Elucidation of Enzyme Mechanism

**basic principles**

- **Binding site** - the substrate must fit in the active site, so the active site must provide proper charge distribution, hydrophobic distribution, proper orientation, etc.
- **General acid/base catalysis** - if a proton needs to be added or removed, there is usually a general acid or general base in the active site to do it (or to hold a water molecule to do it).
- **Nucleophilic catalysis** - when nucleophilic catalysis is in force, a nucleophile is provided, or enhanced.
- **Other destabilization/stabilization of intermediates** - metal ions may be present for binding, for polarizing bonds, etc. Some metals may add or remove electrons.

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**Xylose Isomerase**

**Reaction:** Catalyzes what appears to be a hydride transfer, or movement of a carbonyl group from C1 to C2. Interconverts Xylose and Xylobiose:

\[
\text{H}_2\text{C(OH)}\text{CH}_2\text{OH} \rightleftharpoons \text{CH}_2\text{OH} \text{H(OH)}\text{C(OH)}\text{CH}_2\text{OH}
\]

**also accepts glucose, which it converts to fructose (which could be but isn’t called “gluculose”).** So it can be used to produce high-fructose corn syrup from starch hydrolysates.

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**Xylose Isomerase**

**things you know how to find out**

- **Sources/Size:**
  - Available from many microbial sources (See table 13.1, p. 360 in Wong).
  - Most are tetramers of ~120-180 kDa, with identical subunits (How could you tell? Measure M, under native and denaturing conditions, end-group analysis to determine if the protomers are the same, separation (if necessary/possible) and sequencing).
  - The E. coli enzyme is a dimer of 44 kDa. X-ray structure shows two domains: α/β barrel and a 5-helix-containing external loop. The symmetry indicates that the structure is a dimer of dimers.
  - pH & T:
    - Stable between pH 4 and 11; pH<sub>opt</sub> 7.5-8.0.
    - Most are relatively heat-stable
  - From *Bacillus stearothermophilus*, it is especially so, with T<sub>mp</sub> of ~80°C

- **Inhibitors:**
  - Sugar alcohols.

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**Xylose Isomerase**

**things you know how to find out**

- **Other features:** Requires a divalent cation Mg<sup>2+</sup>, Co<sup>2+</sup> (Mn<sup>2+</sup>, Fe<sup>2+</sup>, Ni<sup>2+</sup>, Cu<sup>2+</sup>, Zn<sup>2+</sup> and Cu<sup>2+</sup> either don’t work or produce less activity than Mg<sup>2+</sup> or Co<sup>2+</sup>.

- **How to tell?** Remove divalent cations using EDTA, then add back small amounts of soluble salts of other metal ions, check for activity.

- **How to compare?** The metal stabilizes the protein's structure as measured by T-stability or resistance to denaturants.

- **What exactly does the metal do?** Aid in binding? Aid in catalysis?

- **Look at V<sub>max</sub> (K<sub>cat</sub>) and K<sub>M</sub> with xylose as substrate using Mg<sup>2+</sup>, Co<sup>2+</sup> and Mn<sup>2+</sup>.**

<table>
<thead>
<tr>
<th>Cation</th>
<th>Mg&lt;sup&gt;2+&lt;/sup&gt;</th>
<th>Co&lt;sup&gt;2+&lt;/sup&gt;</th>
<th>Mn&lt;sup&gt;2+&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>K&lt;sub&gt;cat&lt;/sub&gt;</td>
<td>8.4</td>
<td>3.9</td>
<td>3.1</td>
</tr>
<tr>
<td>V&lt;sub&gt;max&lt;/sub&gt;</td>
<td>1.2</td>
<td>2.2</td>
<td>2.7</td>
</tr>
<tr>
<td>K&lt;sub&gt;M&lt;/sub&gt;</td>
<td>10.3</td>
<td>12.5</td>
<td>11.3</td>
</tr>
</tbody>
</table>

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**Chemical modification** (X-ray/NMR)

Some examples follow…
**Xylose Isomerase**

**things you might not know about**

Sequence information:
If many sequences for evolutionarily related enzymes are available, residues that are important in binding and catalysis will be conserved. Of course, many others may also be conserved. Any residues we propose to be important in binding and catalysis had better be conserved, but this is not proof that they are important.

Physical studies of the metal-binding sites (techniques such as epr spectroscopy of CuII, Mn++, Fe++, Ni++) optical spectroscopy (especially for CuII and Cu ion), resonance Raman spectroscopy and Mossbauer spectroscopy (for Fe ion), X-ray absorption spectroscopy and EXAFS (extended x-ray absorption fine structure) can be used to study the coordination geometry, nature of ligands, etc.

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**Xylose Isomerase**

**active site**

Two metal binding sites per monomer. Both must be occupied for the enzyme to be active. In all XIs studied, Mg2+ ion has the same Kp for both sites. In some, but not all of the XIs studied, Co2+ has different Kp's for the two sites: the two sites are therefore not identical. Site I: the metal is octahedral (6 ligands), with 4 ligands from the enzyme (two Glu-COO- and two Asp-COO-). The other two sites are occupied by oxygen atoms from the substrate (or probably water in the absence of substrate). The substrate is bound in the open-chain form, and a threonine also binds the substrate, according to one study.

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**Xylose Isomerase**

**suggested mechanism**

(You can never prove a mechanism, just disprove other alternatives)

Two reasonable hypotheses

1) proton abstraction leading to an enediol intermediate
   This mechanism is also possible for triosephosphate isomerase

2) a concerted hydride transfer which requires metal ions and is therefore not possible in triosephosphate isomerase

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**Sulphhydryl Proteases**

**Papain**

Sources/Site:
- Papaya latex of unripe fruit (note tissue specificity and growth-stage specificity. Many related (but "different" enzymes are found in plants).
- From a GRAS source, widely used in the food and beverage industry.
- 212 AA's, Mr = 23,350, monomer

Reaction, Substrate specificity:
- Not very specific for any particular residues.
- Relevant crystallographic studies indicate binding sites for seven sidechains, each with its own preferences. Overall substrate specificity is an aggregate of these. Their relative importance and preferences can be assessed by comparing Kp/kcat for different substrates.
- Also hydrolyzes esters
- Good for bunnies

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**Sulphhydryl Proteases**

**Papain**

Other features:
- Seven Cys, three S-S bonds (leaves one free SH).
- Two clear domains, one of helices and coil, the other of sheet.

Inhibitors:
- Thiol-specific reagents inactivate. Several classes of polypeptide inhibitors exist

T and pH:
- Quite T-stable at neutral-to-alkaline pH.
- Unstable at pH < 4. pHopt 5.5-7.5. kcat/Km shows a bell-shaped curve, optimum at pH 6 and pK's of 4.3 and 8.5
- pH-dependence is misleading - NMR spectroscopy sheds light ...
**NMR Spectroscopy and His**
**brief digression**

NMR is very useful for measuring the pKa of a Histidine sidechain. When the imidazole nitrogen is protonated, the ring is “more aromatic”, which gives the CH proton resonances chemical shifts that are more like those of benzene (i.e., they move downfield at lower pH). The change in chemical shifts of the nitrogens themselves is even greater.

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**Sulfhydryl Proteases**
**suggested mechanism**

1. The His imidazole is weakly basic, but can help deprotonate an incoming water molecule.
2. The water attacks the thioester intermediate.
3. The tetrahedral intermediate breaks down to form the acid product and to regenerate the enzyme.

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**Serine Proteases**
**chymotrypsin - kinetics**

We could, but won’t, discuss Occurrence, purification, properties, M, pI, AA composition, etc. But, let’s cut to the chase, and look at the reaction it catalyzes.

**Kinetics**

- First, choose a substrate and an assay; there are many and studies date back to the 30s. Possibilities include:
  - peptides of various composition and lengths
  - measurement of new ω-amino groups, loss of substrate or appearance of product by HPLC, TLC, etc.
  - model compounds
  - amide - measure ammonia using Nessler's reagent, etc.
  - ester - measure pH, etc.
  - with a chromogenic leaving group (e.g., nitrophenol)

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**Sulfhydryl Proteases**
**Papain**

1. NMR spectroscopy of papain shows that His is the residue with a pKa of ~8.5. Abnormally high.
2. The one free CysSH is conserved among several related proteins.
3. The Cys-SH ionizes to an anion, which stabilizes the charged form of the His imidazole (so it remains protonated at a higher pH than normal).
4. S-modified papain (can you design an affinity reagent that would inactivate it?) still shows a pKa near 4. In this form, the pKa of the Imidazolium is unusually low!
5. A thio-acyl intermediate has been inferred by several methods. The most direct is the observation of the intermediate using 14C-NMR at very low temperature (insufficient energy to convert the intermediate to the next intermediate in the sequence.
6. What would the pKa's of His be in the acylated enzyme?
Chymotrypsin

**kinetics & specificity**

You could vary and compare
- Substrates with different chain length (Y and X)
- Analyze products: is it an exopeptidase, an endopeptidase or does it care?
- The nature of R (the carboxyl donor in the peptide bond) and the nature of R' (the amino donor in the peptide bond).
- The nature of the carboxyl bond (is it an esterase as well as an amidase?)

To determine where in a protein does the enzyme hydrolyze bonds and why AND what other kinds of bonds it will hydrolyze.

Chymotrypsin

**rapid kinetics (pre-steady-state)**

Rapid mixing experiments show early part of the reaction, perhaps before the rate-limiting step.
- (some called “Stopped-flow” experiments)
- Chymotrypsin shows “initial burst” followed by slow phase.
- Magnitude of initial burst = E(t)
- Rate of slow phase = k2
- Suggests covalently intermediate is formed with release of first product, and RLS is hydrolysis of E P1 to regenerate free enzyme.

**Reversibility**

Substrate analogs without a hydrolyzable bond (e.g., indole, β-indolylpropionate).

Formyl-L-tryptophan and formyl-L-phenylalanine are also inhibitors: the use of 18O water shows that the enzyme catalyzes the exchange of 18O into the inhibitor (“pseudosubstrate”), suggesting more strongly that they are bound at the active site. Isotope exchange is a “partial reaction” catalyzed by the enzyme.

**Irreversible**

Diisopropylfluorophosphate irreversibly inhibits the enzyme as do some acylating agents. Ser 195 is found to be phosphorylated by the reagent. Toluenesulfonfyl-L-phenylalanylchloroketone (TPCK) is an affinity reagent that inactivates chymotrypsin and is found to modify His 57.

Photosoxidation of histidine also inactivates the enzyme.

Chymotrypsin

**kinetics & specificity**

Answers:
- Chymotrypsin is not an exopeptidase.
- It is specific for amides and esters in which
  - the carboxyl group is provided by an aromatic amino acid.
  - Substituted phenylalanine and tyrosyl esters and amides are hydrolyzed more slowly.
- Amides and esters of other large hydrophobic residues are also substrates but are hydrolyzed slowly.
- The enzyme is not particularly selective for the nature of the alcohol or amine moiety.

Chymotrypsin

**pH dependence**

- Hydrolysis of N-acetyl-tryptophan amide as a function of pH at low [S] is bell-shaped, with pKa’s near 7 and 9. At high [S], the low pH ionization is also observed. Kd is found to increase at pH 8.5, accounting for the basic ionization. However, the change in activity on the basic side is complex. It appears that the enzyme may undergo a conformational change with a pKa of 8.5.

- The nature of the carboxyl bond, donor in the peptide bond, and the nature of R' (the amino donor in the peptide bond)

- Kinetics shows us the steps leading to the RLS
  - Formation of the acyl enzyme intermediate
    - Is slow for esters - the RLS is hydrolysis of the acyl enzyme.
  - There is no initial burst for amide substrates.
  - Hydrolysis of the acyl enzyme intermediate is the same, irrespective of substrate, but it is rate limiting for ester substrates, so its pH dependence is observed.
  - It is not rate limiting for amide substrates, so its pH dependence is silent.
**Chymotrypsin 1° Structure**

- Compare the sequences of "related" proteins
- Ser 195 and His 57 are conserved
- Suggests they are essential

**Chymotrypsin 3° Structure**

- Solve structure in presence of formyl-Trp-amide
- Pseudosubstrate binds near Ser 195 and His 57
- Also implicates Asp 102

**Muscle Aldolase**

Bernard Horecker’s mechanism

Horecker found that the strong reducing agent sodium borohydride irreversibly inactivated the enzyme in the presence of either DHAP or F-1,6-bisP. If the borohydride was tritiated, tritium appeared in the inactive enzyme. The tritiated enzyme could be cut enzymatically and the resulting peptides could be mapped to determine where the tritium was attached. The tritium and a reduced derivative of the DHAP were attached to the ε-amino group of a lysine, implying the presence of a DHAP-Lys imine intermediate, which was trapped by the reduction.
Take a Breath

More Mechanisms

So far, except for a brief reference to bound metal ions, we have only considered reactions that can be catalyzed by amino acid sidechains. There are only a few types of sidechains, and they can’t do everything, so enzymes enlist the aid of non-protein components, "cofactors".

Vitamins and Minerals

Cofactors

B-vitamins are water soluble, essential compounds, most of which enable enzymes to carry out more complex reactions. Many of them contain AMP.

Pyridine Nucleotides

NAD+/NADH, Coenzyme 1, Coczymase
NADP+/NADPH (from niacin)

Hydride (H-) transfer reactions, i.e., 2-electron oxidations/reductions
Including most “dehydrogenase” reactions
Examples: Lactate dehydrogenase, Alcohol dehydrogenase
Reduced form is fairly strong reducing agent, $\epsilon$° = -400mV
UV Spectra
Flavins

Flavin “mononucleotide”, FMN/FMNH$_2$

(Vitamin B$_2$, Riboflavin, coenzymes)
1-electron transfer reactions
2-electron transfer reactions
Many reactions involving O$_2$ involve a flavin and an iron ion.

Flavins

Flavin Adenine “Dinucleotide”, FAD/FADH$_2$

UV/Visible Spectra - titration with oxidizing agent

There are three oxidation states
Oxidized
Reduced
Semiquinone (one-electron gained or lost, paramagnetic)

UV/Visible Spectra - titration with oxidizing agent

Flavins and pyridine nucleotides
Sometimes work together

A typical series of reactions in metabolism follows the sequence:

FAD $\rightarrow$ FADH$_2$

$R$-$\text{CH}_2$-$\text{CH}_2$-$R$ $\rightarrow$ $R$-$\text{CH}_2$-$\text{CH}_2$-$R$ $\rightarrow$ $R$-$\text{CH}_2$-$\text{CH}_2$-$R$

$[+ \text{H}_2\text{O}]$

In the Krebs Cycle, these compounds are succinate, fumarate, malate and oxaloacetate. In fatty acid oxidation, they are fattyacyl groups. In fatty acid synthesis, the reactions are in reverse order, and the compounds are fattyacyl Acyl-Carrier Protein derivatives and the coenzyme is NADP$^+$. There are many exceptions; sometimes, flavins participate in oxidations of alcohols to ketones and sometimes pyridine nucleotides participate in desaturations.

Thiamine Pyrophosphate

Thiamine, vitamin B$_1$, “cocarboxylase”

Acyl transfer reactions, oxidative decarboxylations.
Examples of enzymes: Pyruvate decarboxylase portion of pyruvate dehydrogenase complex, acetoin synthetase, transketolase.

Thiamine Pyrophosphate
**Thiamine Pyrophosphate**

**General Mechanism**

The C-H is “active”, and the C- is a good nucleophile. The C- attacks a carbonyl carbon, typically α to a carbonyl group. The adjacent carboxyl, etc., can expel the coenzyme-bound fragment, which is a good leaving group because it can accommodate the electrons from the broken bond. The fragment can attack another electrophile (e.g., transketolase), or give the electrons to an acceptor, or pick up a proton.

**Lipoic Acid**

**Acyl transfer reactions**

Examples of enzymes: Lipoic reductase/transacetylase portion of pyruvate dehydrogenase. Reaction to be shown later.

**Coenzyme A**

**Acyl transfer and condensation reactions**

Vitamin form: calcium pantothenate.

**Coenzyme A**

**two kinds of reactions**

Activates acyl groups because it is a good leaving group.

Activates acyl groups because it is a good leaving group.

**Pyruvate dH**

E3 dimer

E1 dimer
**Biotin carboxylation reactions**

Examples of enzymes:
- Pyruvate carboxylase
- Acetyl-CoA carboxylase
- Propionyl-CoA carboxylase

Forms an amide bond with protein; bound form called biocytin.

Suggested mechanism for pyruvate carboxylase

Strongly bound by a protein from egg called avidin; avidin inhibits all biotin enzymes.

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**Pyridoxal Phosphate (pyridoxine), vitamin B₆**

Decarboxylation, dehydration of amino acids, sulfur incorporation.

Examples of enzymes: Histidine decarboxylase

Pyridoxal phosphate... is held in the active site as an imine

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**S-Adenosyl Methionine**

Methyl donor (only methyl, not other oxidation states)

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**Folate Cofactors**

*Folic acid*

1-carbon transfer reactions (various oxidation states)

Example: Thymidylate synthetase (methylation of dUMP to dTMP)

1-carbon unit generally held here, e.g., N⁵,N¹⁰-Methylene FH₄

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**Coenzyme Q**

*Ubiquinone, Q-10*

an electron carrier
**Vitamin C**

ascorbic acid

Strong 2-e reducing agent ($e^- = \text{mV}$)

![Chemical structure of Vitamin C]

**Metal Ions**

**Ions**

Zn$^{+}$, Mo$^{5+}$, Ni$^{+}$, etc.

Usually tetrahedral, occasionally octahedral, coordinated by N or O atoms from the protein, substrate, or water (when there is no substrate).

*Example:*
The zinc atom of alcohol dehydrogenase

**Metal Clusters**

Iron and a few other metals sometimes occur in clusters

Non-heme iron proteins: ferredoxin, high-potential iron proteins: aconitase

Mixed metal clusters exist - FeMo of Nitrogenase

**More Metal Ions**

Cu$^{+}$/Cu$^{2+}$

Oxidases, superoxide dismutase, polyphenol oxidase, plant nitrite reductase.

Tetrahedral, as shown in the recent X-ray structure of nitrite reductase (stereo pair on the left).

**More Metal Ions**

Mo ($\text{Molybdenum}$)

Nitrogenase, nitrite reductase. Exists in clusters, but more commonly bound to a cofactor known as molybdopterin.

![Chemical structures of metal clusters]

**Metal Clusters**

Iron and a few other metals sometimes occur in clusters

Non-heme iron proteins: ferredoxin, high-potential iron proteins: aconitase

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**Hemes**

$O_2$ binding, 1-e/Fe transfer rx, disproportionation

(not a vitamin)

Examples: Catalase, peroxidase, cytochrome P-450

Structure (iron protoporphyrin IX, there are others)

Extraplanar ligation from

*Enzyme (usually His) or substrate (usually O) or water

![Chemical structures of hemes]

**More Metal Ions**

Mo ($\text{Molybdenum}$)

Nitrogenase, nitrite reductase. Exists in clusters, but more commonly bound to a cofactor known as molybdopterin.

![Chemical structures of metal clusters]

(For more info [at](http://metallo.scripps.edu/PROMISE/MOCOMAIN.html))