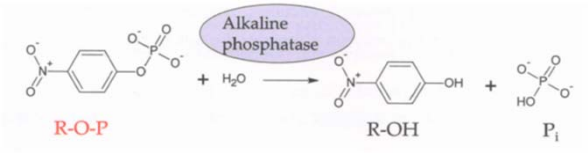
Enzyme kinetics of alkaline phosphatase



The class

- Alkaline phosphatase kinetics
- Brief background of steady state kinetics and the logic behind the measurements
- Assays – parameters to think about when designing assay, how this assay differs from glucose oxidase assay etc.
- Other assays

Learning outcomes

- What is an enzymatic assay
- Measuring products generated by an enzyme
- Basis of design of assays
- Determination of kinetic parameters

Alkaline phosphatase

- Alkaline phosphatase is an enzyme that hydrolyses esters of phosphoric acid into the corresponding alcohol and phosphoric acid
- Can be isolated from bone, kidney, intestine, plasma, liver, spleen, plants and microorganisms
- The kinetics of enzymes is often studied with model substrates that yield a color, so the progress of the reaction can be followed with a spectrophotometer

Assay nitty-gritties

- How do you quantitate *p*NPP ?

Ans: Calibration curve - graph showing the analytical response as a function of the known quantity of analyte

- What are you calibrating ?

Ans: The color due to reducing sugars

- How ?

Ans: By running standards of reducing sugar (known quantity of analyte) and measuring absorbance (analytical response)

Then plotting the analyte response as a function of reducing sugar

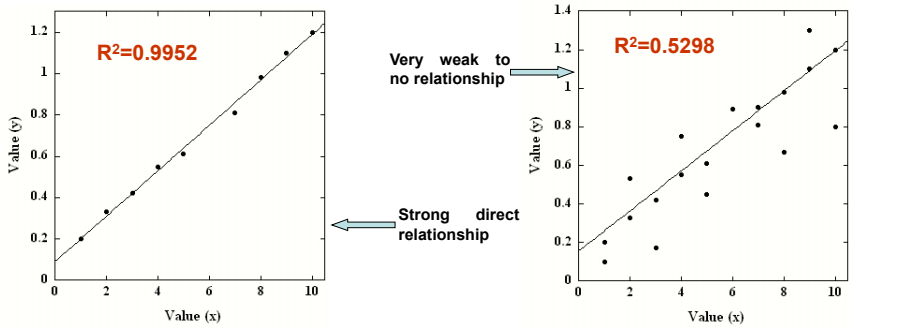
Calibration curve prep

- Here, known is the concentration of sugar you prepare
- Unknown is the absorbance due to enzyme assay
- Record the absorbance of the alcohol
- Plot Abs vs ester
- Use the Abs of *p*NPP generated to determine the concentration or amount of reducing sugar in unknown

Calibration : Best fit

R^2: compares the sums of the variations for the y-values to the best-fit line relative to the variations to a horizontal line.

- R^2 x 100: percent of the variation of the y-variable that is explained by the variation of the x-variable.
- A perfect fit has an $R^2 = 1$; no relationship for $R^2 \approx 0$



99.5% of the y-variation is due to the x-variation

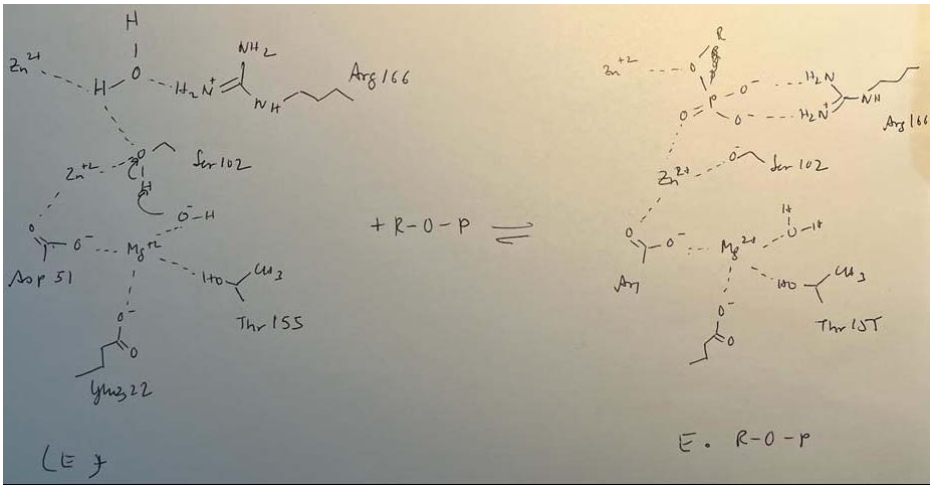
53.0% of the y-variation is due to the x-variation

Assay

- First determine the calibration curve by measuring absorbance of the known and plot
- Then measure the concentration of unknown and convert the absorbance to the known to quantify the unknown
- How will you use to determine the MM kinetics ?

The reaction mechanism

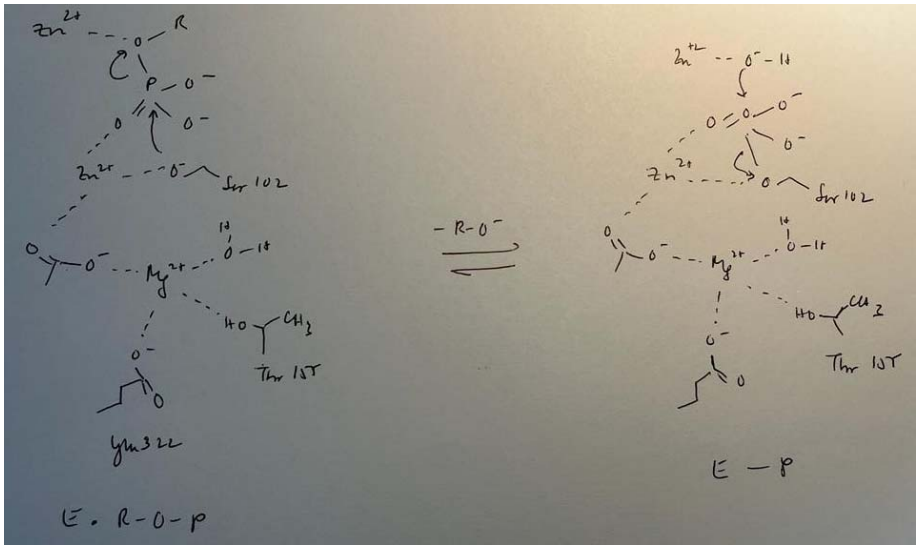
Step 1- Note the active site water, hydroxyl ion, Zn and Mg ion. Hydroxyl ions are formed under alkaline conditions



Comment to previous page:

- In the first step, preceding page. Negatively charged substrate replaces the water and associates with enzyme through the positively carged Arg166 and two Zn^{+2}
- Enzyme substrate complex is formed

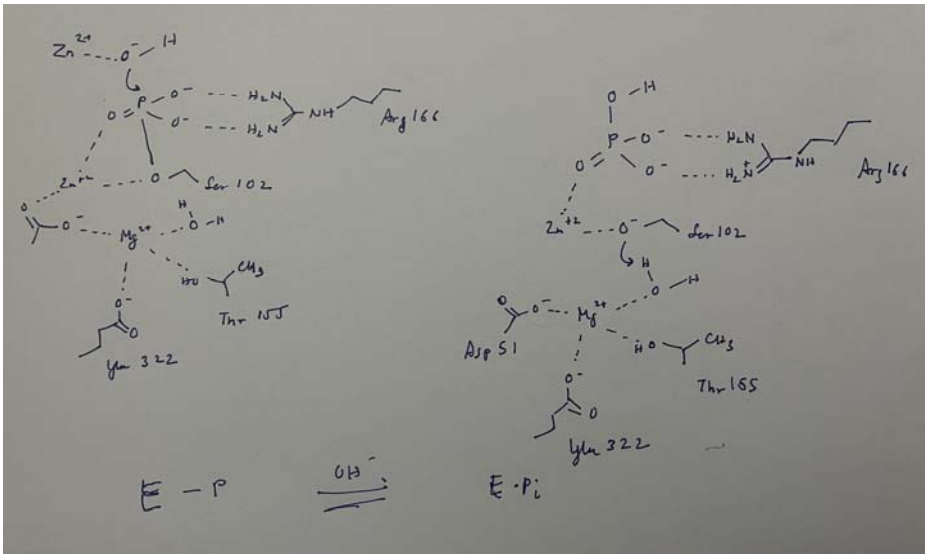
Step 2: Deprotonated Ser102 is a strong nuclephile that substitutes *p*-nitrophenol in a nucleophilic substitution reaction. Pi remains covalently bound to Ser102



Comments to prev slide

- You can see that the Zn^{2+} associates with the hydroxyl ion
- The enzyme product complex is formed

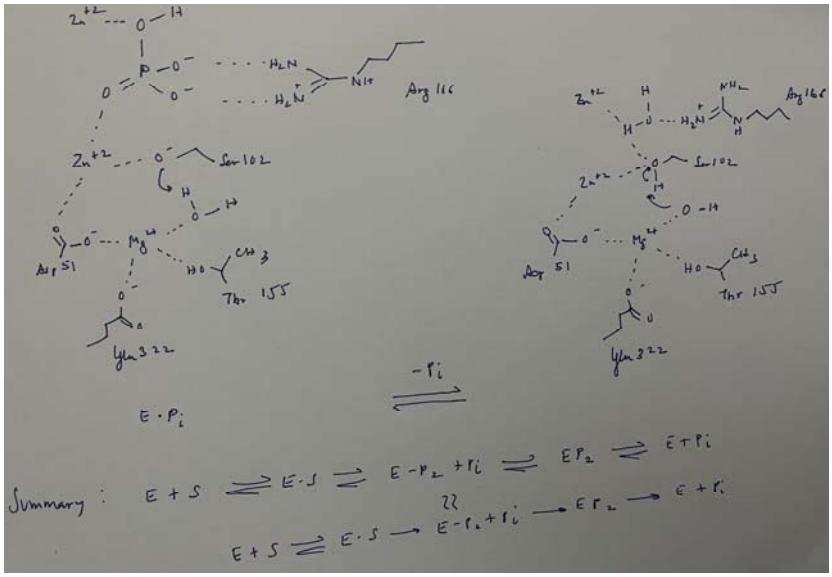
Step 3: the hydroxyl ion carries out a nucleophilic substitution reaction at the phospho-serine



Comments to prev slide

- Observe that the bond between the phosphate and Ser102 breaks.
- The inorganic hydroxy phosphate is loosely attached, hence the E dot Pi nomenclature.

Step 4: hydroxy phosphate is replaced by water



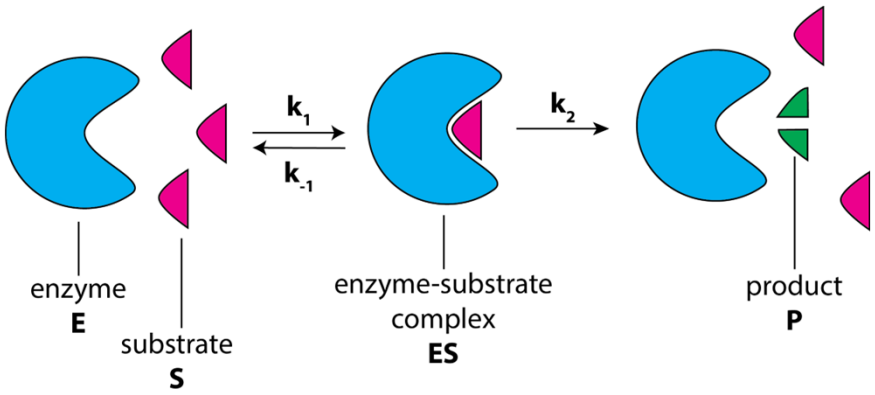
Comments to prev slide

- Now the active site is as before and ready for the second reaction. So what is broken step by step is for one turnover (remember kcat). The enzyme catalyzes many such turnover (substrate to product)

- A simple organic chemistry happening at th eenzyme active site. Difference is the amino acid side chain spatial arrangements and the cofactors
- Ignoring the formation of ester from alcohol and phosphate is one approximation. If you approximate further by assuming that the ester bond breaking and Pi formation is the reate limiting step, you can simply the mechanism further to

  E+S ⇔ ES -> E + P …. MM kinetics

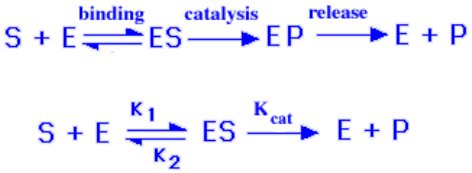
Steady-state enzyme kinetics



Assumptions of Michaelis-Menten kinetics:

1. The reaction is at equilibrium
2. The reaction is at steady-state

Michaelis-Menten Enzyme Kinetics



Steady-state Model:

- Formation of ES = Disappearance of ES

$k_1[E][S] = k_2[ES] + k_{cat}[ES]$

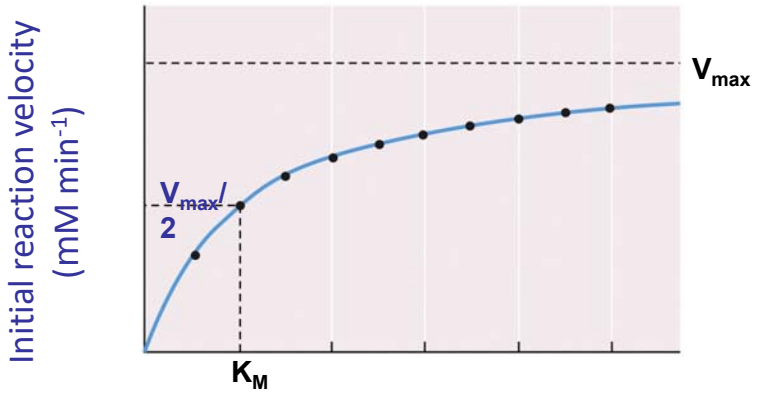
- Define a new constant, K_m (Michaelis constant):

$K_m = (k_2 + k_{cat})/k_1$

K_m reflects the affinity of the enzyme for the substrate:

- low K_m = high affinity
- K_m = [S] that produces V= $1/2V_{max}$

An enzyme's response to substrate can be visualized using the Michaelis-Menten plot



Michaelis-Menten Equation $v_o = \frac{V_{max}[S]}{K_M + [S]}$

Simplifying M & M analysis using a Lineweaver-Burk Plot

- A **double-reciprocal** representation of V vs. [S] data: plot 1/V vs. 1/[S]
- Take the inverse of both sides of the M-M equation to get the L-B equation, which specifies a **line**

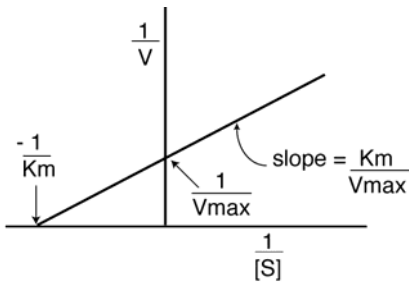
M-M $V = \frac{V_{max}[S]}{K_m + [S]}$

Inverse M-M $1/V = (K_m + [S]) / V_{max}[S]$

Rearranging

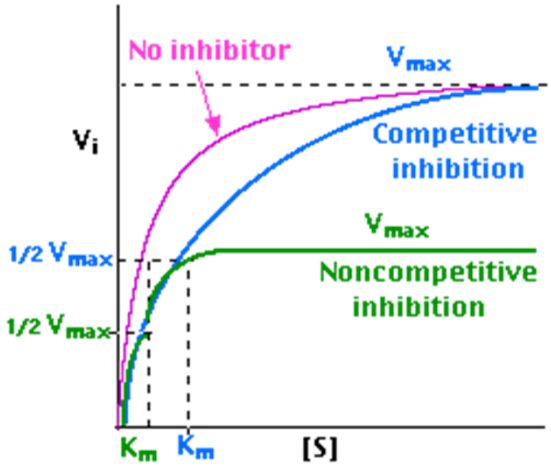
L-B $1/V = 1/V_{max} + K_m/ V_{max} (1/[S])$

Determining Kinetic Parameters from an L-B Plot



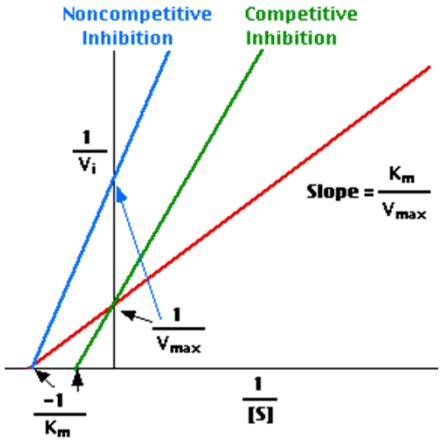
$1/V = 1/V_{max} + K_m/ V_{max} (1/[S])$

Michaelis-Menten Kinetics with Inhibition

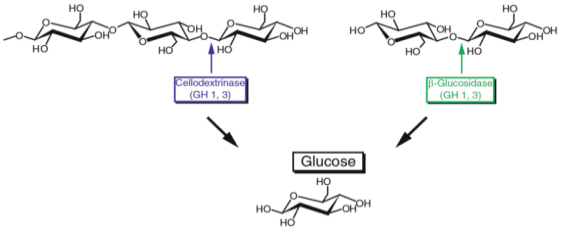


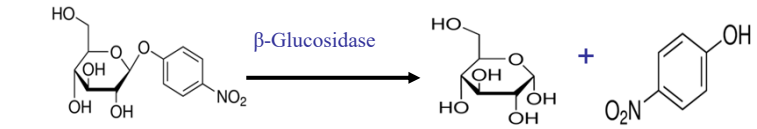
Just to give you an idea of how the plot changes in presence of various kinds of inhibitors

Lineweaver-Burk Plots with Inhibitors



Another type of colorimetric reaction





p-Nitrophenyl β-D-glucopyranoside

Glucose

p-NP

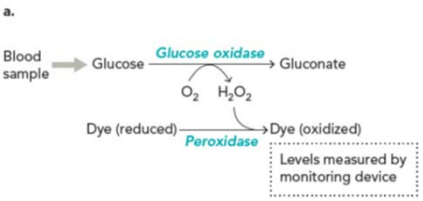
Why *p*-Nitrophenyl β-D-glucopyranoside and not glucose ?

- Colorimetric assays are simple to perform and easy to measure

- pNP gives a yellow color
- 1 mole of pNP = 1 mole of glucose
- Measure amt of *p*NP => Know amount of glucose
- How will you measure amount of pNP ?

Ans: Calibration curve !

Glucose oxidase test – enzyme based test for glucose



glucose oxidase converts glucose to gluconate and hydrogen peroxide (H_2O_2).

The enzyme peroxidase then catalyzes a redox reaction in the presence of H_2O_2 that changes a colorless reduced dye into a colored oxidized product.

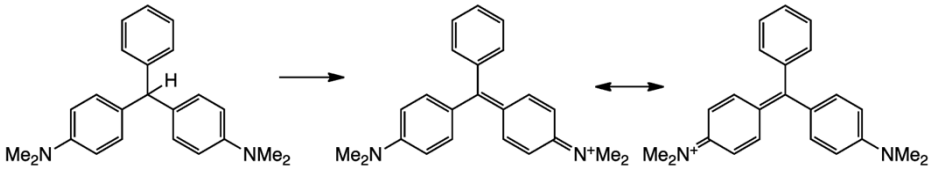
- The level of oxidized dye in the sample is measured using a blood glucose

Quantification of the released phosphate with Malachite Green and ammonium molybdate

P_i + $(NH_4)_2MoO_4$ $\xrightarrow{H^+}$ $H_3PMo_{12}O_{40}$ (yellow)

H^+ ↓ $+HMG^{2+}$ (yellow, λ_{max} 446 nm, Malachite green)

$[(MG^+)(H_2PMo_{12}O_{40}) + 2H^+$ (green coloured complex, λ_{max} 640 nm)]



Malachite green

Assay

- how much product is formed over a given time or, in some cases, how much substrate has been used up
- a method for measuring either product or substrate in the presence of the other is required.
- '**stopped assays**' - stopping the reaction after a fixed time, then measuring how much product has been formed.
- chemical, enzymatic to bioassay ….. the simplest is chosen provided it is reliable.
- phosphate release from a phosphate ester can be measured by the standard phosphomolybdate procedure ….. No separation needed.
- Stopping the reaction - denature the enzyme, such as strong acid, alkali or detergent; heat;

When to stop a stopped reaction?

- How do we decide the time of the reaction ?
- these methods provide only one single point and the velocity must be calculated from the slope of a line connecting this point with the blank before starting the reaction.
- control measurements at different reaction times must be undertaken to establish the linearity of the assay and appropriate choice of time point.

Stopped assay

- Where no direct signal for the conversion of substrate or product can be found, the reaction must be stopped after a defined time and the amount of product formed or substrate converted must be analysed
- Instead of a continuous progress curve these methods provide only one single point and the velocity must be calculated from the slope of a line connecting this point with the blank before starting the reaction.
- control measurements at different reaction times must be undertaken to establish the linearity of the assay and appropriate choice of time point.