Enzymes as Catalysts

1. **Overview**
   - (a) discovery of enzymes
   - (b) function & importance
   - (c) catalytic power & specificity
2. **Characteristics and Properties**
   - (a) What is a catalyst?
   - (b) relation to free energy diagrams
   - (c) enzyme - substrate interactions
     - (i) lock & key model
     - (ii) transition state model
     - (iii) induced fit model
   - (d) enzymes as proteins
   - (e) non-protein cofactors
     - (i) metal ions
     - (ii) organic cofactors
3. **Nomenclature / Classification**
4. **Enzyme Purification and Assay**
   - (a) activity measurements
   - (b) enzyme units
   - (c) turnover number and properties
   - (d) purification and purity
   - (e) initial velocity measurements
   - (f) assay conditions

A. Historical Aspects

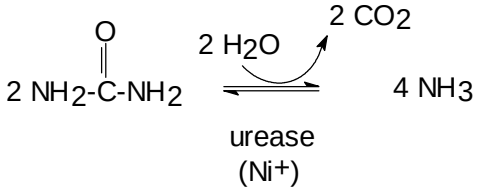
Enzymes are catalysts: increase the rate of reaction without undergoing change
-1 reaction: 1 or more enzymes
-*E. coli*--3000 enzymes
-eukaryotic cell--50,000 enzymes
"**Enzyme**"--means "in yeast" (Greek)
-used by man--fermentation of alcohol and cheese

1.(a) discovery of enzymes

- enzyme first used in 1878 by Kühne to indicate yeast were organized systems capable of fermentation

- shown by Büchner in 1897 that yeast extracts could also ferment sugar to alcohol; nevertheless, the term enzyme (<u>in</u> yeast) adopted and now refers to biological catalysts produced by living organisms

- attempts to purify enzymes began, resulting in the crystallization of urease by Sumner in 1926 $\Rightarrow$ enzyme consisted of protein!
  -urease catalyzes the hydrolysis of urea to form CO_2 and NH_3



- ultracentrifuge indicated most enzymes had large size (M_r of 10^4 - 10^7) $\Rightarrow$ also showed each enzyme was a homogeneous population.

- chemistry of enzymes first required an understanding of their structure (1st amino acid sequence (ribonuclease) obtained in 1960), three-dimensional organization (x-ray crystal structure of lysozyme in 1965) and some knowledge of their mechanism of action (Fischer's lock & key model - 1894) by detailed kinetic investigation.

- present day can easily synthesize proteins by recombinant DNA methodology (1st chemical synthesis in 1969) and manipulate the structure using site-directed mutagenesis to examine important sections of the

enzyme.

1.(b) function and importance

- enzymes are biological catalysts

- primary function is to catalyze all metabolic processes within a cell. An understanding of enzymes therefore leads to an understanding of the relationships and control of cellular function.

- today, enzymes are also used as analytical reagents, eg. ELISA; gene reporters

- they have been applied to medicine

  (i) measurement of key enzymes as an indication of disease e.g., phenylketonuria

  (ii) genetic disorders

  (iii) treatments, e.g., cystic fibrosis

- are important for industry / biotechnology
  -eg., invertase (sucrase)

  sucrose + H_2O ⟶ glucose + fructose

1.(c) catalytic power and specificity

- catalytic power is illustrated by the ability of enzymes to catalyze reactions under conditions of 37°C, pH 7.2, 1 atm

  $N_2 + 8H^+ \rightleftharpoons 2NH_3 + H_2$

<u>industry</u>	<u>nitrogenase</u>
800K	300K
900 atm	1 atm

Fe	Fe, Mo
acid	neutral

- **specificity** relates to binding of substrate to enzyme

- group specific enzymes can use a variety of substrates, each containing a certain functional group which is modified

- absolute specificity utilize only one substrate (or specific pair) in one reaction

2.(a) What is a catalyst?

- any molecule that increases the speed (or rate) of a chemical reaction without undergoing a permanent change in its structure.

2.(b) relation to free energy diagrams: catalysts reduce the energy of activation for a reaction (see figures)

2. (c) enzyme-substrate interactions

(i) lock & key (Fischer: 1894)

(ii) induced-fit (Koshland: 1958)

(iii) transition state stabilization

2. (d) enzymes as proteins

- composed of amino acids linked by peptide bonds

- important feature is 3-dimensional organization (2°, 3°, 4° structures)

- destroyed by heat / other denaturants (guanidinium HCl, urea, organic solvents, detergents)
  -mechanical disruption
  -keep at low temperature (ice): (i) proteolysis, (ii) thermal denaturation

- synthesized by cell from gene

  1 gene = 1 enzyme (protein)

  -regulation of production at level of gene
  -stability of mRNA $\rightarrow$ enzyme levels

2. (e) non-protein cofactors

- biological materials cannot induce certain reactions because proteins don't contain certain molecular features

- therefore, assisted by binding of either

  (i) metal ions, or

  (ii) organic cofactors

active enzyme (holoenzyme)	=	protein + cofactor (apoenzyme)

3. Nomenclature / Classification

$\Rightarrow$ all enzymes placed into 6 categories based on reaction that is being catalyzed
$\rightarrow$ many enzymes given trivial names, eg., catalase; suffex "ase" added to the substrate name
$\rightarrow$ systematic naming: Enzyme Commission (1955)
$\rightarrow$ Web site: HTTP://www.expasy.ch/sprot/enzyme.html

E.C. #^{A}. #. #. # (Reference: Palmer Chapter 1 and web site, see above)

#^{A} = enzyme class or type of reaction that is catalyzed (see types 1-6 below); the next # - often refers to the subclass or type of substrate; the third # refers to the subsubclass: more precise determination of the reaction being catalyzed; the fourth # is the enzyme serial number assigned by the Enzyme Commission.

1. **oxidoreductase <u>or</u> dehydrogenase**, reductase, oxidase ($A = O_2$)
   - oxidation—reduction reactions

     $$DH_2 + A \rightleftharpoons D + AH_2$$

   - substrate: oxidase
   - substrate: dehydrogenase

2. **transferase**
   - group transfer reactions

     $$DX + A \rightleftharpoons D + AX$$

   - substrate: transferase

   donor: acceptor group-transferase.

3. **hydrolase**
   - hydrolytic reactions

     $$A - X + H_2O \rightleftharpoons X - OH + HA$$

     substrate + "-ase" = substratase
   - substrate: group hydrolase

4. **lyase**
   - elimination reactions

   non-hydrolytic removal of a group leaving a double-bond (usually)

   <u>or</u>

   addition of a group over a double bond.

5. **isomerase <u>or</u> racemase,** epimerase, tautomerase, mutase, cis-trans isomerase, cyclo-isomerase
   - isomerization reactions

6. **ligase**
   - reactions in which two molecules are joined at the expense of an energy source

     $$X + Y + ATP \rightleftharpoons X - Y + ADP + P_i$$

     X:Y ligase (ADP-forming)

Examples

1. lactate dehydrogenase

$$\text{L-lactate} + NAD^+ \Leftrightarrow \text{pyruvate} + NADH$$

- systematic name is: L-lactate:NAD^+ oxidoreductase, which denotes the fact that L-lactate acts as the electron donor and NAD^+ acts as the electron acceptor

(a) class $\Rightarrow$ oxidoreductase $\therefore$ EC 1
(b) acts on CH-OH group of donors $\therefore$ 1.1
(c) with NAD^+ or $NADP^+$ as acceptor $\therefore$ 1.1.1
(d) 1.1.1.27 represents the classification with the serial number for lactate dehydrogenase (27)

2. hexokinase

$$\text{ATP} + \text{D-hexose} \Leftrightarrow \text{ADP} + \text{D-hexose 6-phosphate}$$

(a) class $\Rightarrow$ transferase $\therefore$ EC 2
(b) transferring phosphorus-containing groups $\therefore$ 2.7
(c) phosphotransferases with an alcohol group as acceptor
(d) 2.7.1.1 assigned serial number of hexokinase

name: ATP: D-hexose 6-phosphotransferase

3. adenosinetriphosphatase

$$\text{ATP} + H_2O \Leftrightarrow \text{ADP} + \text{orthophosphate}$$

(a) class $\Rightarrow$ hydrolase $\therefore$ EC 3
(b) acting on acid anhydrides $\therefore$ 3.6
(c) in phosphoryl-containing anhydrides $\therefore$ 3.6.1
(d) 3.6.1.3 is the EC serial number for adenosinetriphosphatase

name: ATP phosphohydrolase

4. fructose-bisphosphate aldolase

D-fructose 1,6 bisphosphate $\Leftrightarrow$ D-glyceraldehyde phosphate + dihydroxyacetone 3-phosphate

(a) class $\Rightarrow$ lyases $\therefore$ EC 4
(b) carbon-carbon lysases $\therefore$ 4.1
(c) aldehyde-lyases $\therefore$ 4.1.2
(d) EC serial number for fructose-bisphosphate aldolase is 4.1.2.13

name: D-fructose 1,6 bisphosphate D-glyceraldehyde 3-phosphate-lyase

5. triosphosphate isomerase

D-glyceraldehyde 3-phosphate $\Leftrightarrow$ dihydroxyacetone phosphate

(a) class $\Rightarrow$ isomerase isomerase $\therefore$ EC 5
(b) intramolecular oxidoreductases $\therefore$ 5.3
(c) interconverting aldolases and ketoses $\therefore$ 5.3.1
(d) EC serial number for triosphosphate isomerase is 5.3.1.1

systematic name: D-glyceraldehyde 3-phosphate ketol-isomerase

6. isoleucyl-tRNA synthase

ATP + L-isoleucine + $tRNA^{Ile}$ $\Leftrightarrow$ AMP + pyrophosphate + L-isoleucyl-$tRNA^{Ile}$

(a) class $\Rightarrow$ ligase $\therefore$ EC 6
(b) forming carbon-oxygen bonds $\therefore$ 6.1
(c) ligases forming aminoacyl-tRNA $\therefore$ 6.1.1
(d) EC serial number for isoleucyl-tRNA synthetase is 6.1.1.5

name: L-isoleucine: $tRNA^{Ile}$ ligase (AMP-forming)

4. Enzyme Purification and Assay

(a) activity measurements

- enzymes use substrates and produce products
- the reaction rate depends on a number of variables:
  i. amount of substrate
  ii. amount of enzyme
  iii. temperature
  iv. pH
  v. presence of interfering substances

- at the start of a reaction, amount of substrate begins to decline
  $\Rightarrow$ rate of decline is typically non-linear
- later in the reaction, substrate concentration decreases at a slower rate.

- same is true for formation of product.

$\Rightarrow$ to measure reaction rates, an enzyme assay is used which is directed to measuring the disappearance of substrate or the appearance of product.

But, rates decrease as the reaction progresses. How is activity then measured? Define initial rate as the speed of conversion of substrate to product in the absence of product (usually $t = 0$).

- two methods to measure enzyme activity:

  i. fixed time or discontinuous assay
  ii. kinetic or continuous assay

- activity (or rate) is described as the change in substrate (or product) concentration over time.

-most often: $\text{Rate} = \frac{\text{moles of P formed}}{\text{time interval}} = \Delta P/\Delta t$

- however, rate is dependent on enzyme and substrate concentration!
- to account for enzyme amounts, another term, specific activity, is generally used.

(b) enzyme units

- enzyme solutions described in terms of arbitrary units because active at very low concentrations and purity is frequently unknown

Unit

-one international unit - amount of enzyme catalyzing the transformation of 1 μmol of substrate per min under defined conditions

-based on measurements of initial velocity

- greater the purity of an enzyme, the greater the fraction of total protein that is actually enzyme protein; relationship defined as "**specific activity**"

- "**specific activity**" - number of enzyme units per amount (mg) of total protein = **U/mg protein**

- **total activity** = U/mg prot x total mg prot. in the fraction

or

- **total activity** = U/mL of frac. x total vol. (mL) of frac.

- I.U.B. proposed katal (1972)
- **katal** is amount of enzymatic activity that catalyzes the transformation of 1 mole of substrate per second under defined conditions
  - 1 U = 10^{-6} moles/60s = 16.7 x 10^{-9} mol/s
  - 1 U = 16.7 nkatal
  - 1 nkatal = 0.06 U
  - 1 katal = 6 x 10^{7} U
- concentration of enzyme in terms of katals is **"molar activity"**, defined as the number of katals per mole of enzyme

(c) turnover number (TN, k_{cat})

- number of moles of substrate transformed into product per unit time per mole of enzyme under optimal conditions **(molecular activity)**

- number of moles of substrate transformed into product per unit time per mole of catalytic site (active site) under optimal conditions **(catalytic center activity)**

- **ratio of V_{max} for enzyme to total enzyme concentration**

- TN = $V_{max}/[E_t]$ = μmol(S→P) x min^{-1} x mL^{-1}/(μmol enzyme or cat. site x mL^{-1}) = min^{-1} or $\mathbf{t^{-1}}$

- typical values fall in the range of 50 - 10^7 min^{-1}

(d) purification and assessment

- purification is the process by which an enzyme is separated from all other cell components.
- 3 categories of purification methods

i. **Precipitation** ⇒ differential solubility
- salting out with $(NH_4)_2SO_4$ most common.
- salts neutralize exterior charge of protein allowing aggregation.
- isoelectric precipitation uses same principle.

ii. **Chromatography**

various types, including:
gel filtration
ion-exchange
hydrophobic
adsorption
affinity

iii. **Electrophoresis**

polyacrylamide or agarose
native or denaturing

for interest only: possible to separate proteins by SDS/polyacrylamide gel electrophoresis (denaturing process), stain protein, cut protein of interest out of gel and renature. Does not work for most enzymes.

⇒ presentation of purification data is vital for analysis of purity and recovery.
- aim is to calculate yield and purification factor.
- need 3 measurements:

i volume of sample (ml)
ii protein content (mg/ml)
iii enzyme activity (units/ml)

- all other quantities derived by calculation:

sample vol (ml) $\times$ protein concn (mg/ml)	=	**total protein (mg)**
sample vol (ml) $\times$ activity (units/ml)	=	**total activity (units)**
$\frac{\text{total activity (units)}}{\text{total protein (mg)}}$	=	**specific activity (unit/mg)**

$\Rightarrow$ the higher the yield, the greater the recovery, the fewer times you'll need to get in the cold room and purify the enzyme.

$\Rightarrow$ the higher the purification factor, the purer the enzyme.

- the purification factor still doesn't give information about how pure it really is!

$\Rightarrow$ need several analytical methods for this determination.

$\Rightarrow$ What properties of any enzyme should be determined?

(i) molecular weight

(ii) subunit structure

(iii) amino acid composition

(iv) amino acid sequence

(v) three dimensional structure

- methods vary in difficulty, with the 1st three given typically provided once an enzyme is purified.

Molecular weight:

native - gel filtration, sedimentation

subunit - SDS/PAGE

Subunit structure:

both native & subunit molecular weights plus densitometry of SDS/PAGE.

(e) initial velocity measurements

- a typical enzyme rxn decreases and levels off as incubation time increases
  - denaturation of enzyme, product inhibition, decrease of enzyme saturation, inactivation of coenzyme, and increase of reverse rxn as product builds up

- critical that **velocity be determined at the very beginning of the rxn**
- initial velocity is the slope of the curve depicting S or P changes with time

  - $v = d[P]/dt = -d[S]/dt$

  - in practice: $v = \Delta[P]/\Delta t = -\Delta[S]/\Delta t$ <u>provided</u> that these parameters are measured at the <u>beginning</u> of the rxn where the rate of S consumption and P formation is linear with time

(1) Why must initial velocities be used?

- consider an expmt to measure the rate of rxn versus [E]

- get varying curves depending on the time at which the rxn is measured

- not linear with [E] except when measuring initial velocity (slope extending from the origin)

- consider another eg.: rate of rxn versus T (temperature)

- problem arises because by choosing an arbitrary incubation time, the incubation time itself now is a variable

- can be avoided by measuring initial velocities
- true initial rate increases exponentially with T

(f) assay conditions

(1) General aspects

- enzyme rxns *in vitro* are assayed by measuring (a) change in S or P with time, or (b) change in a physical or chemical property associated with the disappearance of reactant or appearance of P

- advisable to measure d[P]/dt rather than -d[S]/dt
  - more accurate to measure a change in quantity from zero to some value rather than a decrease in quantity from a large to a somewhat smaller value
- try to assay enzyme under optimal conditions of pH and T so rate (and P changes) are large; i.e., the assay has greatest sensitivity

"**Discontinuous assay**"--fixed time point sampling of kinetic data

"**Continuous" assay**"--rxn is analyzed continuously using a monitoring technique

- for the discontinuous assay the rate of P formation must be linear over the incubation time chosen
- for the continuous assay one must use the initial part of the curve for dP/dt
- for both continuous and discontinuous assays one must use [E] that falls within the range in which P formation varies linearly with [E]

(2) Assays of Enzymes or Substrates

Two types of assays

(a) Measure the amount of E present

- E is present at much lower concentration than S
- E is present in limiting amounts and S is present in excess
- assay conditions correspond to levelled off portions of v versus [S] curves
- thus velocity is proportional to [E]

(b) Measure the amount of S present

- [E] is high enough to convert all S $\rightarrow$ P
- S is limiting reagent

- assay conditions correspond to those of initial part of v versus [S] curves

(3) Coupled Reactions
- some reactions difficult to assay because of absence of a readily measurable compound or property
- couple one rxn to another which produces a measurable product

e.g.,

$$A \xrightarrow{E_1} B \qquad \text{Reaction 1}$$

$$B \xrightarrow{E_2} C \qquad \text{Reaction 2}$$

- if Rxn 1 is one of interest, it can be coupled to Rxn 2

- if rxn conditions for assaying E_2 are compatible with those of E_1 then both stages can be measured simultaneously

- if not as above, then the 2 stages of assay must be separated

  - rxn with E_1 is run first and stopped (boiling, change pH, etc.)

  - second rxn (with E_2) is added and rxn proceeds until all of B is converted to C

  - for such "coupled reactions" E_2 must be in sufficient concentration so that all of B is converted to C

    - the relative amounts of E_1 and A depend on the nature of assay

    - if E_1 is being measured then A should be in high concentration

    - if A is being measured then A should be in low concentration

End of Section I