Previous Class

Term test I discussions

Today

Detection of enzymatic intermediates: chymotrypsin mechanism
Mechanistic Understanding of Enzyme-mediated Reactions

**Ultimate goals:**

- Identification of the intermediates, and conformational states of enzyme
- Rates for the formation and decomposition of these states determined
- Determination of type of chemical catalysis that are involved
- Elucidation of three dimensional structures of enzyme-substrate and enzyme-intermediate(s)
- Biological consequence of enzyme-mediated reaction
- Mechanisms that regulate the reaction in the cell
Detection of Intermediates

Rules of Thumb for Proof of an Bonne Fide Intermediate:

• The intermediate is isolated and characterized
• The intermediate is formed sufficiently rapidly to be on the reaction pathway
• The intermediate reacts sufficiently rapidly to be on the reaction pathway

Requires pre-steady state measurements for intermediate formation and decomposition rates

Requires steady state measurements to compare intermediate rate constants with the overall catalytic activity of the enzyme reaction
Detection of Intermediates

Chymotrypsin- a serine protease family member

Biological function – Hydrolyzes proteins and peptides at the peptide bond – digestive enzyme
Peptide bond cleavage is a special case of amide bond hydrolysis.

$$\begin{align*}
\text{H} & \quad \text{O} & \quad \text{H} \\
\text{H}_3\text{N} & - \text{C} & - \text{N} & - \text{C} & - \text{CO}_2^- & + & \text{H}_2\text{O} & \rightarrow & \text{H}_3\text{N} & - \text{C} & - \text{CO}_2^- & + & \text{H}_3\text{N} & - \text{C} & - \text{CO}_2^- \\
\text{R}_1 & & \text{H} & & \text{R}_2 & & & & & & & & & & \text{R}_1 & & \text{R}_2
\end{align*}$$
Serine Proteases

Specificity:

Chymotrypsin - carboxy side of aromatic a.a. (Y, F, W)

Trypsin - carboxy side of basic a.a. (R, K)

Elastase - carboxy side of small aliphatic a.a.
Specificity determined by fit into binding pocket. Chymotrypsin has a large binding pocket for side chain.
Trypsin has a similar binding pocket, but it contains as aspartate to interact with Lys and Arg.
The triad is H-bonded: **Asp** is H-bonded to **His** to keep it oriented. **His** is H-bonded to **Ser**. This increases the nucleophilicity of the Ser oxygen.

**His** acts as a general base, abstracting a proton from Ser

**Ser** attacks the carbonyl carbon of the peptide bond of the substrate.

Forms first tetrahedral intermediate

**His** now acts as a general acid by transferring a proton to nitrogen of first tetrahedral intermediate

This causes the peptide bond to break, and the first product leaves.
The acyl enzyme remains. This is a covalent intermediate in which the Ser is esterified. His now acts as a general base- activates attack of the Ser ester by water OH.

Second tetrahedral intermediate forms. His transfers its proton back to Ser to break down intermediate.

The second product is released- The original enzyme is regenerated.
Mechanism of Chymotrypsin

Covalent Intermediates:
1) Tetrahedral intermediate 1.
2) Acyl enzyme intermediate
3) Tetrahedral intermediate 2.

http://www.kingsu.ab.ca/~hank/Course%20files/Biochemistry/chymotrypsin.htm
Mechanism of Chymotrypsin
Mechanism of Chymotrypsin
Mechanism of Chymotrypsin
Transition state stabilization is provided by 2 backbone NH groups to stabilize the negative charge of the oxygen in the tetrahedral intermediates.

Further evidence of TS stabilization is provided by experiments in which all three catalytic residues were changed to Ala. Rate enhancement over uncatalyzed rate was observed, even in the absence of catalytic residues.
Detection of Chymotrypsin Intermediates

para-nitrophenylacetate as a synthetic substrate for chymotrypsin.

\[
\text{PNPA} + \text{H}_2\text{O} \rightarrow \text{CH}_3\text{COO}^- + \text{PNP}^- - 2\text{H}^+
\]

para-nitrophenol (PNP) - chromophoric
Evidence of Intermediates-Product

"Burst"

$A_{400 \text{ nm}}$ PNP− release

Burst
1 mol/mol of enzyme

Minutes
Evidence of Intermediates-Product “Burst”

Burst experiments:
the stoichiometric release of one of the products that is much faster than the steady state Vmax

The p-nitrophenol released in the initial pre-steady state 'burst' is stoichiometric to the concentration of active sites present (mole:mole)

Rate then slows down to Vmax

Suggests an initial phase of rapid acylation of chymotrypsin then a slow hydrolysis of acylenzyme intermediate (rate determining step)
Measurement of the acylation rate constant

Chromophoric leaving group: PNPA amino acids (acetyl-Phe ethyl ester)
Use excess substrate to ensure the enzyme is constitutively acylated
\[ d[E\text{-Ac}]/dt = 0 \]
Rate of acylation \((k2)\) is determined from the rate of appearance of the nitrophenol (PNP) (exponential phase)
Often acylation rate is too fast for stopped-flow measurement
Measurement of the acylation rate constant

Chromophoric inhibitor displacement: Proflavin (pg 220-221)

Proflavin is competitive inhibitor of chymotrypsin

Absorbs at 465nm when bound to active site

Stopped-flow experiment – acetyl-Phe ethyl ester mixed with chymotrypsin-proflavin solution

Initial rapid displacement of some proflavin (↓ in Abs$_{465}$) (dead time)

As acylenzyme is formed equilibrium breaks down – proflavin is completely displaced (no affinity for acylenzyme) (↓ Abs$_{465}$ #2)

Absorbance stays constant until acylenzyme breaks down (↑ Abs$_{465}$)

Acylation rate constant obtained from the exponential second phase of absorbance decrease
Measurement of the deacylation rate constant (k3)

Chromophoric inhibitor displacement: Proflavin

Excess of enzyme is used in stopped-flow experiment

Substrate is consumed and the acylenzyme is formed rapidly (dead time)

Slow hydrolysis of acylenzyme is monitored by the rebinding of proflavin to the free enzyme being produced (k3)
Characterization of Intermediate

Previous experiments demonstrate that intermediates exist

Proof that catalytic Serine (Ser-195) is the residue acylated:

\[ \text{DFP} \]

Organophosphate is \textbf{irreversible inhibitor}

After inhibition enzyme was hydrolyzed and the peptide containing the phosphate ester was identified – Ser195

Further Proof: X-ray crystallography – observe three dimensional structure of acylenzyme (non-specific and specific)
Next Class

Protein Tyrosine Phosphatase Mechanism and Intermediates