Welcome to lecture 3

Enzyme-based biosensors

Plan for today:

We will talk about enzyme sensors and go through some undergraduate material that will be included in the final exam

For most of you the latter should be very familiar but, if not, **it is your responsibility to catch up** – there are books in the library and plenty on the Internet

But first let's take a look at a few more cool sensors

Cool biosensors

that I did not see before last Thursday class

- More sensors for early diagnosis of various diseases – similar to what we discussed last time
- Another great allergy-meter: monitors histamines in general and alerts users via cell phone (RFID); especially useful for folks with allergy to pollen, etc
- A biosensor to detect ovulation: great for couples who want a baby or for ladies who don't use contraceptives. Cool!

  The suggested design was a belt – perhaps something more discrete would be better? ☺
- Low progesterone during early pregnancy is associated with high risk of miscarriage – would it not be cool... It definitely would!

Not so cool – bio?

- BIOSENSOR FOR MESURING BLOOD OXYGEN LEVEL: This sensor shines light through one side of a finger and detector measures the light that comes through the other side
- Biosensor is a built-in-a-watch medicine reminder for high blood pressure patients: it detects specific substances (such as ACE) which are responsible for high blood pressure
- Multifunctional Household Biosensor for measuring body temperature and blood pressure
- Peptic ulcer biosensor: ITS LIKE A SQUARE BOX AND WHEN YOU KEEP THIS BOX ON YOUR ABDOMEN AFTER 15 MINS OF MEAL IT WILL DETECT DISTURBED SECRECTION OF GASTRIN – is it a "listening" device?
- Blood Viscosity Sensor, pH and salinity meters, etc

More cool biosensors

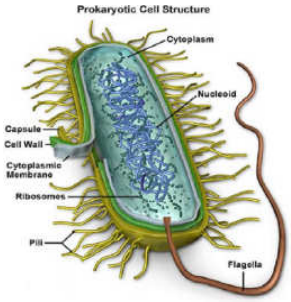
- A biosensor in babies bottle to check that milk is OK
- Detection of malaria in saliva (currently done in blood)
- All-in-one tooth brush with biosensor for plaque detection – apparently there is deposition of dextransucrase that can be detected enzymatically
- "Bad-breath-meter" – a small enzyme-based device to detect sulfur-compounds produced by mouth bacteria
- Identity biosensor: why use passwords, scan retina or check fingerprints, when you can just spit into a biosensor? Cool!!!
- And one more sensor to be presented by the author

Pheromatch is a cool sensor but is it a good business?

Think it over and we will discuss it next Thursday

At the last lecture

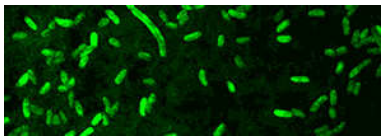
We discussed some pretty cool bacterial biosensors

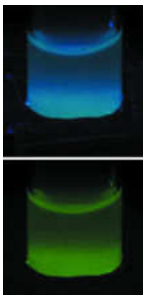


Vancomycin Erythromycin

bacterium

UO_2^{2+}





Stress Signal

Cell

Stress-inducible promoter

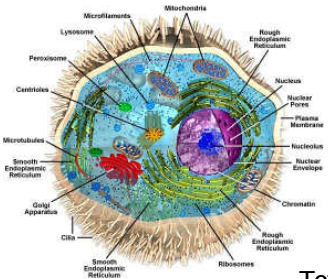
DNA reporter

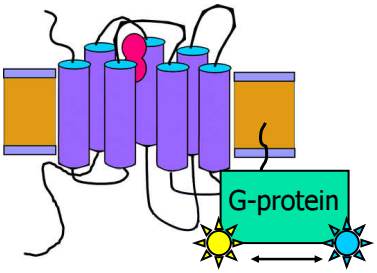
Stress response activated

Bacteria "flashes" the warning

Also at the last lecture

mammalian cell-based biosensors and technologies

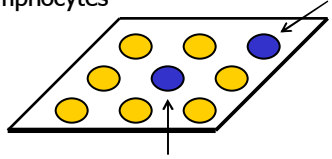




Conformational change

Lymphocytes

Toxin



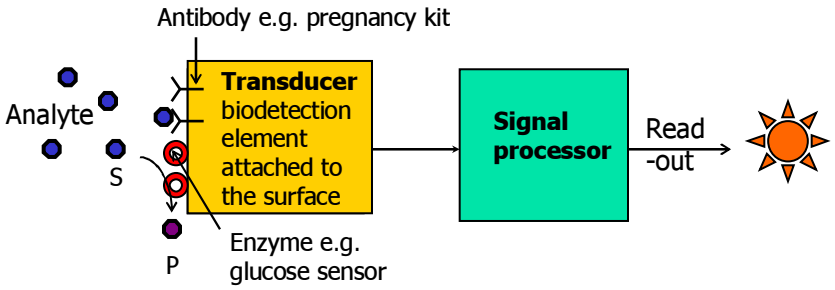
Pathogen

We also talked about biosensor's performance characteristics

Main topic today: **ENZYMES**

But first…

Generalized biosensor

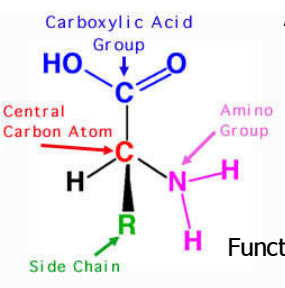


In most enzyme-based sensors analyte is a substrate for the enzyme and the reaction product is detected to generate a signal

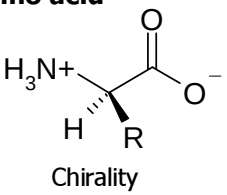
It is important to remember that the pretty circles on the diagram are actually rather complex **protein molecules**

Protein structure

Proteins are linear co-polymers of 20 natural L-amino acids



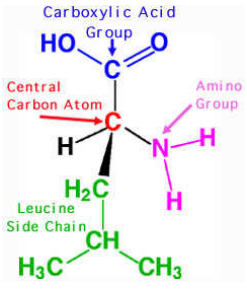
Amino acid



Chirality

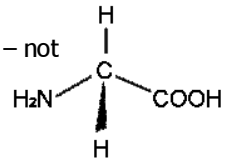
Functionality

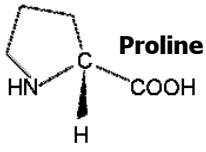
Side chains



Exceptions:

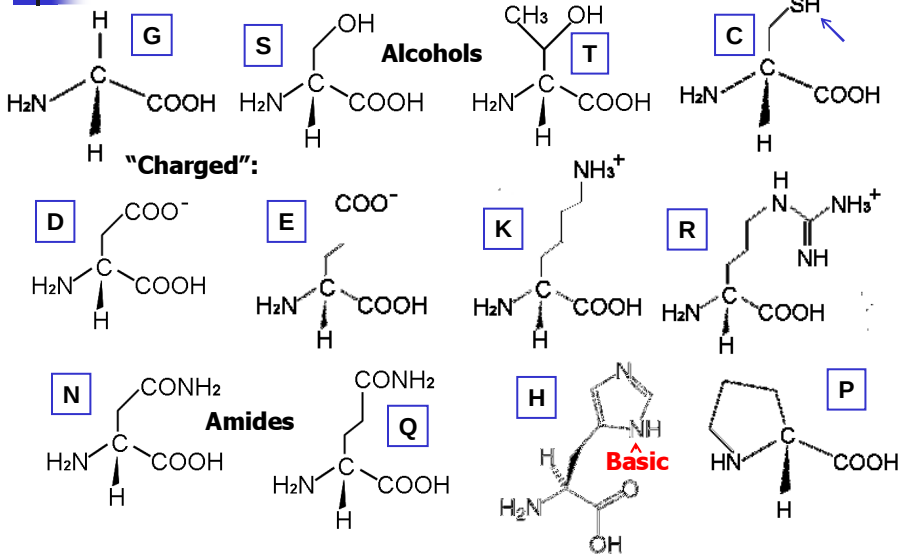
Glycine – not chiral



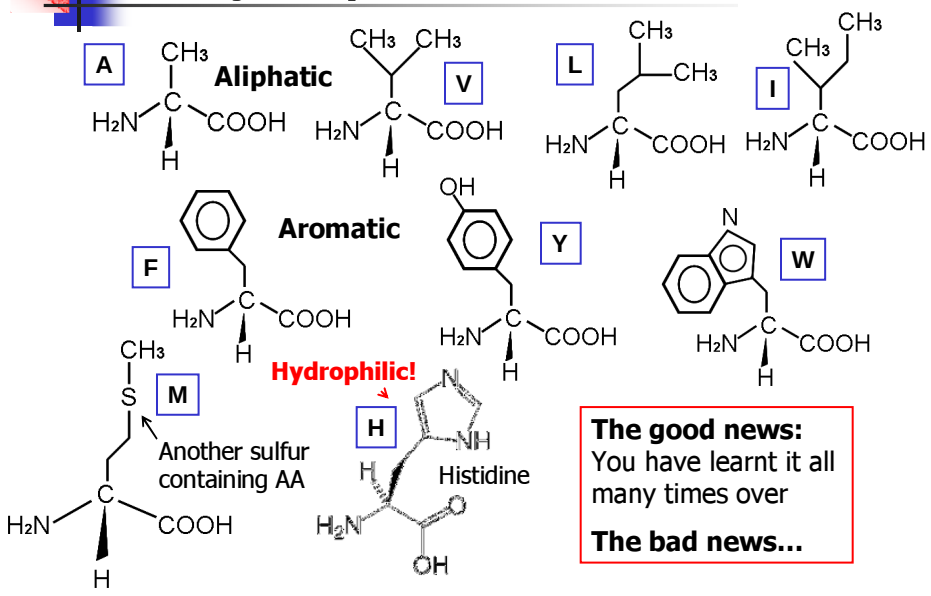


Proline – no NH_2 group

Hydrophilic amino acids



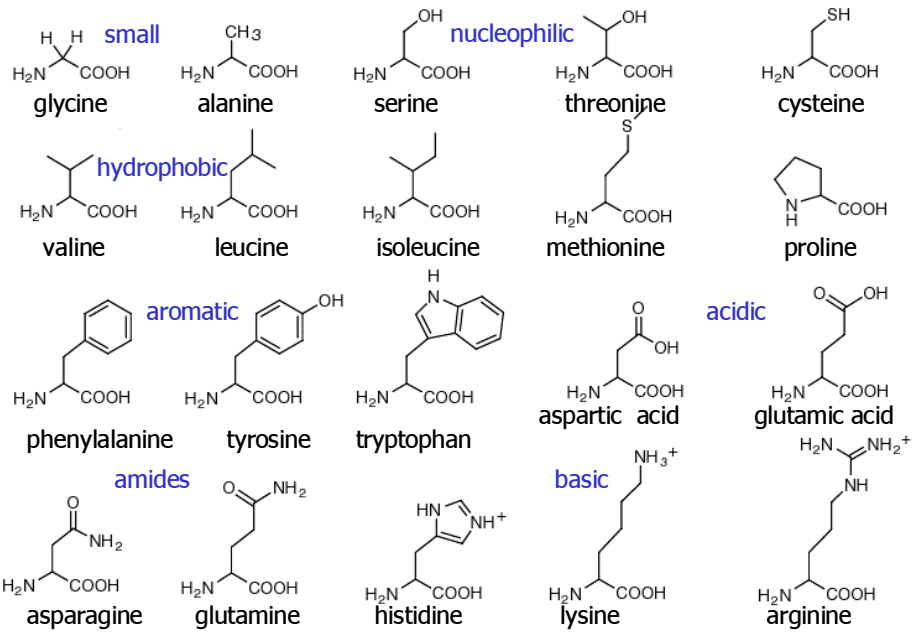
Hydrophobic amino acids



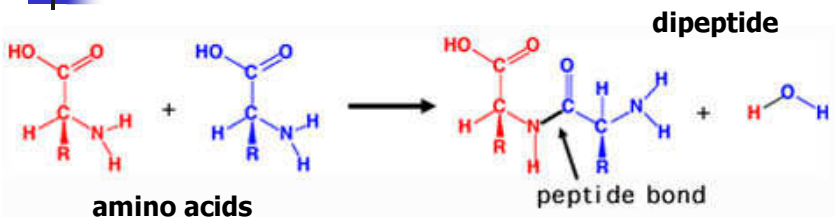
The good news:
You have learnt it all many times over

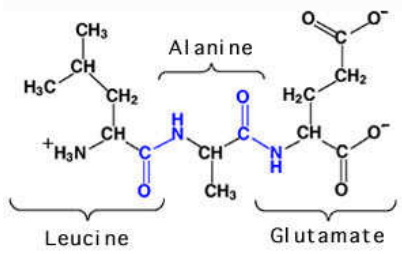
The bad news...

You'll need to know the structure of all 20 for the exam

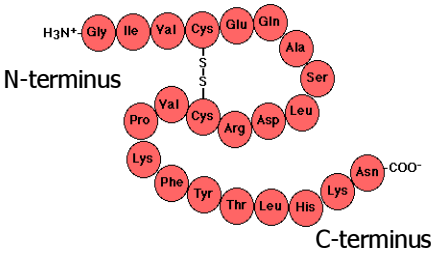


Peptide bond formation





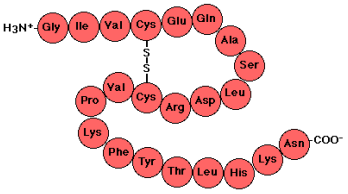
tripeptide



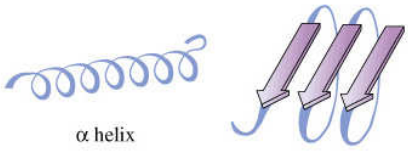
polypeptide

Protein Structure

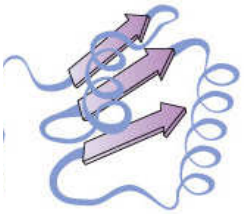
Primary structure



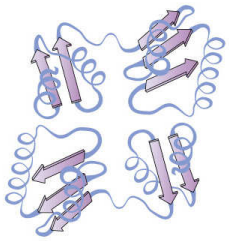
is **the sequence** of amino acids in a peptide chain



Secondary structure



Tertiary structure

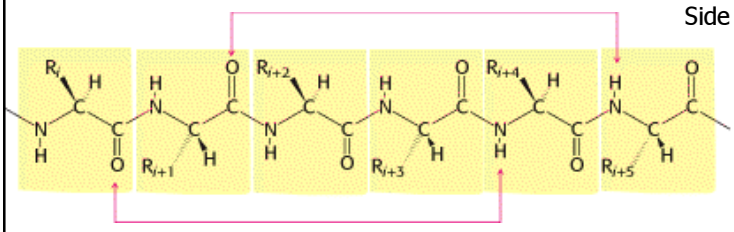


Quaternary structure

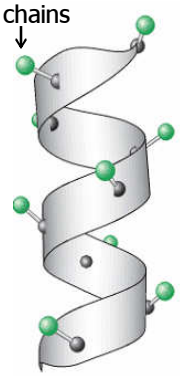
Secondary structure

Describes H-bond connectivity in the AA sequence

α-helix is a common motif in the secondary structure of proteins



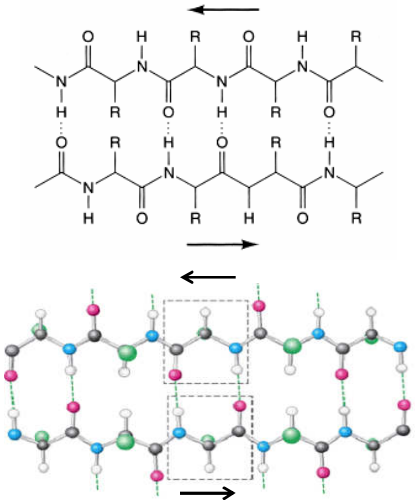
- The CO group of residue *n* forms a hydrogen bond with the NH group of residue *n*+ 4
- Except for amino acids near the ends of an α-helix, all the main-chain CO and NH groups are hydrogen bonded



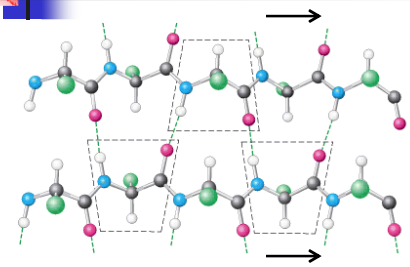
Often depicted as ribbons or rods

Secondary structure: β-strands

- β-strand (a polypeptide chain in β-sheets) is a stretch of amino acids whose peptide backbones are almost fully extended (distance between adjucent AA is 3.5Å vs 1.5Å in α-helix) and where the peptide carbonyls point in alternating directions relative to the plane of the sheet.
- **β-strands are directional:** In the antiparallel arrangement (N-terminus of one strand is adjucent to C of another), the NH and CO group of each amino acid are respectively hydrogen bonded to the CO group and the NH group of a partner on the adjacent chain

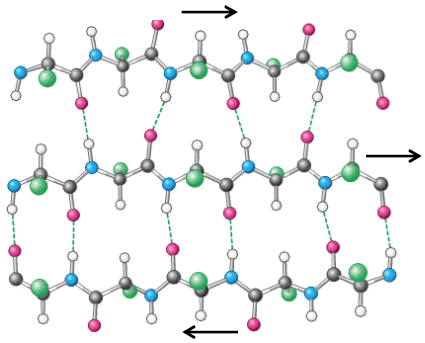


β-strands and β-sheets



Parallel β-strands

A little more complicated: For each amino acid, the NH group is H-bonded to the CO group of one amino acid on the adjacent strand, whereas the CO group is hydrogen bonded to the NH group on the amino acid two residues farther along the chain

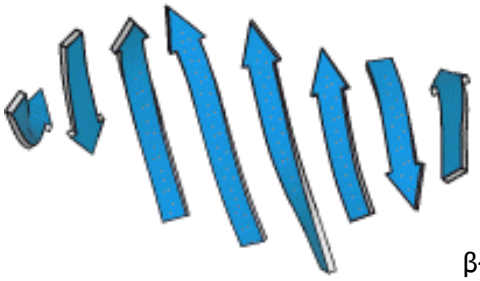


Many strands (e.g. 4-5 sometimes 10 or more), can come together to form β sheets. β-sheets can be purely anti-parallel, purely parallel, or mixed (as above). Typically the distance between β-strands in a sheet is about 4.5-5.0Å

β-sheets are very diverse!

β-sheet are critical structural element in many proteins

A β-sheet comprising 8 strands

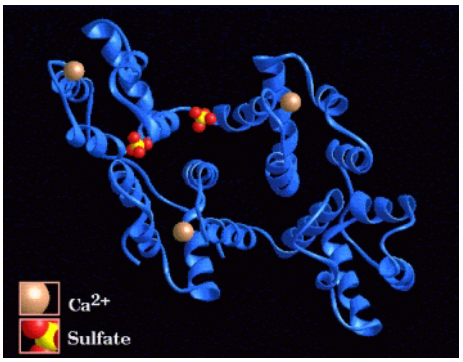


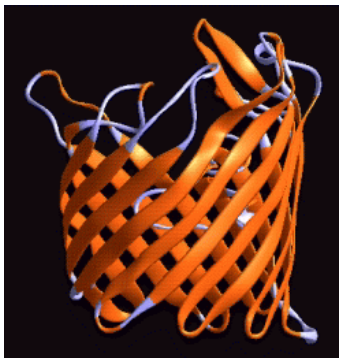
β-strands forming a protein core

- β-strands are usually depicted by broad arrows pointing in the direction of the carboxyl-terminal end

Secondary structures in proteins

Proteins may consist of almost exclusively α-helix or β-sheets, although such "exclusive" structures are rare

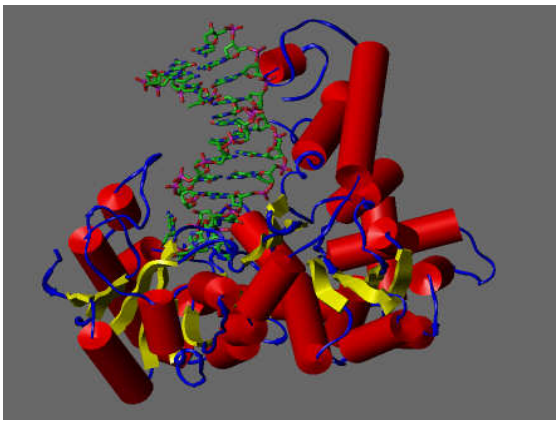




Which one is likely to be more stable? ☺

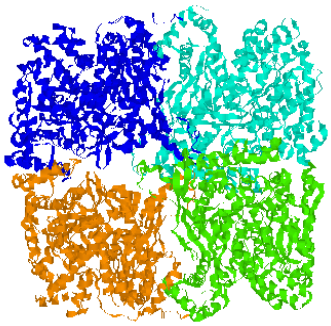
Tertiary structure

Tertiary structure describes the folding of the polypeptide chain to assemble the different elements of secondary structure in a particular arrangement

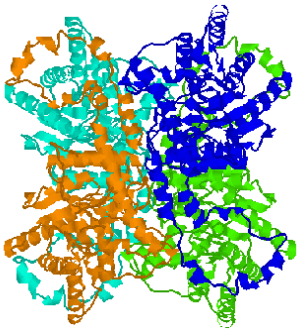


Quaternary structure: subunits

Describes the assembly of tertiary structures



phosphorylase

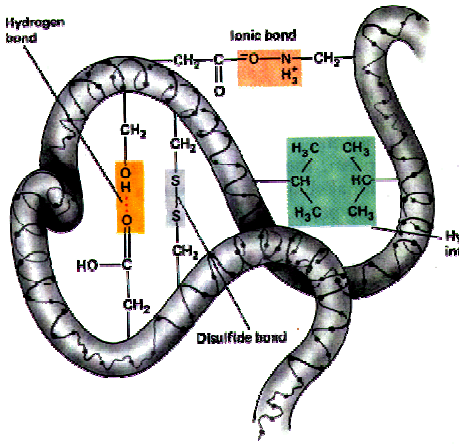


glucose isomerase

Subunit are held together by non-covalent forces

In each case the active site is formed by a pair of sub-units
Note the interwoven arrangement of polypeptide chains in GI

Keeping it all together



- Hydrophobic interactions in the core of protein globules are often critical for maintaining the integrity of the fold

- Proteins are active only if their unique tertiary/ quaternary structure is maintained
- This is achieved by a variety of covalent and non-covalent interactions
- In water soluble proteins the hydrophilic amino acids are on the surface, whereas the hydrophobic groups are shielded from the aq environment in the interior of the protein

Next quiz will have Qs on protein structure

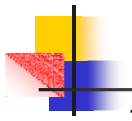
Why is this important?

The knowledge of protein structure provides a wealth of useful information about your receptor

- Single subunit *vs* multi-subunit protein (stability)
- Overall fold (stability)
- Amino acid residues at or near the binding/catalytic site (immobilization chemistry)
- The position of C- and N-terminus (immobilization methods)
- Performance improvements through protein engineering

Where can I get protein structures from?

PROTEIN DATA BANK!

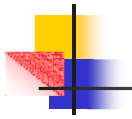


Home Work

Illustrate your cool biosensor with a protein structure from PDB and mark/describe elements of second/tert/quat structure

The rules of the game:

- You probably don't yet know whether you would use an enzyme, an antibody or else. In fact, the protein you need may not even exist – it was an **imaginary sensor** after all ☺
- However, you should identify a protein which is relevant (or make up the relevance!) to your sensor. For example, if your biosensor monitors a hormone you can find an enzyme that convert it to something else or metabolize e.g. some "hormone oxidase" and **justify your choice**
- You can also look for proteins that bind or interact with your target e.g. "cockroach pheromone receptor" or even the target itself e.g. cancer biomarker protein or a similar/related protein and **justify your choice**
- If your sensor is so cool that finding anything reasonably relevant is difficult, make up a plausible connection and **justify it**. Justifications like "I chose Dystrophin because my name start with D – David" won't work!



Enzyme-based biosensors

Enzymes have dominated the biosensors field in the 90s; in commercial terms it is still by far the biggest success

Why are enzymes so useful in biosensors?

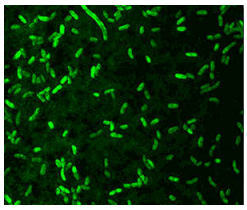
- **S**ensetivity
- **S**electivity
- **S**tability/Reproducibility
- Ease of integration with major types of transducers

Let's see how it works

Sensitivity

Luciferases are enzymes that convert energy to light

560nm



490nm

Luciferin + ATP → Luciferyl adenylate + PPi

Luciferyl adenylate + O2 → Oxyluciferin + AMP + light

FMNH2 + RCHO + O2 ⟶

FMN + RCOOH + H2O + light

How can we use this enzyme analytically?

- To determine early stage cell breaking e.g. monitor the quality of erythrocytes in blood banks
- To look for bacteria e.g. hygiene monitoring

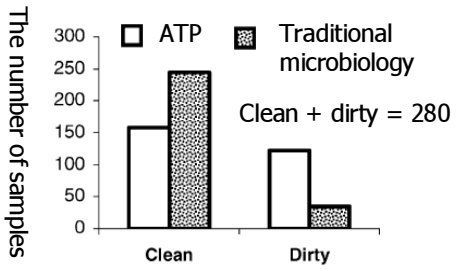
Hygiene monitoring

A sampling kit

Cotton swab: is used to wipe across or "swab" a surface and pick up bacteria for analysis

It takes at least a day to get the result in traditional microbiological assay, while biosensors gives it mins

Comparative analysis of 280 spots and equipment at hospital kitchens



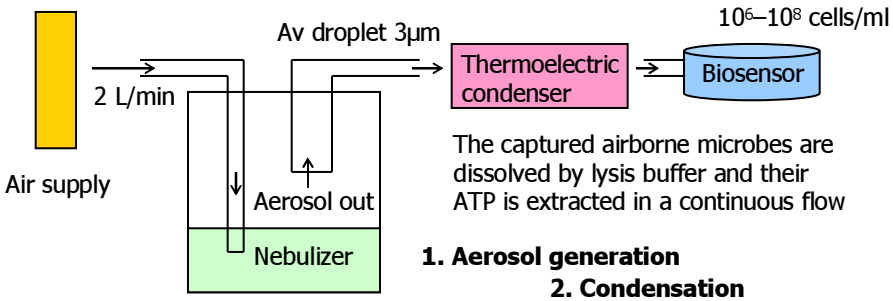
Int. J. Hyg. Environ.-Health 209 (2006) 203–206

and there is more...

Airborne bacteria detection

A new real-time detection system to measure ATP extracted from airborne microorganisms:

1. Condensation system to concentrate aerosol
2. Collection of bacteria and ATP extraction
3. Bioluminescence detection of bacterial ATP



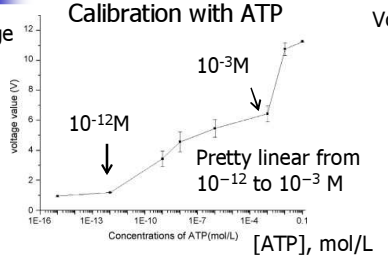
The captured airborne microbes are dissolved by lysis buffer and their ATP is extracted in a continuous flow

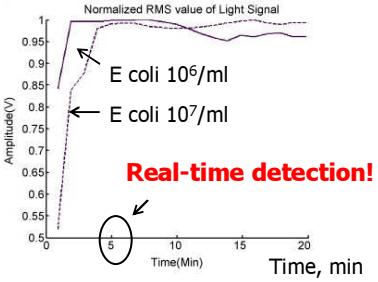
1. Aerosol generation
2. Condensation
3. Detection

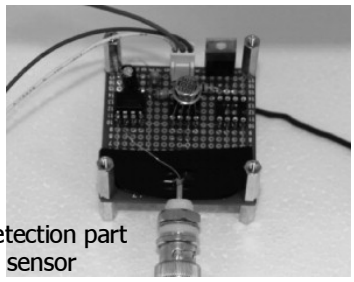
Lee et al (2008) Sensors and Actuators B: Chemical, 132, 443-448

Detection of airborne microbs

Calibration with ATP







the detection part of the sensor

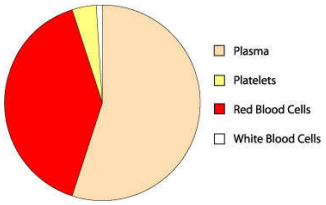
Applications:

- Military purposes
- In/out-door air quality control systems
- Air pollution monitoring in public transportation systems

Lee et al (2008) Sensors and Actuators B: Chemical, 132, 443-448

Selectivity

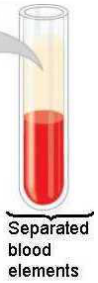
Glucose in a blood sample



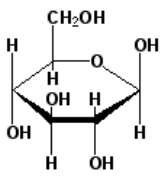
Can we chemically detect glucose in this sample? **YES**

Without separating it out? **NO**

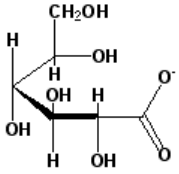
Plasma 55%	
Constituent	**Major functions**
Water	Solvent for carrying other substances
Icons (blood electrolytes Sodium Potassium Calcium Magnesium Chloride Bicarbonate	Osmotic balance pH buffering, and regulation of membrane permeability
Plasma proteins Albumin Fibringen Immunoglobulins (antibodies)	Osmotic balance, pH buffering Clotting Defense
Substances transported by blood Nutrients (such as glucose, fatty acids, vitamins) Waste products of metabolism Respiratory gases (O_2 and CO_2) Hormones	



Glucose sensor



Glucose Oxidase



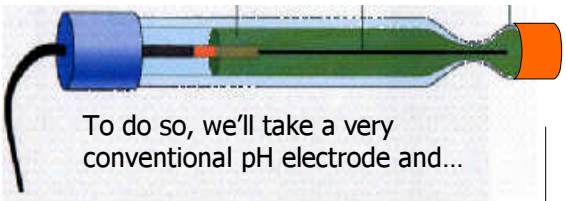
at neutral pH

H^+

A product with ionizable group is generated, which at neutral pH will dissociate to give COO^- and H^+, hence we can follow the reaction by monitoring the pH…

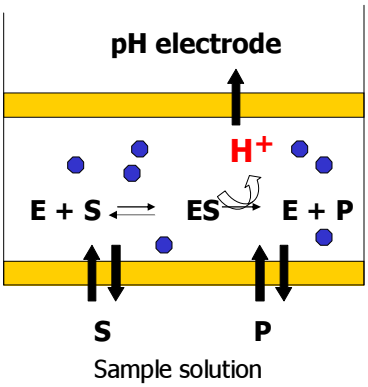
All we need to do then is to somehow modify the tip of the electrode so that it will measure only the enzyme reaction

Enzyme electrode



… add a little extra

To do so, we'll take a very conventional pH electrode and…



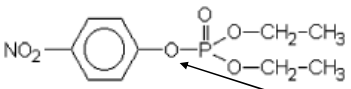
Glucose oxidase (●) or another enzyme (E) is located between the two membranes

The bottom membrane must be permeable to S and P but not to E →

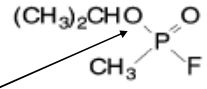
S - glucose or other analyte

P – gluconic acid or other product

Useful for other enzymes?



YES



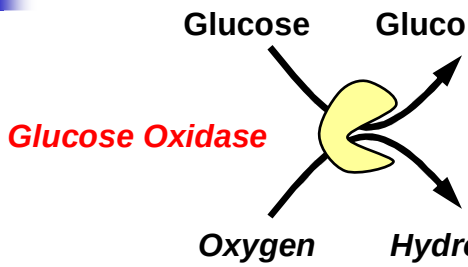
Paraoxon: organo-phosphorus pesticide

hydrolysis

Sarin: chemical warfare agent

- Organophosphate hydrolase (Aryldialkylphosphatase EC 3.1.8.1) is capable of hydrolyzing P-O bonds in most presently known organophosphorus neurotoxins
- Since the hydrolysis products are strong acids, the detection of organophoshates can be done with a pH-sensitive device
- Many other hydrolytic, group-transfer, etc enzymes can be assayed in this manner too, although this is not the only way to generate a signal

Compatibility with transducers

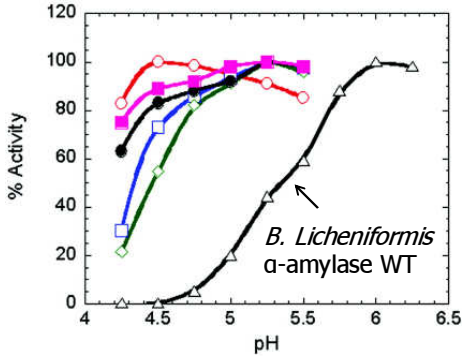


- Oxygen measurement: $[O_2]$ electrochem
- Gluconic acid measurement: pH elctrochem/optical
- Peroxide measurement: $[H_2O_2]$ electrochem
- Coupled to peroxidase rxn: [Dye] optical
- Thermal: enthalpy T

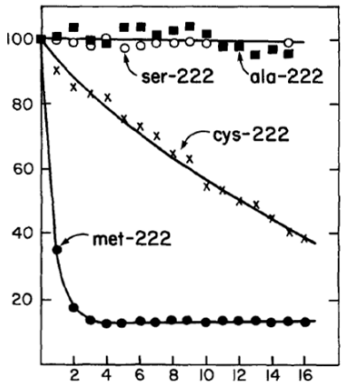
Stability

Enzymes are very labile. Right?

Comparison of WT and recombinant amylase activity at **95-105°C**



Practically no loss of activity for recombinant enzymes at pH 4.5



Inactivation of WT subtilisin and its mutants by **1M H_2O_2** at pH 9.5 (25°C)

Enzymes in biosensors

If **S**elective, **S**ensitive and **S**table enzyme is available – great

If not, the desired property can be engineered

- **Rational protein engineering:**

Knowledge-based structural alterations by substitution of selected amino acids e.g. the replacement of oxidation sensitive Met in subtilisin with Ala or Ser

- **Directed evolution**:

Introduction of a VERY LARGE number of mutations (or changing the AA sequence in other ways), followed by high-throughput screening (HTS) to select proteins with the desired characteristics e.g. temp-based screening of vast library of amylases

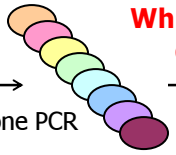
Let's take a brief look at directed evolution

Is this really possible?

Randomly introduce zillion of mutations

E

e.g. error-prone PCR

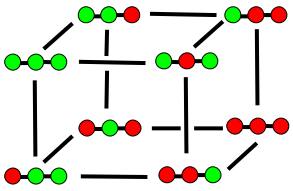


Which one is good?

Assay them all?

Let's check the numbers:

3 positions, 2 amino acids



2^3 = 8 combinations
3 dimensions

50 positions, 20 amino acids

20^{50} combinations
= 1.23×10^{65}

Thousands of Dimensions

Screening 1 combination per second:
10^{57} years – not a useful timeframe

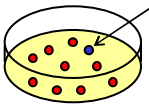
You can NOT screen them all!

But you can still accomplish a lot provided that

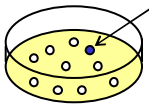
1. Libraries are constructed smartly rather than randomly to increase the hit rate
2. Have great HTS capabilities (10^6 sample a day is now routine)
3. Only look at what you want – 99.9% of mutations will be useless anyway…

We know what we want but how do we pick them?

I am good – you must check me ☺



This would be easy if the bad guys die (or stay invisible)



Why is it called "evolution"?

Darwinian Evolution

- Adaptation
  - Mutation (random)
  - Sex/Recombination
- Selection
  - Survival
  - Reproduction
- Opportunistic [Non-directional]

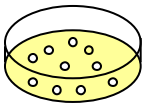
Directed Evolution

- Adaptation
  - Random point mutations in target gene
  - In vitro recombination of related genes
- Selection through repeated rounds of screening for **desired** phenotype (directed, by experimentor)
- Prior knowledge of the protein is not required

Directed evolution

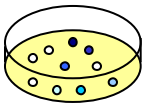
for thermostability

Generated library



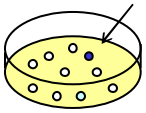
Plenty of recombinant enzymes to check

"Normal" assay



Some enzyme are more active than other

Assay at T above the normal T inactivation



Very few will "survive"

Now we can pick this guy, use it generate another library which will be assayed at even higher temperature. And do it again, and again, and again

e.g. 40°C → 45°C → 50°C → 55°C → 60°C

For example: LOX sensor

Lactate oxidase is used in biosensors to measure the concentration of lactate in the blood and other body fluids

$$\text{L-Lactate} + O_2 \xrightarrow{\text{LOX}} \text{Pyruvate} + H_2O_2$$

- Lactate levels in serum are indicates of oxygenation state of tissues can be a warning of ischemic condition
- Lactate sensors are sometimes used during life-threatening surgery, intensive therapy and in other critical conditions
- Lactate sensors are also used in sport-related applications – athletes' blood monitoring and others

The half-life of this enzyme at 70°C was increased ~36 times that of the WT and this significantly prolong the lifetime of LOX-based biosensors

Biosensor for D-amino acids

Enzymes can also be evolved for sensitivity and selectivity (or the lack thereof)

Is selectivity always good?

D-Amino acids sensor can be useful for some biomedical applications and in food analysis e.g. product quality, ripening or deterioration

D-amino acid + O_2 + H_2O → α-keto acid + H_2O_2 + NH_3

However, in most cases one would like to know the **total content of D-amino acids in a sample**

Is it realistic to have an enzyme active on all 20 D-aa?

Possibly – there are pretty non-specific enzyme but…

Will the sensor's response be same in different AA mixtures?

e.g. will the signal be the same for 10:1 ratio of D-AA1 to DAA2 and D-AA2 to DAA1?

Defining enzyme selectivity

Can we quantitatively define enzymes' selectivity to get a better understanding of enzyme-based sensors performance?

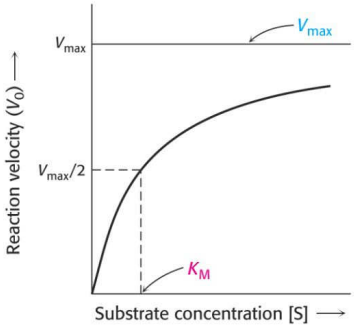
The answer to this question and much more - after the break

Now it's **quiz time** ☹

The bad news: to quantitatively compare the selectivity of different enzymes, we need to know some kinetic parameters

Enzyme kinetics

The kinetics of most enzymatic reactions is described by **Michaelis-Menten** equation



$$v = \frac{Vmax[S]}{Km + S}$$

- Michaelis-Menten equation provides a relationship between the reaction rate and substrate concentration
- In the majority of enzyme sensors the signal is a direct function of the rate

Hence, understanding this relationship and factors affecting the rate is critical for achieving good performance of enzyme-based biosensors

Derivation of the MM equation

Consider an enzyme reaction:

$$E + S \underset{k\text{-}1}{\overset{k1}{\rightleftharpoons}} ES \xrightarrow{k_{cat}} E + P$$

In a steady state the rate formation of the ES complex and the rate of its decomposition (both way – towards P and S) are the same:

Hence, $V = k1\,[E]\,[S] = k\text{-}1\,[ES] + kcat\,[ES]$ (1)

formation of ES — decomposition of ES

Substituting [E] in eq (1) we get $k1\,([Eo]–[ES])[S] = k\text{-}1[ES] + kcat[ES]$ or

$k1[Eo][S] = k\text{-}1[ES] + kcat[ES] + k1[ES][S] = [ES]\,(k\text{-}1 + kcat + k1[S])$ (2)

and from (2) $[ES] = k1\,[Eo]\,[S]\,/((k\text{-}1 + kcat + k1[S])$

Derivation of the MM equation

[ES] = k1[Eo][S] / ((k-1 + kcat +k1[S]) can be rearranged as

$$[ES] = \frac{[Eo][S]}{(k\text{-}1+kcat)/k1 + S}$$

Because (k-1 + kcat)/k1 = K_M (this is a constant called Michaelis-Menten constant or "K_M" for short, we get the following equation:

$$[ES] = \frac{[Eo][S]}{Km + S}$$

and because $v = \frac{d[P]}{dt} = kcat\,[ES] = \frac{kcat\,[Eo]\,[S]}{Km + S}$

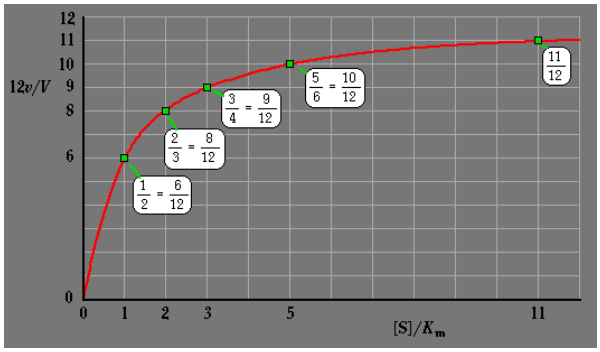
given that Kcat x [Eo] = Vmax, we get the following **FINAL** equation ⟶

$$\mathbf{v = \frac{Vmax[S]}{Km + S}}$$

Approximations

Note that v does not reach the limit *Vmax* at any attainable value of [S], and remains noticeably far from it even at relatively high values of [S]

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

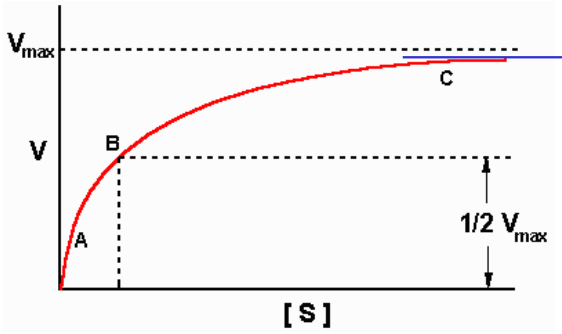


For example, if [S] = 11 *K*m, then *v/V* = 11/12, or just over 90%

MM equation: partial solution 1

What if S>>Km?

then $v = \frac{V_{max}[S]}{K_m+[S]} \sim \frac{V_{max}[S]}{[S]} = V_{max}$



This is effectively a **zero order** reaction – a type of reaction where the **rate does NOT** depend on the concentration of reactants

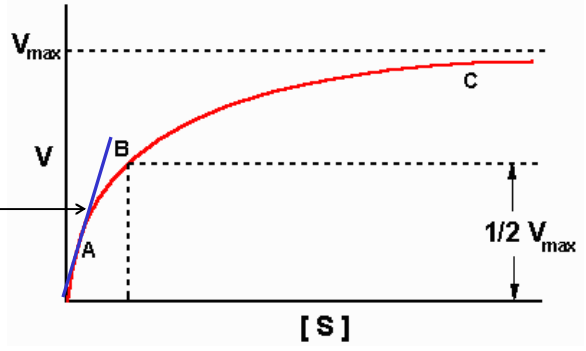
MM equation: partial solution 2

What if Km>>S?

then $v = \frac{V_{max}[S]}{K_m+[S]} \sim \frac{V_{max}[S]}{K_m} = K_{app}[S]$

this is now a constant

This is effectively a first order reaction - a type of reaction where the reaction **rate is proportional** to the reactant's concentration



Determination of K_m and V_{max}

Michaelis-Menten equation is a hyperbola but for experimental determination of Km it is much handier to have a linear function

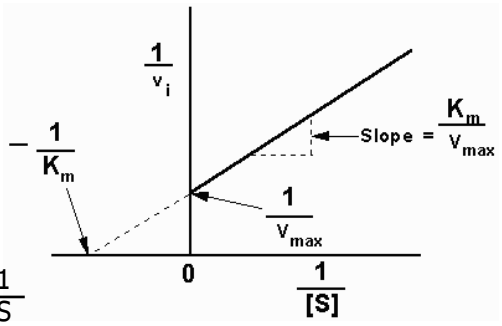
Hence, $v = \frac{V_{max}[S]}{K_m+[S]}$

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

and in the final form:

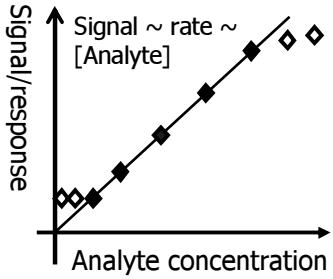
$$\frac{1}{v} = \frac{1}{V_{max}} + \frac{K_m}{V_{max}}\frac{1}{S}$$

e.g. Lineweaver-Burk Plot (or others)



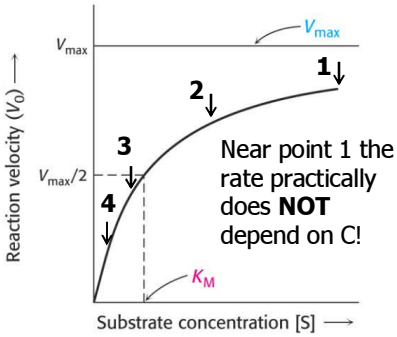
Why are we talking about this?

Suppose we have an enzyme sensor for analyte X, which works by measuring the **concentration of X via the reaction rate** i.e. the more X in a sample, the higher the rate and the bigger the signal



Much below K_M (e.g. point 4) so that the rate would be approximately linear with the analyte concentration

Where do you have to be on the MM plot?

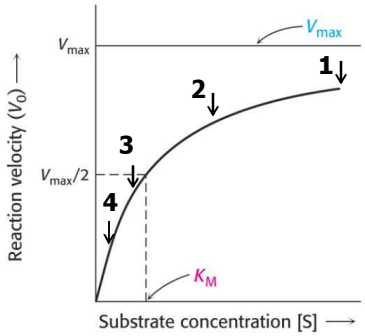


Near point 1 the rate practically does **NOT** depend on C!

Hence, we need an enzyme with Km above the detection range

The other way around?

However, one can imagine a biosensor where it would better to do measurements at [S]>>Km (point 1 and up)



- Suppose we have a sample containing an analyte of interest
- But instead of measuring the rate of reaction as before ([A] ~ V_{enzyme} ~ Signal), we determine the analyte's **concentration**
- For example, our enzyme make a mol of colored product per mol of analyte i.e. [A] = [P] ~ Signal
- We'd want our E to work at a max!

Any ideas: what kind of measurement would benefit from the analysis under the [S]>>Km condition?

Enzyme with low Km and high Vmax would be much better

A sensor for two substrates

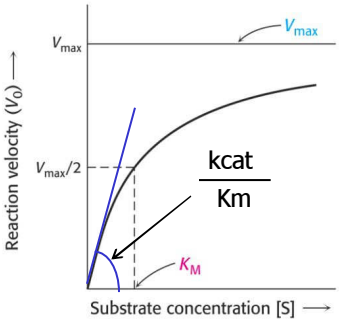
Suppose an enzyme can convert two substrates S_A and S_B

$$E + S_A \underset{k\text{-}1A}{\overset{k1A}{\rightleftharpoons}} ES_A \xrightarrow{kcatA} E + P_A \qquad \textbf{For } S_A$$

$$E + S_B \underset{k\text{-}1B}{\overset{k1B}{\rightleftharpoons}} ES_B \xrightarrow{kcatB} E + P_B \qquad \textbf{For } S_B$$

$$\text{then} \quad \frac{va}{vb} = \frac{d[Pa]}{d[Pb]} = \frac{(kcat/Km)_A}{(kcat/Km)_B}$$

where $\frac{kcat}{Km}$ is a "**specificity**" **constant**



- The ratio of the specificity constants for two substrates is a quantitative measure of how efficient the enzyme is in converting these substrates; it applies to any number of substrates
- Also enables to compare the catalytic efficiency of different enzymes

The problem with D-AA sensor

	D-Alanine			D-Aspartate		
	V_{max} (min^{-1})	K_m (mM)	V_{max}/K_m	V_{max} (min^{-1})	K_m (mM)	V_{max}/K_m
Wild-type	3900[a]	0.9[a]	4330[a]	40	33.1	1.2
M213R[b]	630	17.8	35	235	2.0	118
First round of error-prone PCR						
Q144R (1-7)	2685	0.8	3200	56	12.8	4.3
L118H (3-382)	3620	0.4	8415	31	12.8	2.4
D242V/Q253R/D304V (2-41)	4555	2.3	1980	40	16.3	2.4
Second round of error-prone PCR starting from His-Q144R						
T60A/Q144R/K152E (4-903)	3660	0.6	5800	53	7.9	6.7
Q144R/G199D/Y223C/H329R (5-249)	5740	0.4	13660	71	9.8	7.2

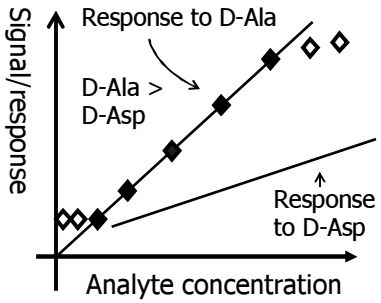
	D-Arginine		
	V_{max} (min^{-1})	K_m (mM)	V_{max}/K_m
Wild-type	640	2.8	228
M213R[b]	Below detection limit		
First round of error-prone PCR			
Q144R (1-7)	470	3.5	135
L118H (3-382)	880	2.1	410
D242V/Q253R/D304V (2-41)	920	4.6	198
Second round of error-prone PCR			
T60A/Q144R/K152E (4-903)	745	3.4	210
Q144R/G199D/Y223C/H329R (5-249)	880	2.4	366

✓Improvement in catalytic efficiency

❖Comparable (kind of) response with neutral and acidic amino acids

☹ Similar response of neutral, acidic and basic amino acids? **NO!**

How does it affect performance



What would the response to D-Asp look like under the same conditions?

It would be flatter - how much flatter will depend on the specificity constants for the two substrates

Suppose now we want to measure D-Ala in the presence of D-XX and for simplicity let's assume D-XX is **NOT a substrate** for D-AA oxidase

What would the response to D-Ala look like?

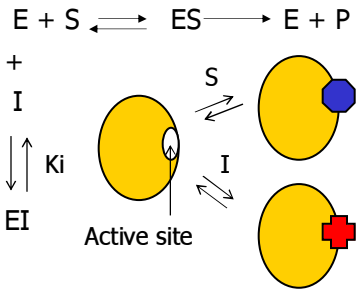
It may be flatter too – the fact that D-XX is not a substrate **does NOT mean it is not an inhibitor** ☺

Enzyme Inhibitors

Definition: An inhibitor is a substance that interferes with enzyme catalysis by reducing the reaction rate

- Inhibitors can be reversible or irreversible
- Reversible inhibitors affect Km or Vmax or both depending on the type
- The type of reversible inhibition can often be discerned from double-reciprocal plots e.g. Lineweaver-Burk, which also enable to determine Ki – the **inhibition constant**
- Ki (analogous to Km) is the concentration of inhibitor at which it occupies half of the sites on the enzyme; it is conceptually equivalent to the dissociation constant of the enzyme (or enzyme-substrate complex) and the inhibitor
- Ki can be determined in a series of experiments with varying concentrations of substrates and/or inhibitors

Competitive inhibition

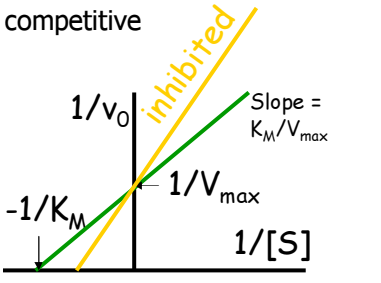


Inhibitor binds to the same site as substrate, but can be competed away by adding more substrate, so

1. V_{max} is not affected
2. Apparent K_m will increase with inhibitor concentration

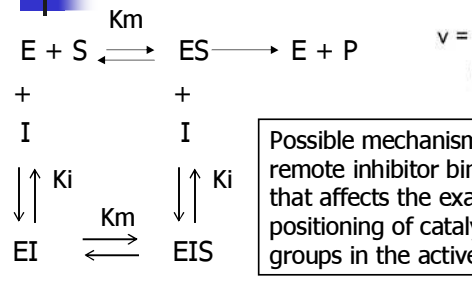
$$\frac{1}{V_0} = \left(\frac{\alpha K_m}{V_{max}}\right)\frac{1}{[S]} + \frac{1}{V_{max}}$$

where $\alpha = 1 + [I]/K_i$



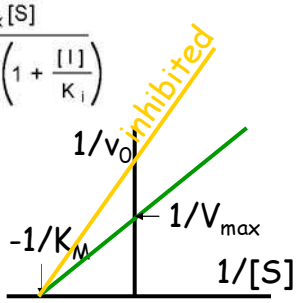
Qualitatively - if [S] >> [I], the inhibitory effect is negligible

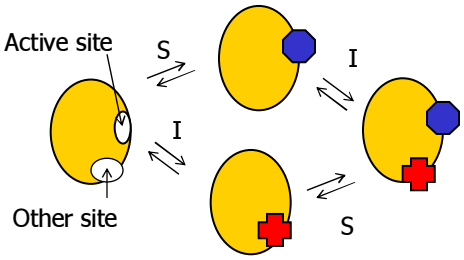
Non-competitive inhibition



$$v = \frac{V_{max}[S]}{\left([S] + K_m\right)\left(1 + \frac{[I]}{K_i}\right)}$$

Possible mechanism: remote inhibitor binding that affects the exact positioning of catalytic groups in the active site

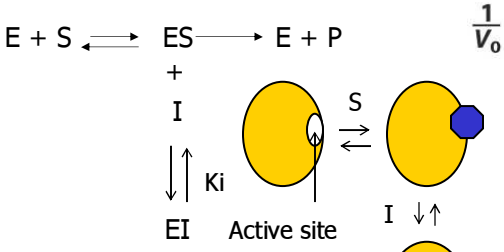




Affect rate without affecting binding of the substrate; hence, lower Vmax and unchanged Km

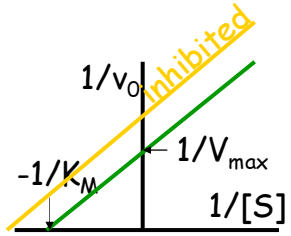
Vmax = kcat[E0]; it can be lowered by reducing kcat – (as above) or by reducing the amount of active enzyme (irreversible inhibition

Uncompetitive inhibition



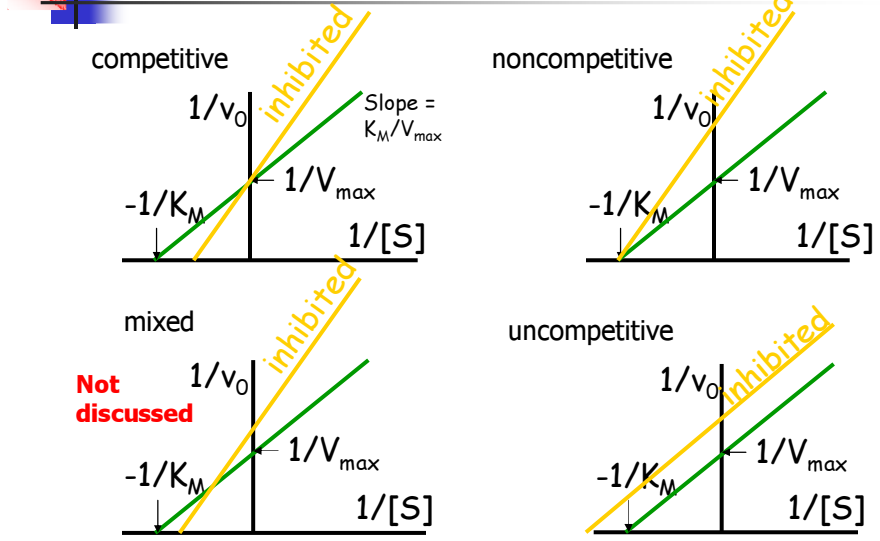
$$\frac{1}{V_0} = \left(\frac{K_m}{V_{max}}\right)\frac{1}{[S]} + \frac{\alpha'}{V_{max}}$$

α' = 1 + [I]/Ki



- Inhibitor binds only to ES complex - increased [S] cannot overcome it
- The inhibitor eliminates a fraction of ES from participation in the reaction
- Vmax is decreased and Km is proportionally decreased

Types of inhibitors: Summary



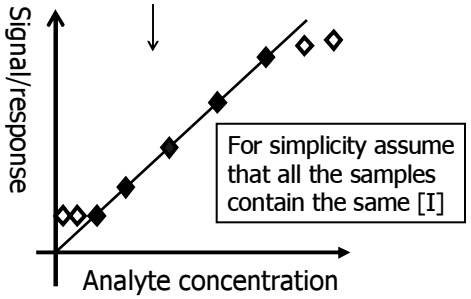
Effect of inhibitors

Suppose your sensor has a good response in a model sample

However, in real-life samples you have a significant amount of strong competitive or non-competitive inhibitor

What would happen to the curve?

Perhaps, but it's up to you to work out ☺



Home Work

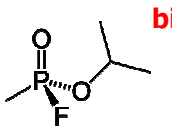
Do NOT email it to me – it'll be a Quiz question

What will happen to the response curve if your samples contain a non-competitive inhibitor and **IMPORTANTLY** what can you do to get the response curve back to what it was?

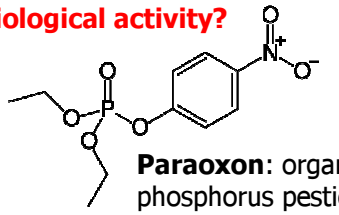
No protein engineering is allowed – you must use the same E and S

Is inhibition always bad news?

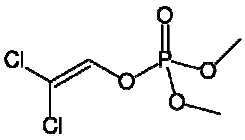
What do the nerve gas sarin, pesticide paraoxon and insectcide dichlorvos have in common in terms of their biological activity?



Sarin: chemical warfare agent



Paraoxon: organo-phosphorus pesticide



Dichlorvos: insecticide

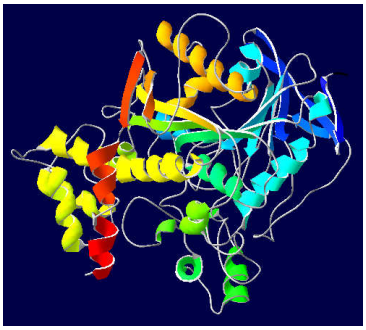
Sarin, paraoxon and dichlorvosas well as many other warfare agents, pesticides and insecticides (not just organophosphates) inhibit or inactivate acetylcholinesterase

Knowing this, can you suggest how to make a biosensor to rapidly detect these guys?

Biosensor based on <u>INHIBITION</u> of AChE

Cholinesterase

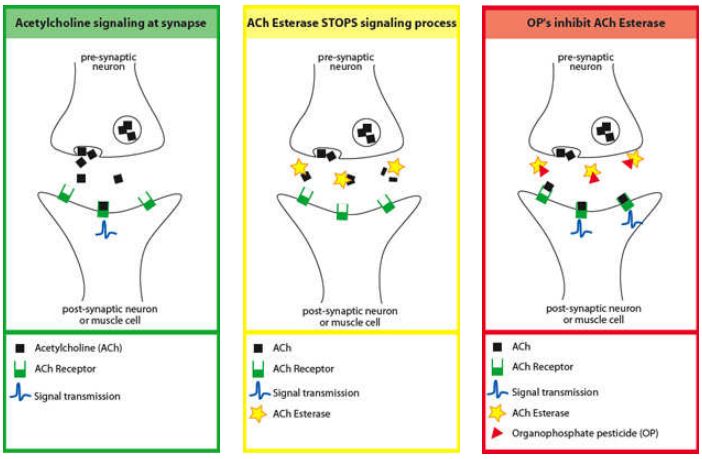
AChE is an enzyme which catalyzes the hydrolysis of the neurotransmitter acetylcholine into choline and acetic acid



- AChE is is a typical serine esterase with a catalytic triad comprising serine, histidine, and glutamic acid residues
- Extremely fast enzyme
- Has a vital physiological function – hydrolysis of neorotransmitter acetylcholine

AChE: biological function

Neurotransmission and inhibition of AChE



Neurotransmission of impulse by a parasympathetic neuron to another neuron, muscle or organ

AChE Biosensors

The principle of biosensors using AChE as a biorecognition element is based on the inhibition of the enzyme's activity by the agent being detected

Areas of applications:

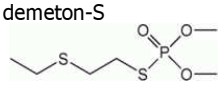
- Defense
- Environmental and food analysis

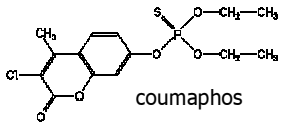
Note: for clinical diagnostics red blood cell cholinesterase (RBC) levels and plasma pseudocholinesterase* levels are determined

*Pseudocholinesterase or butyrylcholinesterase is a glycoprotein enzyme produced by the liver and circulating in the plasma; it hydrolyzes exogenous choline esters but its physiological function is unknown

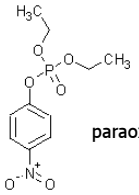
Inhibition of AChE

demeton-S

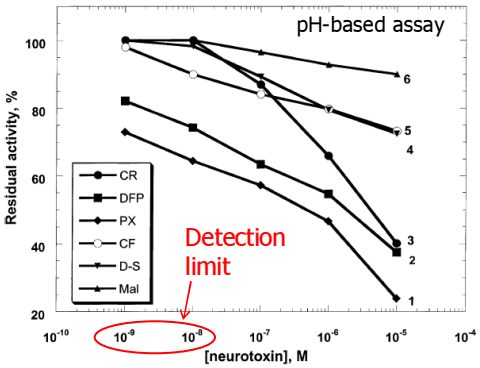




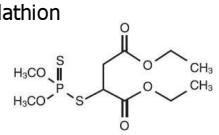
coumaphos



paraoxon



malathion

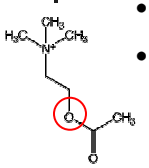


diisopropyl fluorophosphate

Inhibition of ACHE by different neurotoxins. CR: carbaryl; DFP: diisopropyl fluorophosphate; PX: paraoxon; CF: coumaphos; D-S: demeton-S; Mal: malathion.

Simonian et al (2001) Analytica Chimica Acta 444, 179

AChE in pesticide analysis

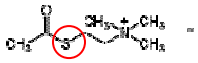


- Many common organophosphate and carbamate pesticides
- Both optical and electrochemical transducer can be used

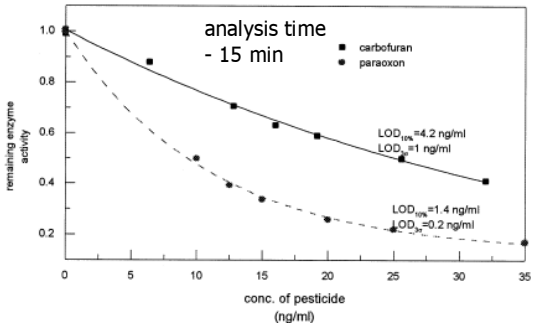
Acetylthiocholine $\xrightarrow{\text{AChE}}$ thiocholine + CH_3COOH

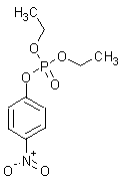
Thiocholine $\xrightarrow{\text{DTNB}}$ 5-thio-2-nitrobenzoate

Yellow color 410nm

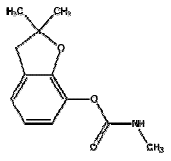


Detection limit in a 1 ng/ml range or ~10^{-9}M





paraoxon



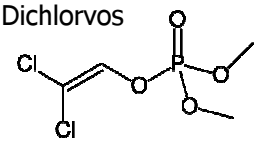
carbofuran

But one can do much better than that…

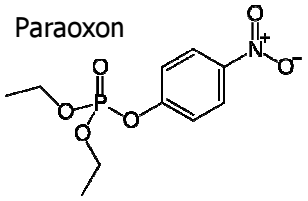
Pogacnik and Franko (1999) Biosensors & Bioelectronics 14, 569

Engineering for sensitivity

Dichlorvos



Paraoxon



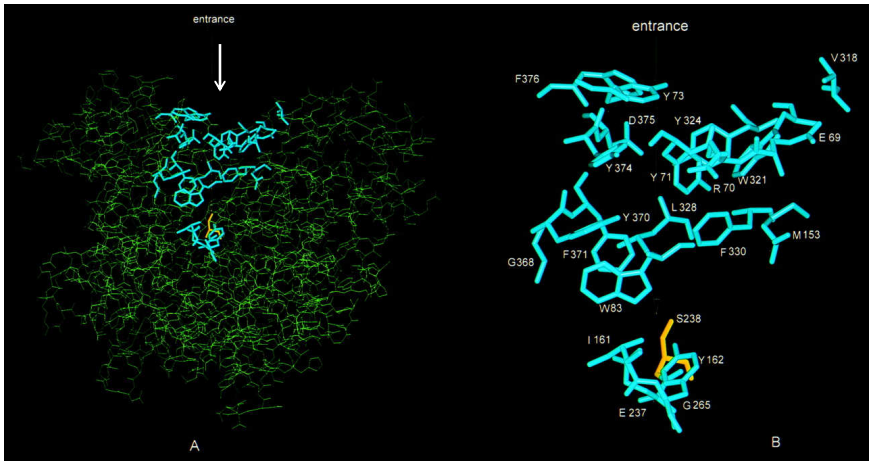
- Dichlorvos (2,2-dichlorovinyl dimethyl phosphate) is a highly volatile insecticide
- The United States Environmental Protection Agency has been considering a ban on its use for years

- Paraoxon is the active metabolite of a highly toxic insecticide parathion
- It is banned/restricted in many countries and will probably be banned completely from all use

Both Dichlorvos and Paraoxon can be detected with great sensitivity using engineered Acetylcholine esterase (AChE)-based biosensors

Engineering AChE

3-D structure of *Drosophila melanogaster* AChE



Mutated amino acids are highlighted in the active site gorge

Enhancement through PE

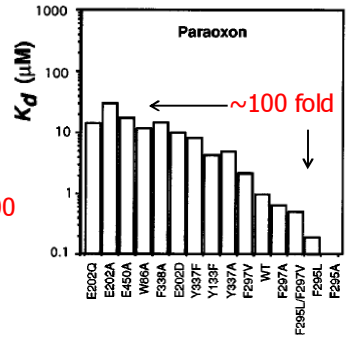
Drosophila AChE - dichlorvos

AChE mutants	k_i $\mu M^{-1}min^{-1}$
E69Y, Y71D	**487**
E69Y, Y71D, F330L	309
E69Y, 374A	205
E69W, 318D	132
E69Y, F330L,Y370F	120
E69W, Y370F	81
E69W, Y370A	64
E69Y, Y370F	14
Drosophila WT	**1.8**
Electric eel	**0.026**

~300 fold

Biosensors and Bioelectronics 20 (2005) 2347–2352

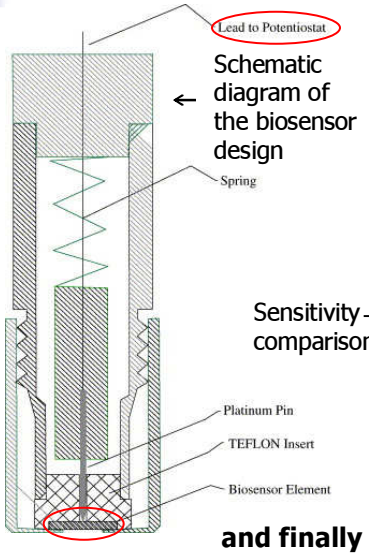
Engineering of **human AChE** for better sensitivity to paraoxon



Effects of mutations at the active site of HuAChE on the *Kd* values for paraoxon

Ordentlich et al (1996) JBC 271, 11953

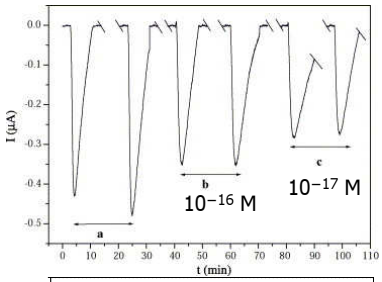
Biosensor's performance



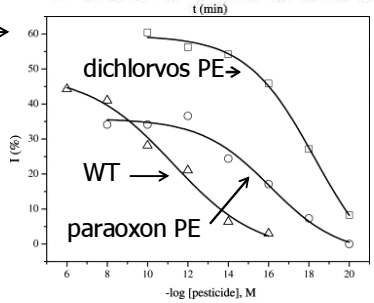
Schematic diagram of the biosensor design

and finally

Biosensor's response to dichlorvos

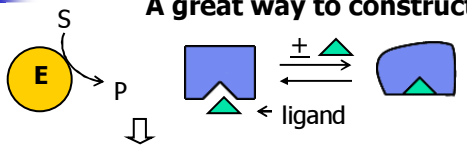


Sensitivity comparison

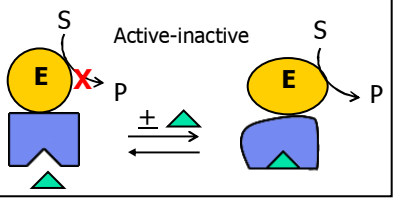


Domain fusion/insertion

A great way to construct molecular switches

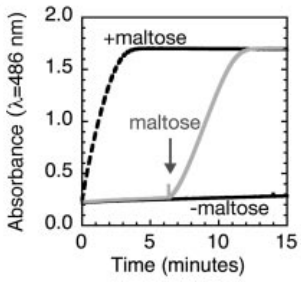


Binding of ligand switches the enzyme activity on and off



A switch recognizes an input signal (e.g. ligand concentration) and changes output signal (e.g. enzyme activity) in response – **great signal amplification too!**

Binding protein (blue) changes conformation



Maltose binding protein (MBP) and β-lactamase (BLA) switch: when maltose binds to MBP BLA activity decreases 600 fold

Very cool!

Guntas (2005) PNAS 2005, 102, 11224

In conclusion

Today we

- Talked about enzyme technology in biosensors and discussed how enzyme performance can be improved by directed evolution and protein engineering
- Briefly reviewed protein structures and basic enzyme kinetics and discovered that all this textbook stuff has some relevance to real life ☺
- Note that basic enzyme kinetics will be included in the final exam too ☹
- The protein databank homework, including the picture of your protein and justification of your choice must be submitted to poly603@gmail.com by 10am next Thursday

Any questions?

Have fun and see you next Thursday