Enzymes II

Mechanism of enzyme reaction, metalloenzymes, kinetics, activity, enzymes in medicine

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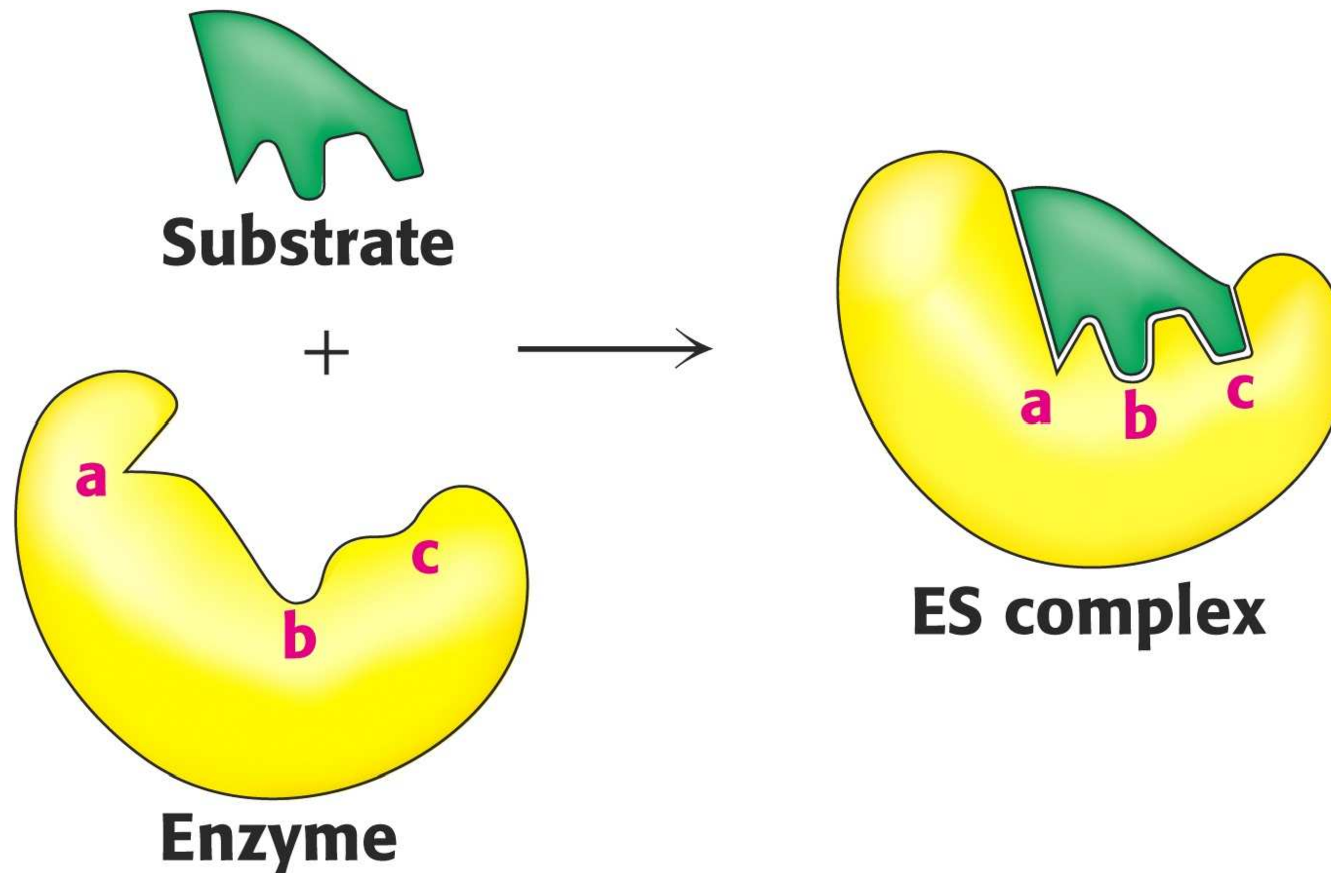
Active site of enzyme

- small part of molecule, deep cleft or pocket on the surface
- three-dimensional environment shields the substrate from water
- in oligomeric enzymes - interface between subunits
- made by side chains of AA which are distant in primary structure
- the site is really ***active*** - protein flexibility facilitates the conformation changes fitting to substrate
- substrate is bound relatively weakly

Binding substrate to enzyme

- binding a substrate induces the conformational changes in enzyme molecule (induced fit model)
- an enzyme-substrate (ES) complex is formed



Example: The active sites of three proteinases

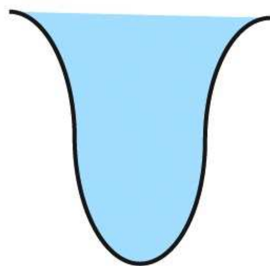
Certain AA residues determine the substrate specificity of these enzymes.

The peptide bonds preferably hydrolyzed:

by **chymotrypsin -** near residues with large, hydrophobic, uncharged side chains (Phe, Trp),

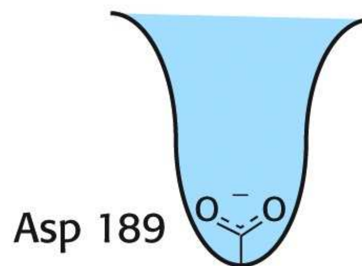
by **trypsin –** near residues with long, positively charged side chains (Arg, Lys),

by **elastase** - near residues with small hydrophobic side chains (Gly, Ala).



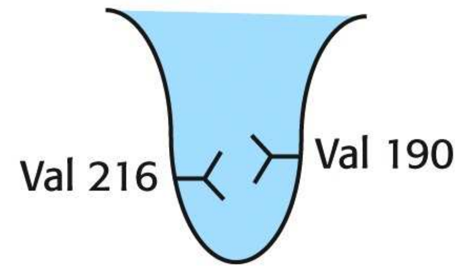
Chymotrypsin

A deep, relatively hydrophobic pocket



Trypsin

At the bottom of the deep pocket there is an acidic residue (Asp)

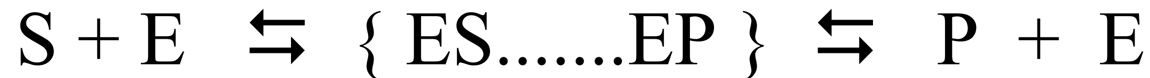


Elastase

Two residues of valine close off the mouth of the small hydrophobic pocket

Catalytic mechanism depends on the number of substrates

- **Monosubstrate reactions**



$$S + E \leftrightarrows \{ ES.......EP \} \leftrightarrows P + E$$

- **Bisubstrate reactions (more common)**

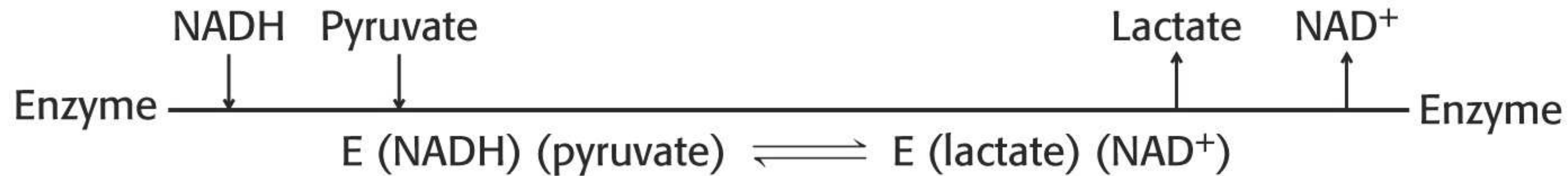
$$S_A + S_B \leftrightarrows \{ ES_{AB}.......EP_{AB} \} \leftrightarrows P_A + P_B + E$$

Sequential reaction: both substrates bind to enzyme, chemical conversion proceeds, and both products are released

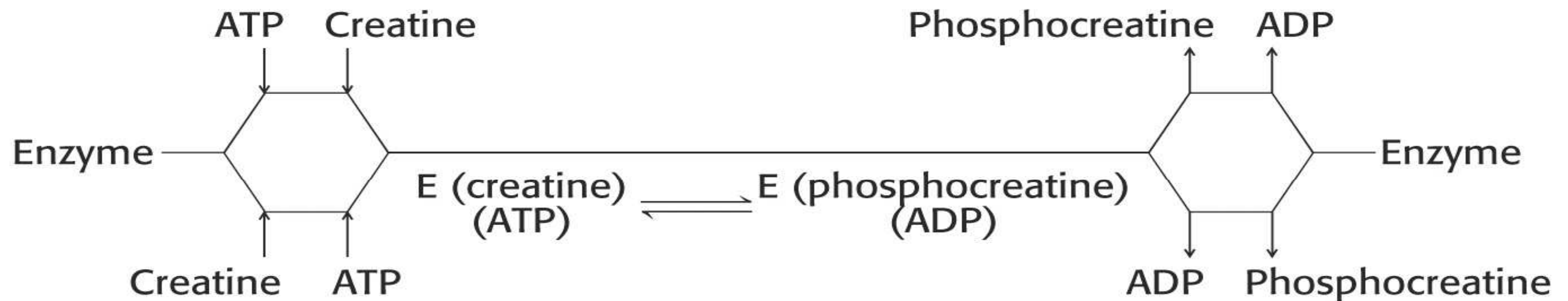
Ping-pong reaction: typical for aminotransferases, one substrate is attached, converted, and released, then the same events occur with the second substrate

Sequential reaction:

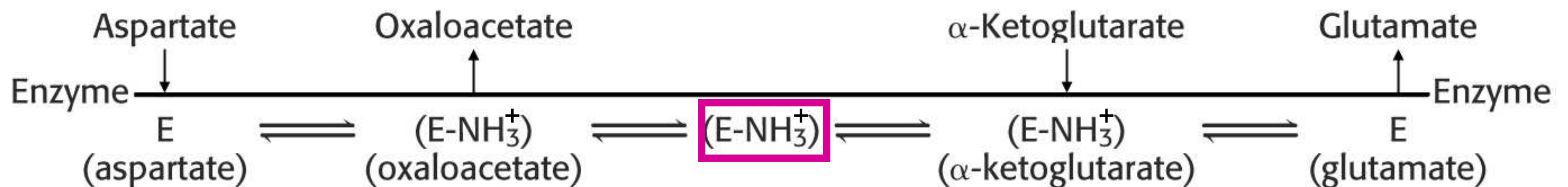
- type **ordered** - the substrates bind the enzyme in a defined sequence



- type **random** – the order of addition of substrates and release of products is random:



Ping-pong reaction:



Catalytic groups

- located in active site
- involved the chemical conversion of substrate
- nucleophilic (cysteine -SH, serine -OH)
- acidic (Asp, Glu), basic (His, Arg, Lys)

Examples of different types of catalytic mechanisms:

- Catalysis through proximity and orientation effects (strained reactants)
- Covalent catalysis – formation of transitory covalent bonds between enzyme and substrate
- Acid-base catalysis – protonization of substrates or catalytic groups of enzyme
- Metal ion catalysis mediating redox reactions or shielding negative el. charges
- Electrostatic catalysis (after excluding water from the active site by binding a substrate)

Examples of proteinases

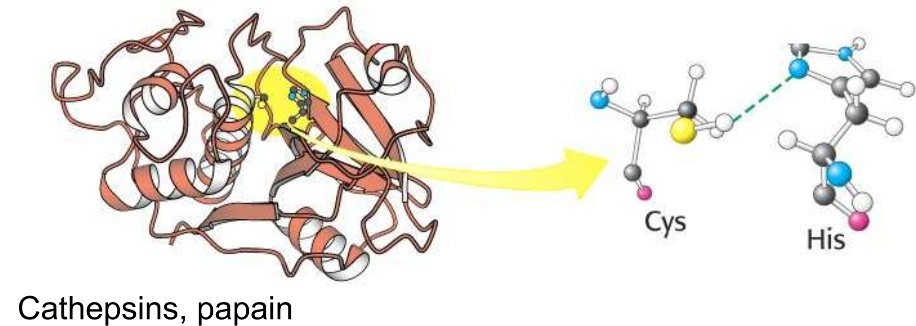
Due to the different arrangement

of active sites, they exhibit

• **different substrate specificities**

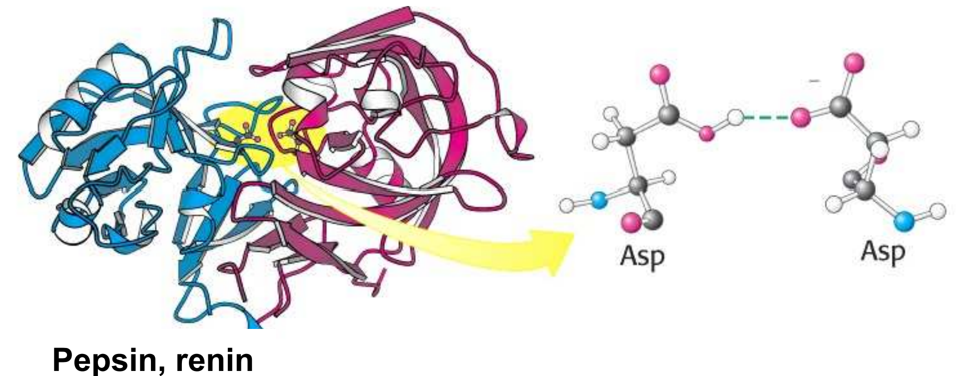
• **different catalytic mechanisms**

CYSTEINE PROTEINASES



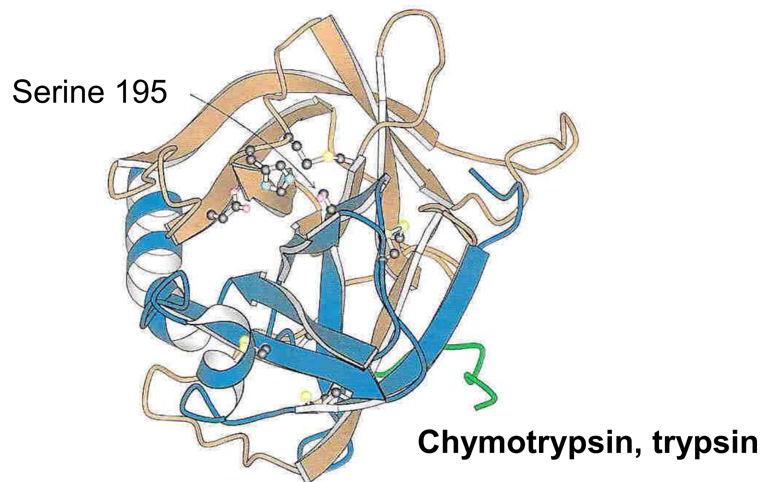
Cathepsins, papain

ASPARTATE PROTEINASES



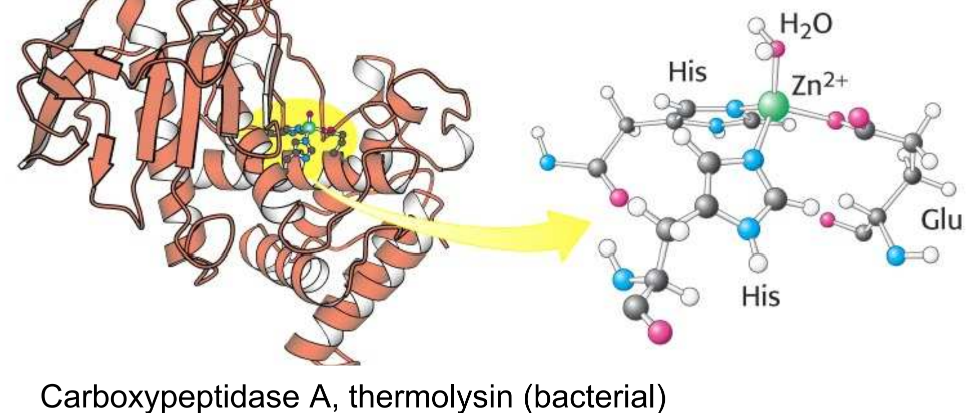
Pepsin, renin

SERINE PROTEASES



Chymotrypsin, trypsin

METALLOPROTEINASES

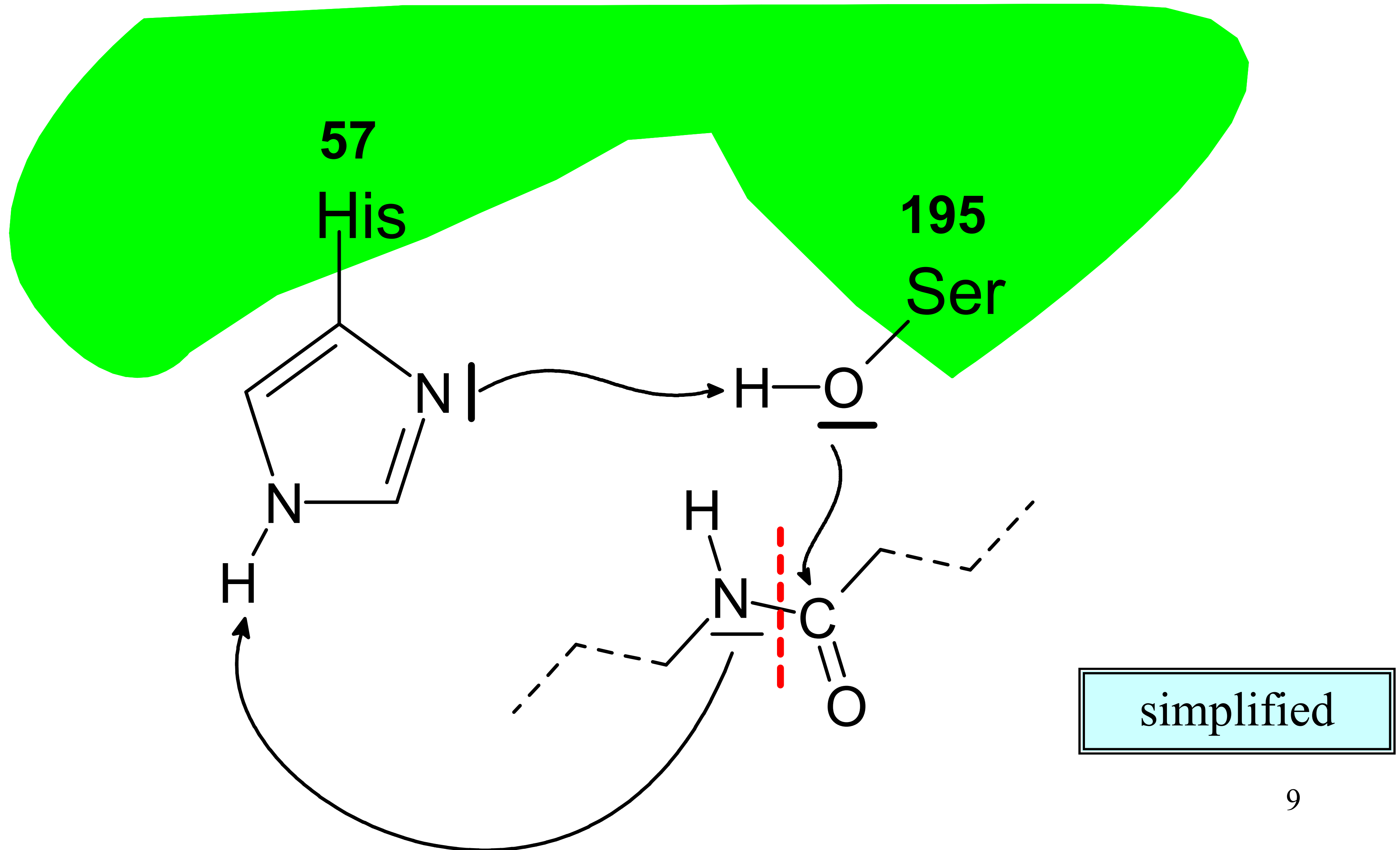


Carboxypeptidase A, thermolysin (bacterial)

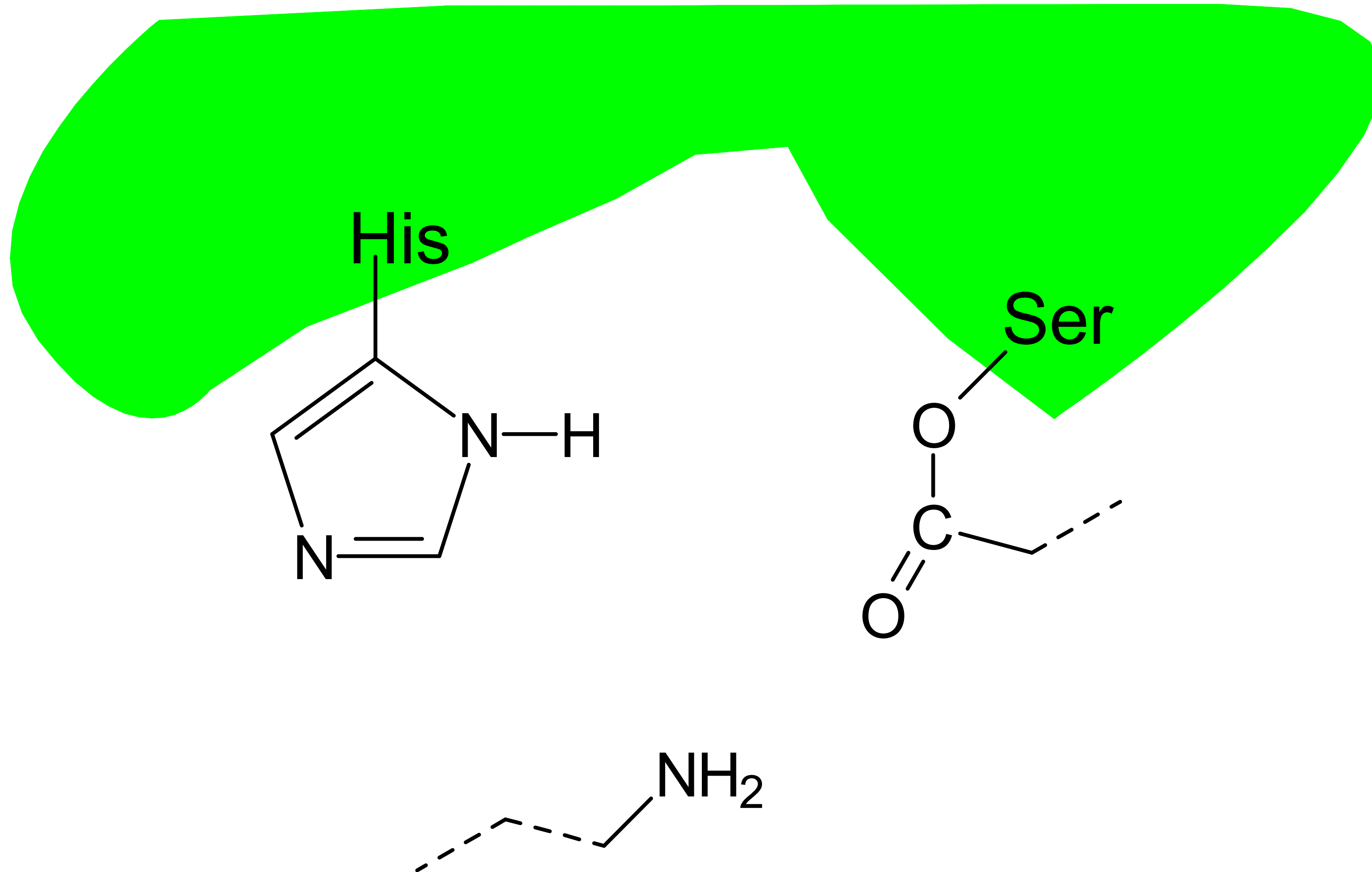
Example: active site of chymotrypsin

Nucleophilic attack of serine -OH to carbonyl carbon of peptide bond

$\Rightarrow$ serine protease



Active site of chymotrypsin: the cleavage of peptide bond

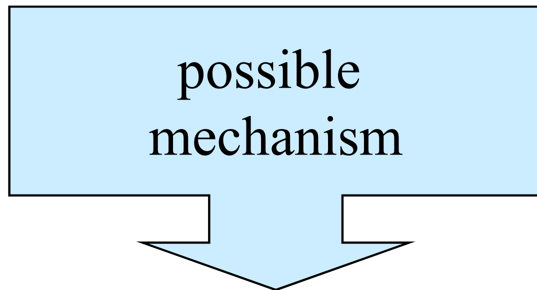


Metalloenzymes

- contain **functioning metal cations** (prosthetic groups) directly involved in catalyzed reaction, metal ions attached tightly (Enz-M)
- some enzymes need metal ions just for **activation**, they are associated relatively weekly (Enz …M), e.g. Ca^{2+} (coagulation factors), Mg^{2+} (kinases)

Metal ion is a part of ternary complex

- three components make a ternary complex: enzyme (Enz), substrate (S), and metal ion (M)
- different types of complexes: Enz-S-M, Enz-M-S, or cyclic complexes



- vacant metal orbitals accept electron pair of nucleophil to make σ-bond
- metal ions make chelates with some groups of enzyme/substrate ⇒ deformation of structure ⇒ strain ⇒ facilitates chemical conversion
- coordination sphere of metal creates a three-dimensional template ⇒ stereospecific control of reaction

Molybdenum (Mo)

- Some oxidoreductases
- cofactor – molybdopterin
- **Xanthine oxidase** (xanthine → uric acid)
- **Sulfite oxidase** (sulfite HSO_3^- → sulfate SO_4^{2-})

Mo in food: legumes, wholemeal cereals

Zinc (Zn)

- Many enzymes
- **Alcohol dehydrogenase** (ethanol → acetaldehyde)
- **Carbonic anhydrase** ($H_2O + CO_2 \leftrightarrows H_2CO_3$)
- **Carboxypeptidases** (cleavage of polypeptides from C-terminal)
- **Cu, Zn-superoxide dismutase** (cytosolic isoform)

  ($2 \bullet O_2^- + 2 H^+ \rightarrow O_2 + H_2O_2$)

Zn in food: red meat, lobsters, legumes, seeds, wholemeal cereals

Copper (Cu)

- many oxidoreductases
- **Ceruloplasmin (ferroxidase)** ($Fe^{2+} \rightarrow Fe^{3+}$)
- **Cytochrome-*c*-oxidase** (R.Ch., electron transfer to O_2)
- **Monoamine oxidases** (MAO, inactivation of biogenic amines, side product H_2O_2, see Med. Chem. II, p. 60)
- **Dopamine hydroxylase** (dopamine → noradrenaline)
- **Lysyl oxidase** (collagen maturation, Lys → alLys)

Cu in food: liver, meat, cocoa, legumes, nuts

Manganese (Mn)

- some hydrolases, decarboxylases, transferases
- **Mn-superoxide dismutase** (mitochondrial isoform)
- **Arginase** (arginine → urea + ornithine)
- Synthesis of proteoglycans + glycoproteins

Mn in food: legumes, nuts, wholemeal cereals

Iron (Fe)

- heme and non-heme iron enzymes
- **Catalase** (heme, $H_2O_2 \rightarrow H_2O + \frac{1}{2}O_2$)
- **Myeloperoxidase** (heme, neutrophils)

  $H_2O_2 + Cl^- + H^+ \rightarrow HClO + H_2O$
- **Cytochromes** (heme, transfer of electrons in R.Ch.)
- **Fe-S proteins** (non-heme, transfer of electrons in R.Ch.)

Fe in food: animal blood, red meat, liver, egg yolk, nuts, broccoli

Selenium (Se)

- few enzymes (redox reactions), Se in selenocysteine
- **Glutathione peroxidase**

  $(2\ GSH + H_2O_2 \rightarrow 2\ H_2O + G\text{-}S\text{-}S\text{-}G)$
- **Deiodases of thyronines** (thyroxine T4 → triiodothyronine T3)
- **Thioredoxin reductase** (ribose → deoxyribose)

Se in food: sea products, legumes

Factors determining the rate of enzymatic reaction

Temperature	Reaction rate increases with temperature, optimal around 40 °C, at higher temperatures the rate decreases due to enzyme denaturation
pH	pH value determines ionization of groups in active site and its surroundings, Constant pH of body fluids is maintained by buffer systems Each enzyme has its pH optimum, intracellular enzymes around pH 7 Digestion enzymes differ: pepsin pH 2
Activators	They accelerate enzyme reaction Often divalent metal cations: Ca^{2+}, Mg^{2+}, Zn^{2+}, Mn^{2+}
Inhibitors	Slow down or stop enzyme reaction competitive: similar to substrate, they compete for active site Other, e.g. heavy metal ions, strongly bind to important enzyme groups
Substrate concentration	At high substrate concentration, enzyme is saturated, so the reactions proceeds with maximal velocity, graphically expressed as saturation curve

Basic kinetic terms

- reaction: S $\rightarrow$ P (S = substrate, P = product)
- definition of reaction velocity (rate):

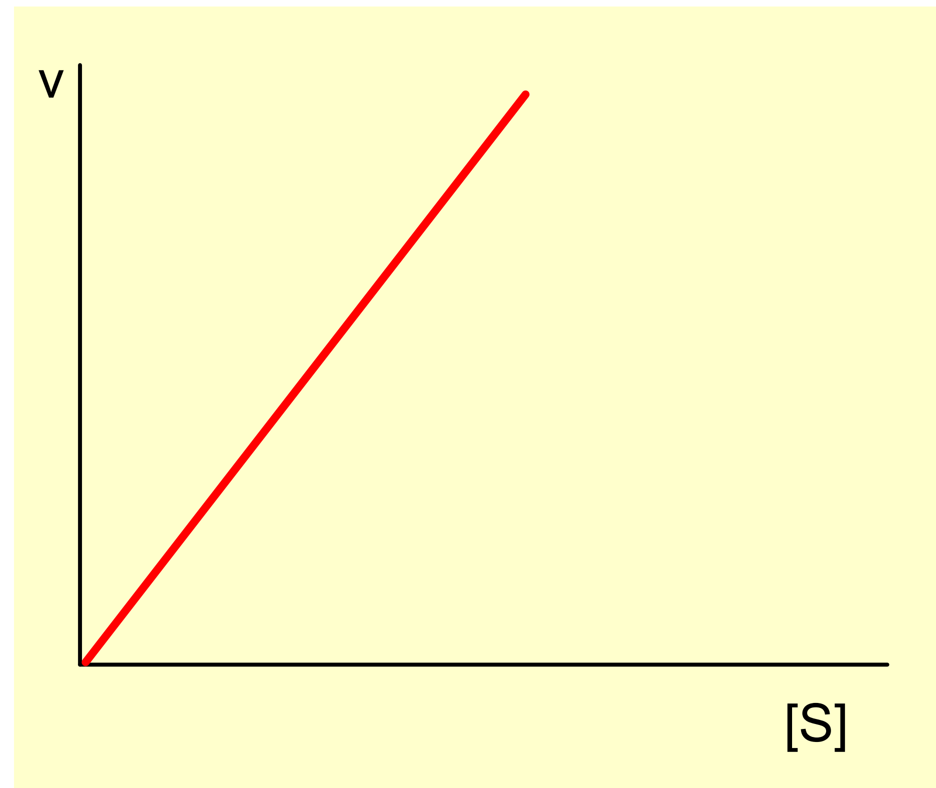
$$v = -\frac{\Delta[\mathrm{S}]}{\Delta t} = \frac{\Delta[\mathrm{P}]}{\Delta t} > 0 \quad [\frac{\mathrm{mol}}{\mathrm{l.s}}]$$

this definiton is for average rate,
instantaneous rate: d[S]/dt

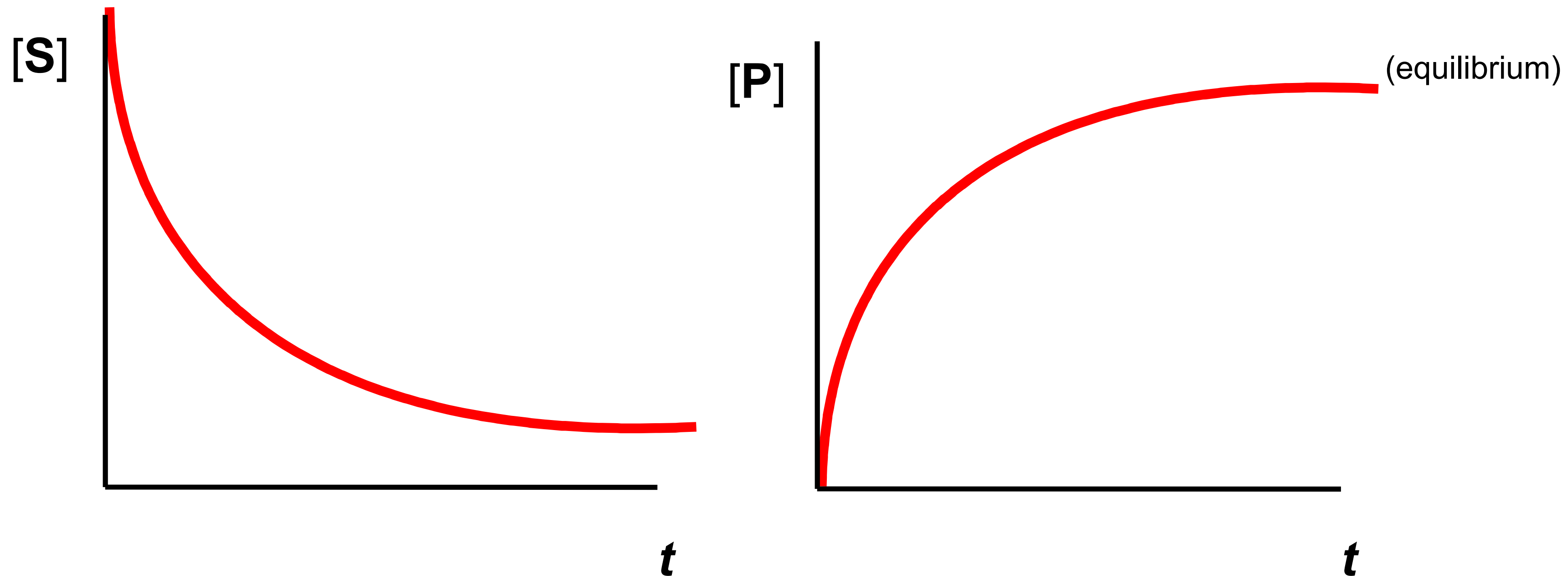
Kinetic equation for reaction S → P

$v = k[S] = k[S]^1 \Rightarrow$ reaction of 1. order

k = rate constant



Kinetic (progress) curves for substrate and product



during reaction:

concentration of substrate decreases

concentration of product increases

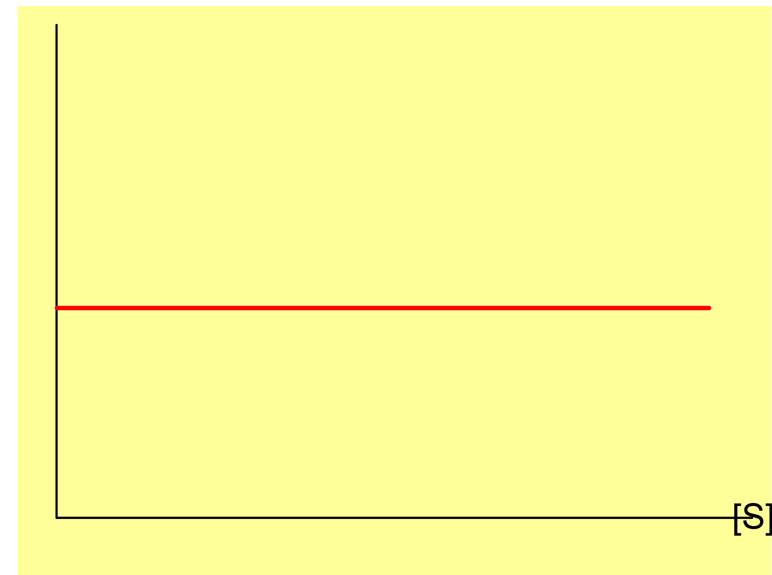
reaction rate is determined from kinetic curves

The instantaneous velocity v_x at any particular time t_x is given by the slope of the tangent to the curve at that time.

Reaction of 0. order

- Reaction rate does not depend on the substrate concentration
- $v = k\,[S]^{0} = k \times 1 = k = \text{constant}$
- At great excess of substrate S

in enzyme rections only in laboratory conditions



Initial velocity v_o

- The highest value of velocity
- It is not influenced by the decrease of substrate
  nor the reverse change of product
- **Determined from kinetic curves at the time $t = 0$**

Initial velocity v_o depends on substrate concentration

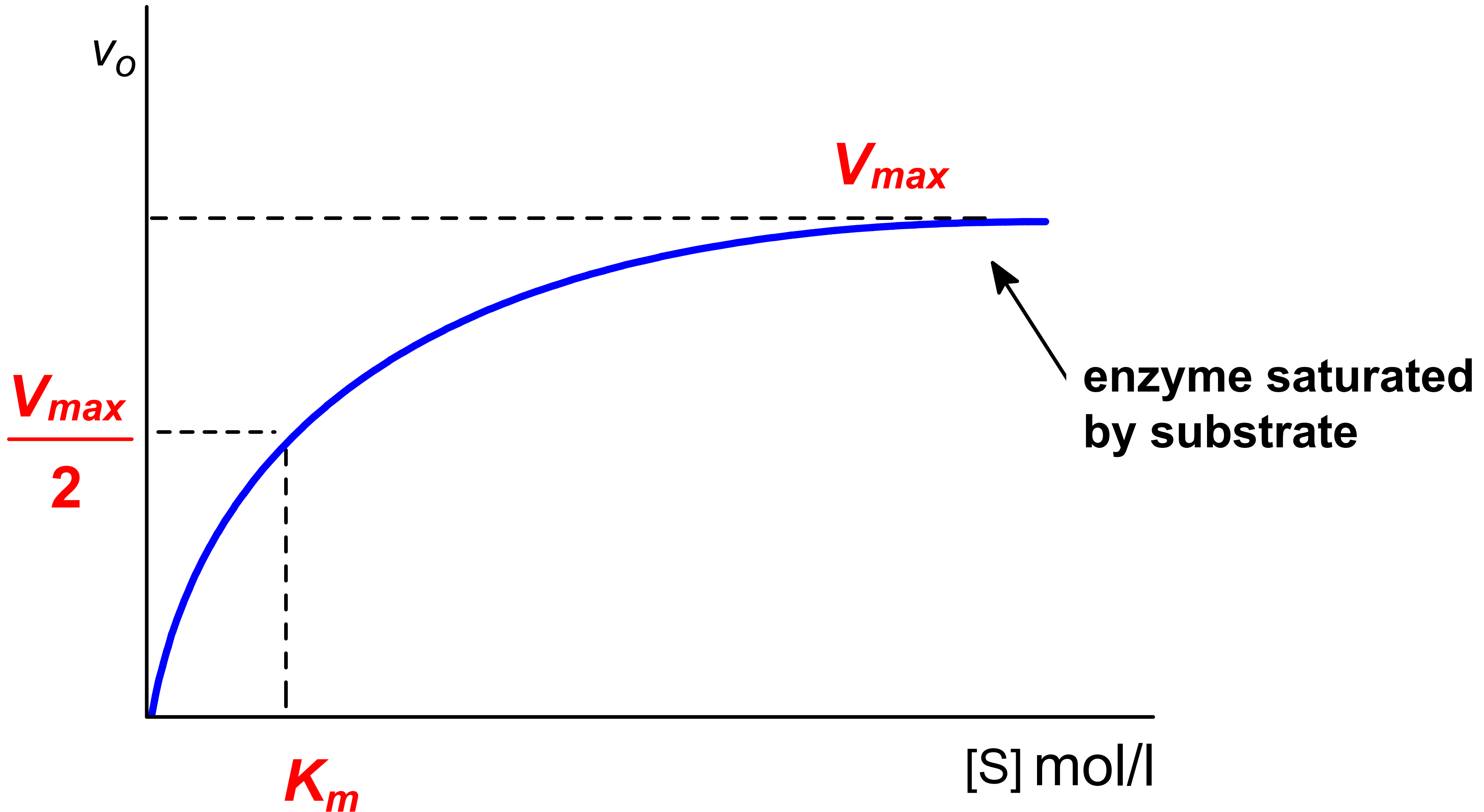
- Michaelis-Menten equation
- for one-substrate reactions

$$v_o = V_{\max} \frac{[S]}{[S] + K_m}$$

$V_{\max}$ = maximal velocity (for the given concentration of enzyme)

K_m = Michaelis constant

The graph of previous equation is saturation curve



If [S] << K_m

$$v_o = V_{max}\frac{[S]}{\cancel{[S]} + K_m} = \frac{V_{max}}{K_m}[S] = k[S]^1$$

at low substrate concentration,

the reaction proceeds by the **1st order kinetics**

If [S] >> K_m

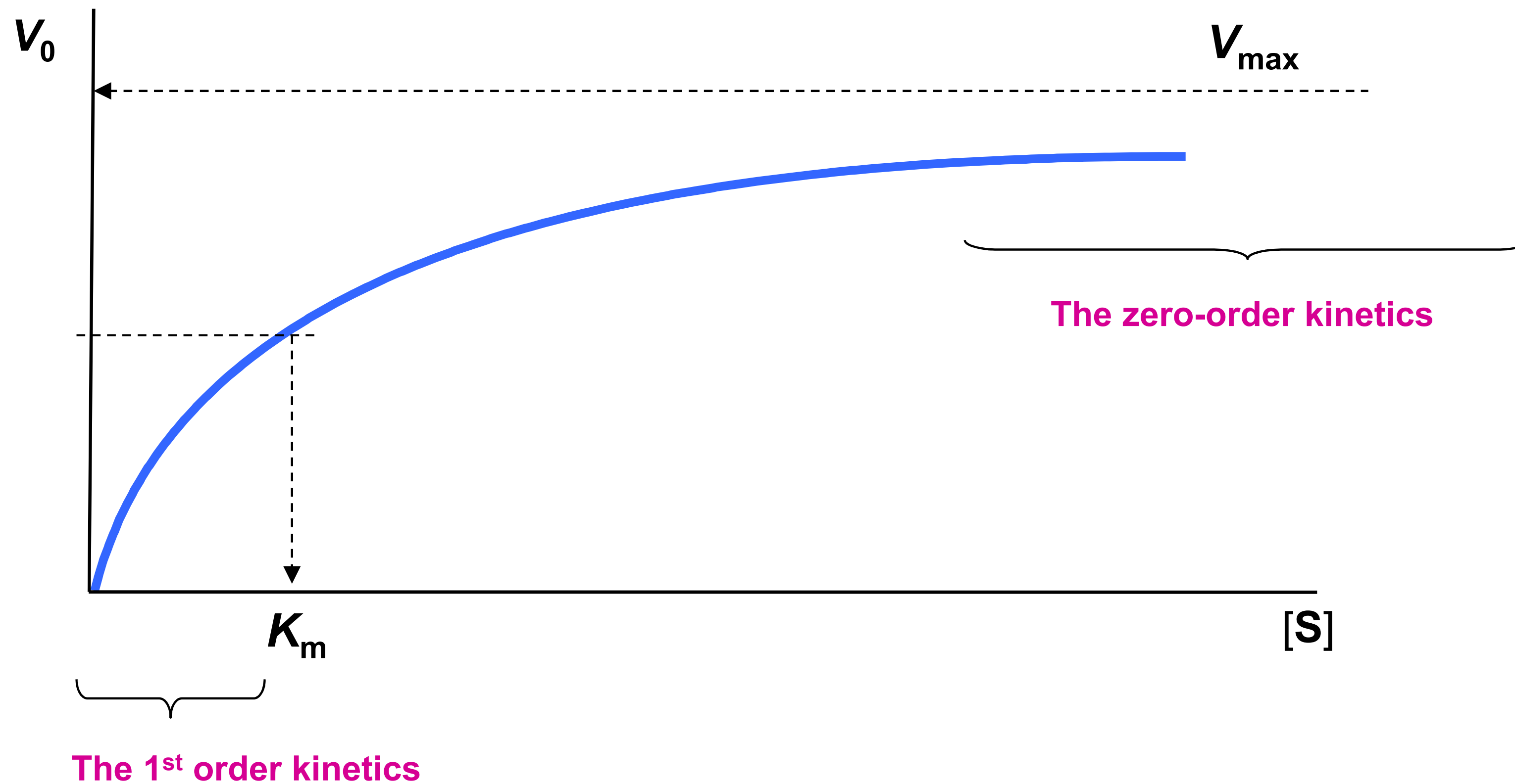
$$v_o = V_{\max} \frac{[S]}{[S] + \cancel{K_m}} = V_{\max} \frac{[S]}{[S]} = V_{\max} = k[S]^0$$

at high substrate concentration,

the reaction proceeds by the **0. order kinetics**

Two parts of saturation curve

compare with pages 21 and 23



If [S] = K_m

$$v_o = V_{\max} \frac{[S]}{[S]+[S]} = V_{\max} \frac{[S]}{2[S]} = \frac{V_{\max}}{2}$$

Significance of K_m and V_{max}

• the Michaelis constant K_m is the concentration of substrate [S] which gives half the maximal velocity V_{max} (50 % saturation of enzyme)

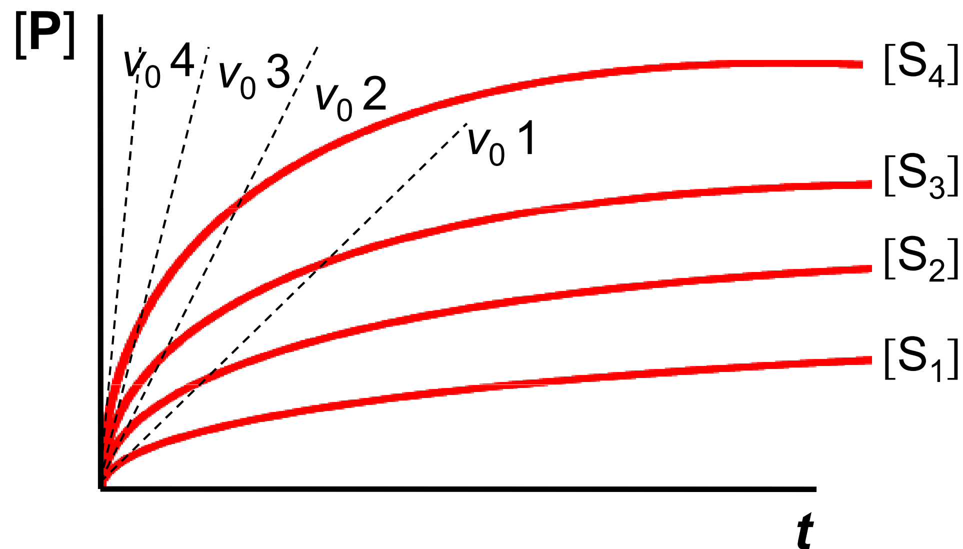
• the K_m has the dimension of concentration (mol/l)

• K_m is inversely related to the affinity of enzyme for its substrate.
If more substrates with similar structure exist, then the best natural substrate is one with the least value of K_m

• if there is a need to measure the activity of enzyme, the substrate concentration has to be at least several times higher than the K_m value.

How to get a saturation curve?

A series of measurements of initial velocity is arranged at a constant enzyme concentration [E] and different substrate concentrations [S] (in the range of 2 - 3 orders).



[E] = const.

From the obtained progress curves, the values v_o are estimated and plotted against the corresponding [S].

The velocity v_o rises linearly as substrate concentration increases, and then begins to level till it reaches a limit value at high substrate concentrations.

Distinguish

Kinetic curve

- time record of **one** reaction
- [S] = f (t) or [P] = f (t)

Saturation curve

- dependence obtained from **many** reactions (see previous page)
- v_o = f ([S])

[S] .... substrate concentration
[P] .... product concentration
f .... function
t .... time
v_o .... initial velocity

V_{max} and K_m describe the kinetic properties of enzyme

- are hardly obtained from saturation curve
- easily obtained from linear double reciprocal plot
- Lineweaver-Burk: **$1/v_o$** is plotted against **1/[S]**

Reciprocal equation

$$v_o = V_{\max} \frac{[S]}{[S] + K_m}$$

$$\frac{1}{v_o} = \frac{1}{V_{\max}} \cdot \frac{[S] + K_m}{[S]} = \frac{1}{V_{\max}} \left(\frac{[S]}{[S]} + \frac{K_m}{[S]} \right)$$

$$\frac{1}{v_o} = \frac{1}{V_{\max}} + \frac{K_m}{V_{\max}} \cdot \frac{1}{[S]}$$

Reciprocal form is the equation of a line (y = a x + b)

$$\frac{1}{v_o} = \frac{1}{V_{\max}} + \frac{K_m}{V_{\max}} \cdot \frac{1}{[\mathrm{S}]}$$

$1/v_o$ .... dependent variable (y)

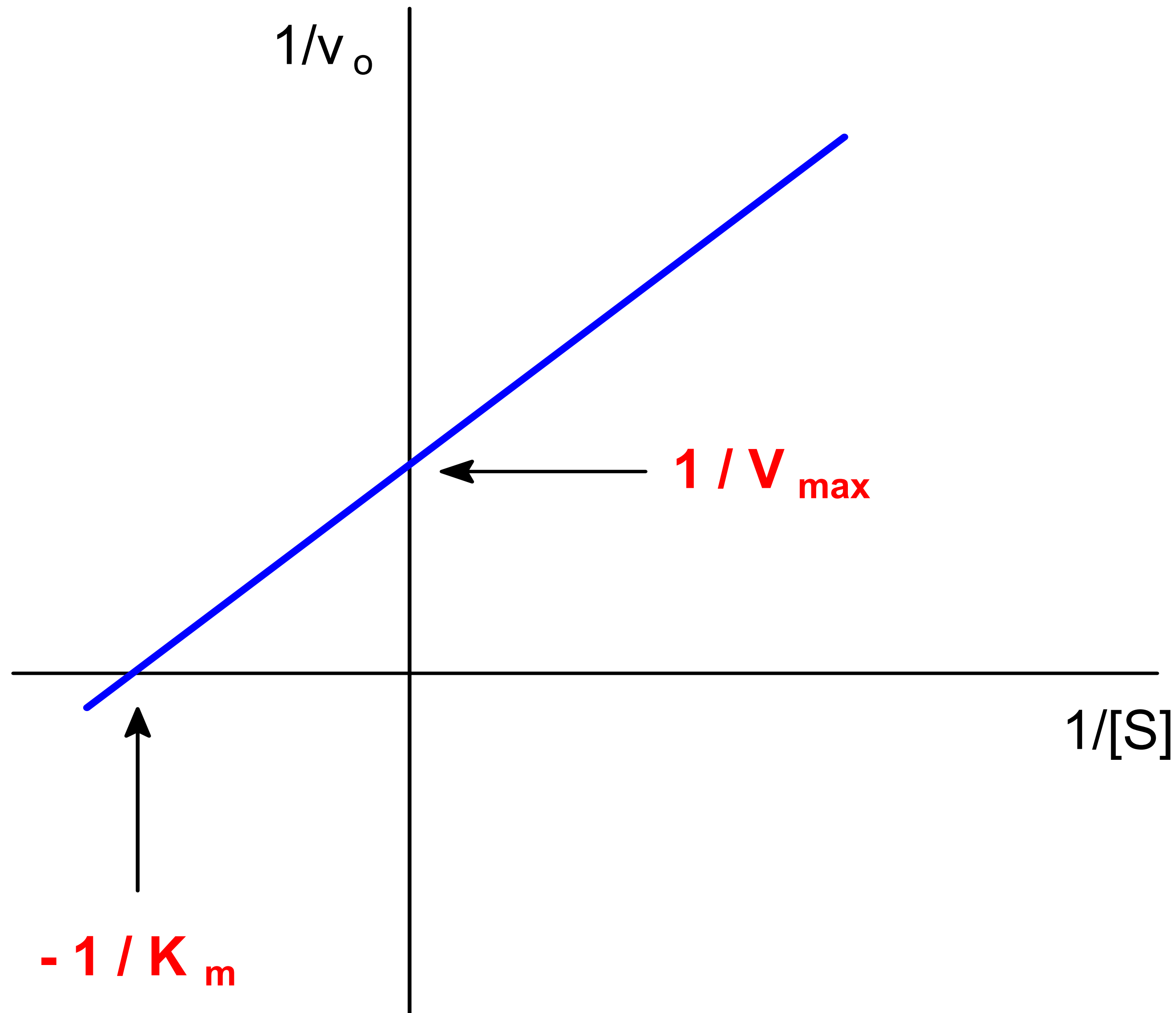
1/[S] .... independent variable (x)

$1/K_m$ ....

$1/V_{\max}$ ....

easily determined from the graph

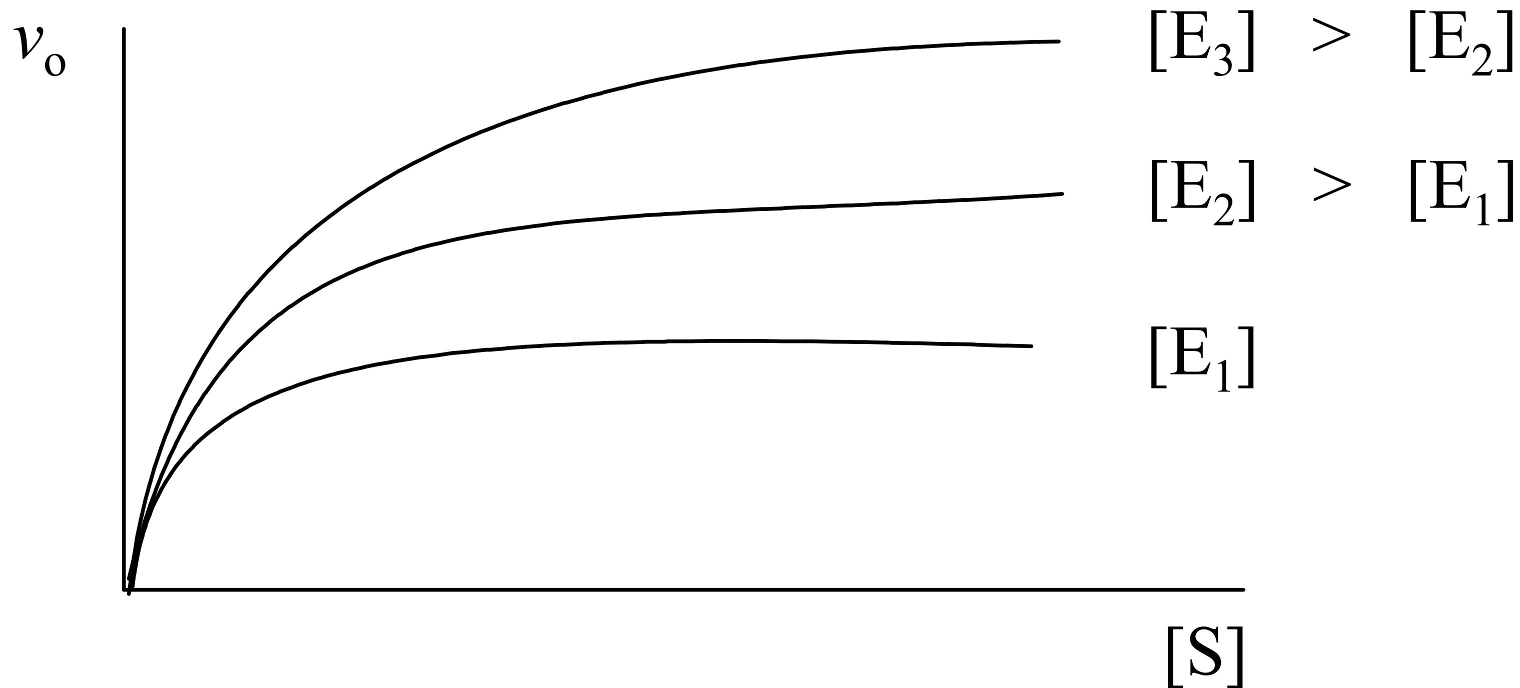
Linear reciprocal plot: $1/v_o$ is the function of 1/[S]



Initial velocity depends on enzyme concentration [E]

saturated enzyme: $v_o = k\,[E]_t$

$[E]_t$ is total concentration of enzyme



K_M does not change, V_{max} increases with increased [E]

How to quantify enzymes in biological material?

- very low concentrations of enzymes
- in very complex mixtures
- in the presence of many other proteins
- simple chemical reactions cannot be used, they are not specific enough to distinguish individual enzymes

Enzyme quantity in a biological sample can be determined by two ways

- **catalytic concentration**
- **µkat/l**
- the product of enzyme reaction is determined

- **mass concentration**
- **µg/l**
- enzyme itself is determined as antigen (immunochemical assay)

Catalytic activity of enzyme

- unit **katal**, 1 kat = mol/s
- such amount of enzyme that catalyzes the conversion of one mole of substrate per one second

IU (international unit), 1 IU = μmol/min

1 μkat = 60 IU, 1 IU = 16.6 nkat

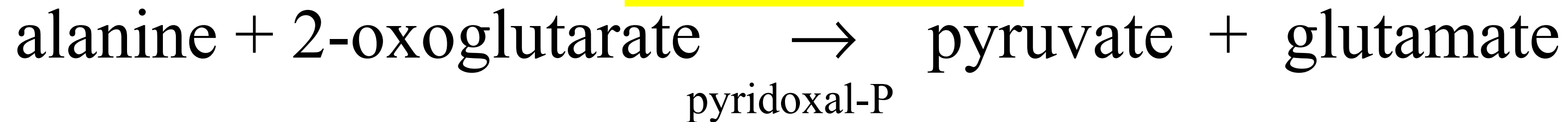
Catalytic concentration of enzyme

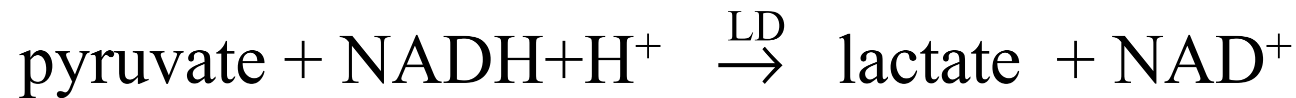
- activity related to the **volume of body fluid** (blood serum)
- typical units: mkat/l, μkat/l

Distinguish: unit and its dimension

Quantity	Unit	Dimension
Catalytic activity	kat	mol/s
Catalytic concentration	kat/l	mol/l.s

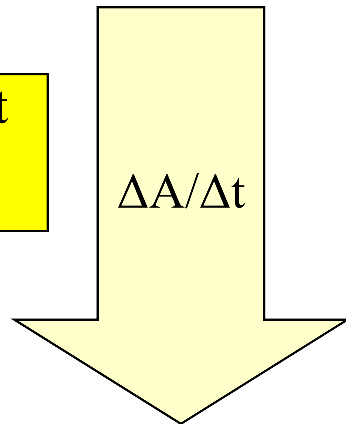
Determination of catalytic activity of ALT: two coupled enzyme reactions





optical (UV) test

Seminars, p.18



the decrease of absorbance at 340 nm is followed in time

Determination of catalytic activity *in vitro*

- optimal conditions: temperature, pH

  the presence of all necessary cofactors

  the absence of all (known) inhibitors

- **excess of substrate** ⇒ 0. order kinetics: **[S] >> K_m** ⇒

  saturated enzyme, reaction rate is constant and close to V_{max}

- Δ[S] or Δ[P] is followed during time

Two methods for catalytic concentration

Feature	Kinetic method	Constant-time method
What is measured	[S] or [P]	[P]
How	continually (e.g. in 10 seconds)	after some time (e.g. 10 min) the reaction is stopped by enzyme inactivation
Kinetic curve needed	yes	no
What is determined	initial velocity v_o	average velocity
Evaluation of method	exact	less exact

Problem 1

Enzyme sample (0.1 ml) was added to substrate solution. After 5 min, 0.2 mmol of product was determined.

What is catalytic concentration of enzyme?

$$\frac{\text{amount of product (mmol)}}{\text{time (s)} \times \text{volume (l)}}$$

Problem 1 - Solution

$$\frac{0{,}2\ (\text{mmol})}{300\ (\text{s}) \times 0{,}0001\ (\text{l})} = \mathbf{6{,}7\ \ mkat/l}$$

Problem 2

Reaction mixture contains:

2.5 ml buffer

0.2 ml solution of NADH (optical UV test)

0.1 ml blood serum

0.2 ml substrate solution

After 60 s, the decrease of NADH absorbance is $\Delta A = 0.03$

ε_{NADH} = 6220 l/mol.cm, cuvette width l = 1 cm.

What is catalytic concentration of enzyme?

Problem 2 - Solution

Serum sample was diluted: $V_{final}/V_{initial} = 3{,}0 / 0{,}1 = 30$

Lambert-Beer law: $\Delta A = \varepsilon \Delta c\, l$

changes of absorbance and concentration expressed per time $\Delta t \Rightarrow$

$\Delta A/\Delta t = \varepsilon \Delta c\, l/\Delta t$ $\qquad \Delta t = 60$ s

$$\frac{\Delta c}{\Delta t} = \frac{\Delta A}{\varepsilon \times l \times \Delta t} = \frac{0{,}03}{6220 \times 1 \times 60} = 8.10^{-8} \text{ mol/l.s}$$

Multiplied by dilution: $30 \times 8 \times 10^{-8} = 2{,}4 \times 10^{-6}$ mol/l.s $=$

$2{,}4 \times 10^{-6}$ kat/l = **2,4 μkat/l**

Consider that

catalytic concentration = rate of chemical reaction

kat/l = mol/l.s

Inhibitors reduce enzyme activity

Irreversible inhibitors

Irreversible inhibitors are usually compounds not of biological origin,

bind onto enzyme mostly **covalently** and make substrate binding impossible.

Heavy metal ions, organophosphates, cyanides

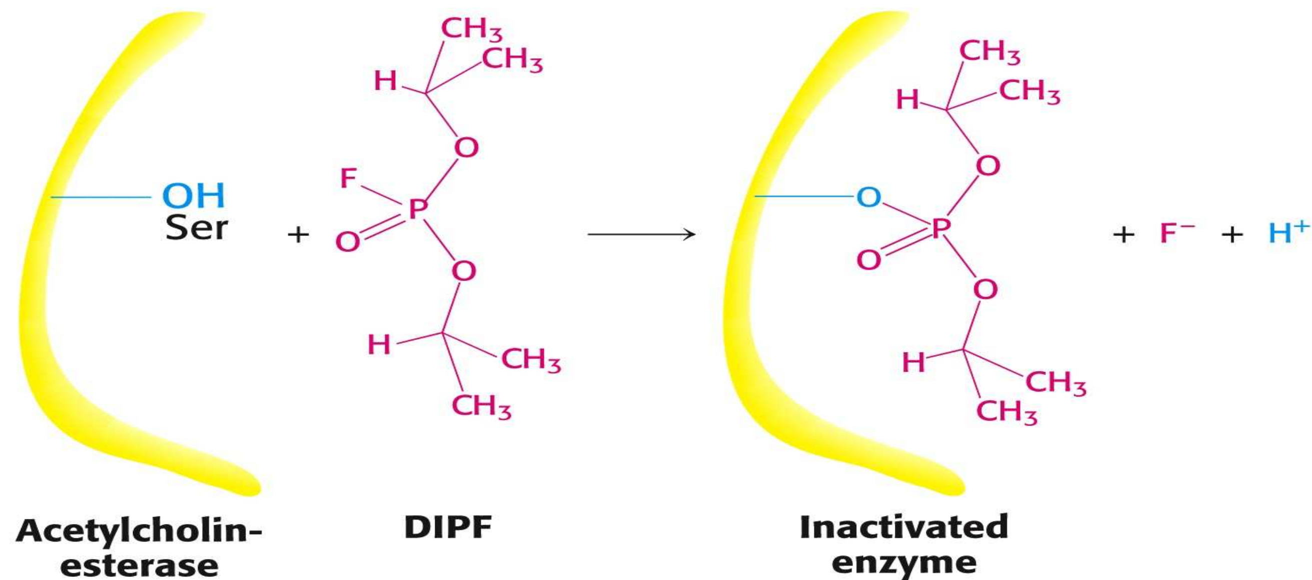
bind and inhibit irreversibly enzymes during isolation.

Reversible inhibitors

In contrast with irreversible inhibitors, reversible inhibitors bind to the enzyme loosely and can rapidly dissociate from the enzyme-inhibitor complex.

These inhibitors are classified as **competitive**, **non-competitive**.

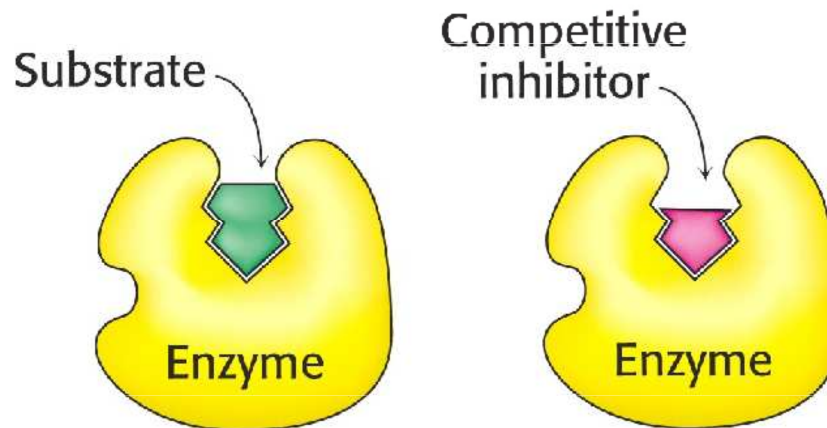
Example of irreversible inhibition



Diisopropyl fluorophosphate (and similar pesticides and nerve gases) inhibits acetylcholine esterase by phosphorylation of a crucial serine residue.

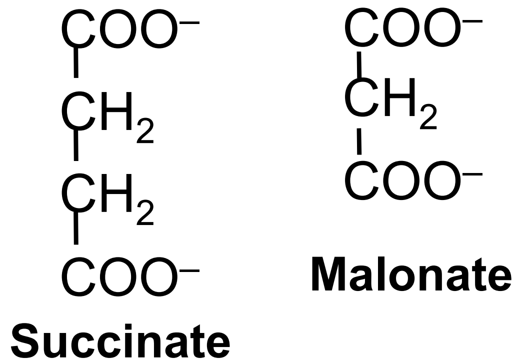
Competitive inhibitors

- resemble the substrates (similar shape of molecule)
- bind to the active sites, but the complex is non-reactive
- they compete with normal substrates for the active sites

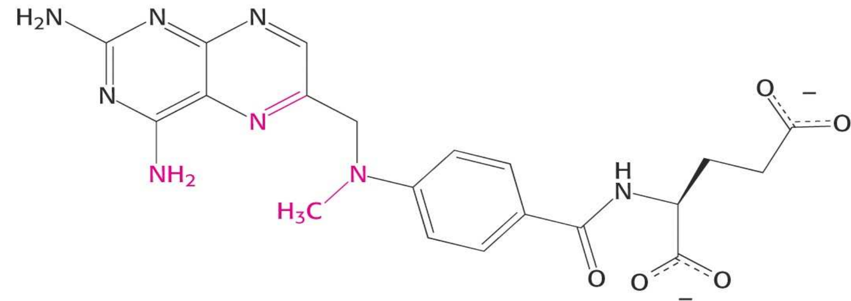


Examples

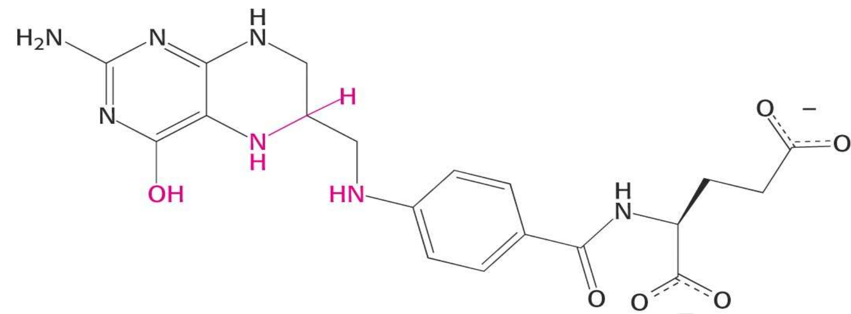
Malonate competitively inhibit succinate dehydrogenase



Methotrexate competitively inhibits active sites for tetrahydrofolate of the dihydrofolate reductase in the synthesis of purine and pyrimidine bases of nucleic acids. It is used to treat cancer.

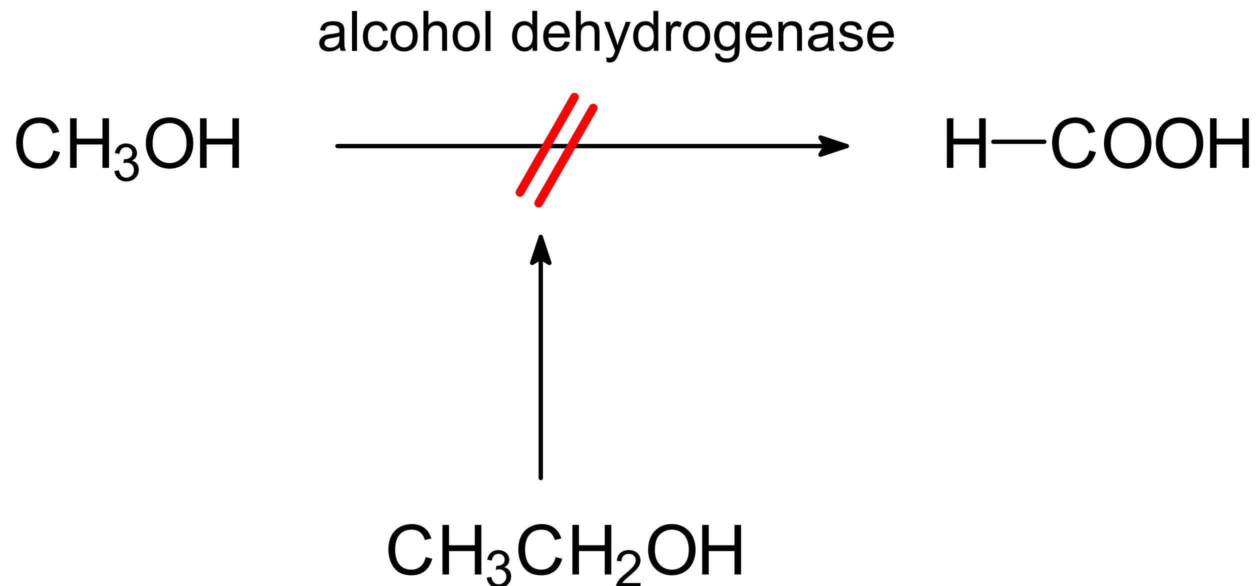


Methotrexate



Tetrahydrofolate

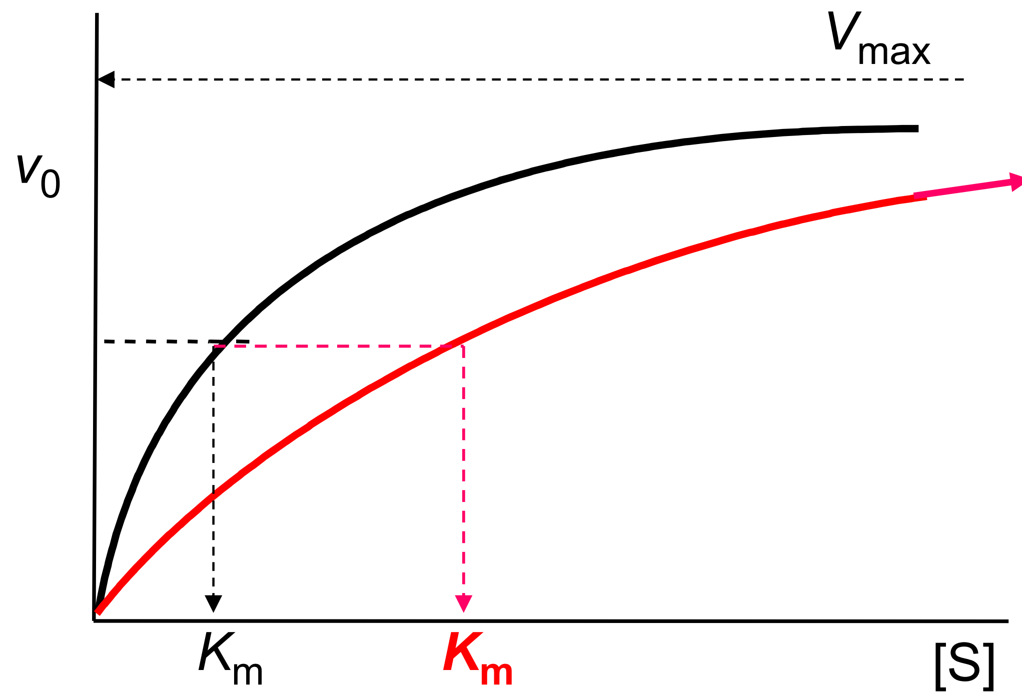
Methanol poisoning is treated by ethanol



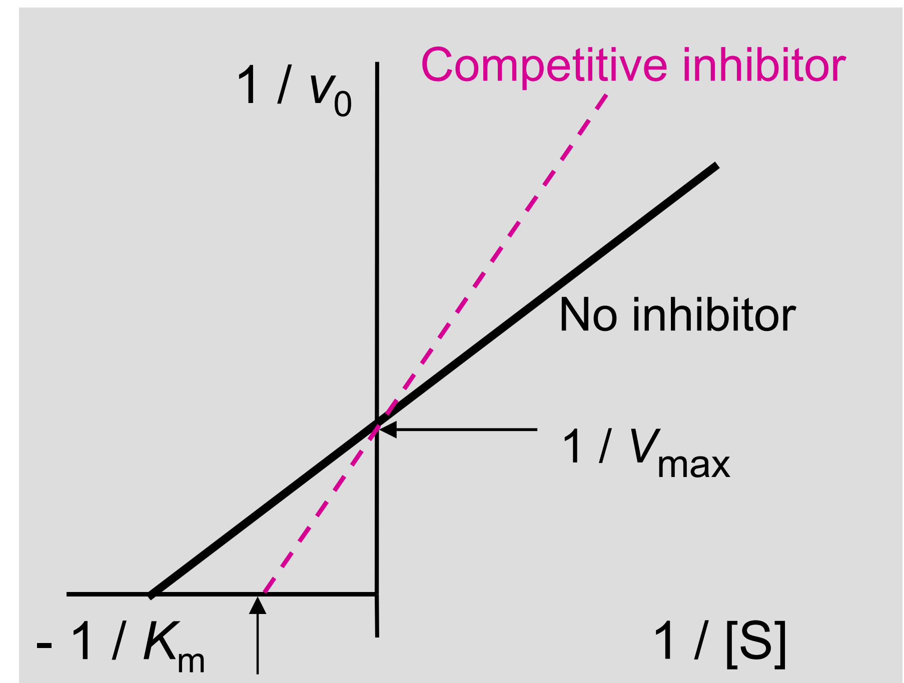
ethanol and methanol are similar molecules

they compete for active site in enzyme

Competitive inhibitors increase K_m without any change in V_{max}

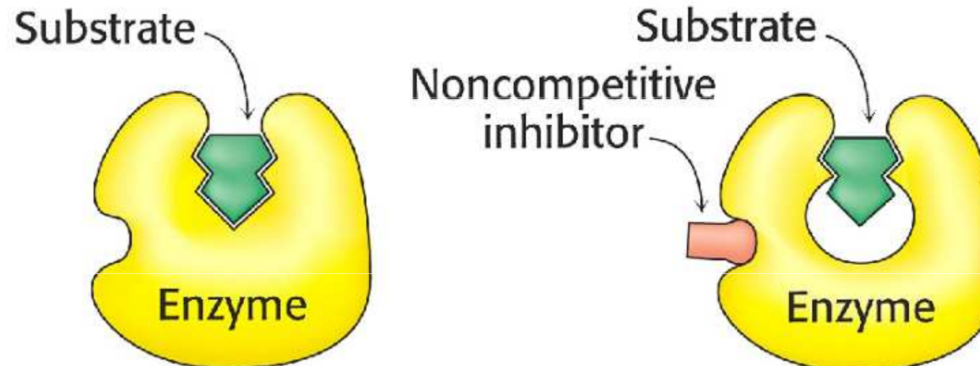


The V_{max} can be reached even in the presence of inhibitor, but at much higher concentrations of [S] that have to overcome the competing inhibitor concentration.



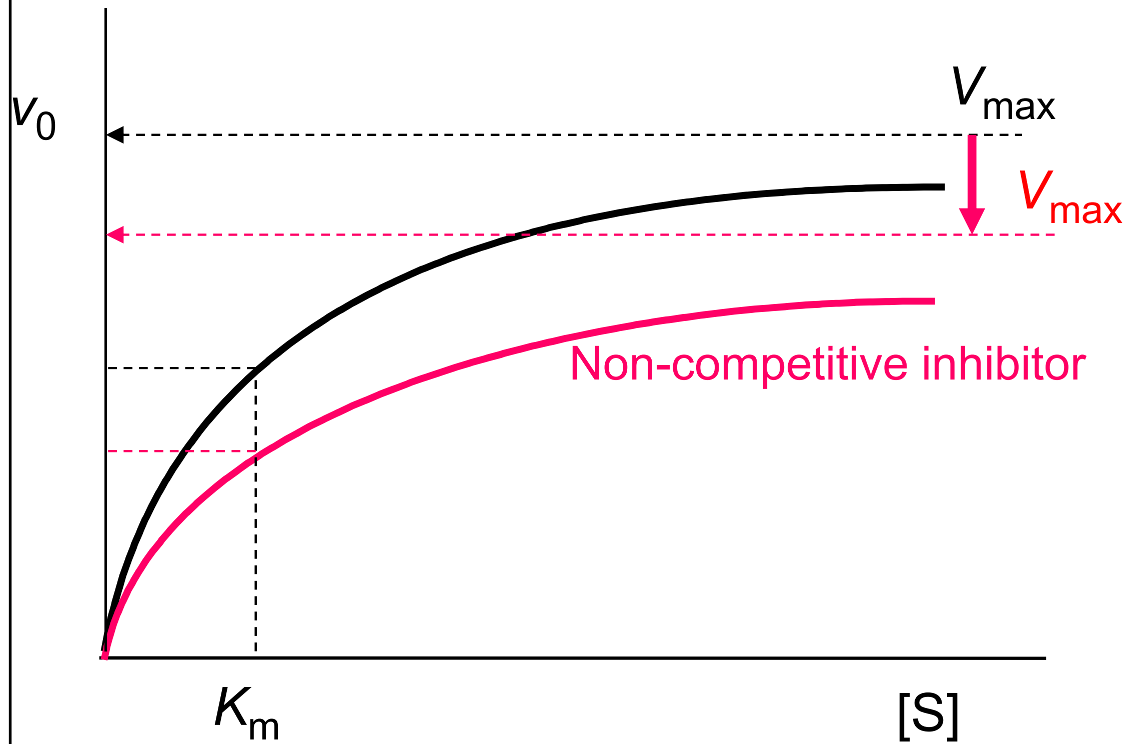
Non-competitive inhibition

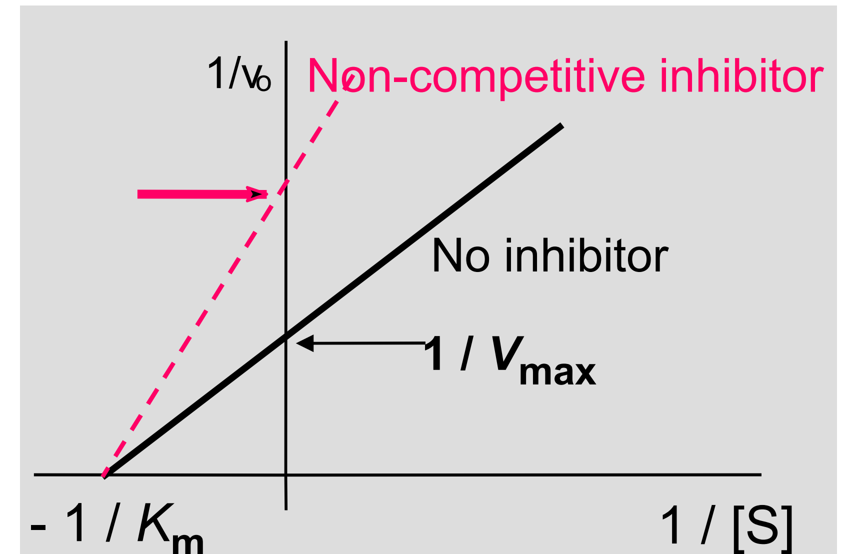
Non-competitive inhibitors bind to both free enzyme and enzyme-substrate complex, but in contrast to competitive inhibitors, **not in the active site** (the structure of inhibitor is distinct from that of substrate).



Non-competitive inhibition cannot be overcome by increasing the substrate concentration. The non-inhibited remaining molecules of the enzyme behave like a **more diluted solution of the enzyme.**

Non-competitive inhibitors decrease V_{max} without any change in K_m





Many drugs are inhibitors of enzymes

- Acetylsalicylic acid, ibuprofen (cyclooxygenase) - see Seminars, p. 67
- Statins (HMG-CoA reductase) inhibit cholesterol synthesis (e.g. lovastatin)
- ACE Inhibitors (angiotensin-converting enzyme) – treatment of hypertension
- Reversible acetylcholinesterase inhibitors (e.g. neostigmine) – myastenia gravis, post-surgical atonia,
- Brain acetylcholinesterase inhibitors (rivastigmine, galantamin) – Alzheimer d.
- Many antibiotics inhibit bacterial enzymes:

Penicillins – inhibit bacterial transpeptidases (formation of bacterial cell-wall)

Tetracyclins, macrolides, chloramphenicol – inhibit bacterial proteosynthesis

Fluoroquinolones (ciprofloxacin) – inhibit bacterial topoisomerase

Regulation principles in enzyme reactions
(three general aspects)

1. Regulation of enzyme **quantity**

2. Regulation of enzyme **activity**

3. Availability and concentration of substrate and/or cofactor

   (*in vivo* less important, *in vitro* critical condition)

Regulation of enzyme quantity

- **Controlled enzyme proteosynthesis**
  constitutive and induced gene expresion,
  regulation of transcription rate,
  posttranscriptional RNA processing,
  regulation of translation rate and posttranslational modifications

- **Controlled enzyme degradation**
  specific intracellular proteinases determine biological half-life of enzymes

Regulation of enzyme activity

- Activation by partial and **irreversible** proteolysis
- **Reversible** covalent modification
- Allosteric regulation

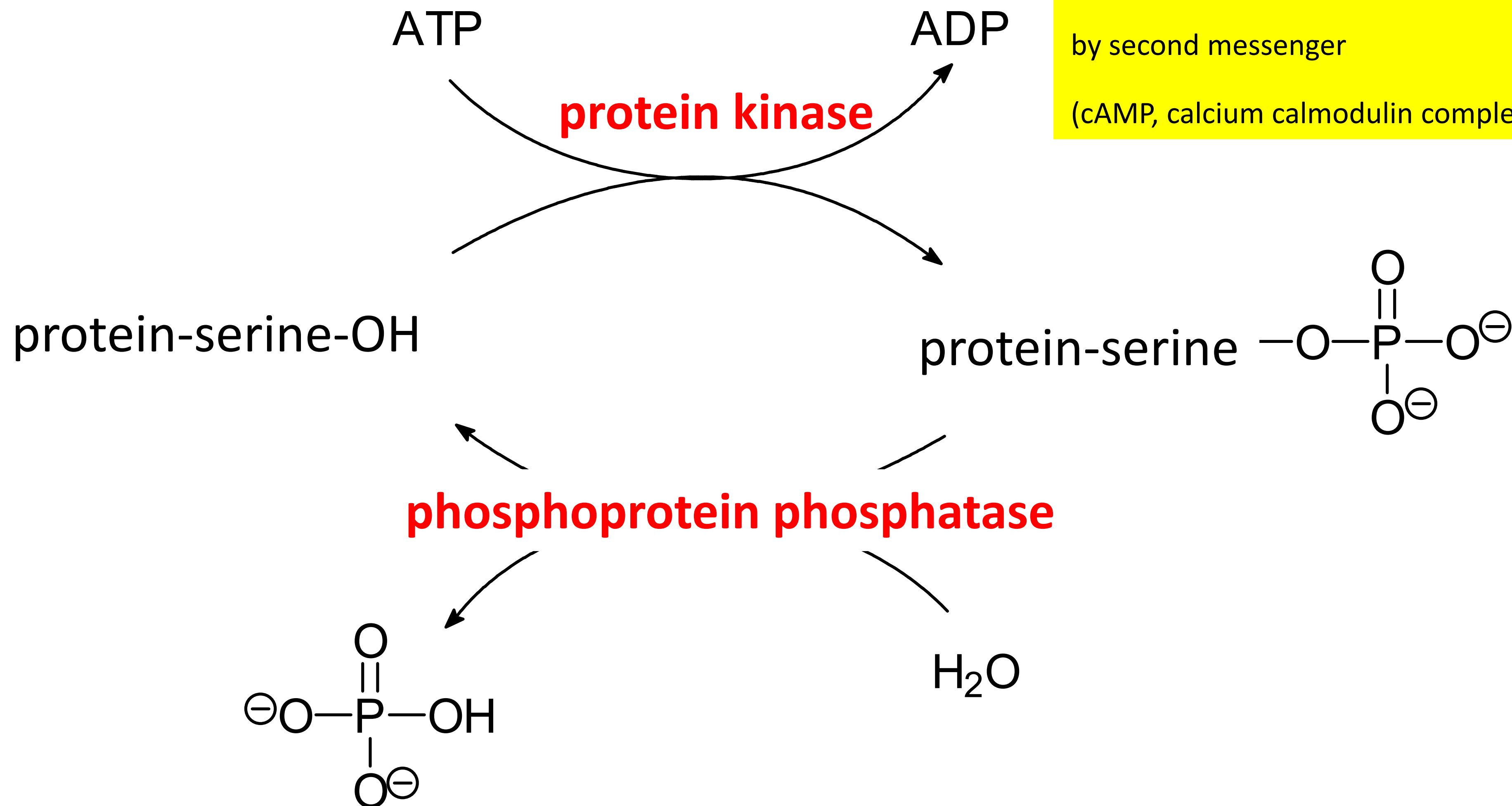
Enzyme activation by partial proteolysis

- Active enzyme is formed by **irreversible** cleavage of certain sequence from **proenzyme** (zymogen) molecule
- Proteinases in digestion (pepsinogen → pepsin)
- Factors of blood coagulation
- Proteinases caspases in apoptosis

Reversible covalent enzyme modification

- **phosphorylation**, catalyzed by kinases
- transfer of phosphoryl $-PO_3^{2-}$ from ATP to -OH group of enzyme (Ser, Thr, Tyr)
- reversible process, dephosphorylation catalyzed by **phosphatase**, hydrolysis of phosphoester
- Other modifications: carboxylation, acetylation, prenylation ...

Phosphorylation and dephosphorylation of enzyme alters its activity



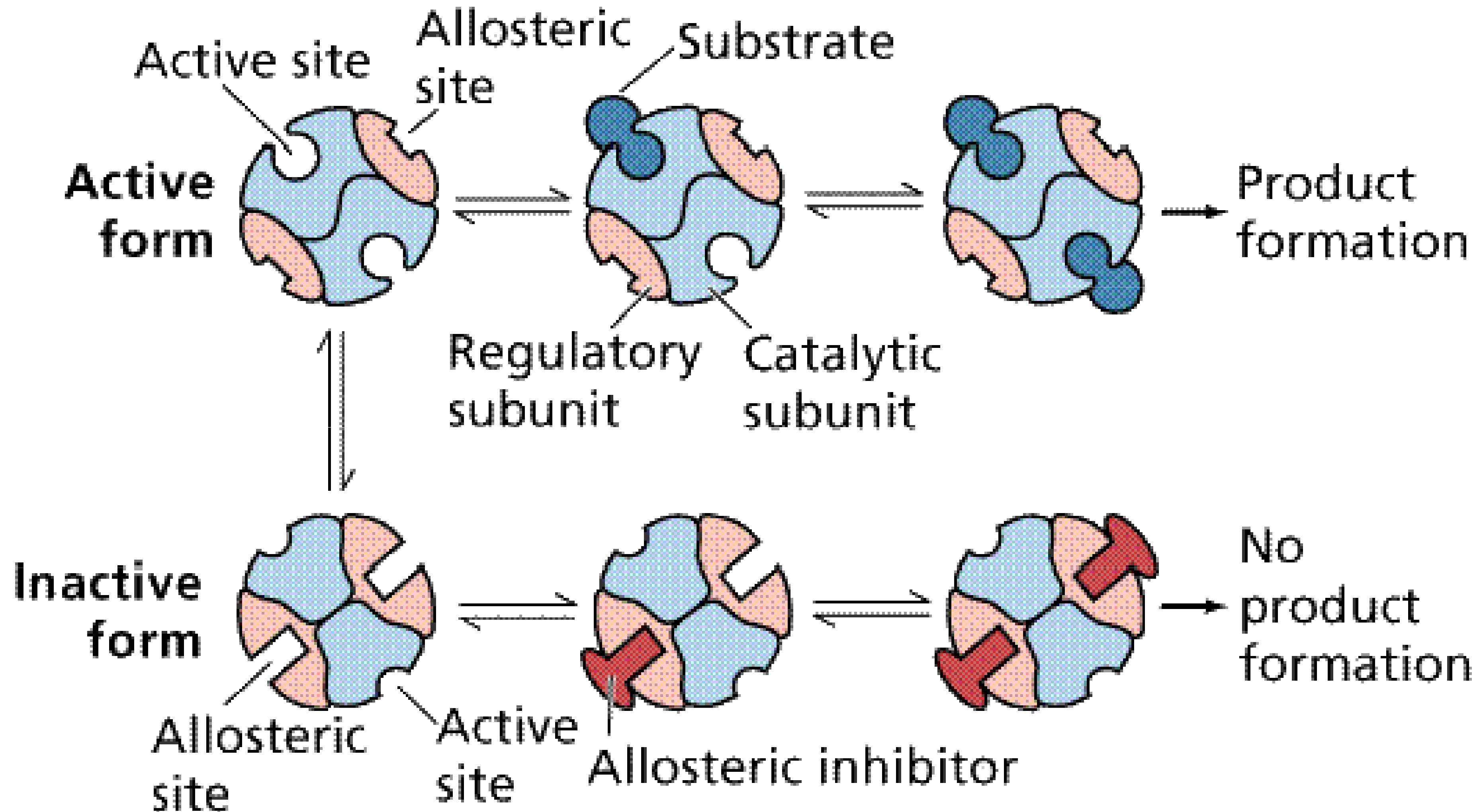
The consequences of phosphorylation/dephosphorylation in two antagonistic enzymes

Feature	**Glycogen phosphorylase**	**Glycogen synthase**
Enzyme catalyzes	glycogen degradation by energy-poor phosphate (P_i)	glycogen synthesis from energy-rich UDP-glucose
By phosphorylation, enzyme is	**activated**	inhibited
By dephosphorylation, enzyme is	inhibited	**activated**

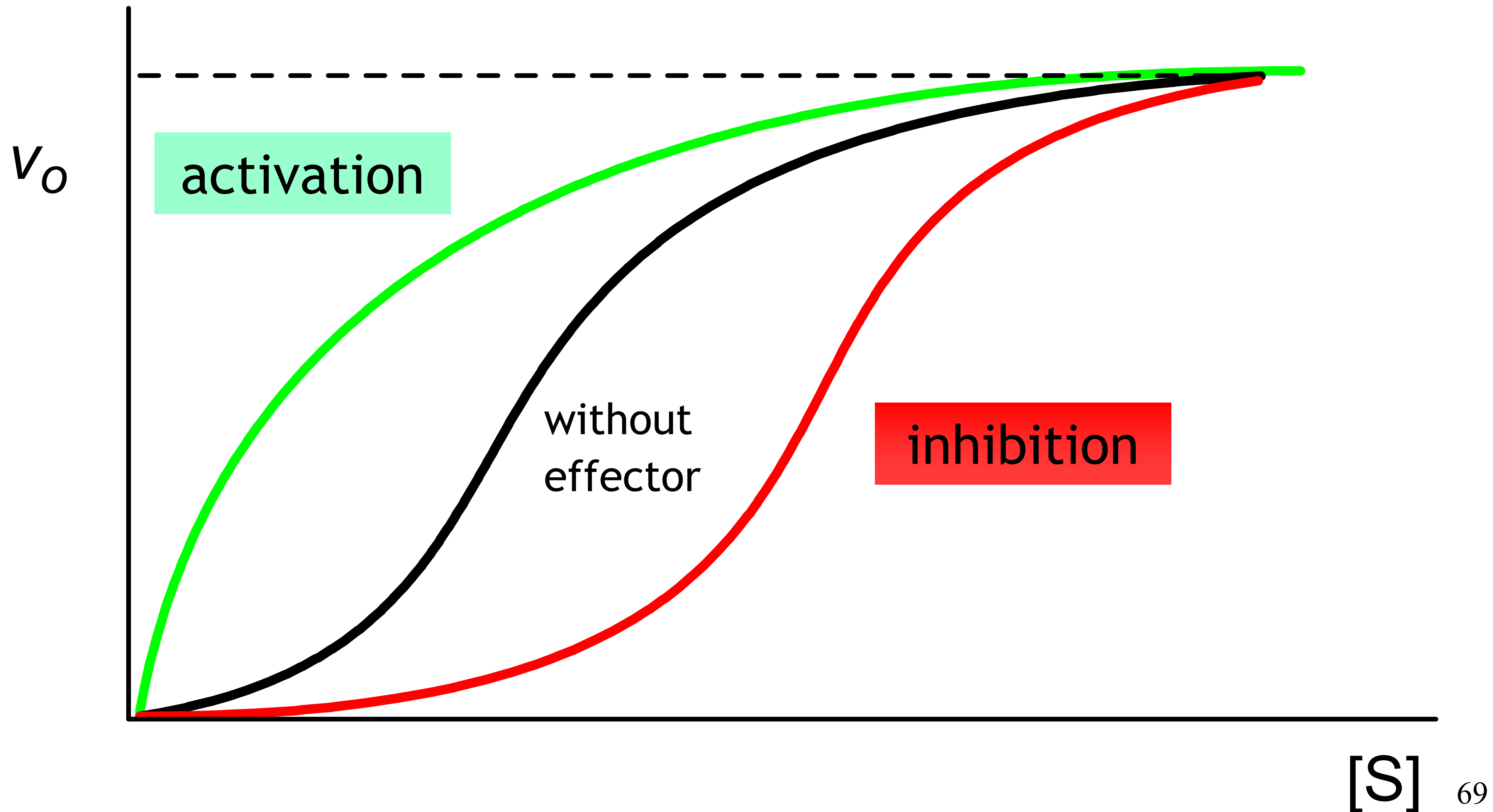
Allosteric enzymes are oligomeric

- more subunits, often regulatory and catalytic
- **effector**, structurally different from substrate - often product, binds to enzyme to **allosteric site** (other than active site)
- binding effector triggers the changes in conformation and activity $\rightarrow$ allosteric activation / inhibition

Allosteric enzymes are oligomeric



Saturation curve of allosteric enzymes is sigmoidal

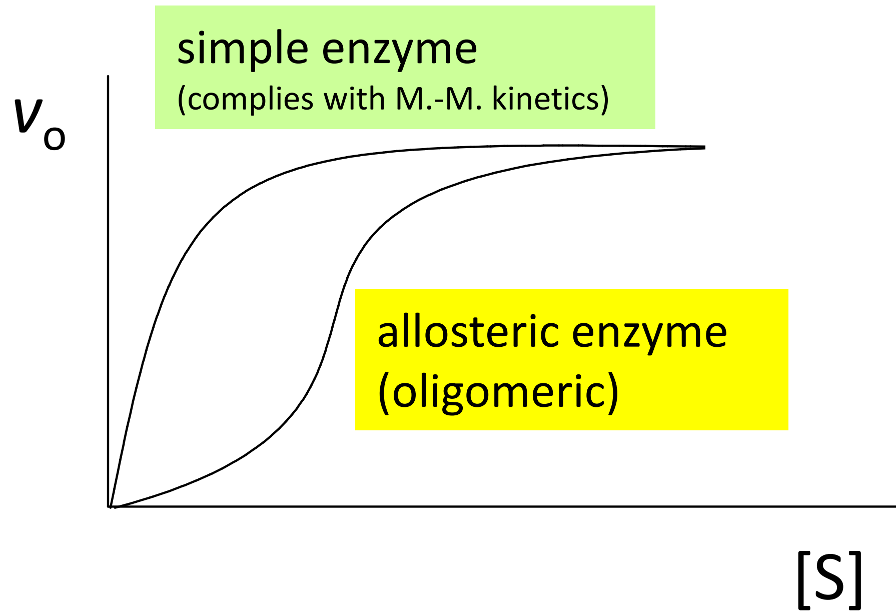


Cooperative effect

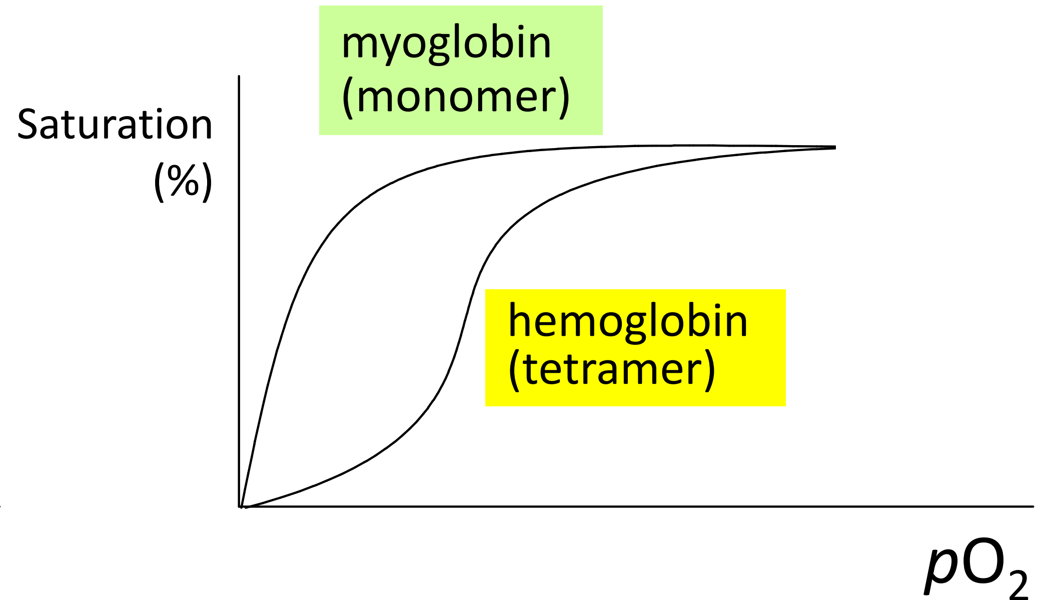
- in oligomeric enzymes and non-catalyzing proteins (e.g. Hb)
- more subunits = more active sites
- binding substrate (or O_2 to Hb) to one subunit/active site induces conformation changes in other subunits/active sites so that other substrate (or O_2) molecules bind more easily (or more hardly)
- example: hemoglobin (tetramer) × myoglobin (monomer)

Compare

Saturation of enzyme by substrate



Saturation of hemoglobin by oxygen



Three utilizations of enzymes in medicine

1. enzymes as **indicators** of pathological condition
2. enzymes as **analytic reagents** in clinical chemistry
3. enzymes as **drugs**

Examples of enzymes in clinical diagnostics

In cell damages, activity of **intracellular** enzymes in **extracellular** fluid (blood serum) is elevated

Enzyme	Reference values	Elevation in serum indicates
ALT	up to 0,9 µkat/l	hepatopaties
CK	up to 4 µkat/l	myopaties, myocardial infarction
PSA	up to 4 µg/l	prostate cancer

ALT alanine aminotransferase, CK creatine kinase,

PSA prostate specific antigen

Isoenzymes/Isoforms

- catalyze the same reaction
- differ in primary structure, physical, and kinetic properties
- often have different tissue distribution
- determined mostly by electrophoresis and/or by immunochemical assays

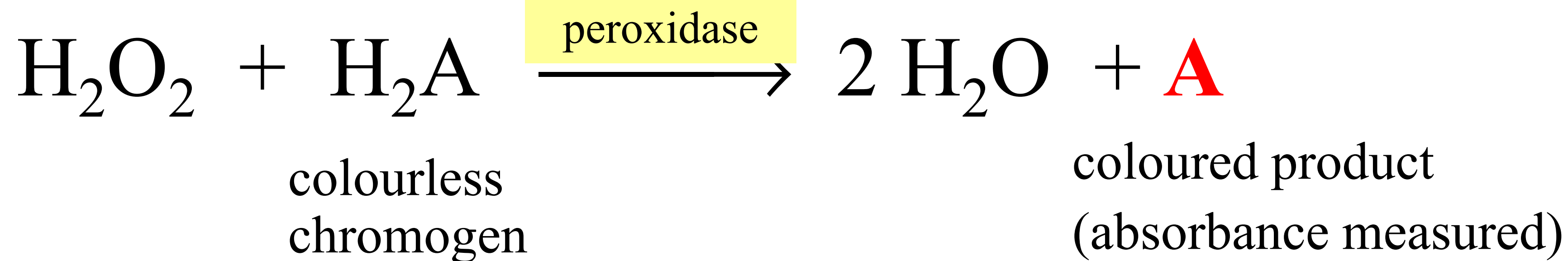
Creatine kinase (CK) is a dimer and makes three isoenzymes

Isoenzyme	Organ	% of total activity in blood plasma	Elevated value
CK-MM	muscles	94-96 %	muscle trauma
CK-MB	heart	up to 6 %	infarction
CK-BB	brain	traces	brain damage

Enzymes as analytic reagents

Enzyme	**Enzyme origin**	**Assay for**
Glucose oxidase	*Aspergillus niger*	glucose
Peroxidase	horse radish (*Armoracia* sp.)	glucose
Lipase	*Candida* sp.	triacylglycerols
Cholesterol oxidase	*Pseudomonas* sp.	cholesterol
Uricase	*Candida* sp.	uric acid
Bilirubin oxidase	*Myrothecium* sp.	bilirubin
Urease	*Canavalia* sp.	urea
Lactate dehydrogenase	*Pediocus* sp.	ALT, AST
Taq polymerase	*Thermus aquaticus*	PCR method

Enzymatic determination of glucose



The principle of glucose determination in biochemical analyzers and in personal glucometers

Personal glucometer

(Practice in 4. semester)

Pancreatic enzymes in therapy

- enzyme mixtures (lipase, amylase, proteinases) of animal origin
- indication: insufficient secretion of pancreas, cystic fibrosis
- 3 × times daily a capsule during meal

gastro-resistant capsules, they survive the passage through stomach, soluble and active in duodenum

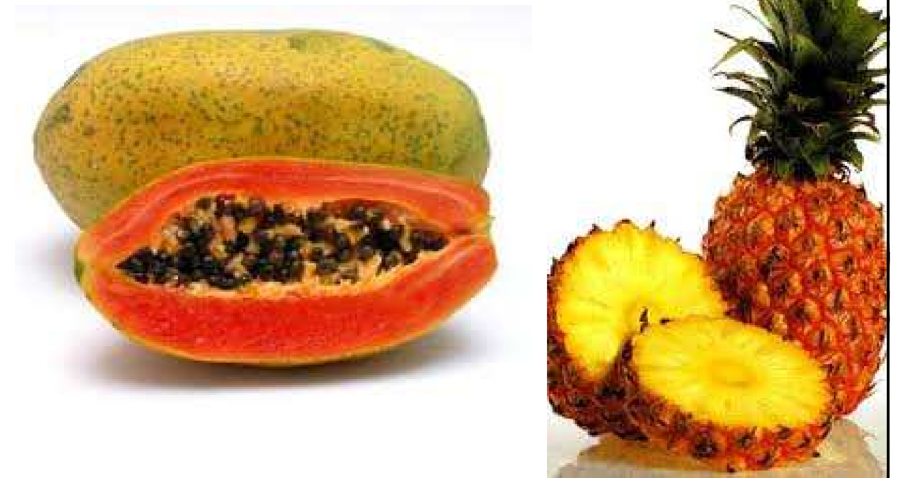
Asparaginase in leukemia treatment

- Catalyzes the hydrolysis of asparagine amide group (deamidation)
- Asn + H_2O $\rightarrow$ Asp + NH_3
- L-asparagine is necessary for the proteosynthesis of some cancer cells
- Hydrolysis of Asp reduces the cell proliferation (see also Seminars, p. 19)

Enzyme fibrinolytics

- thrombolytic drugs, dissolve blood clots in veins
- **urokinase** (human enzyme, serine protease)
- converts plasminogen to plasmin $\rightarrow$ which degrades fibrin $\rightarrow$ thrombolysis
- venous thrombosis, pulmonary embolism, acute myocardial infarction

Proteases in enzyme therapy



Local treatment

- fibrinolysin, chymotrypsin, collagenase and other
- degrade necrotic tissue, clean wounds, decubital ulcers etc.

Systemic treatment

- trypsin, chymotrypsin, papain (papaya), bromelain (pineapple)
- anti-inflammatory agents
- sports injury, trauma, arthritis, other kinds of swelling, arthritis etc.
- Wobenzym, Phlogenzym and other