Chemical Mechanism of Enzymes

Enzyme Engineering

5.2 Definition of the mechanism

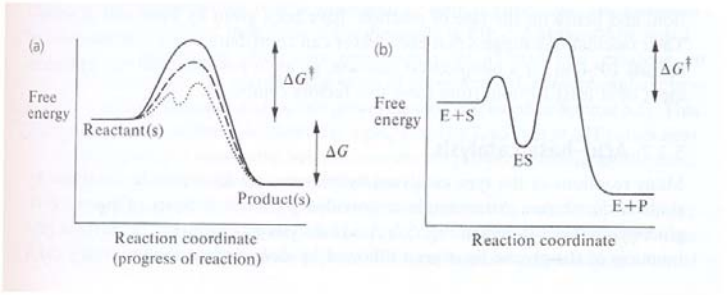
1. The sequence from substrate(s) to product(s) : Reaction steps
2. The rates at which the complex are interconverted
3. The structure of the complex

5.3 Background to catalysis

- Reaction : the energy barrier must be overcame

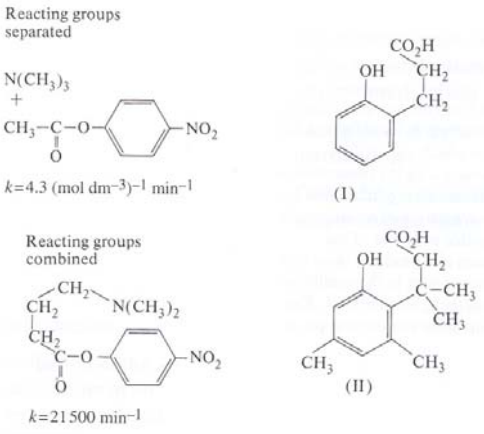
$$k' = \frac{kT}{h} e^{-\Delta G^{\ddagger}/RT} = \frac{kT}{h} e^{-\Delta H^{\ddagger}/RT} \cdot e^{\Delta S^{\ddagger}/R}$$

- Transition state : the highest point of energy
- Catalyst lowered the Gibbs free energy in the transition state
- One example : making a intermediate (covalent catalysis)



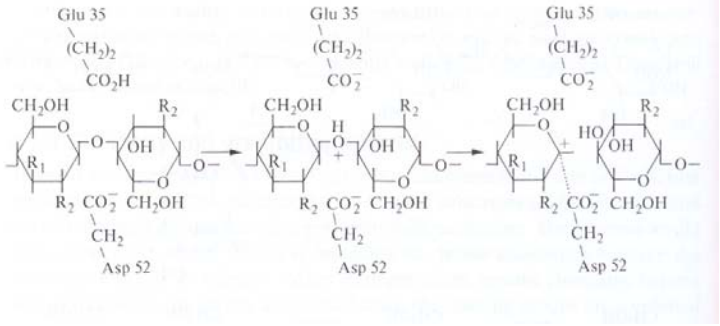
5.3.1 Proximity and orientation effects

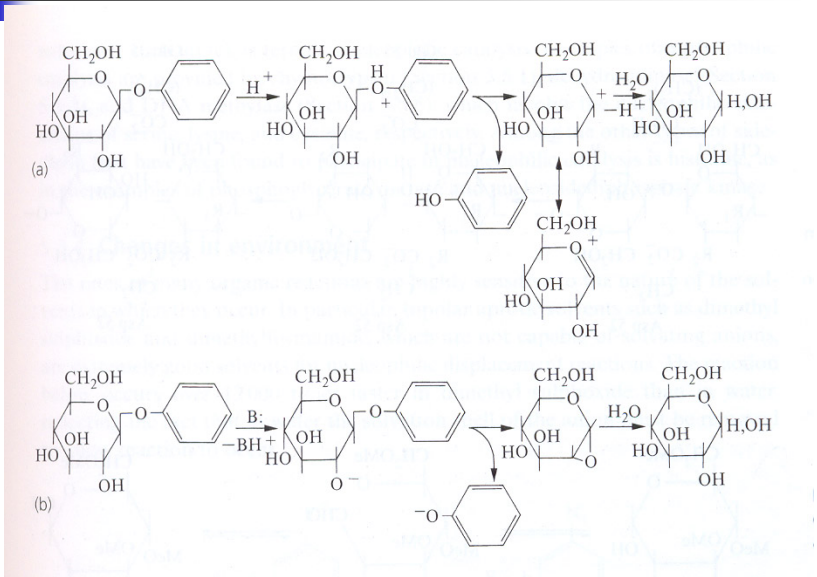
- Enzyme brings the substrates into close proximity and increase the chance of reaction
- The substrates must be in the correct orientation
- Each factors enhance the reaction rate up to 10^4-fold



5.3.2 Acid-base catalysis

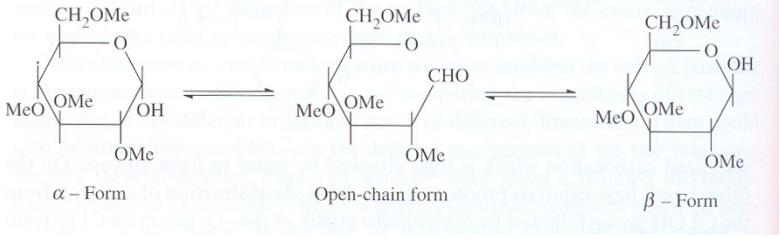
- Acid catalysis provide a proton, while base one abstract a proton
- Glu 35 in lysosome is in highly hydrophobic condition (pK_a goes to 6 from 4.3) → Cleavage occur by ionized Asp 52





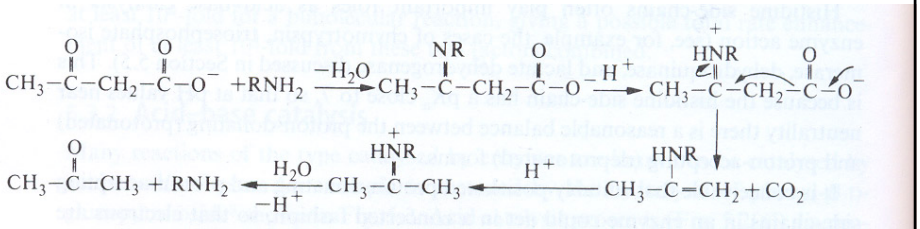
5.3.2 Acid-base catalysis

- Histidine play important roles : pK_a close to 7, balancing between proton donor and acceptor
- Electron instead of proton can be pulled and pushed



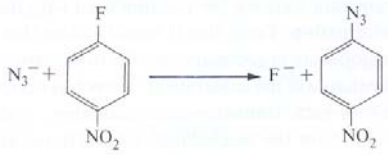
5.3.3 Covalent catalysis

- Formation of intermediates that speed up the reaction
- Schiff base : Rapid formation and breakdown of an imine



5.3.4 Changes in environment

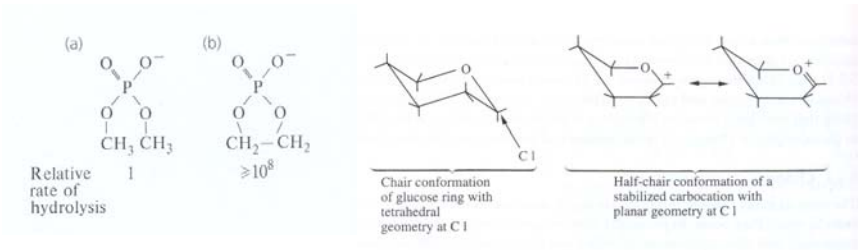
- DMSF, or DMF are good examples of the importance of environment



- 12000 times faster in DMSF than in water
- Enhancement factor is up to 3 X 10^6–fold

5.3.5 Strain or distortion

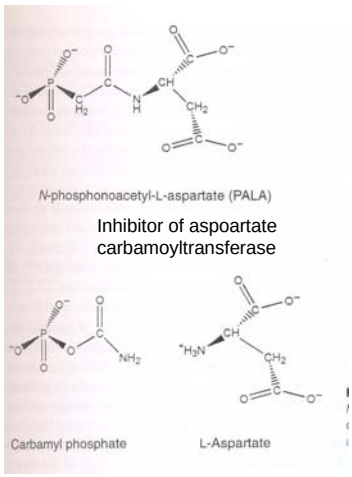
- Strain or distortion of chemical bonds greatly reduce the activation energy, and enhance the rate of reaction
- Often found in many enzymes



Distortion of glucose ring when oligosaccharide is cleaved

5.3.6 Transition-state analogues

- Stably bound to transition state of active sites
- Powerful tool for understanding enzyme mechanisms and for developing inhibitors
- Dissociation constant is slow as well as association constant, reflecting induced fit mechanism



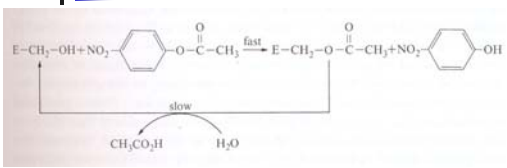
5.3.7 Discussion

- Some other factors, like e-withdrawing effect of metal, may involve in the enzyme catalysis
- Quantitative approach for the factors are being developed
- Some novel enzymes based on the mechanisms were developed
  1. Artificial chymotrypsin was made on oligosaccharide : 5% of activity
  2. Abzymes : antibody + enzyme
  3. New enzymes by changing amino acid sequence

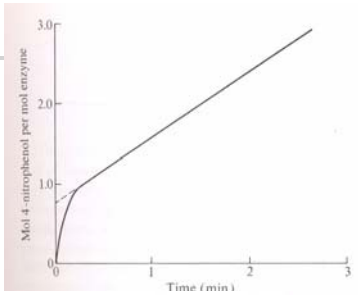
5.4 Experimental Methods

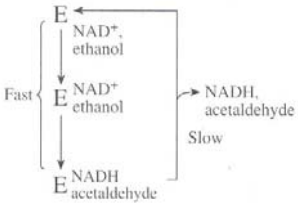
1. Kinetic Studies with changing conditions
   1. Variation of substrate concentrations (complex reactions)
   2. Variation of substrate structure
      - Features of active site can be found (ex. Chymotrypsin, papain..)
   3. With inhibition – Essential structural features can be studied
   4. Variation of pH → Checking pK_a of the enzyme
      Involvement of His can be found
   5. Pre-steady-state Kinetics

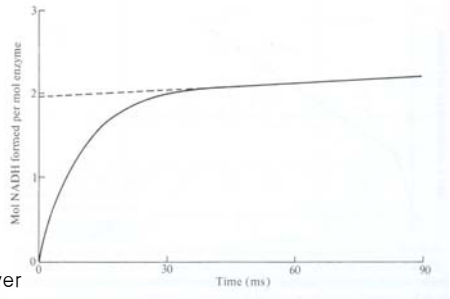
5.4.1 Kinetic studies



Chymotrypsin with 4-nitrophenyl acetate



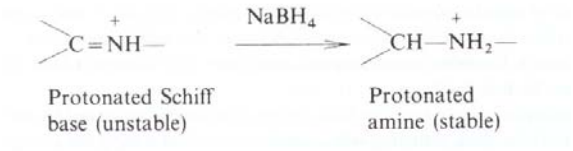




Alcohol dehydrogenase from liver

5.4.2 Determining intermediates

- Acyl-enzyme or NADH-bound enzyme
- Breakage of acyl-enzyme is even slower in low pH → X-ray crystallographic study is possible
- In chymotrypsin, breakage of acyl-enzyme is much faster than formation of complex → Trapping method
- Trapping of imine (Schiff base) using $NaBH_4$

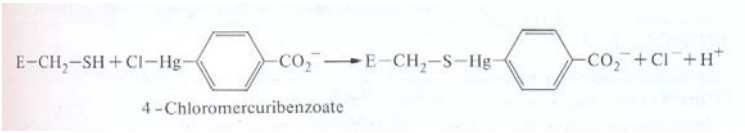


5.4.3 X-ray crystallography studies

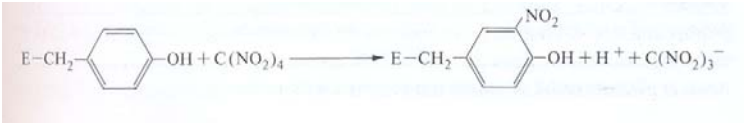
- Long time taken to make crystal : Stable complex between substrate and enzyme is required
- When the equilibrium shifts substrate or product
- when enzyme is bound with poor substrate of inhibitors
- Mutant enzyme with slow kinetics is useful too
- Essential step for proving the proposed enzyme mechanisms

5.4.4 Chemical modification of AA

- Specificity (one Lys among 30 should be modified) is difficult to achieve and must be checked by Mass spec, etc.
- High affinity b/n Mercury & Sulfur or b/n Tyr & $C(NO_2)_4$



4-Chloromercuribenzoate

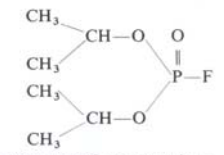


Other reagents for chemical modification

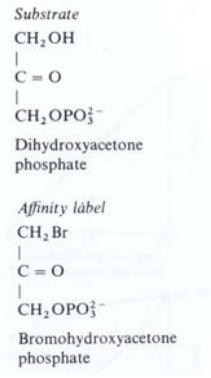
Side-chain	Reagent(s) used
Cysteine	Mercurials, e.g. 4-chloromercuribenzoate Disulphides, e.g. 5,5′-dithiobis-(2-nitrobenzoic acid) Iodoacetamide Maleimide derivatives, e.g. *N*-ethylmaleimide
Lysine	2,4,6-Trinitrobenzenesulphonate Pyridoxal phosphate (± reducing agent such as $NaBH_4$)
Histidine	Diethylpyrocarbonate Photo-oxidation
Arginine	Phenylglyoxal 2,3-Butanedione
Tyrosine	Tetranitromethane *N*-Acetylimidazole Iodine
Tryptophan	*N*-Bromosuccinimide
Aspartic acid or Glutamic acid	Water-soluble carbodiimide plus nucleophile, e.g. glycine methylester

5.4.4 Chemical modification of AA

- Super reactive side chain
  - Some chemicals bind with highly reactive side chain
  - 2,4,6-trinitrobenzenesulphonate with glutamate dehydrogenase
  - Not always with the active site aa.
- Affinity labeling
  - Incoporating structural feature to the reactive chemical
  - Important for pharmaceutical industry



Diisopropylfluorophosphate



5.4.4 Chemical modification of AA

- Interpretation
  - Modification in other place can inactivate the enzyme → Need to be confirmed by chemicals with different size (ex. Cys of creatine kinase)
  - Relationship b/n modification and activity
  - Addition of substrate before chemicals must reduce the inactivation effect

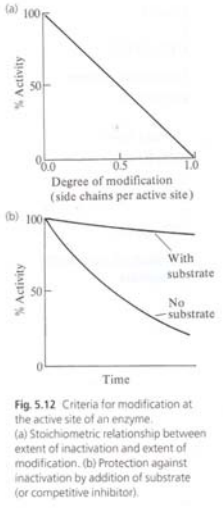
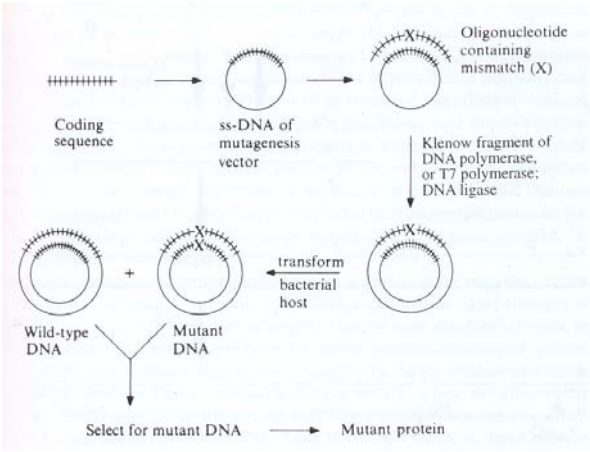


Fig. 5.12 Criteria for modification at the active site of an enzyme. (a) Stoichiometric relationship between extent of inactivation and extent of modification. (b) Protection against inactivation by addition of substrate (or competitive inhibitor).

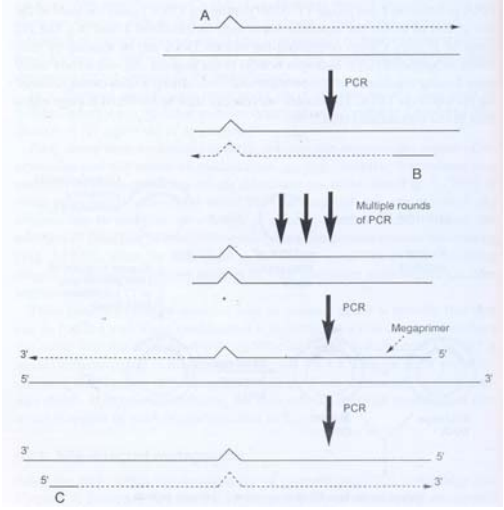
5.4.5 Site-directed Mutagenesis

- Highly Specific
- Method 1 – Using oligonucleotide



5.4.5 Site-directed Mutagenesis

- Method 2 – Using PCR



5.4.5 Site-directed Mutagenesis

- Choice of mutated aa
  - From structural database, candidate aa in active site can be chosen
  - Similar size and polarity with different chemical property
  - If flexibility is questioned, mutate to Pro

Amino acid	Replacement
Ala	Ser, Gly, Thr
Arg	Lys, His, Gln
Asn	Asp, Gln, Glu, Ser, His, Lys
Asp	Asn, Gln, Glu, His
Cys	Ser
His	Asn, Asp, Gln, Glu, Arg
Leu	Met, Ile, Val, Phe
Lys	Arg, Gln, Asn
Ser	Ala, Thr, Asn, Gly
Tyr	Phe, His, Trp
Trp	Phe, Tyr

5.4.5 Site-directed Mutagenesis

- Novel aa (Non-natural amino acid) can be introduced : Mixture of site-directed mutagenesis and chemical modification
- Chemical modification introduces a bulky group, which may alter the enzyme structure (must be checked with other method like circular dichroism spec.)
- Chemical modification is useful to introduce other functionality (heavymetal for X-ray crystallography)
- Site-directed mutagenesis : more specific and no limitation

5.5 Examples

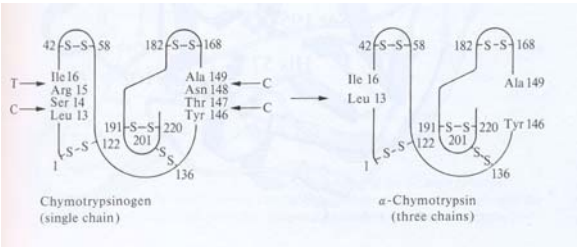
5.5.1 Chymotrypsin

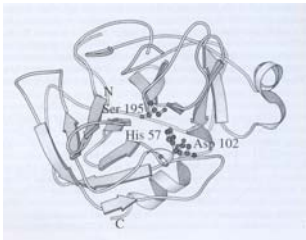
- Synthesized in acinar cells of the pancreas as chymotrypsinogen (precursor) with 230 residues
- Activated by proteolysis, which cut b/n Arg 15–Ile 16 by trypsin, then b/n Leu 13–Ser 14 by chymotrypsin, and cut out Thr 147–Asn 148
- 3 polypeptides connected by 3 disulfide bonds
- Cleavage on amide and ester bonds
- Higher activity and less specificity on ester bond
- Cleavage of peptide bond after hydrophobic side chain (Trp, Tyr, Phe, Leu, Met)

5.5.1 Chymotrypsin

- Two domains (aa 27–112 and aa 133–230)
- Active sites are located between two domains
- X-ray crystallography studies showed

1. The hyper-reactivity of Ser 195

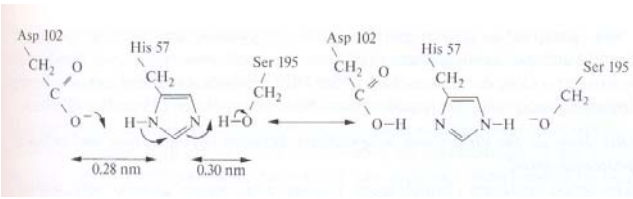


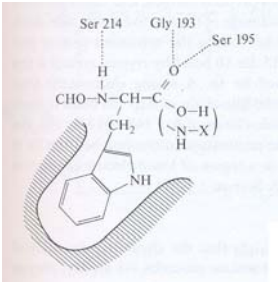


5.5.1 Chymotrypsin

- All serine protease have the same active site
- Asp102 → Asn decreases the activity 5000-fold
- Asp102 is more important on Ser195 than His57

2. Substrate binding
   - Studied by an inhibitor (N-formyl tryptophan)
   - Aromatic side chain binds in the pocket of non-polar side chains

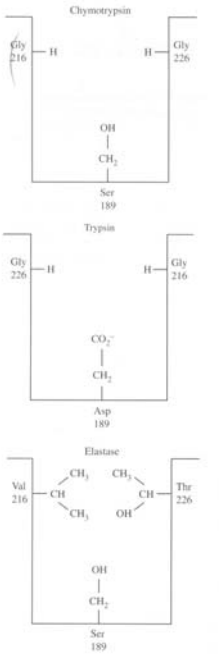




5.5.1 Chymotrypsin

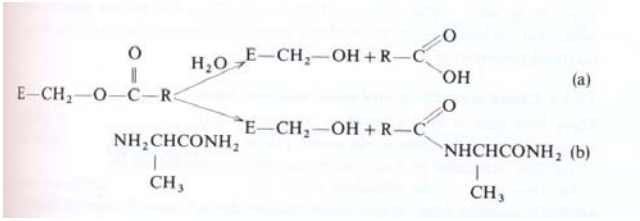
3. The difference of substrate specificity of serine proteases
   - Difference of the aa in the side chain

4. How the zymogen activated?
   - No change of active site
   - Formation of pocket structure by proteolysis : positive charge of Ile16 with Asp192 move other side chains of Arg145 Met192 into the pocket



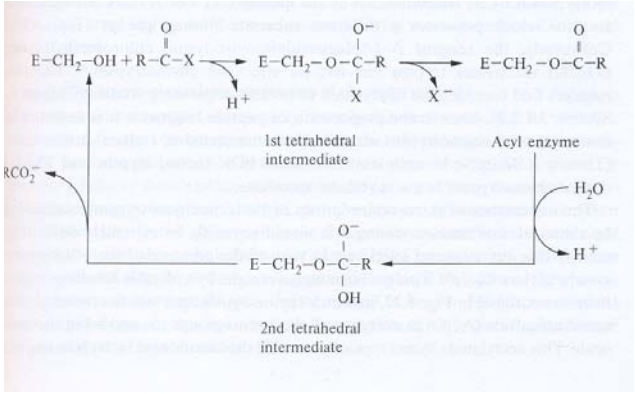
5.5.1 Chymotrypsin

- Detection of intermediates
  - Hydrolysis of ester: deacylation is slower
  - Hydrolysis of amide: deacylation is faster
  - No acylated enzyme detected
  - Acylation is detected by “trapping” method



5.5.1 Chymotrypsin

- Tetrahedral intermediate was also detected
- Oxyanion of the structure is stabilized by NH groups of Gly193 and Ser19 : increasing the activity ~1000-fold

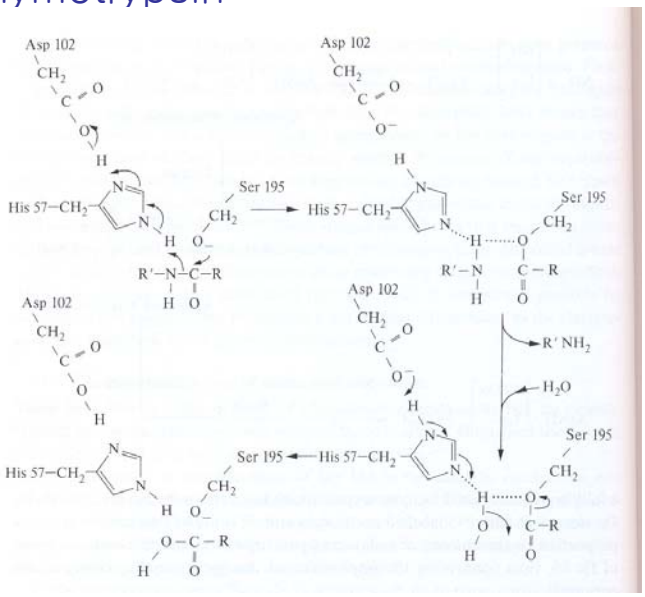


5.5.1 Chymotrypsin

- Chemical modification
  - Ser195 was modified by DISF or PMSF
  - His57 was labeled by TPCK
  - Chemical modification of NH2 was used to reveal the importance of NH3+ of Ile16
- Kinetic study
  - Formation of acyl-enzyme complex
  - Specificity of enzyme to specific aa
  - Kinetic constant of each reaction step

5.5.1 Chymotrypsin

- Proposed mechanism

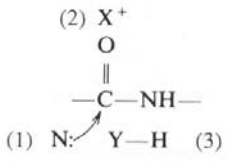


5.5.1 Chymotrypsin

- Comparison with other proteases
  - Every protease have the following three components

1. Nucleophilic group attacking carbonyl group of substrate
2. Positive charge stabilizing oxyanion
3. Proton donor, to make NH- leaving group

- In chymotrypsin, 1 → Ser195, 2 → -NH- of Gly193 and Ser195, and 3 → His57



5.5.1 Chymotrypsin

- Carboxypeptidase
  - 1 → Water activated by Glu270
  - 2 → Zn^{2+} ion
  - 3 → ?? (Tyr248 was proposed but rejected by site-directed mutagenesis)
- Thiolpeptidase(Papain) use cystein instead of serine
- Acid protease (HIV protease) use H_2O activated by Asp