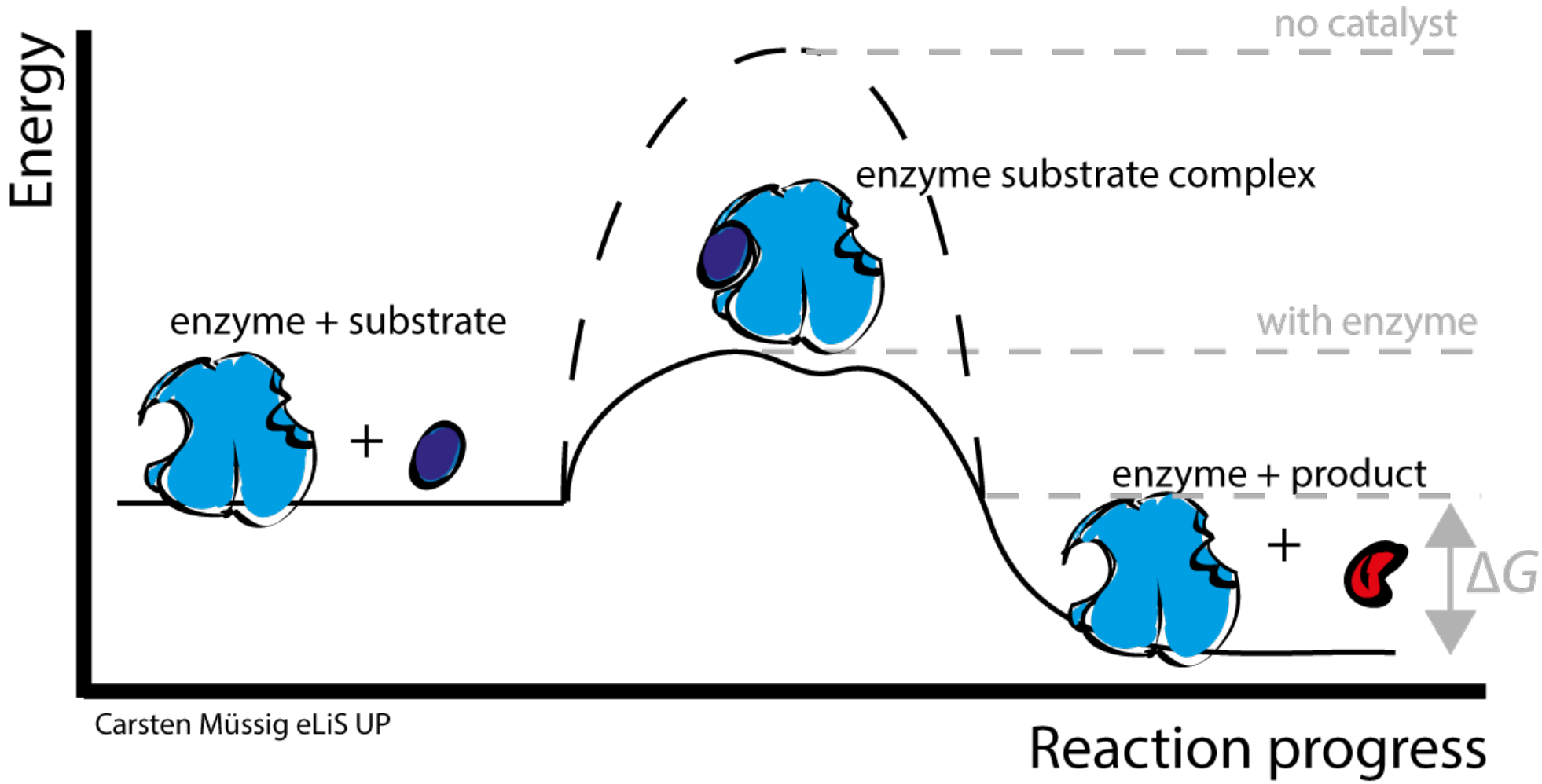
Lect#12: Active Site Investigations

- **(1) Kinetic Studies**
  - **(a) Variation of substrate concentration**
  - **(b) Variation of substrate structure**
  - **(c) Reversible inhibition**
  - **(d) Variation of pH**
  - **(e) Pre-steady state kinetics**
- **(2) Detection of Intermediates**
- **(3) X-ray Crystallographic Studies**
- **(4) Protein NMR**



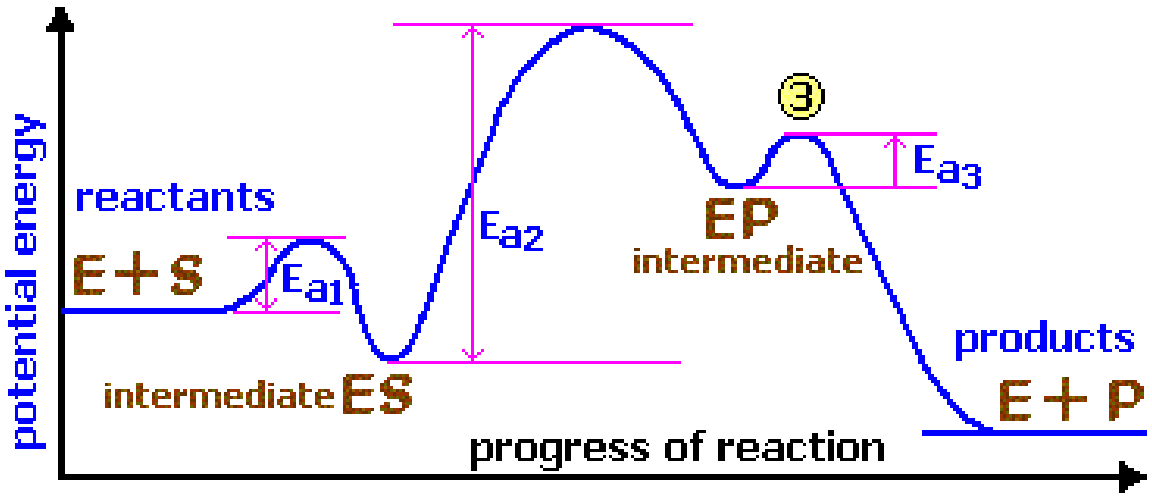
Lecture #12: **Active Site Investigations I**

1. Kinetic Studies

a. Variation of substrate concentration

- steady state kinetic studies indicate that substrate reactions proceed **via the formation** and decay of one or more E-S complexes
- **do not provide information on the temporal sequence** of complexes
- need **substrate binding** and **product inhibition** to distinguish between mechanisms in which a ternary complex is formed in an **ordered** or a **random** fashion

Energetics of Substrate Binding to Enzymes



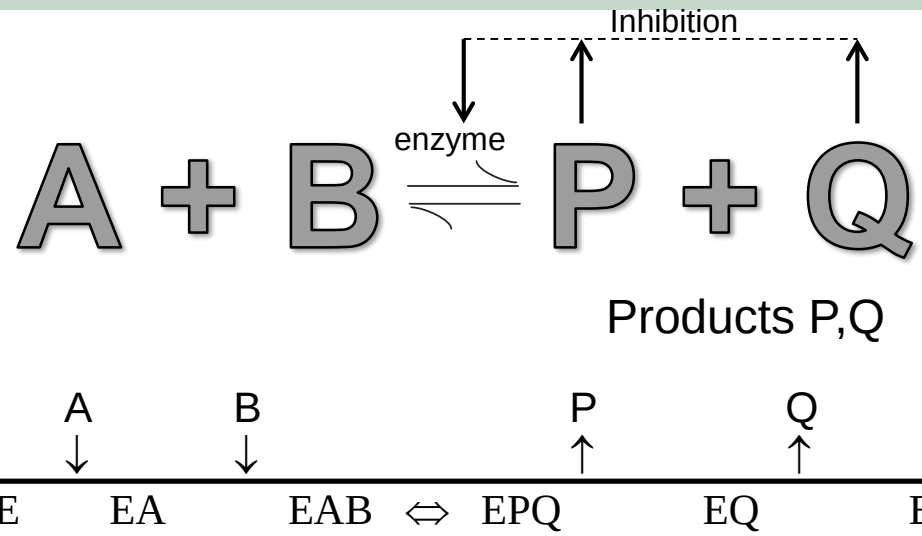
$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} EP \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} E + P$$

$$K_{D1} \qquad K_{D2} \qquad K_{D3}$$

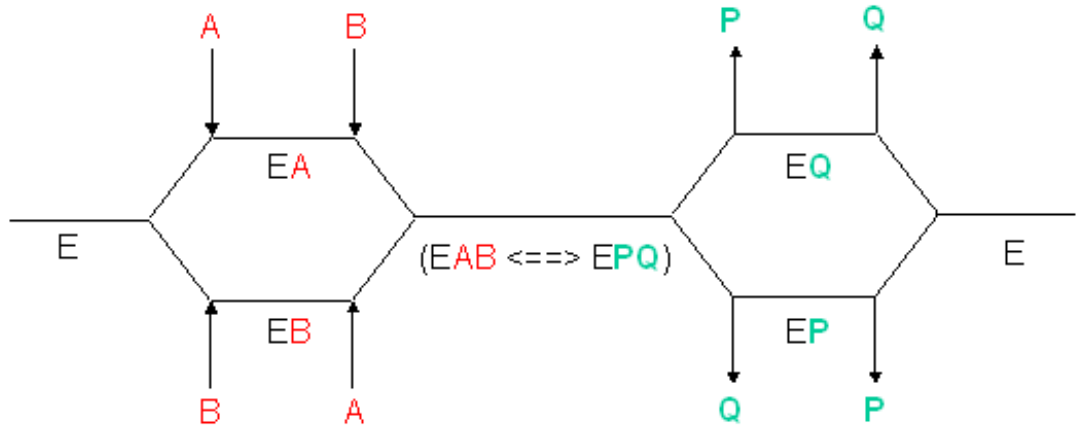
Product Inhibition

- to determine the reaction mechanism type (ordered versus random) and which substrate binds first, second, etc

- **Ordered Bi Bi** mechanism may be experimentally distinguished from the **Random Bi Bi** mechanism
- if only 1 product (P or Q) is added to the reaction mixture, the reverse reaction can't occur but the **product** by **binding to the enzyme** will **inhibit** the forward reaction



- product Q **directly competes** with substrate A (at fixed [B]) **= competitive inhibitor**
- since B combines with EA, not E, then Q is a **mixed inhibitor** of B when [A] is fixed
- product P combines with EQ so it is a **mixed inhibitor** of both A and B substrates



- in a **random mechanism:** both substrate and product binding and release are **random**

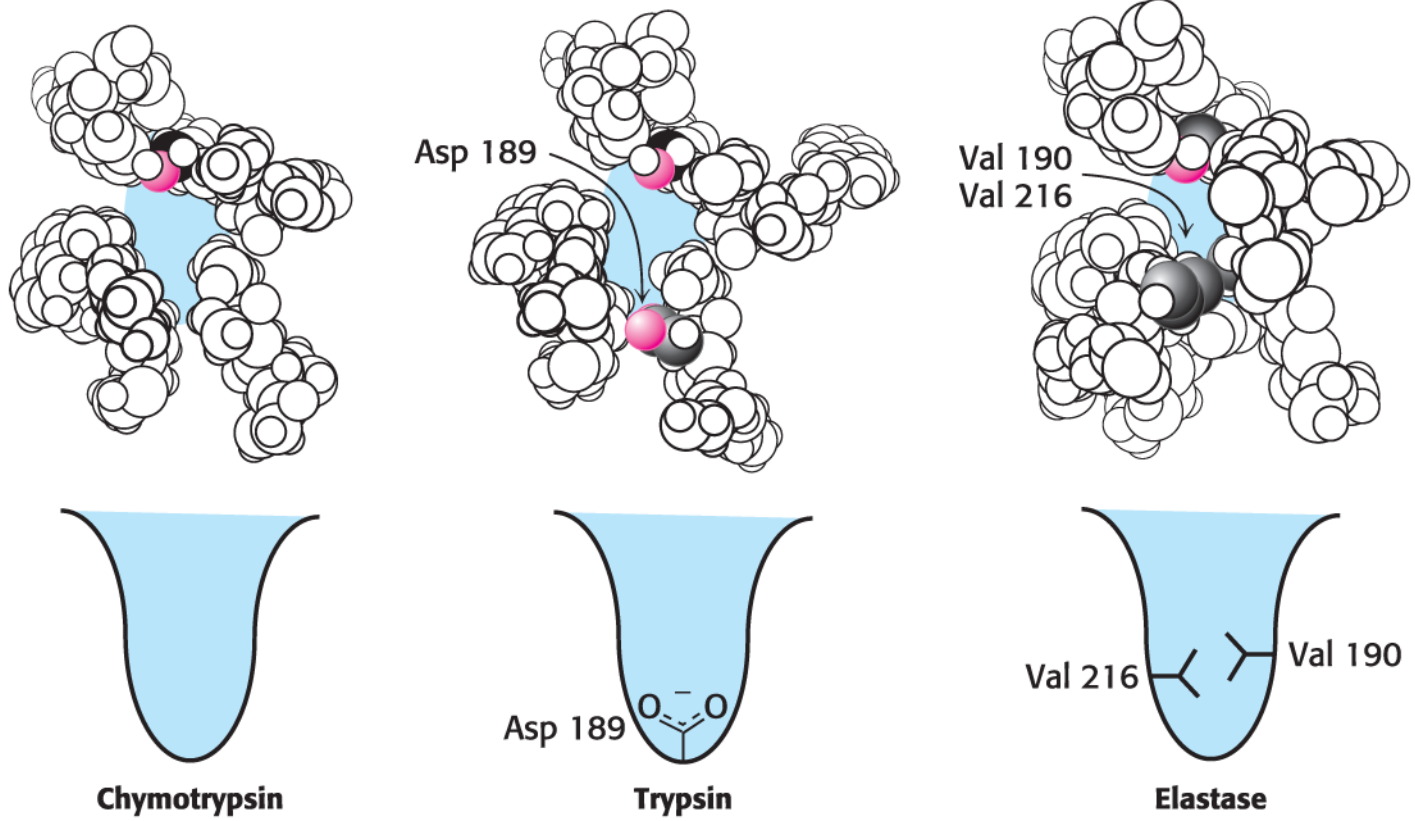
then both P and Q are **<u>competitive inhibitors</u>** of A when [B] is fixed and of B when [A] is fixed

-product inhibition pattern will help to determine the **type of reaction mechanism** in operation for a given enzyme

b. <u>Variation of substrate structure</u>

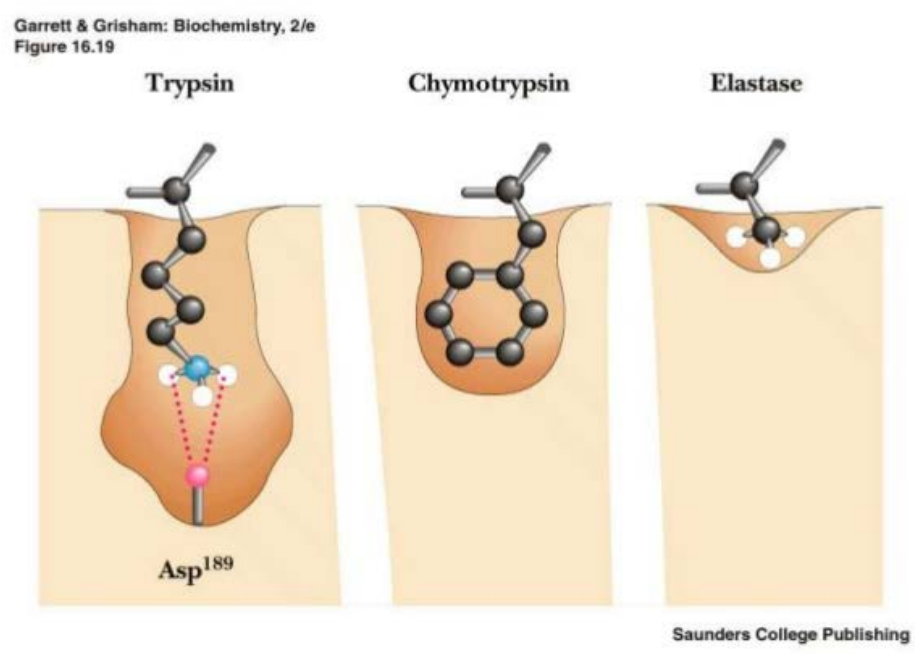
- can learn a great deal about the general features of enzyme active sites by correlating the **rates of reactions** with the **<u>structures</u> of the substrates**

- eg., <u>serine proteases</u>: chymotrypsin, trypsin, and elastase show different specificities for R groups around peptide (amide) bond
    - the substrate-binding sites of these enzymes **must contain features** that account for the observed specificities



Berg et al., *Biochemistry*, 9e, © 2019 W. H. Freeman and Company

G&G3:F14-20



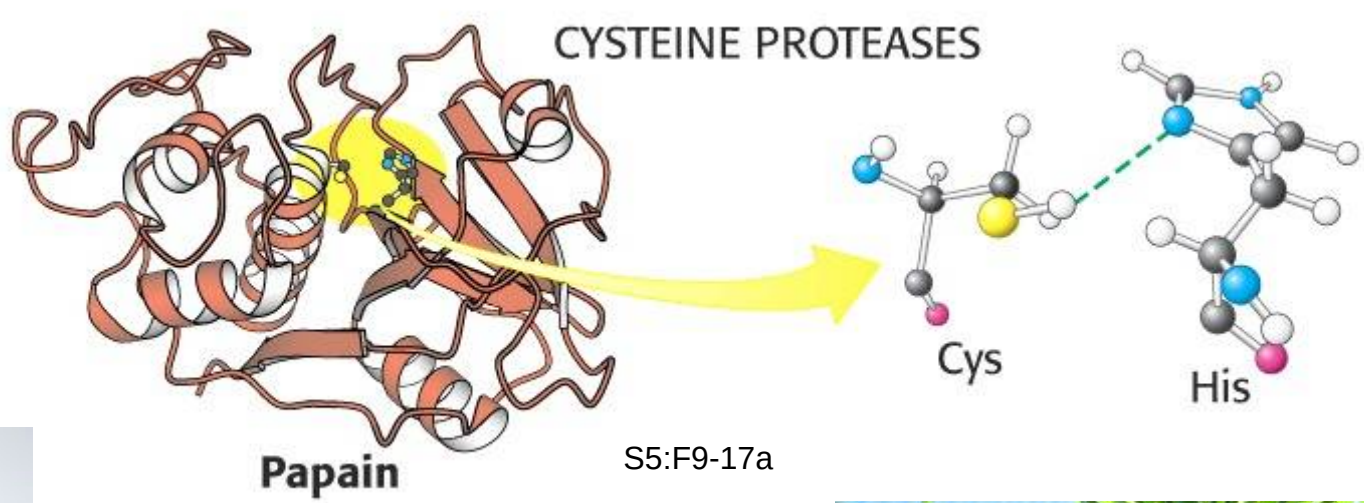
- binding pockets of enzyme matches the residue side chain for chemical, electrostatic and steric complementarity

Papain

- Found in papaya
- **papain**, also known as **papaya proteinase I**
- Broad pH (3-11) and temperature stability
  - For this reason very popular for a variety of food applications.

Used as a meat tenderizer on inferior meat cuts (can also use slice of pineapple on meat)

- The enzyme makes its way into the muscle and hydrolyzes primarily connective tissue proteins (collagen etc.) and softens muscle
- Have to use low amount to prevent liquefaction of muscle
- If you mix raw papaya into Jell-O it will not form a gel



S5:F9-17a

P&S:F5-8

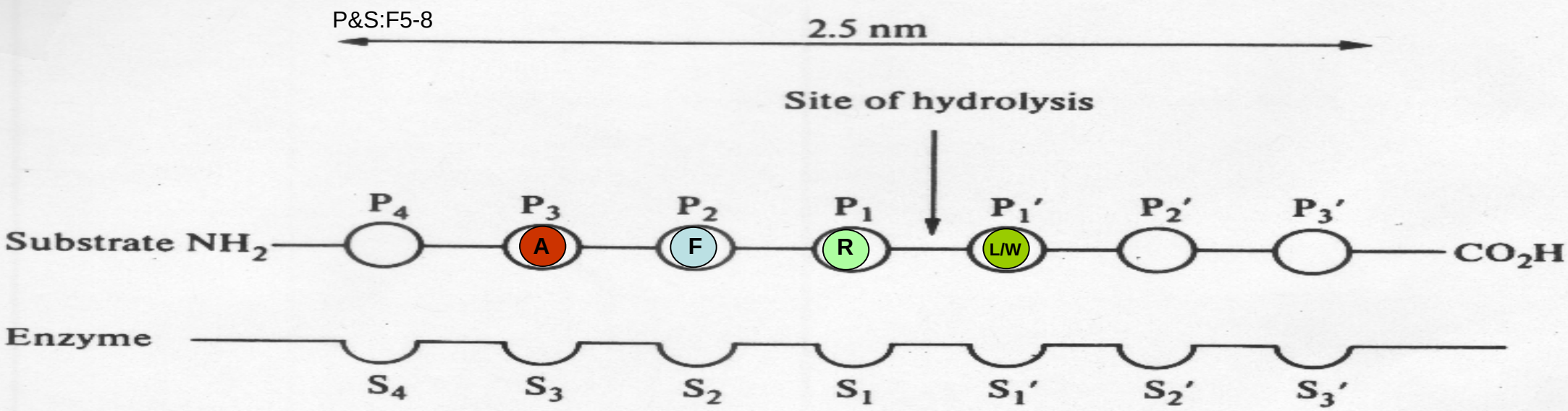
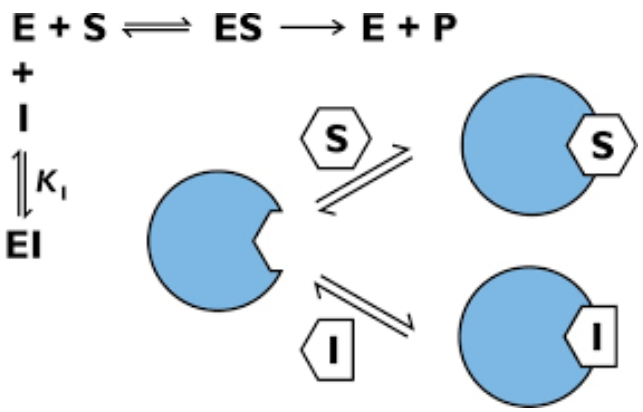
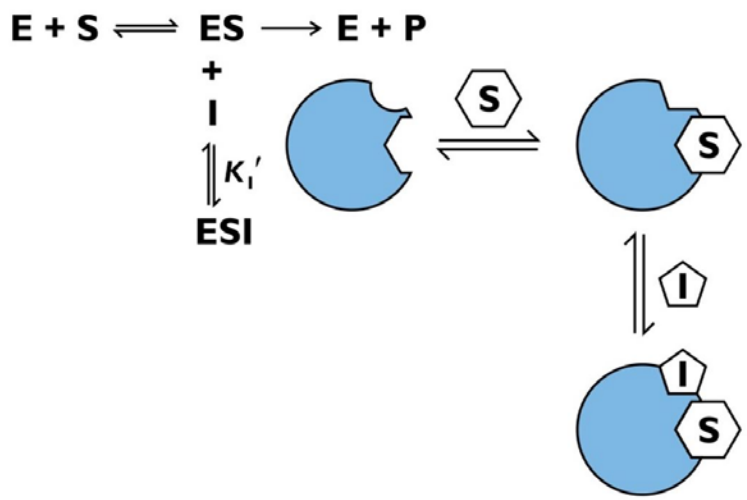


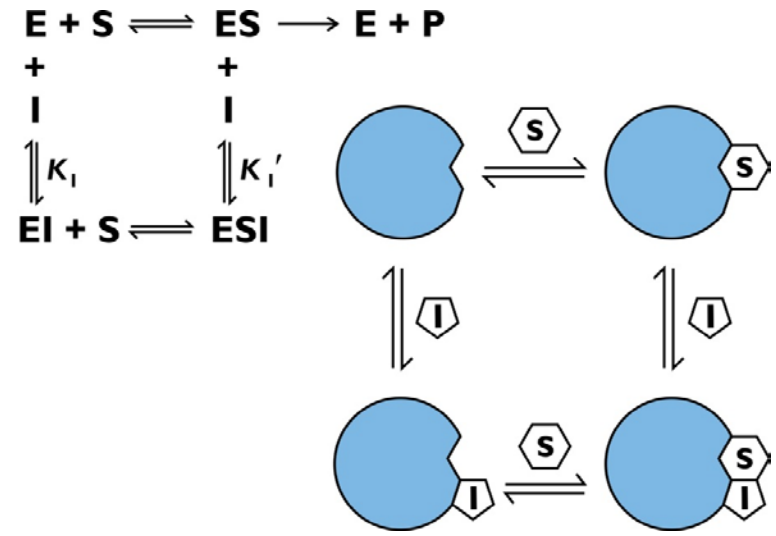
FIG. 5.8. The 'subsites' of papain as revealed by active-site mapping.

- exhaustive studies on **specificity of papain** towards synthetic peptide substrates
  - several **subsites** on the enzyme
  - subsite S_2 interacts specifically with the L-Phe side chain
  - subsite S_1' is stereospecific for L-amino acids with a preference for the hydrophobic side chains of Leu and Trp
  - the tripeptide, **Ala-Phe-Arg** is a powerful **competitive inhibitor** of the enzyme since it occupied the subsites S_3, S_2, and S_1 and can't be hydrolyzed

c. <u>Reversible inhibition</u>--study of inhibition of enzyme-catalyzed reactions can give information on the structures of active sites







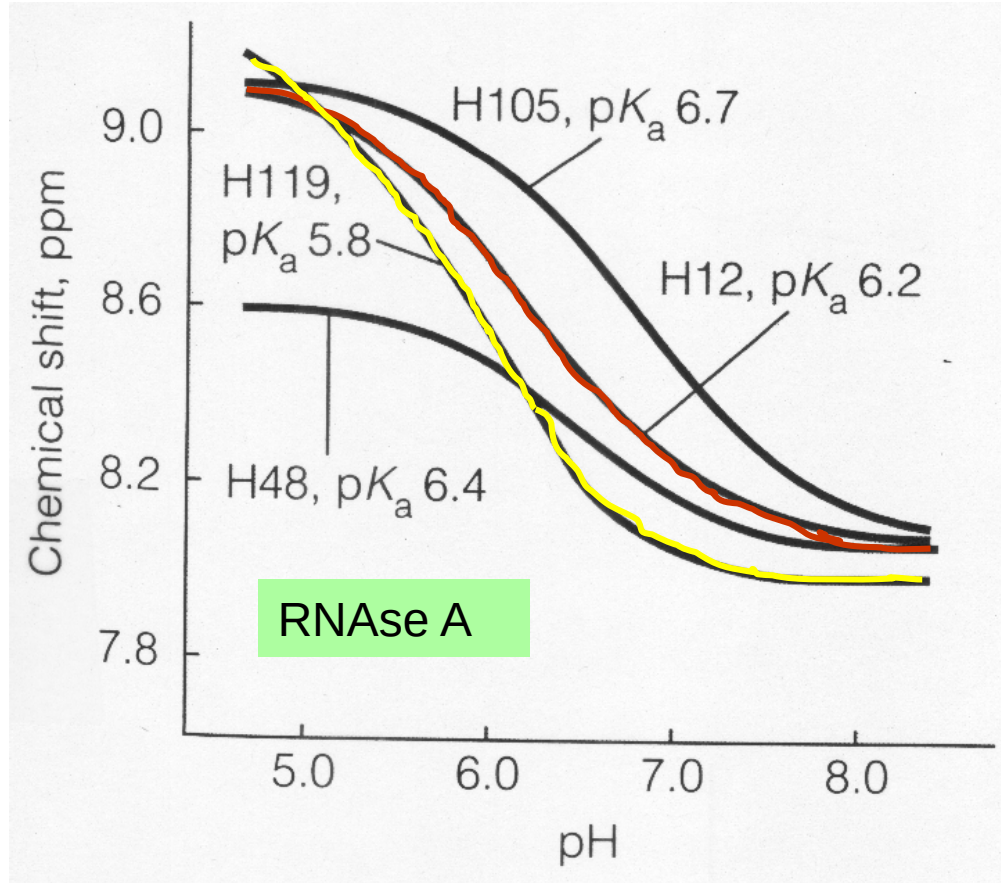
- most likely explanation for **competitive inhibition** is that the substrate and inhibitor bind to the same site on the enzyme

- **compare the structure** of the substrate and inhibitor and deduce the essential structural features of these molecules

- competitive inhibitors are used in **X-ray crystallographic** studies where it is usually difficult to study the E-S complex directly

d. <u>Variation of pH</u>

- catalytic activity of many enzymes is markedly dependent on pH
- due to the ionization of the amino acid side chains involved in the catalytic mechanism
- plots of reaction rate versus pH makes it possible to deduce the pK_a values of the side chains involved in catalysis
- <u>caveat:</u> **microenvironment** of a side chain can shift the pK_a of the side chain by up to four pH units
  - two His side chains shown to be involved the catalytic mechanisms of ribonuclease A, later confirmed by NMR spectroscopy



e. <u>Pre-steady state kinetics:</u> detect enzyme-containing complexes to determine their rates of formation and decay

- enzyme concentration = substrates **<u>but usually requires special techniques</u>** to achieve rapid mixing and rapid detection of the events
- eg., chymotrypsin catalyzed hydrolysis of p-nitrophenylacetate
- production of p-nitrophenol shows a burst phase (1 mol per mol enzyme) then a slower steady state rate
- fast step corresponding to the formation of acyl enzyme and release of p-nitrophenol
- slow step, corresponding to the hydrolysis rate of the acyl enzyme

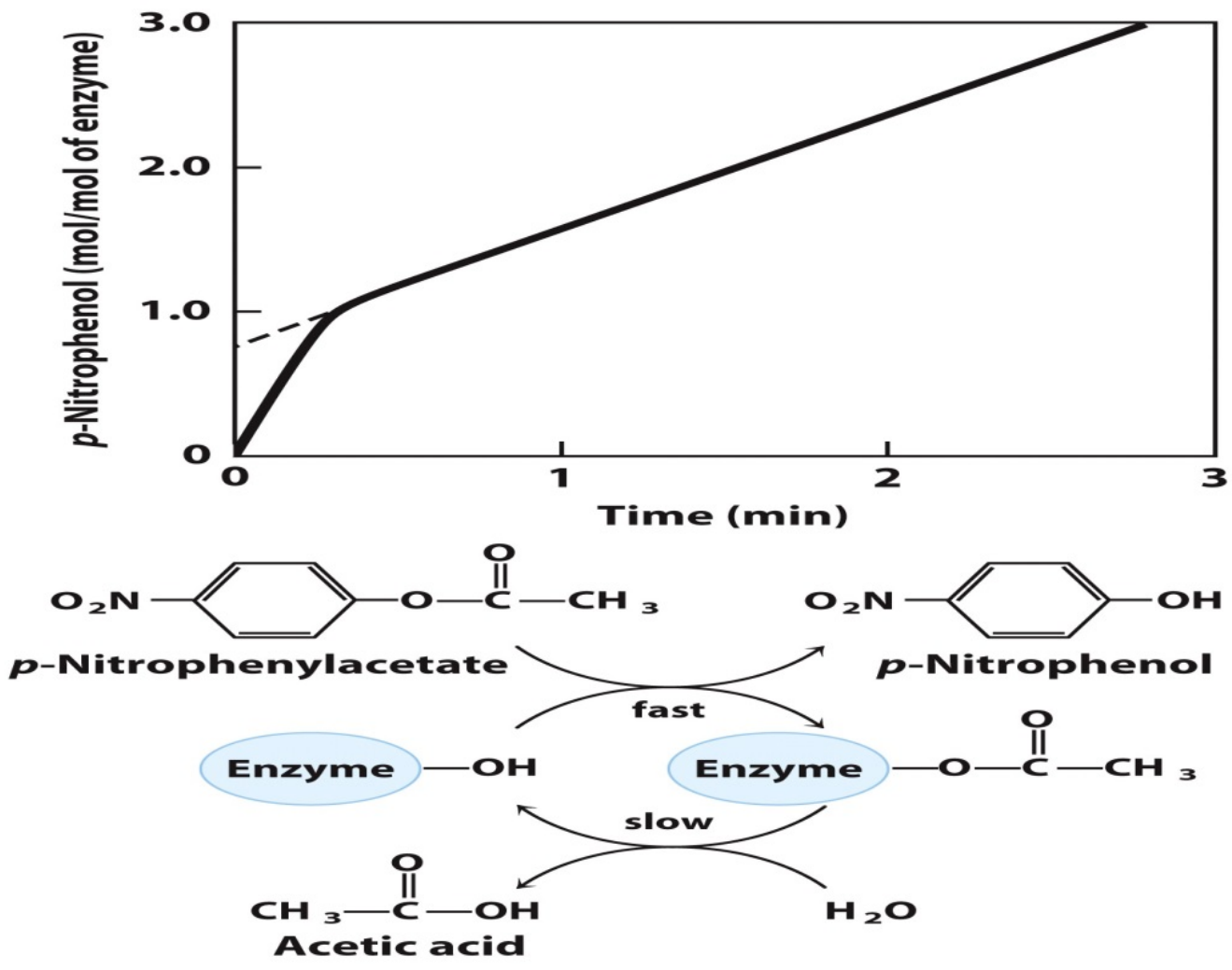
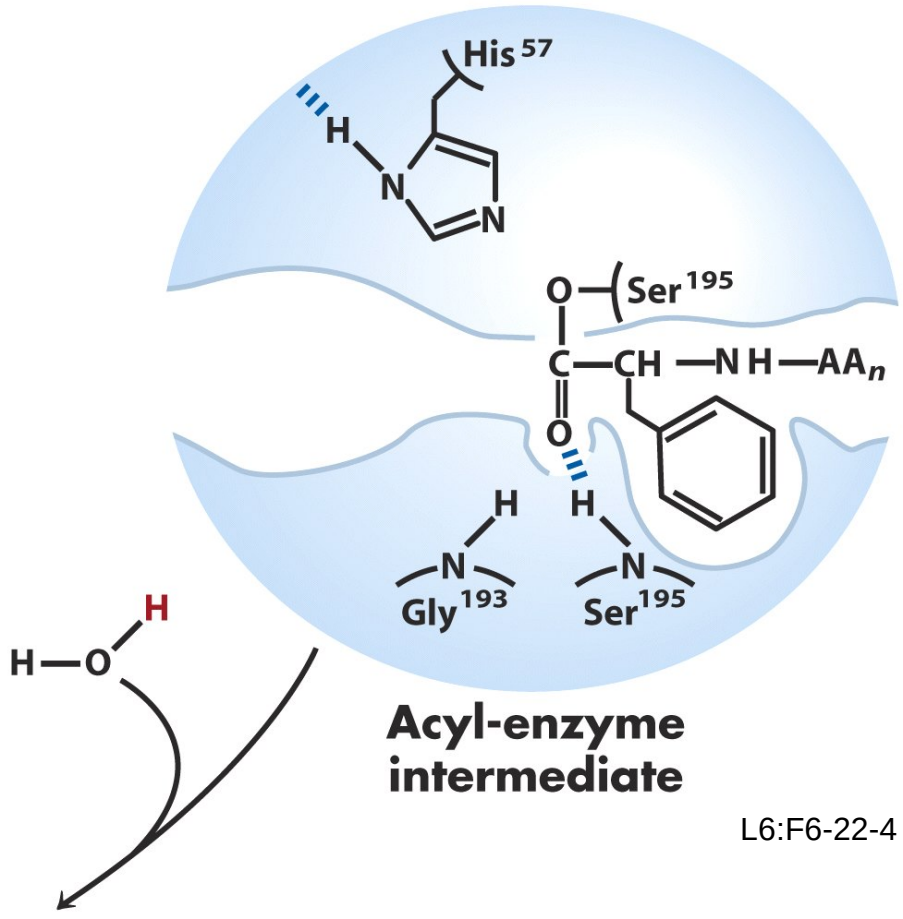


Figure 6-20
Lehninger Principles of Biochemistry, Sixth Edition

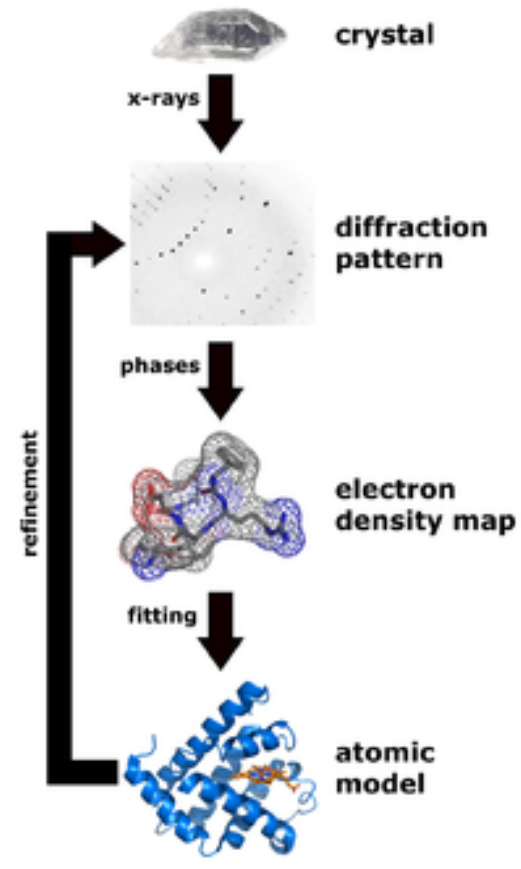
2. <u>Detection of Intermediates</u>

- study of the reaction pathway is to **detect any intermediates** involved in the reaction
- must be sufficiently stable to be isolated and characterized
- may be inferred to exist from spectroscopic studies
- rates of formation and decay of intermediates must be consistent with the **overall rate** of the reaction
- rate of **breakdown** of the acyl enzyme intermediate of chymotrypsin is **very slow** at acid pH
  - intermediate was crystallized by mixing enzyme and ester substrate and rapidly pulsing the solution pH (acidification)

-X-ray structure showed that **Ser 195** becomes **acylated**



L6:F6-22-4



3. <u>X-ray Crystallographic Studies</u>

- collection of diffraction data takes hours and S → P occurs
- Approaches to X-ray structure determination of active site

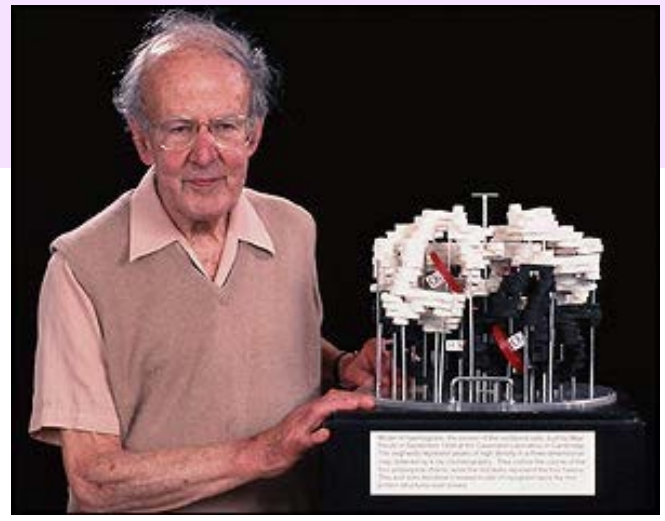
(i) structure of active complex if the **equilibrium** lies to one side

(ii) enzyme structure with poor substrate or **competitive inhibitor**

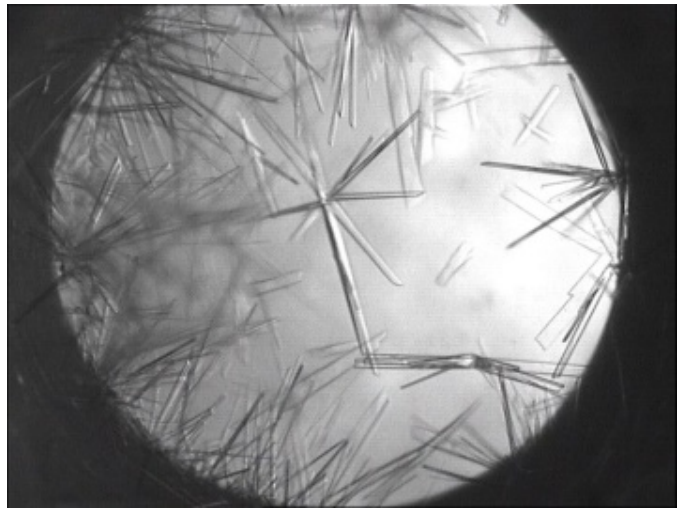
(iii) structure of unstable complexes at **low temperature**

(iv) use synchrotron radiation to reduce data collection time to seconds

(http://www.vega.org.uk/video/programme/132)

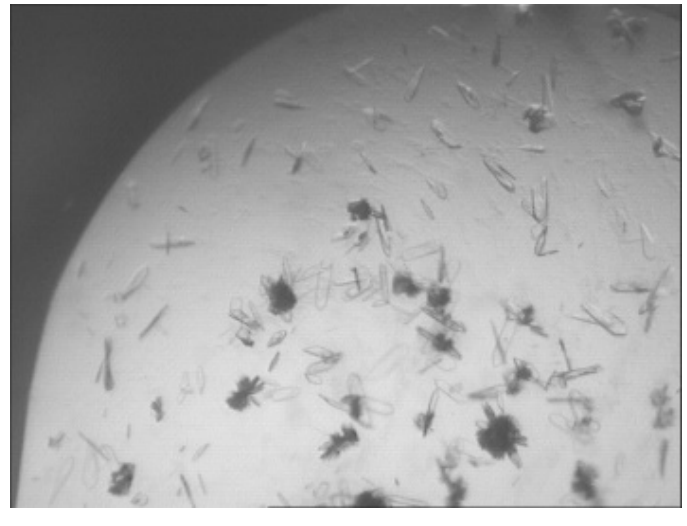


Max Perutz with hemoglobin model



E-P complex for P. A. toxin

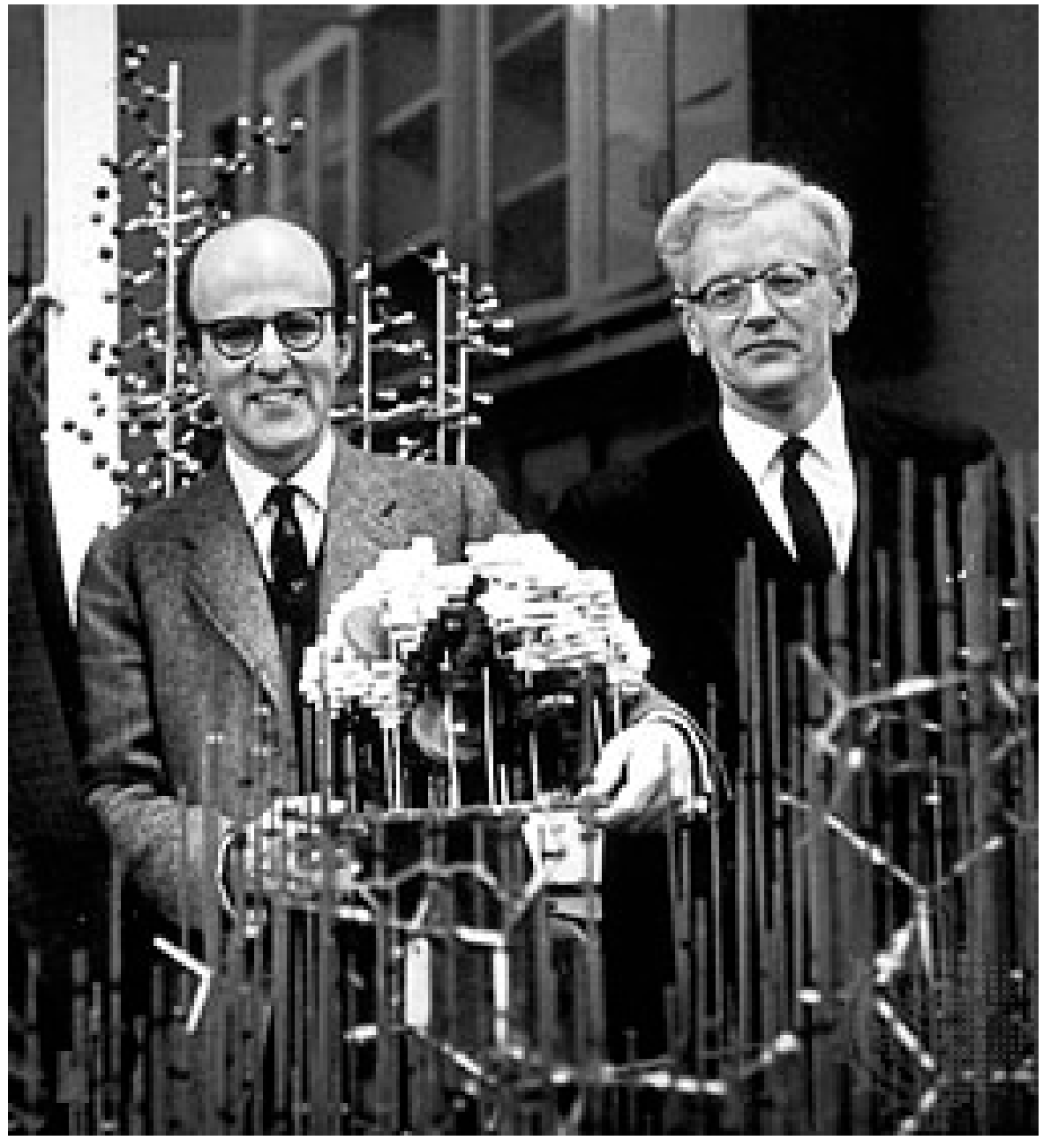
Nature (2005) **436**, 979-984

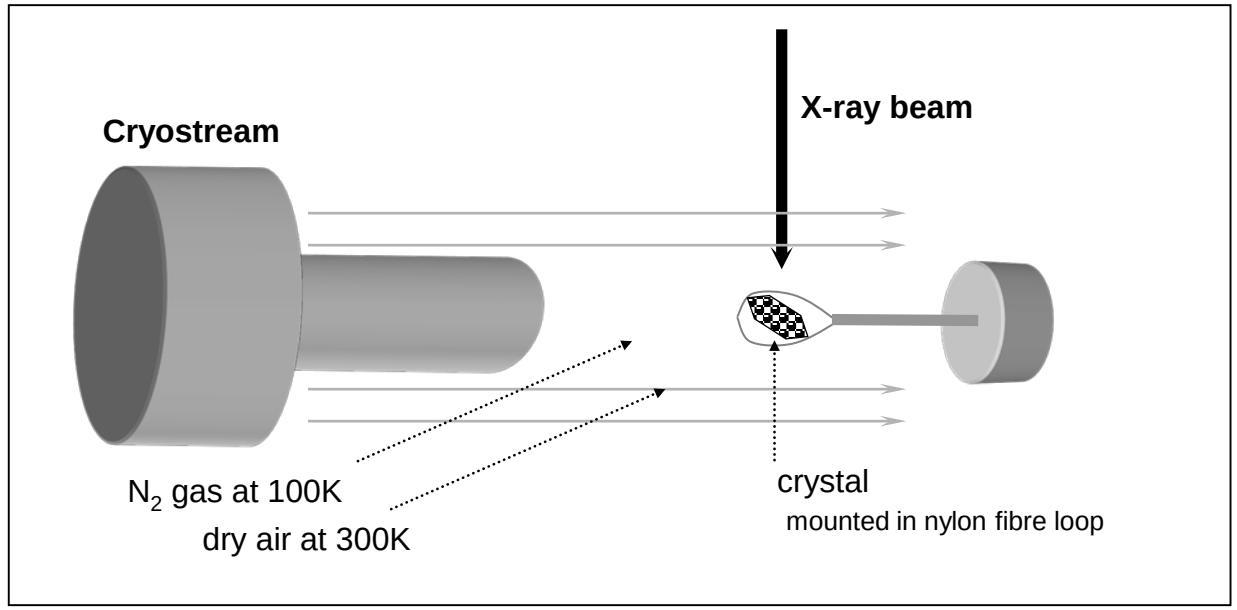


E-I complex for P.A. toxin

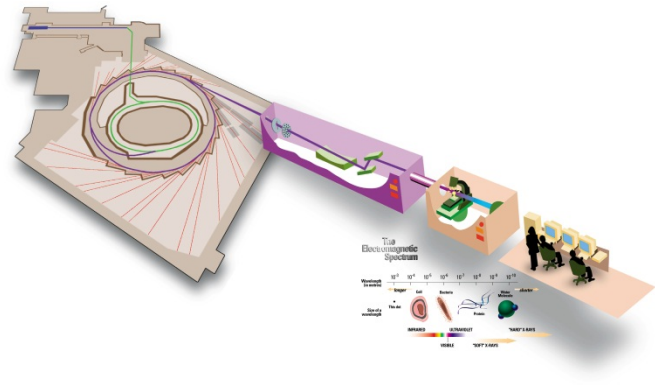
Biochem. J., (2005) 385, 667-675.

Max Perutz
Nobel Prize in Chemistry in 1962

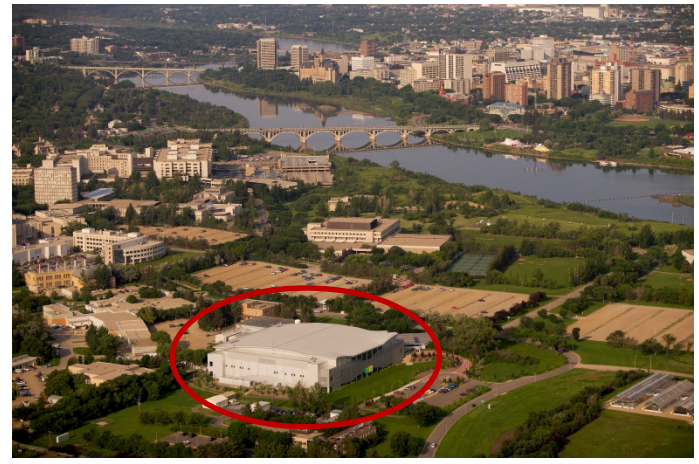




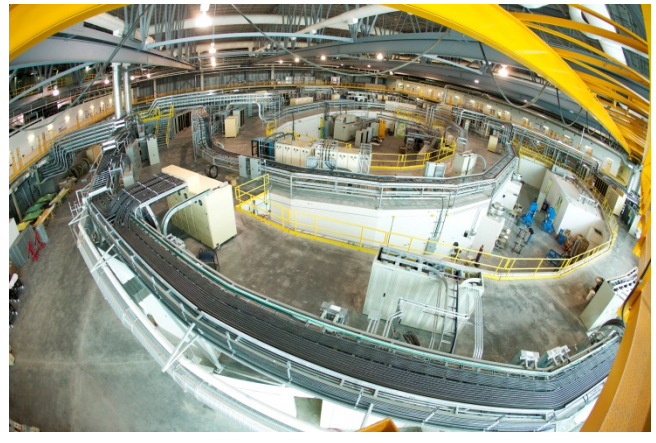
Typical setup for cryogenic crystallography



http://www.lightsource.ca/



Canadian Light Source (CLS)



X-ray crystallography

Advantages:

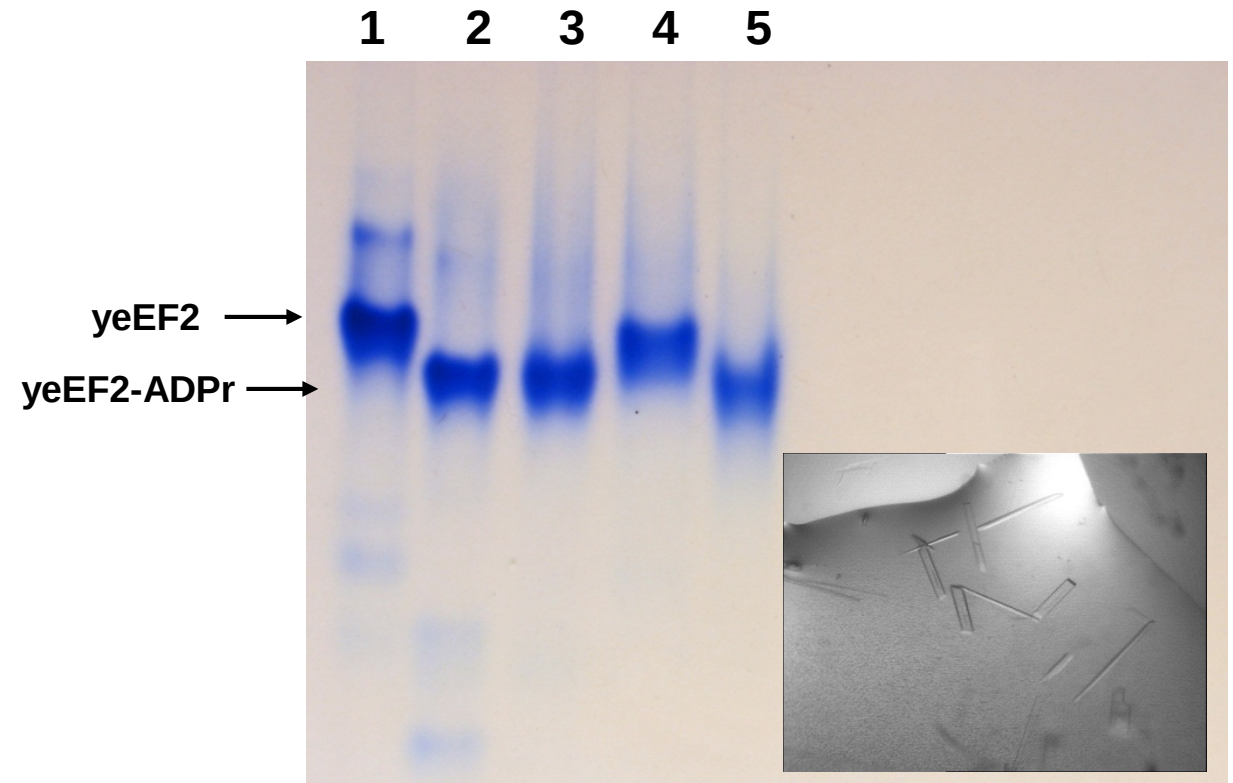
-direct location of active site and information on the nature of the side chain involved in the catalytic mechanism

-examine extent of any structural change accompanying the binding of substrate

Limitations:

-can only provide a **static picture of structure**; danger of differences in structure because of the high ionic strength conditions required for crystallization of protein

-however, *many enzymes are **active** in the crystal state*



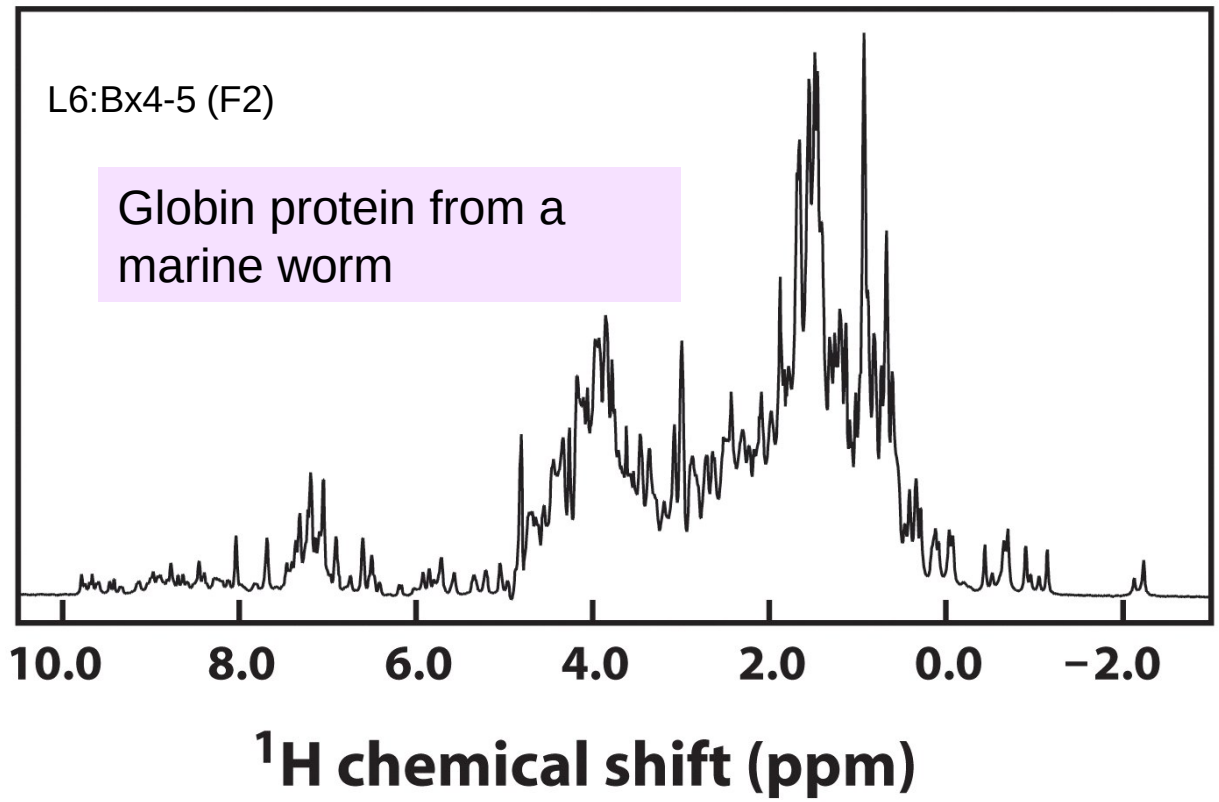
Enzyme is active in the crystal state!

https://www.youtube.com/watch?v=Z5fVZegUhzU

Nature (2005) **436**, 979-984

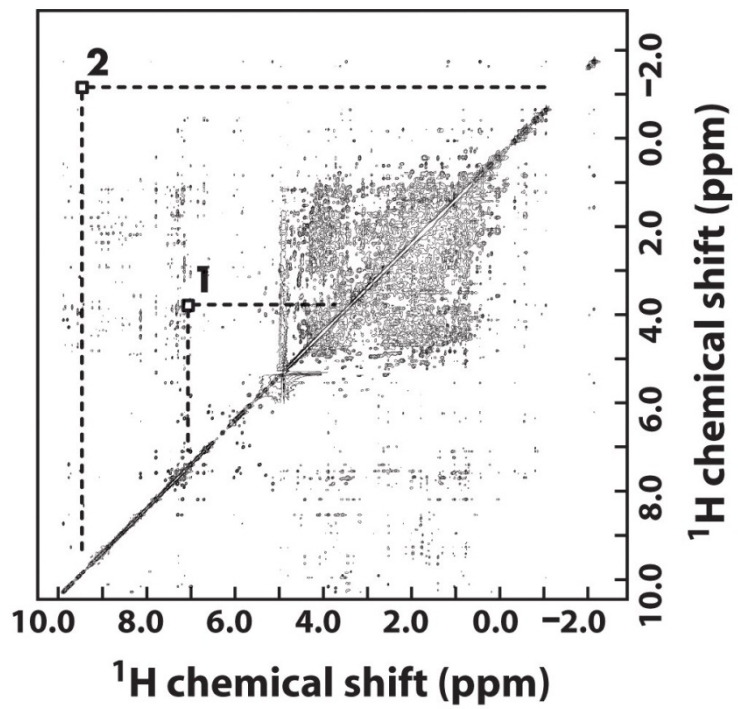
Nuclear Magnetic Resonance for Protein Structure Determination

- Complimentary technique to X-ray crystallography
  - advantage is that the structures by NMR are **in solution**
    - Illuminates the **dynamic side** of protein structure
  - disadvantage is that it requires a **large amount of instrument time** to determine a protein structure
    - Limited to 30-35 kDa (smaller proteins) with 50 – 60 kDa proteins a possibility
- One-dimensional spectra
  - ^{1}H NMR spectra can be complicated because even a small protein has many ^{1}H atoms



•Structural analysis of proteins is possible with 2-D NMR techniques

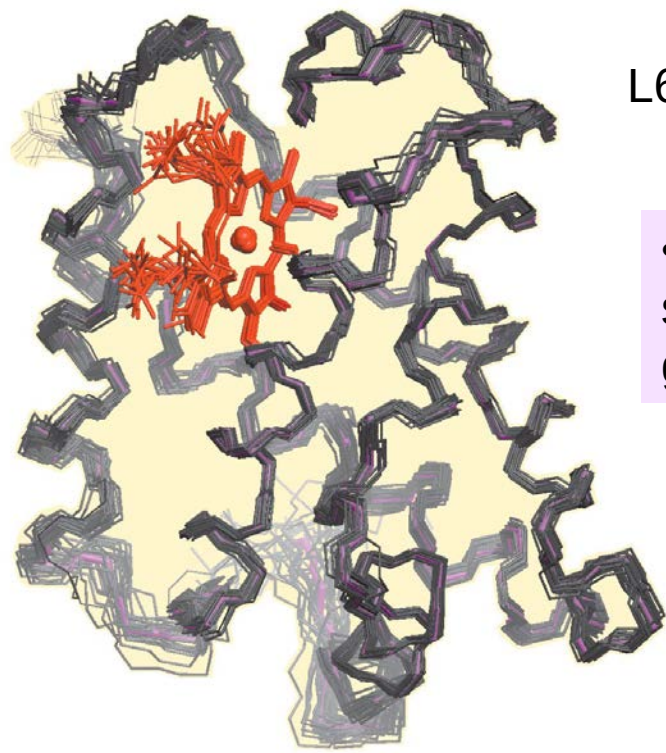
•Allow distance-dependent coupling of nuclear spins in nearby atoms through space (NOE) or connected by covalent bonds (TOCSY)



L6:Bx4-5 (F3a)

•2-D NMR spectrum of marine globin protein

- Translating 2-D NMR spectra into 3-D structure can be laborious
  - NOE signals provide some information about the distances between atoms, but atoms must be identified
  - TOCSY experiments can help in the identification
  - Requires isotope enriched protein samples (^{13}C, ^{15}N)
  - Generate 3-D structure
    - Feed distance constraints into a computer
      - Chirality, van der Waals radii, bond lengths and angles
    - Computer generates a family of closely related structures that represent a **range** of conformations consistent with NOE distance constraints



L6:Bx4-5 (F3c)

- Family of NMR structures for marine globin protein