Structure/Function Studies of Beta Lactamase

BACTERIA & ANTIBIOTICS

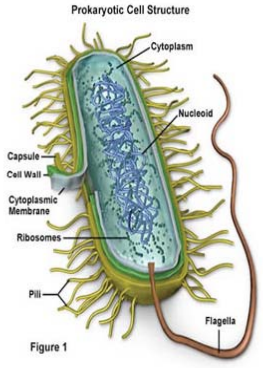
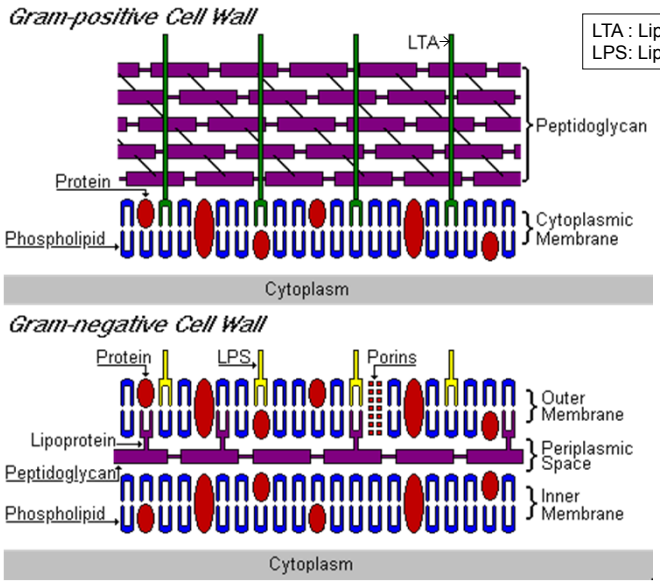


Figure 1

How Antibiotics work:

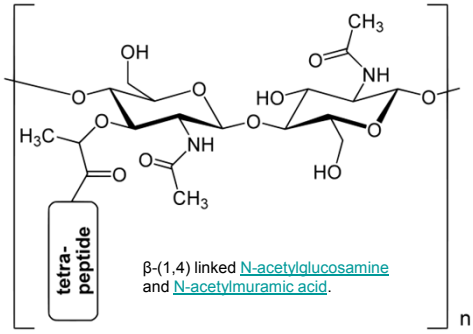
1. Weaken bacterial cell wall (β-lactams, vancomycin, etc)
2. Disrupt cell division/DNA replication
3. Disrupt protein synthesis (Rifampin, Tetracyclines, Macrolides, etc)
4. Disrupt other metabolic processes within bacterial cells

Bacterial Cell Walls

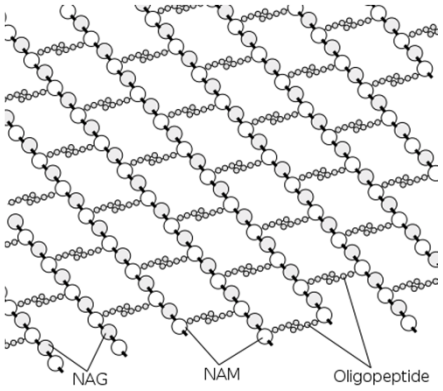


LTA : Lipoteichoic acid
LPS: Lipopolysaccharide

Peptidoglycan

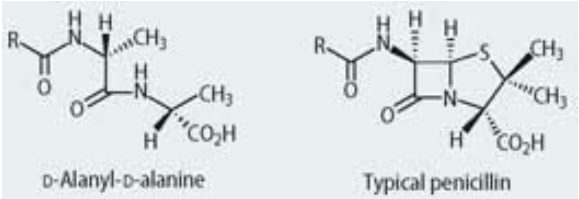


β-(1,4) linked N-acetylglucosamine and N-acetylmuramic acid.



Peptidoglycan synthsesis requires *transpeptidase* enzymes.
Catalyze crosslinking of tetrapeptides.

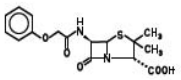
Beta-lactam antibiotics are transpeptidase inhibitors



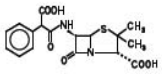
- D-Alanyl-D-alanine is last residue of tetrapeptide and a *substrate* of transpeptidase.
- β-lactam antibiotics (e.g., penicillin) are structurally similar to D-Alanyl-D-alanine. Bind in transpeptidase *active site* (irreversibly) and *inhibit* activity (via suicide inhibition)
  → β-lactams destabilize cell wall and kill bacteria.
- Transpeptidases are also called *Penicillin Binding Proteins (PBS's)*

β-LACTAMS & β-LACTAMASE

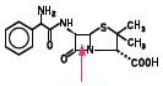
Penicilin V



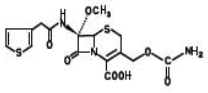
Carbenicilin



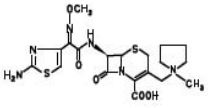
Ampicilin



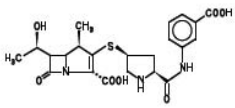
Cefoxitin



Cefepime



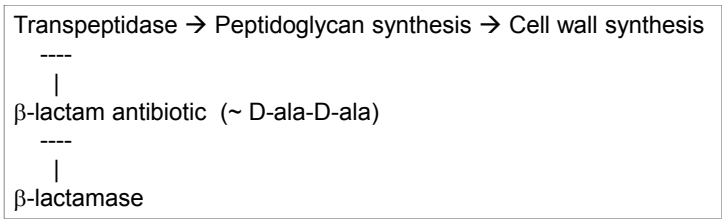
Ertapenem



 : β-lactam

β-lactam resistance mediated by β-lactamase enzymes

- β-lactamase hydrolyzes β-lactam ring
  - Inactivates
- Bacteria have/get genes encoding β-lactamase
  - Expression induced by exposure to β-lactam
  - Obtain gene via plasmid transfer
- What is mechanism?

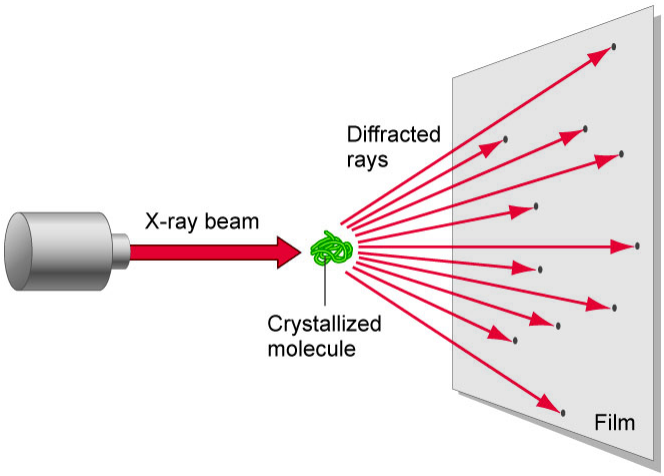


Methods to study enzyme mechanisms

- Structural Analysis
  - With/without substrate/inhibitor
- Activity
  - Kinetics
    - Competitive inhibition
    - Isotope effects
  - Studies of mutant enzymes

Usually, a combination of structural analysis and activity studies are required.

Determination of Protein Structure
X-ray crystallography



Output

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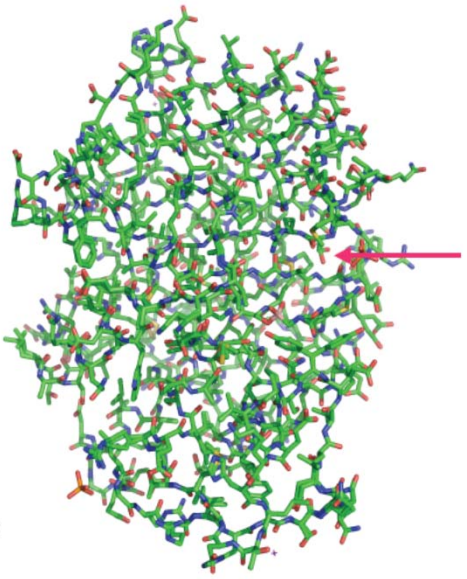
data_r1nymsf
#
_audit.revision_id       1_0
_audit.creation_date  2003-08-26
_audit.update_record     'Initial release'
#
loop_
_refln.wavelength_id
_refln.crystal_id
_refln.index_h
_refln.index_k
_refln.index_l
_refln.intensity_meas
_refln.intensity_sigma
_refln.status
1 1     0     0     6        223.87          11.12 0
1 1     0     0     8       6438.11         252.80 0
1 1     0     0    10       4876.17         200.58 0
1 1     0     0    12       4419.22         182.40 0
1 1     0     0    14       5941.22         223.95 0
1 1     0     0    16       1372.35          56.99 0
1 1     0     0    18      51797.60        2015.90 1
1 1     0     0    20       4997.55         146.55 0
1 1     0     0    22       2050.97          82.98 0
1 1     0     0    24      70057.00        2586.40 0
1 1     0     0    26       2502.86         104.08 0
1 1     0     0    28        501.98          29.19 0
1 1     0     0    30      20797.00         561.40 0
1 1     0     0    32       4295.61         123.87 0
1 1     0     0    34         35.94          16.46 0
```

Output records intensity and position of each “spots”.

In the end, crystallographic analysis generates a set of coordinates for all atoms in the molecule

Need a way to graphically represent this in order to interpret Structure/function relationships

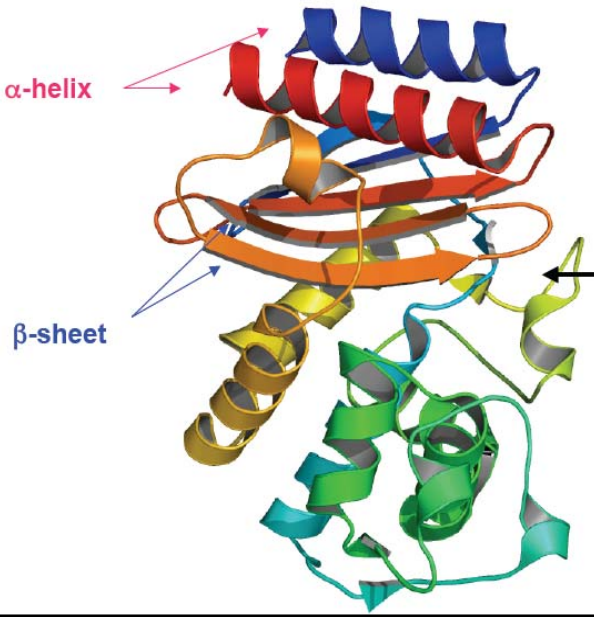
β-Lactamase: Line drawing



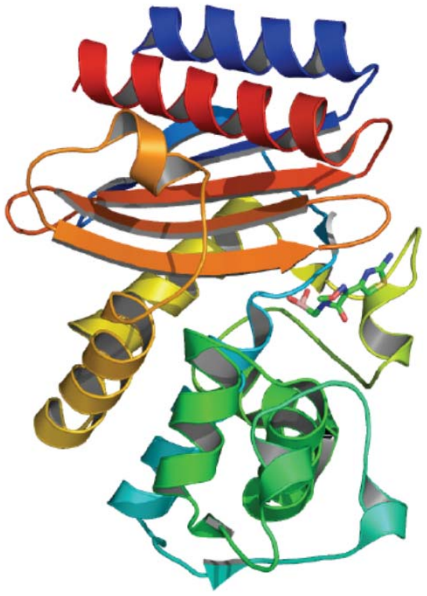
Active site

264 residues
2030 atoms

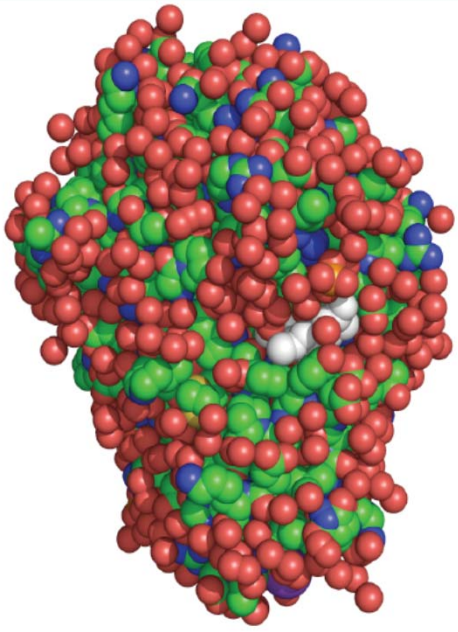
β-Lactamase: Cartoon Drawing



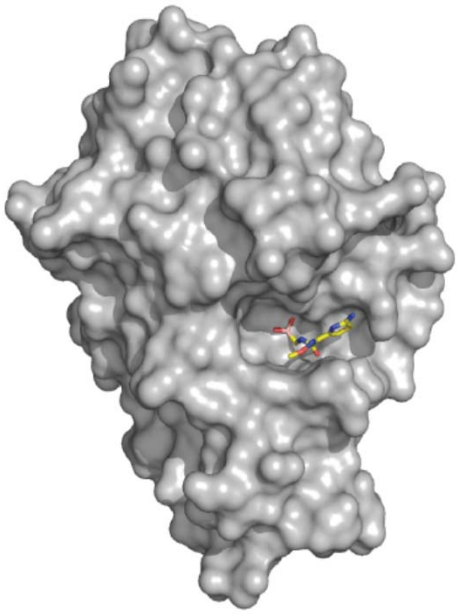
Cartoon Drawing of β-Lactamase-Inhibitor Complex



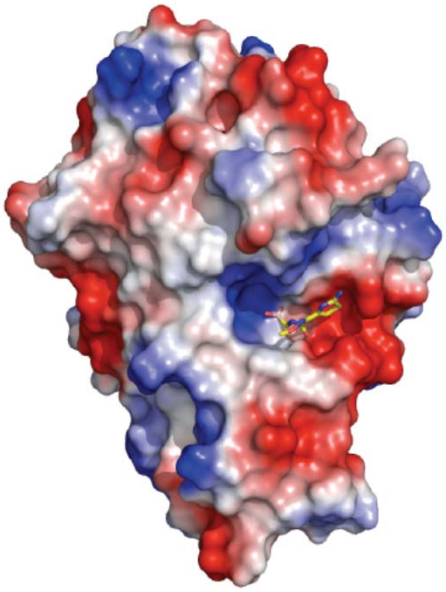
Space-filling (CPK) model

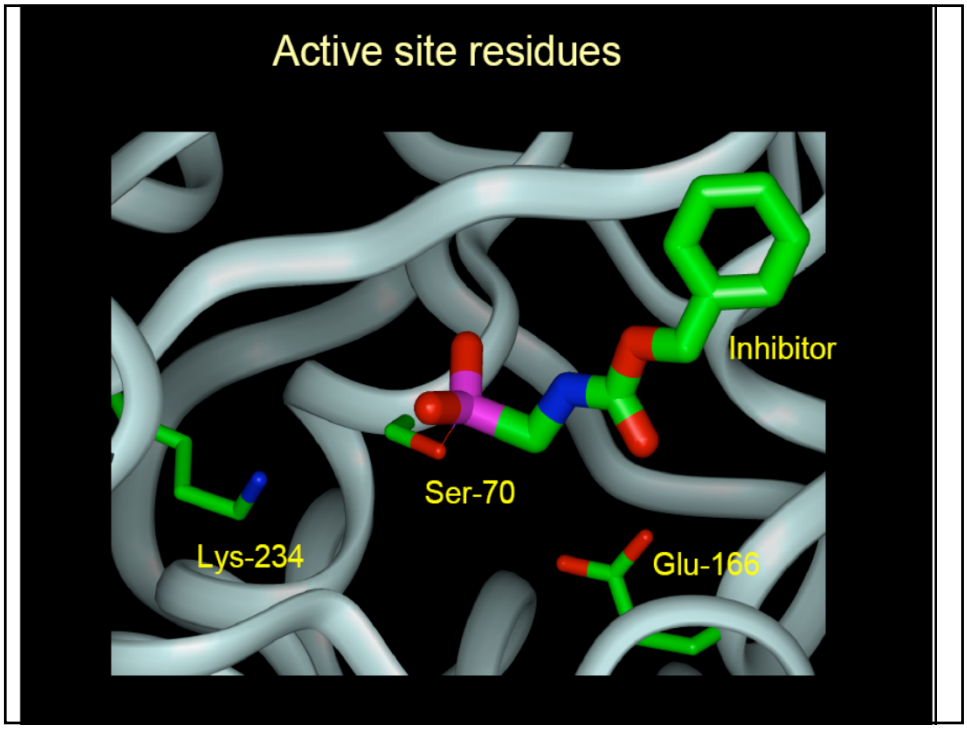


Surface Diagram of β-lactamase-inhibitor



Surface Diagram of β-lactamase-inhibitor





Viewing Protein Structure

1. Find the Protein Data Bank (PDB) code for the structure.
- http://www.rcsb.org/ or in original publication.
e.g.,1ERM : TEM-1 w/ inhibitor BJI

BJI: 1(R)-1-ACETAMIDO-2-(3-CARBOXYPHENYL) ETHYL BORONIC ACID

2. Visualize structure :
- http://firstglance.jmol.org : Good for quick overall look.
* Enter PDB (protein data bank) code in the box.
* Shift+Left click or Wheel = zoom, Ctrl+Right click=translation.
* Menu on the left is self-explanatory. To see details, choose "Vine.." and select "More detail: Show all non-water atoms colored by element".

OR

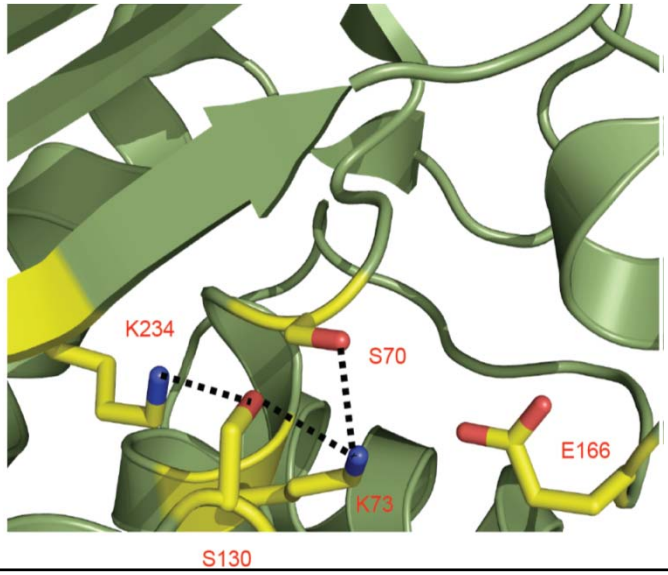
- http://pymol.org/ : Must download the program
- Hide nonbonded to hide water.
- select cat, (res 70+73+130+166+234)
  select tem1, /1ERM//A/1-499
  select inh, (res 500)

Methods to study enzyme mechanisms

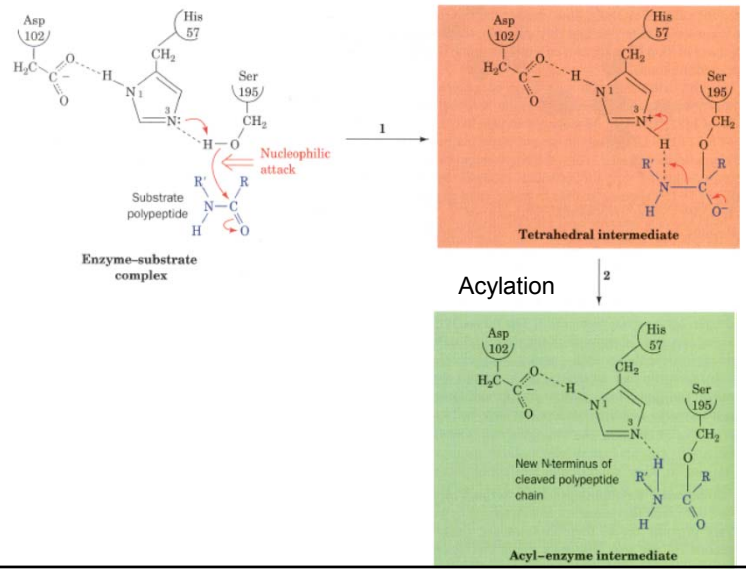
- Structural Analysis
  - With/without substrate/inhibitor
  - Organization/Position of (catalytic) residues in active site
  - Reasons for affinity/specificity
- Activity
  - Kinetics
    - Competitive inhibition
    - (Kinetic) Isotope effects
  - Studies of mutant enzymes

Usually, a combination of structural analysis and activity studies are required.

Active Site Residues of β-Lactamase



Catalytic Mechanism of Serine Proteases: Chymotrypsin



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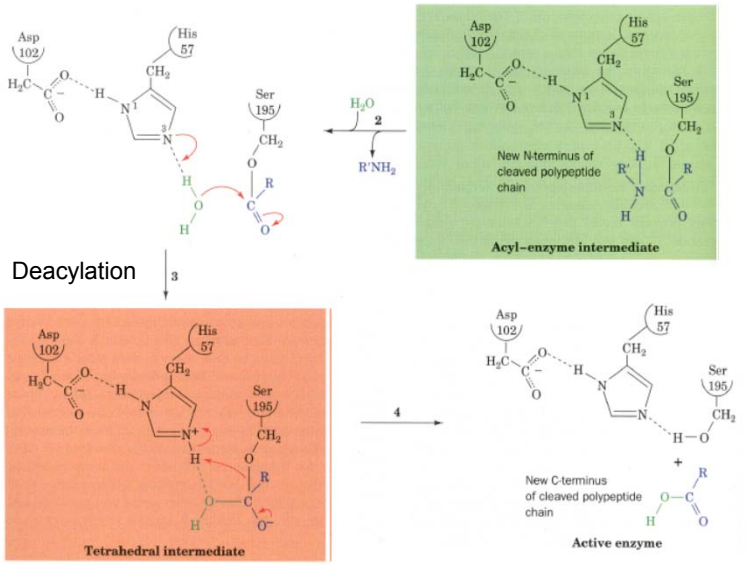

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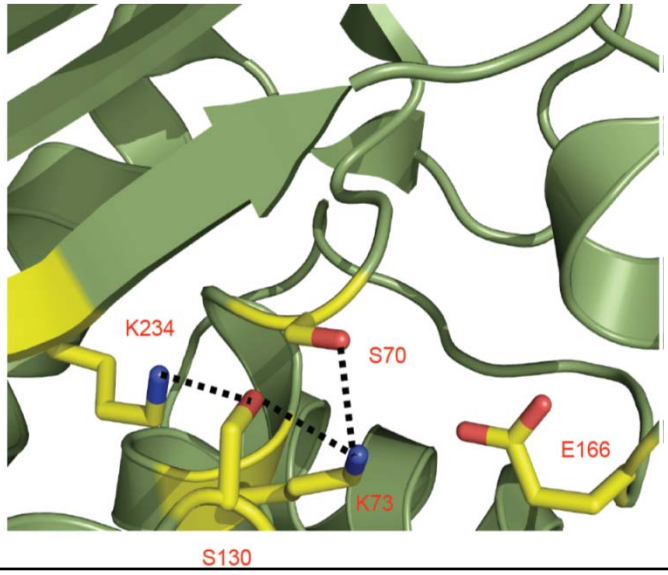

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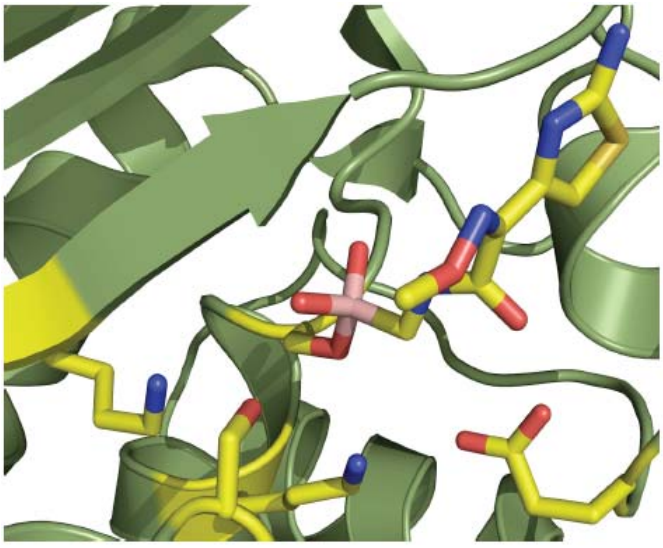
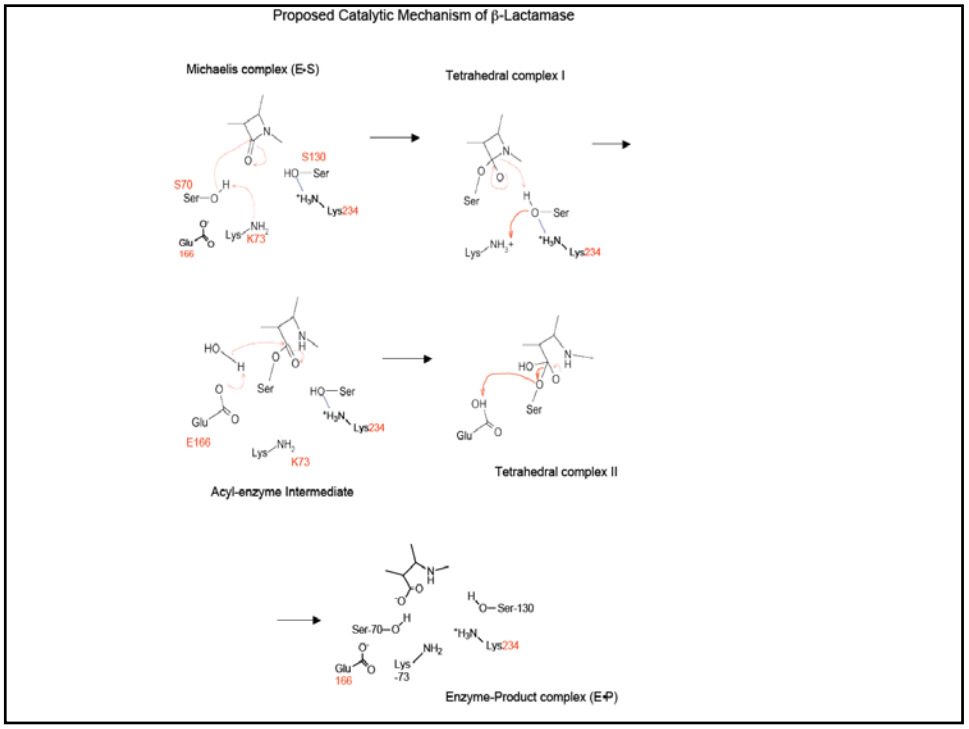
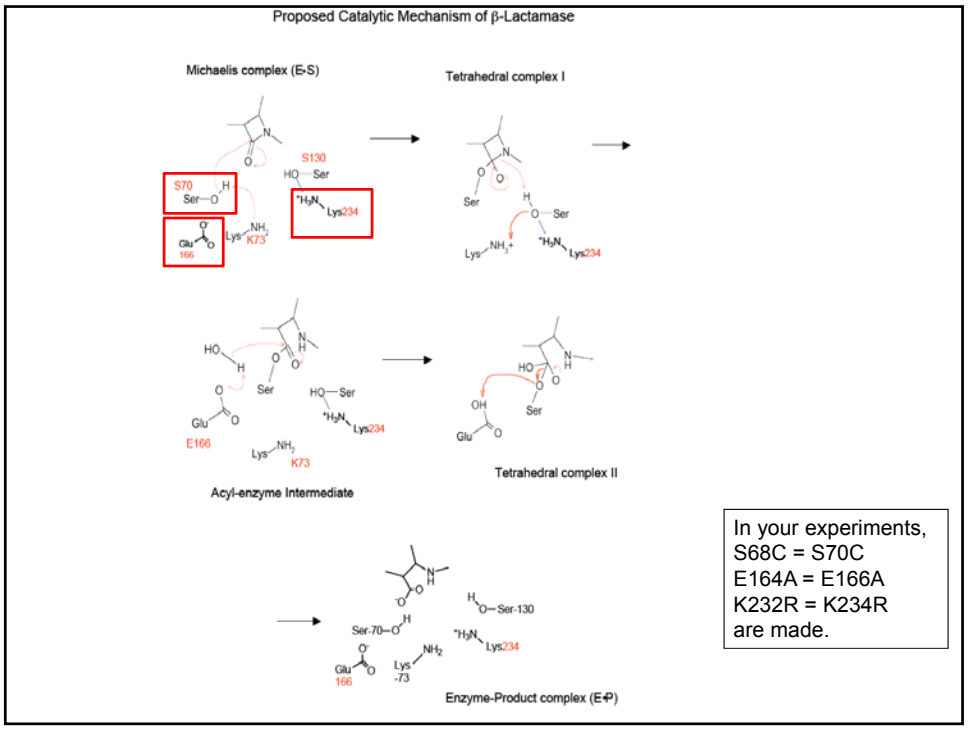
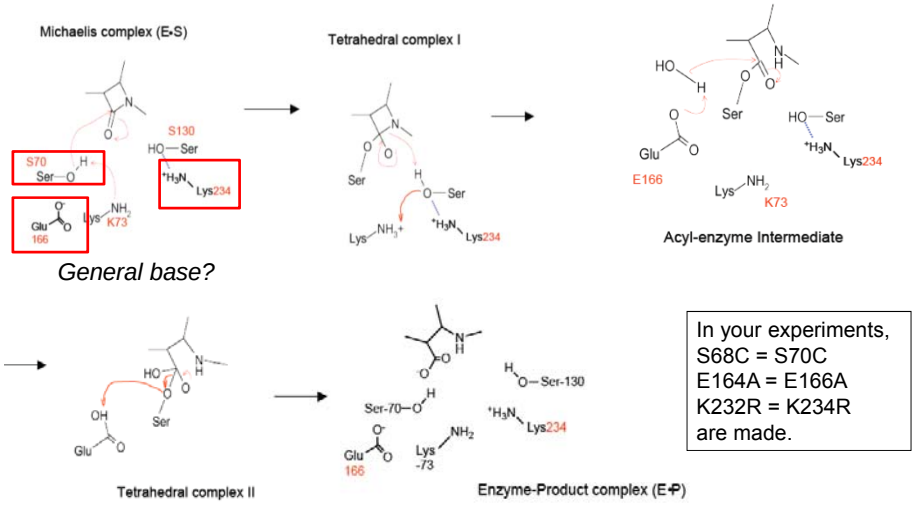






In your experiments,
S68C = S70C
E164A = E166A
K232R = K234R
are made.

Proposed Catalytic Mechanism of β-Lactamase



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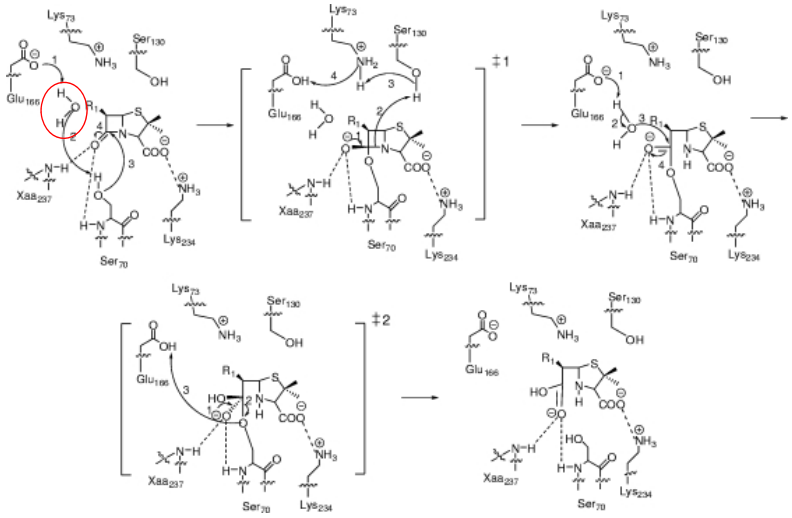


http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2806661/?tool=pubmed

Mutational Analysis

- Effects of Mutations
  - Conservative Mutation – Preserve polarity, charge, etc.
    - E.g., E166D
  - Non-conservative Mutation
    - E.g., E166F
  - Active site
    - Recognition
      - » Steric effects
      - » Electrostatic effects
    - Catalysis
  - Non-active site
    - Surface-exposed vs. core residues
      - » Effects on structure, folding, solubility?

Types of Mutagenesis

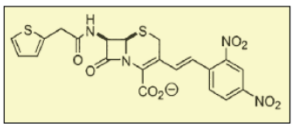
- Point (e.g., E166K)
  - Used w/ *a priori* knowledge of structure/function.
- Combinatorial
  - Random generation of all possible combinations of residues at one or more sites.
  - E.g., used to engineer enzymes w/ altered activity.
- Scanning (e.g., E166A, S70A, S103A, etc.)
  - Used when there is less knowledge about structure/activity.

Overall Experimental Strategy

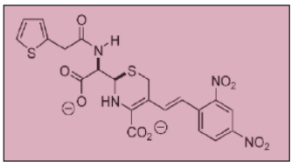
- Use PCR to make desired mutation in gene
- Subclone gene into expression vector
- Transformation into expressing cells (*E. coli*)
- Express/Purify Protein
- Characterize
  - Structural analysis
  - Protein stability
  - Functional analysis: activity, binding, etc.

Characterization: Nitrocefin Assay

Nitrocefin



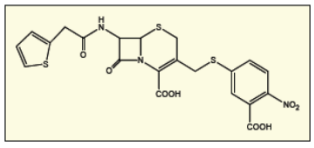
TEM1



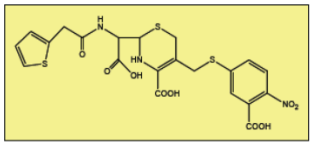
λ_{max} = 390 nm at pH 7.0

λ_{max} = 486 nm at pH 7.0

CENTA™



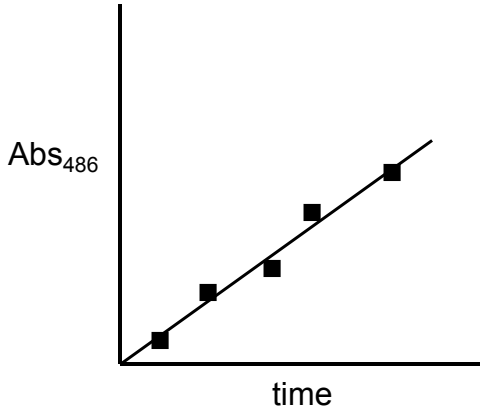
TEM1



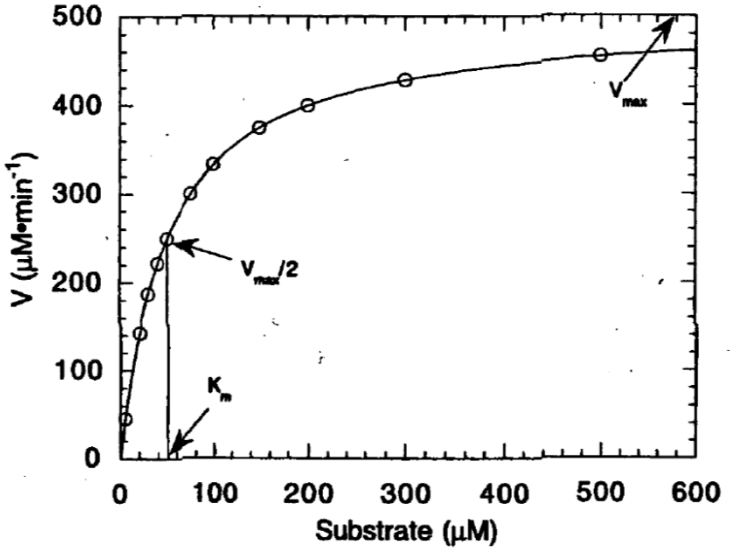
λ_{max} = 340 nm

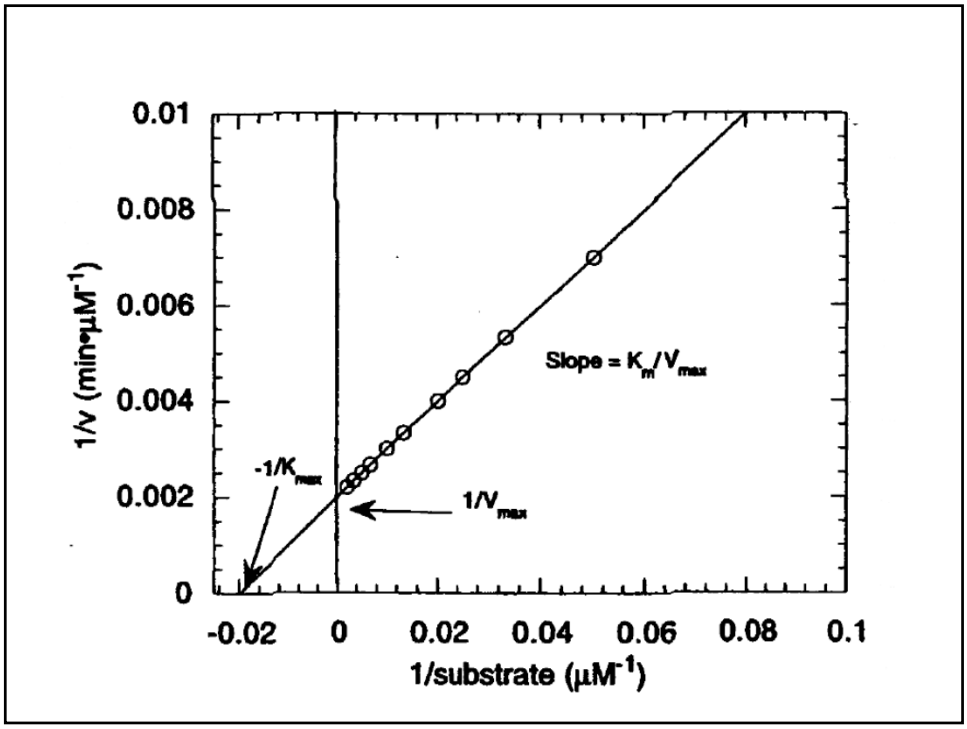
λ_{max} = 405 nm

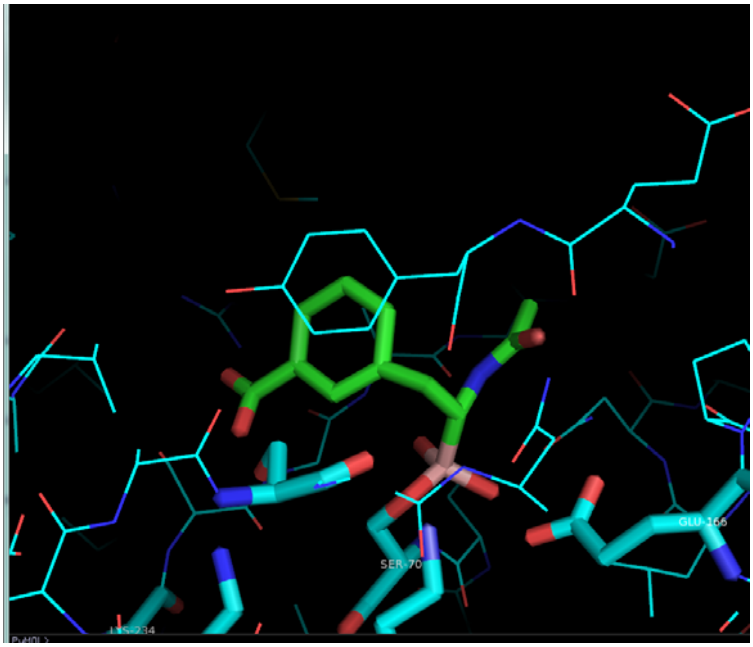
Nitrocefin assay analysis



$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_{cat}} E + P \qquad V = \frac{k_{cat}[E_T][S]}{[S] + K_M}; K_M = \frac{k_{-1} + k_{cat}}{k_1}$$




0.01
0.008
0.006
0.004
0.002
0
1/v (min•μM-1)
Slope = Km/Vmax
-1/Kmax
1/Vmax
-0.02
0
0.02
0.04
0.06
0.08
0.1
1/substrate (μM-1)



Summary

- Molecular Cloning
- Protein expression
- Protein purification
- Enzyme Structure
- Enzyme Kinetic Assay
- Enzyme Mechanism

Some key points

*Understand the reasons behind the experiments and knows how to carry out experiments.

Molecular Cloning

- Do you know the work-flow of the cloning process?
- Can you interpret a plasmid map and the functions of each element?
- Do you know how to prepare pure plasmid DNA?
- Do you know how PCR works?
- Do you know how to design primers for various purposes? E.g., cloning into vector, making mutation.
- Can you set up appropriate PCR conditions to carry out?
- Do you know how to restriction enzymes work and are used for cloning?
- Do you know how to run and analyze DNA on gel?
- How does a ligase work?

Some key points

*Understand the reasons behind the experiments and knows how to carry out experiments.

Protein Expression

- What is the mechanism of IPTG induction?
- What is the T7 or ptac promoter-based system? *DE3 strain
- What are competent cells? How does *transformation* work?
- Do you understand the role of antibiotics and can you properly choose which one to use?

Some key points

*Understand the reasons behind the experiments and knows how to carry out experiments.

Protein Purification

- Can you choose appropriate chromatography methods to purify a given mixture of proteins?
- Do you know what chromatography resins are made of?
- Can you prepare adequate buffers for chromatography? E.g., pH, salt…
- Can you interpret/predict a chromatogram? Do you know how it is recorded? E.g, x and y axis.
- Do you understand how SDS-PAGE works? How the discontinous buffer system help the resolution? How is it different from native PAGE run without SDS?
- How do you ‘see’ proteins in gels, interpret the results? E.g., name of the stain
- How does BCA and Bradford assays work?

Some key points

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Enzyme Structure

- Can you identify residues and name the amino acids based on structural interpretation of the side chains.
- Can you identify active site residues in TEM1?
- Can you identify a hydrogen bond in a structure? *sense of distance, scale

Some key points

*Understand the reasons behind the experiments and knows how to carry out experiments.

Enzyme Kinetic Assays

- What are the basis of kinetic assays? *x and y axis of any plot. Can you construct a M-M plot or L-B plot from a kinetic assay and extract appropriate parameters (Vmax, Km, kcat?
- Given the assay results of a mutant b-lactamase, can you interpret/suggest a potential role of the mutated residue?

*** Beer's law for UV/Vis spectroscopy!!!
: Used in measuring ANY concentration that absorbs light. E.g, DNA at 260nm, Protein at 280nm and other chemicals (e.g., Nitrocefin) in their own absorbing wavelength.

Some key points

β-Lactams and β-lactamase

- What are they? What do they do and How do they work? *Chemical structure of substrate/product
  *Role of side chains
- Gram-negative and positive bacteria
- General base, Activation of Serine

*** Extra Note
-Why you need resistance marker
-Why 5'P is not in the primers you order
-Miniprep buffer and how they work
-Cathode & anode
-Role of APS/TEMED, EDTA, isopropanol in gel making and running.
-Ori, promoter in plasmid
-Size exclusion limit is in the name of the resin. How to read off this limit in the graph Kav vs log MW
importance of isopropanol in SDS-PAGE making
- DNA chemical structure. Name. Charge
- requirement of ligase vs polymerase.
- estimate mw of protein and DNA using 0.1 kDa/aa and 0.5 kDa/bp and gel-separation

The End

Good Luck!