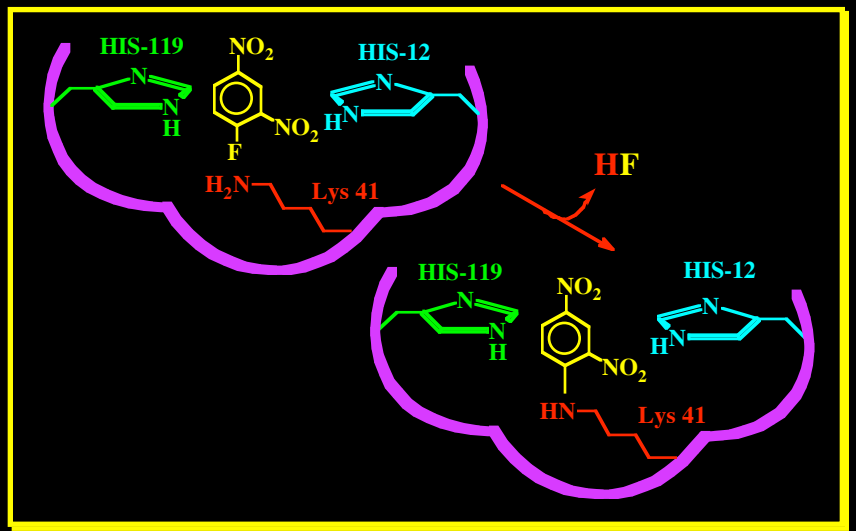
Substrate specificity to pyrimidine base is due to specific hydrogen bonding by Thr 45 at the active site.



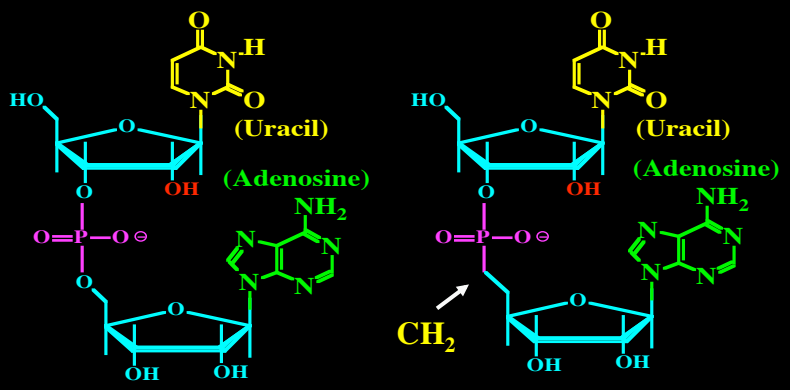
Iodoacetate inactivates RNAse quantitatively at pH 5.5. Carboxymethylation of RNAse is 5000 times faster than that of free Histidine. One mole of ICH_2COOH is incorporated per mole of RNAse. Analysis of inactive enzyme reveals two inactive products - 15% acylated His 12 and 85% acylated His 119.



Fluorodinitrobenzene (FDNB) specifically inactivates RNAse at pH 8.5. The reaction occurs at three Lys residues - Lys-41; Lys-7 and Lys-1 in the given order. Inactivation by FDNB is due to incorporation at Lys-41



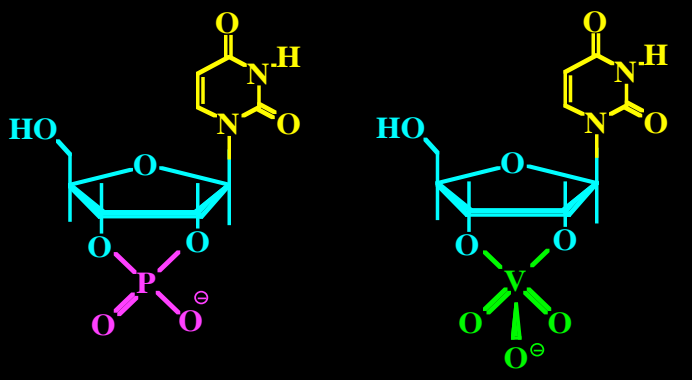
Structure of substrate and a Competitive inhibitor



Dinucleotide substrate (UpA)

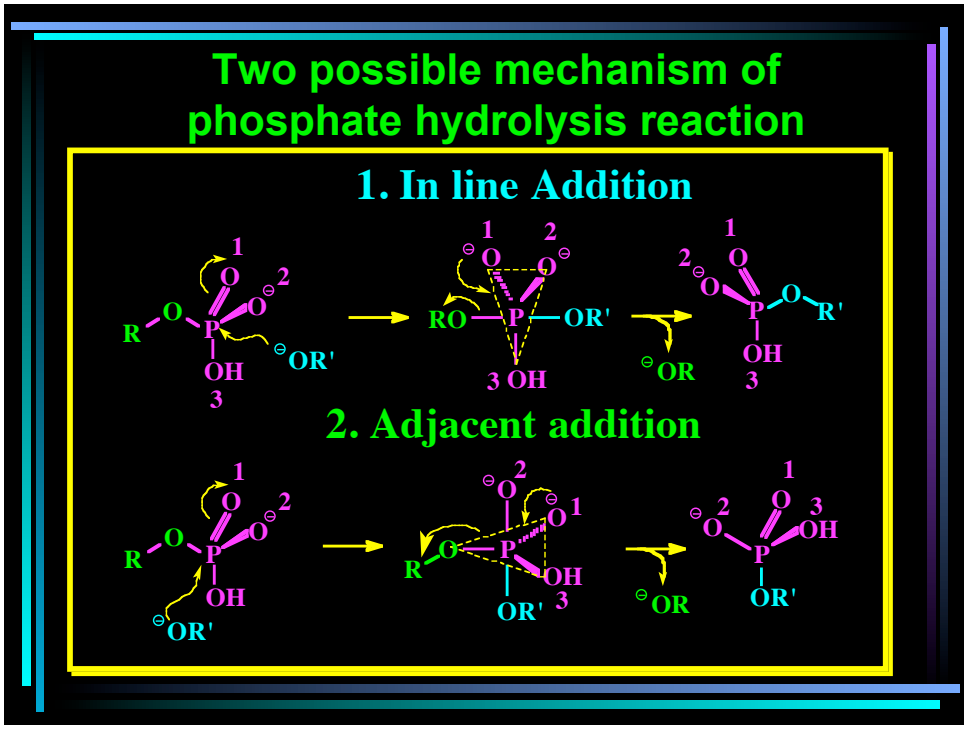
Competitive Inhibitor (UpCH2A)

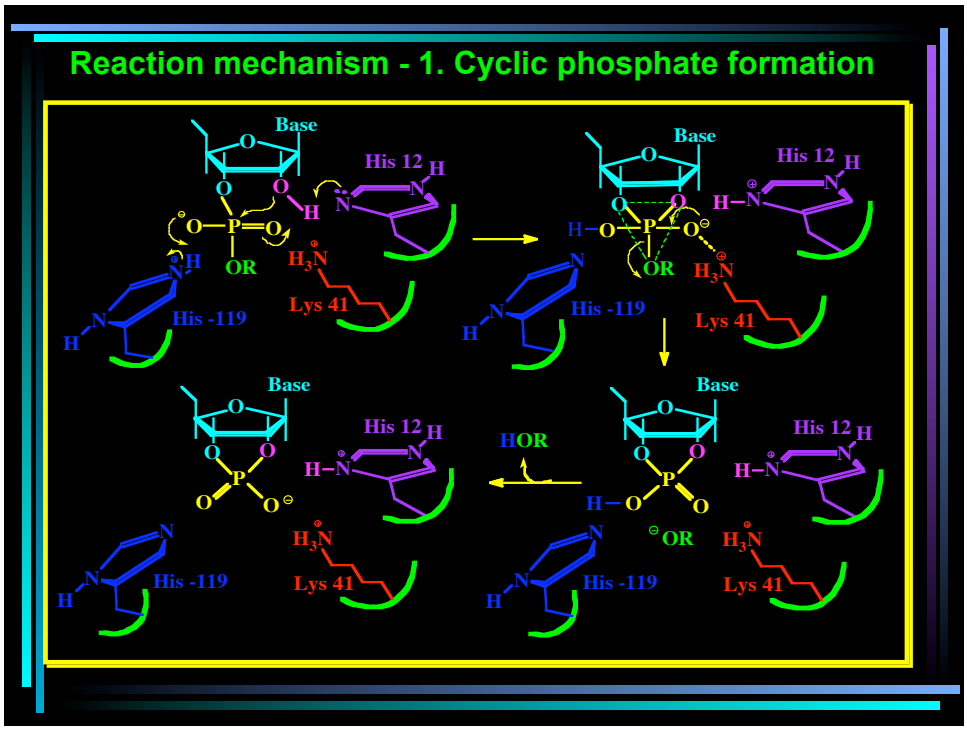
Structures of cyclic intermediate and the transition state analog which binds 10^4 times tighter than the cyclic intermediate.

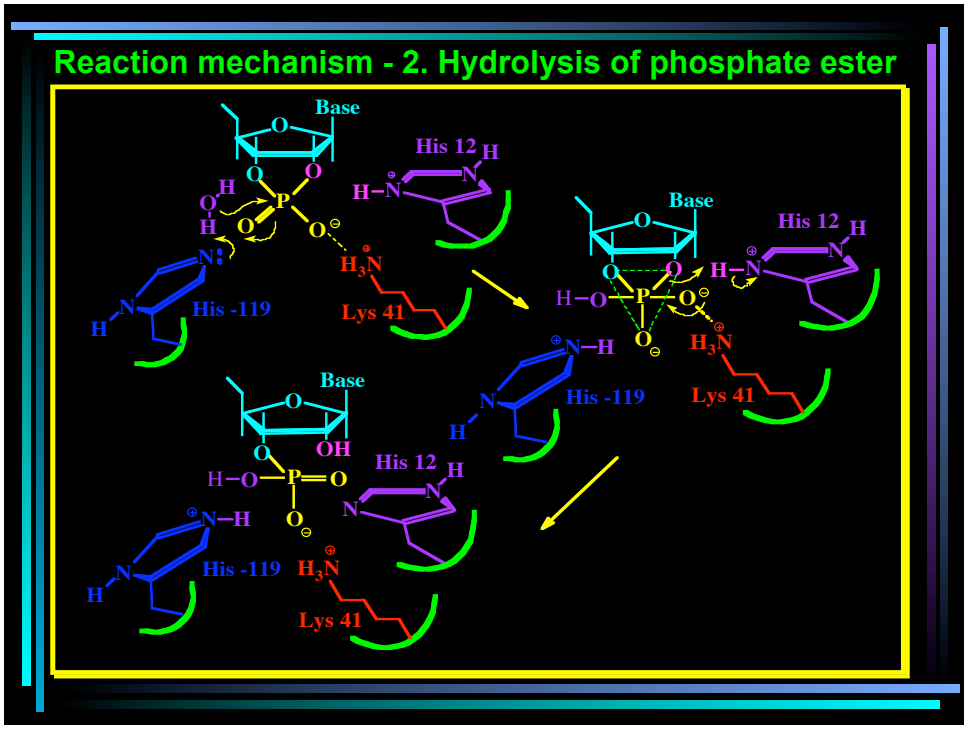


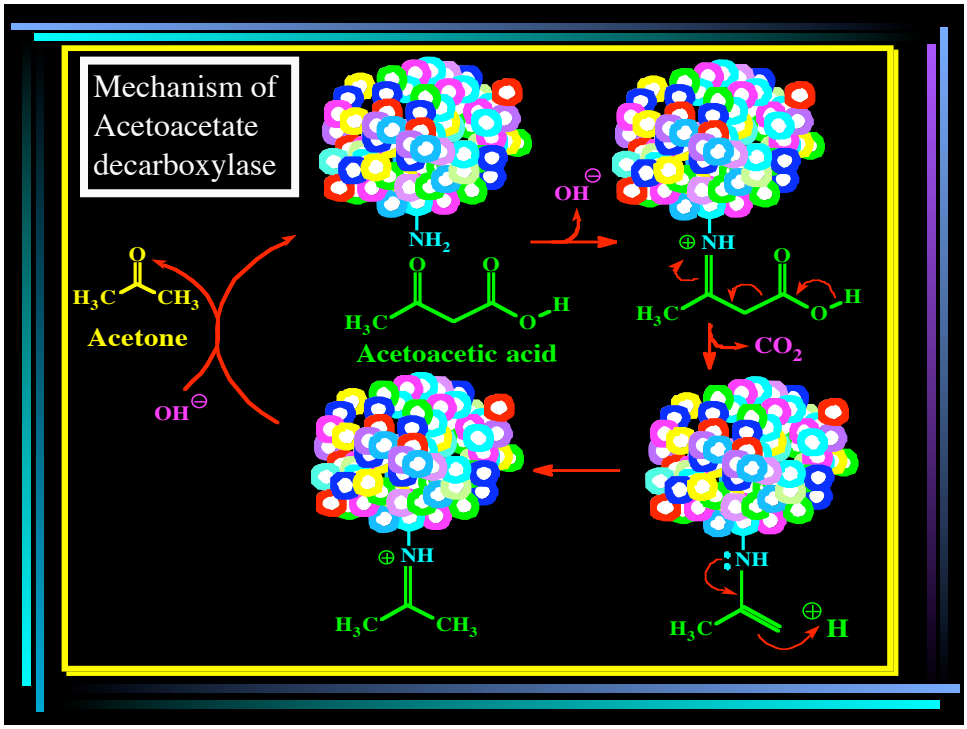
Uridine - 2',3' - phosphate

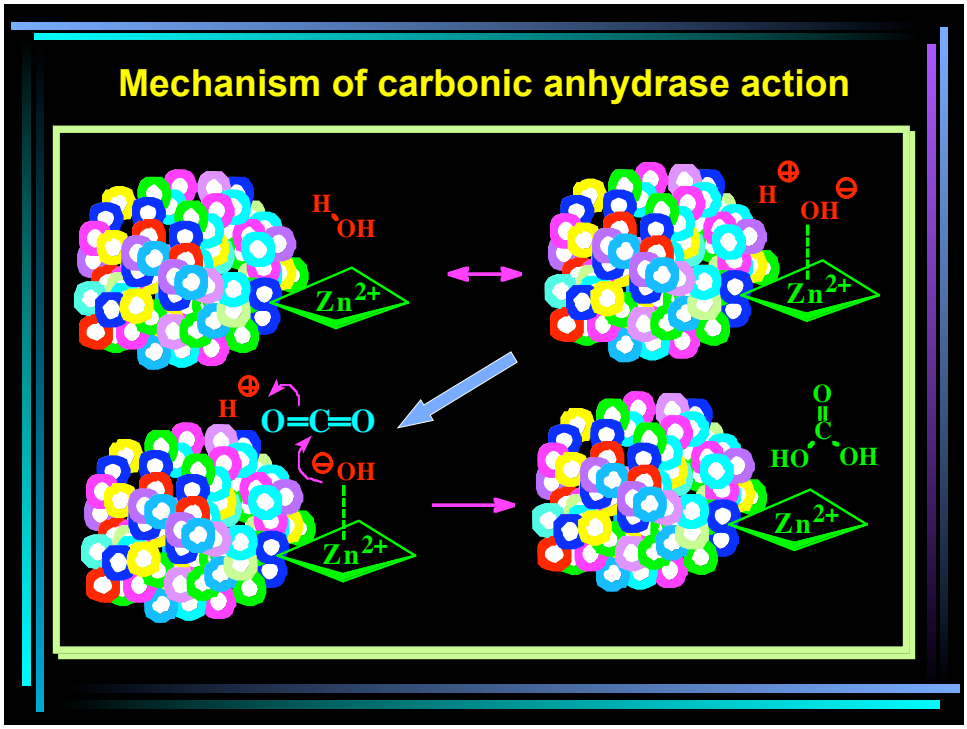
Uridine - 2',3' - vanadate

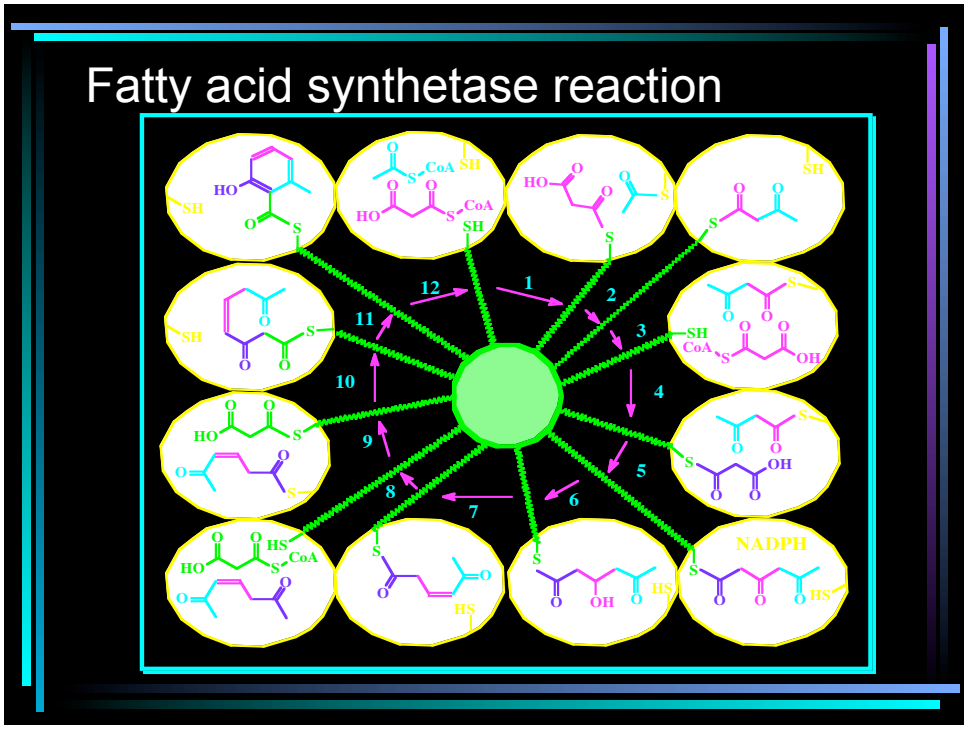

Two possible mechanism of phosphate hydrolysis reaction
1. In line Addition
2. Adjacent addition


Reaction mechanism - 1. Cyclic phosphate formation
Base
His 12
His -119
Lys 41
HOR

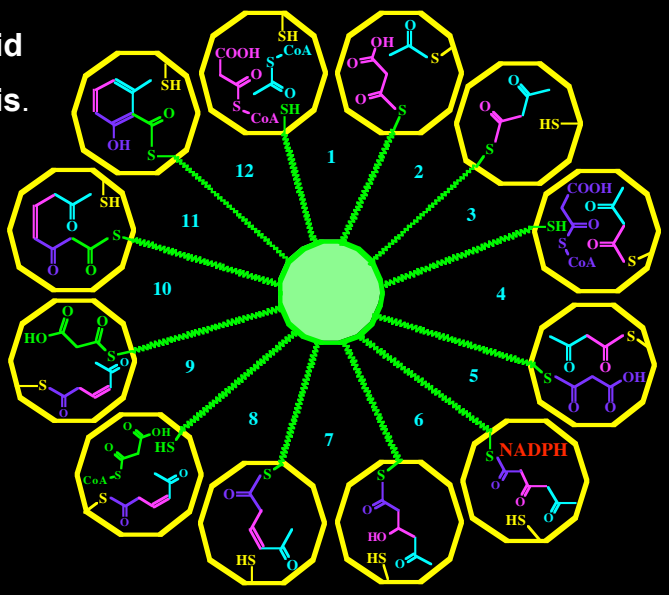

Reaction mechanism - 2. Hydrolysis of phosphate ester
Base
His 12
His -119
Lys 41


Mechanism of Acetoacetate decarboxylase
Acetone
Acetoacetic acid
OH
CO2


Mechanism of carbonic anhydrase action
H
OH
Zn2+
H
O=C=O
OH
HO
OH
O


Fatty acid synthetase reaction
HO
SH
CoA
HS
NADPH
OH
1
2
3
4
5
6
7
8
9
10
11
12

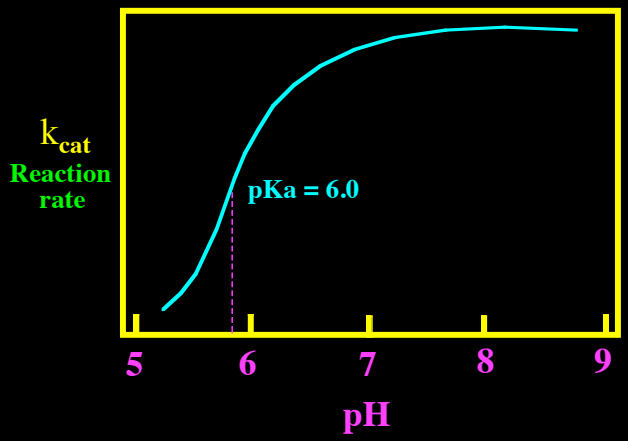
6-methyl salicylic acid biosynthesis.



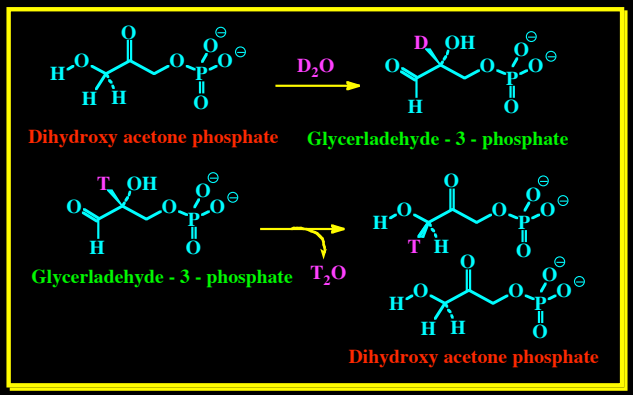
Triose phosphate isomerase

- Catalyzes the interconversion of dihydroxyacetone phosphate and glyceraldehyde-3-phosphate (an 1,2 proton shift reaction).
- Achieved catalytic perfection by having a k_{cat}/K_m of 4 x 10^{8} M^{-1} s^{-1} (as a result of high k_{cat} and low K_m) which is close to the diffusion rate of substrate to active site of enzyme.
- An enediol intermediate is formed during the reaction.
- Second fastest acting enzyme known.

Triose phosphate isomerase rate versus pH profile indicates the presence of a single pKa group. This group was identified to be glutamate at 165 by affinity labeling. In addition, an unusual neutral form of imidazole group is also involved in the catalysis.

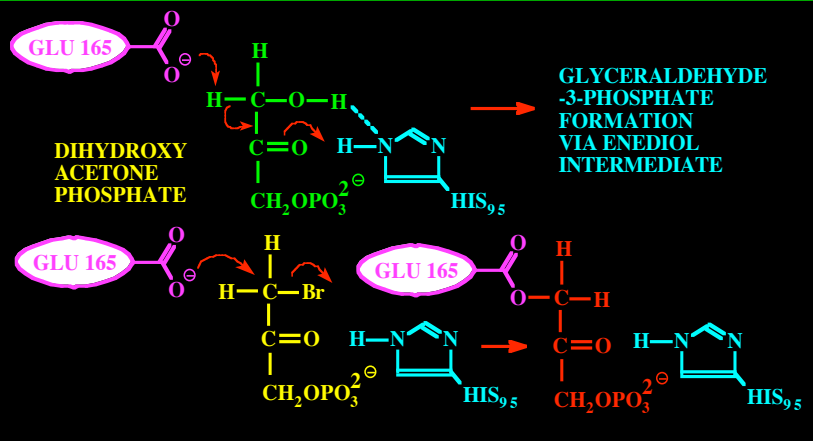


Proton exchange



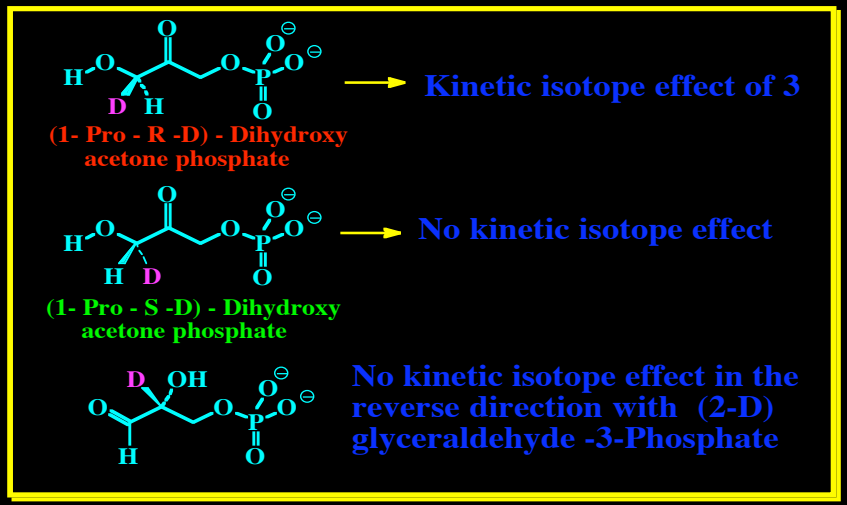
- When the reaction is carried out in D_2O, deuterium gets incorporated into C-2. Thus, there is no direct transfer of hydride from C-1 to C-2.
- In the reverse direction, when C-2 is labeled with tritium some tritium is incorporated into C-1 and some is lost to water. Therefore, the same catalytic base is abstracting and donating the proton.

Affinity labeling with 3-bromoacetol phosphate

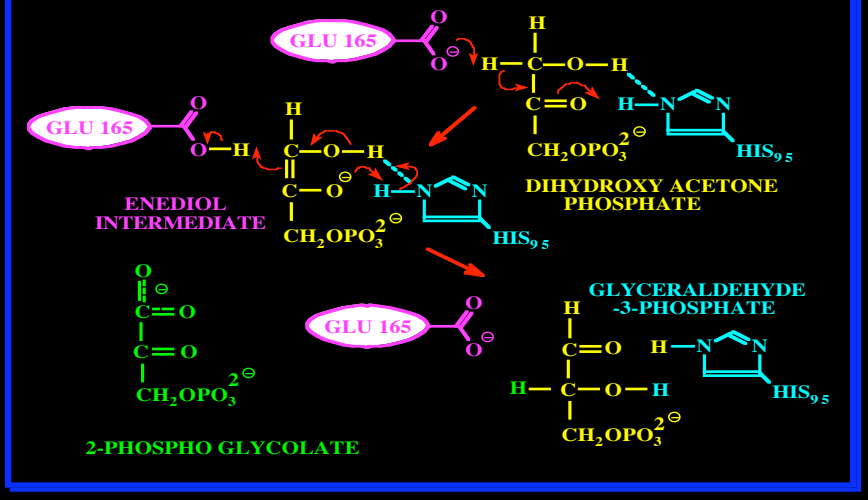


The rate limiting step in forward direction is enediol formation. In the reverse direction, the formation of enediol is not rate limiting (so no kinetic isotope effect).

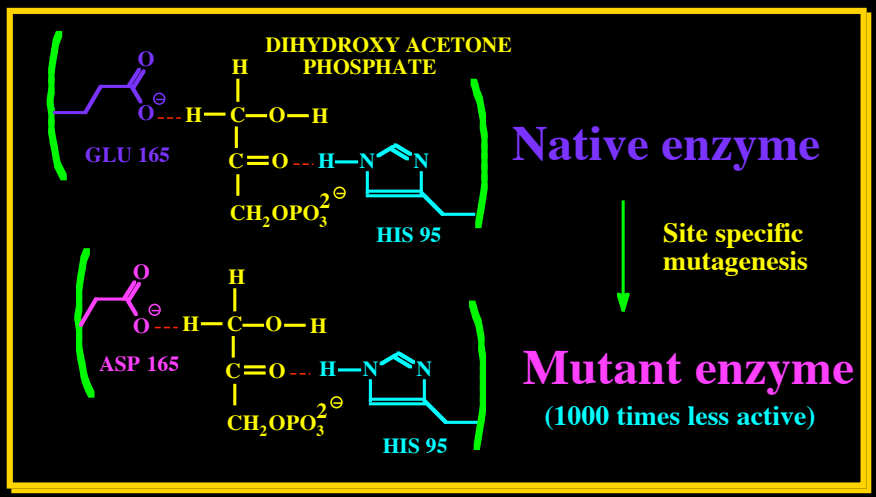
The breakdown of enediol must be rate limiting in this direction.

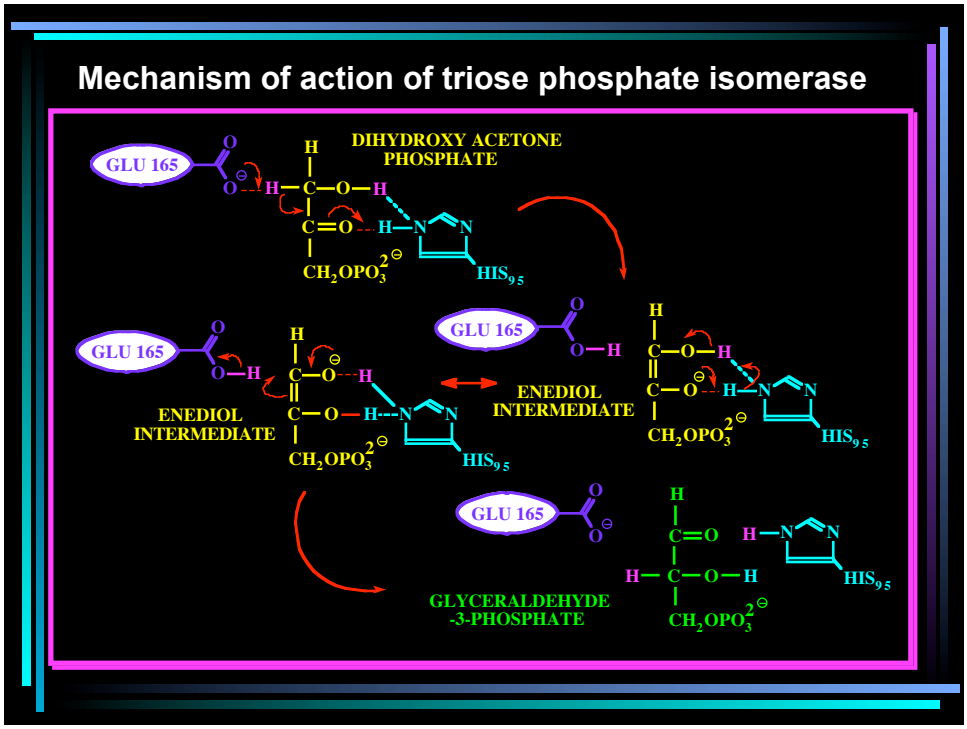


Triose phosphate isomerase tight binding inhibitor - 2-phosphoglycolate



Conversion of Glu 165 to Asp 165 by site specific mutagenesis alters the active site by 1 Å unit. This small change causes the rate to drop by a factor of 1000.




Mechanism of action of triose phosphate isomerase
GLU 165
DIHYDROXY ACETONE PHOSPHATE
$CH_2OPO_3^{2-}$
HIS_{95}
ENEDIOL INTERMEDIATE
GLU 165
ENEDIOL INTERMEDIATE
$CH_2OPO_3^{2-}$
HIS_{95}
GLU 165
GLYCERALDEHYDE -3-PHOSPHATE
$CH_2OPO_3^{2-}$
HIS_{95}