Metabolic engineering some basic considerations

Lecture 8

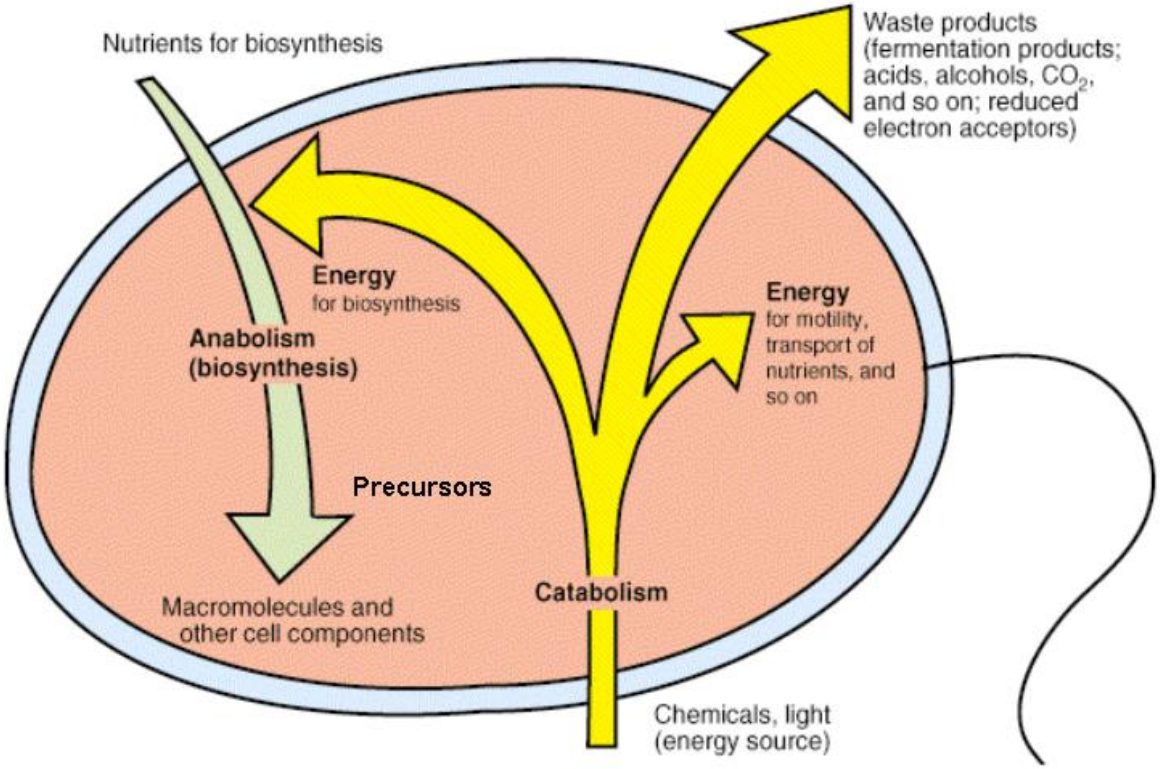
Moving towards metabolic engineering

- Metabolic engineering:
  - Recruiting heterologous activities to perform directed genetic modifications of cell factories with the objective to improve their properties for industrial application
  - „the improvement of cellular activities by manipulation of enzymatic, transport, and regulatory functions…with the use of recombinant DNA technology“ Bailey, 1991

Metabolic engineering versus bioprocess engineering

- Bioprocess engineering targets optimization of processes that utilize living organisms or enzymes (biocatalysts) for production purposes
- Metabolic engineering focuses on optimizing the biocatalyst itself

Microbial metabolism: Basic principles



Microbial metabolism: Basic principles

- About 1,000 anabolic reactions synthesize the macromolecular components that make up functional cells
- 11 intermediates of central carbon metabolism and the cofactors ATP, NADH, and NADPH constitute the core of this biochemical network
- intermediates and cofactors must be supplied through the catabolism at appropriate rates and stoichiometries for balanced growth
- At the same time, intermediates are the precursor molecules for all products derived from cellular metabolism
  - A particular intermediate serving as a precursor for a product will be depleted

Carbohydrate Catabolism

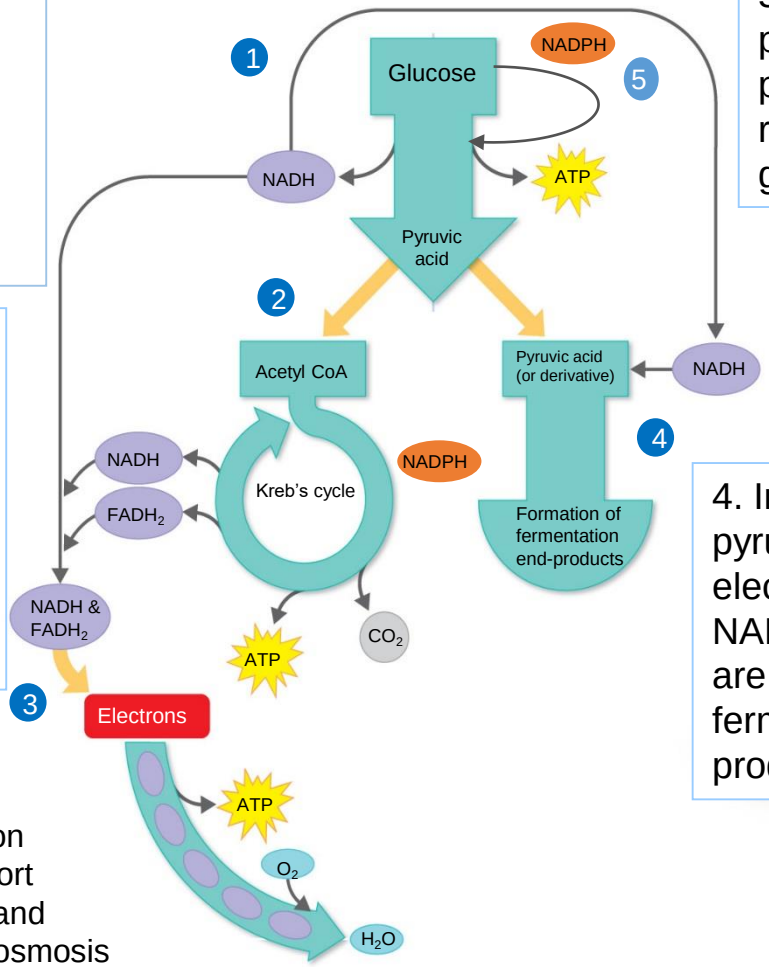
- The breakdown of carbohydrates to release energy
  - Glycolysis produces ATP and NADH
  - Pentose phosphate pathway
    - Uses pentoses and generates NADPH
    - Operates with glycolysis
  - Entner-Doudoroff pathway
    - Produces NADPH and ATP
  - Krebs cycle or TCA cycle
    - Produces NADH, FADH2 and ATP
- Electron transport chain and fermentative pathways regenerate NAD^+

An overview of respiration and fermentation

1. Glycolysis produces ATP and reduces NAD^+ to NADH while oxidizing glucose to pyruvic acid. In respiration, the pyruvic acid is converted to the first reactant in the Krebs cycle, acetyl CoA.

2. The Krebs cycle produces some ATP by substrate-level phosphorylation, reduces the electron carriers NAD^+ and FAD, and gives off CO_2. Carriers from both glycolysis and the Krebs cycle donate electrons to the electron transport chain.

3. In the electron transport chain, the energy of the electrons is used to produce a great deal of ATP by oxidative phosphorylation.

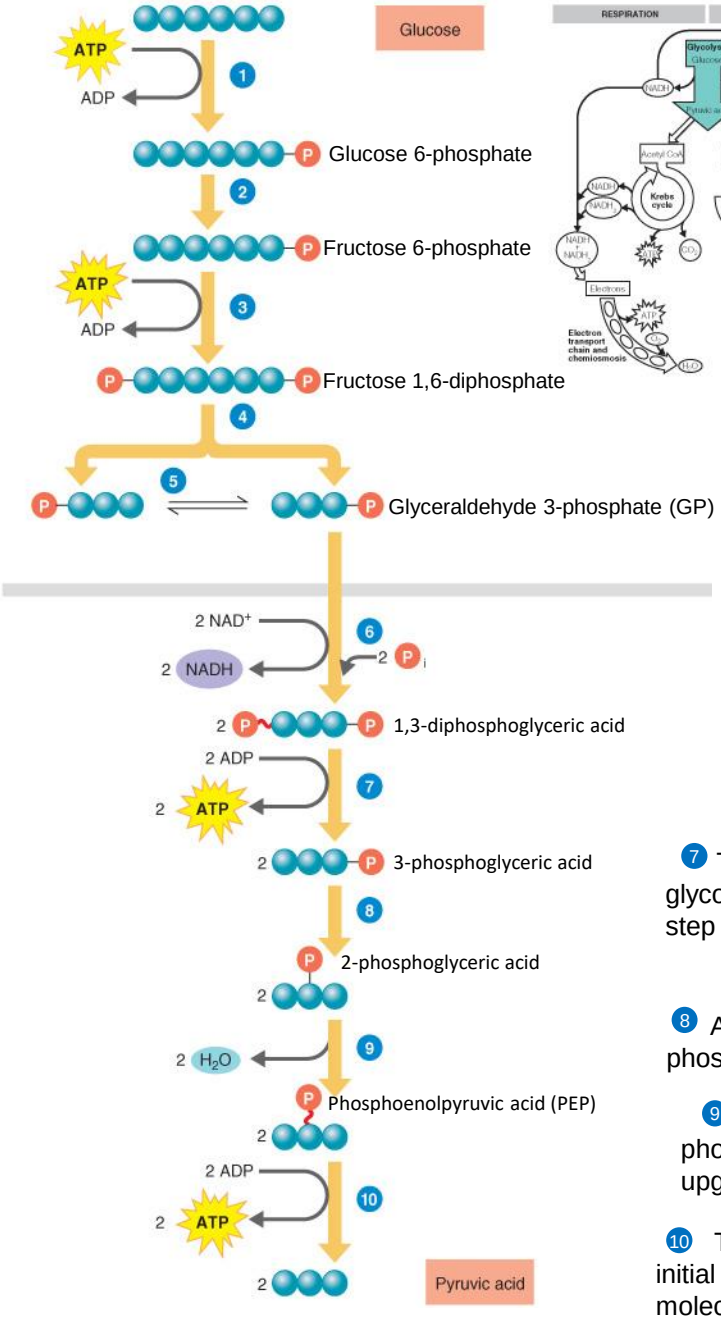


Electron transport chain and chemiosmosis

5. In the pentose phosphate pathway pentoses for anabolic reactions and NADPH is generated.

4. In fermentation, the pyruvic acid and the electrons carried by NADH from glycolysis are incorporated into fermentation end-products.

Outline of the reactions of glycolysis (Embden-Meyerhof pathway).



1 Glucose enters the cell and is phosphorylated. A molecule of ATP is invested. The product is glucose 6-phosphate.

2 2 Glucose 6-phosphate is rearranged to form fructose 6-phosphate.

3 The P from another ATP is used to produce fructose 1,6-diphosphate, still a six-carbon compound. (Note the total investment of two ATP molecules up to this point.)

4 An enzyme cleaves (splits) the sugar into two three-carbon molecules: dihydroxyacetone phosphate (DHAP) and glyceraldehyde 3-phosphate (GP).

5 DHAP is readily converted to GP (the reverse action may also occur).

6 The next enzyme converts each GP to another three-carbon compound, 1,3-diphosphoglyceric acid. Because each DHAP molecule can be converted to GP and each GP to 1,3-diphosphoglyceric acid, the result is two molecules of 1,3-diphosphoglyceric acid for each initial molecule of glucose. GP is oxidized by the transfer of two hydrogen atoms to NAD^+ to form NADH. The enzyme couples this reaction with the creation of a high-energy bond between the sugar and a . The three-carbon sugar now has two P groups.

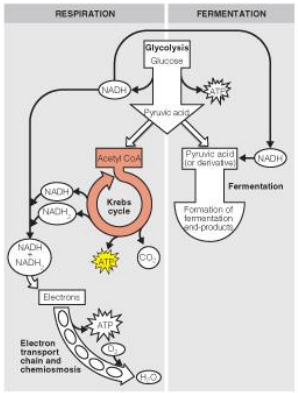
7 The high-energy P is moved to ADP, forming ATP, the first ATP production of glycolysis. (Since the sugar splitting in step 4, all products are doubled. Therefore, this step actually repays the earlier investment of two ATP molecules.)

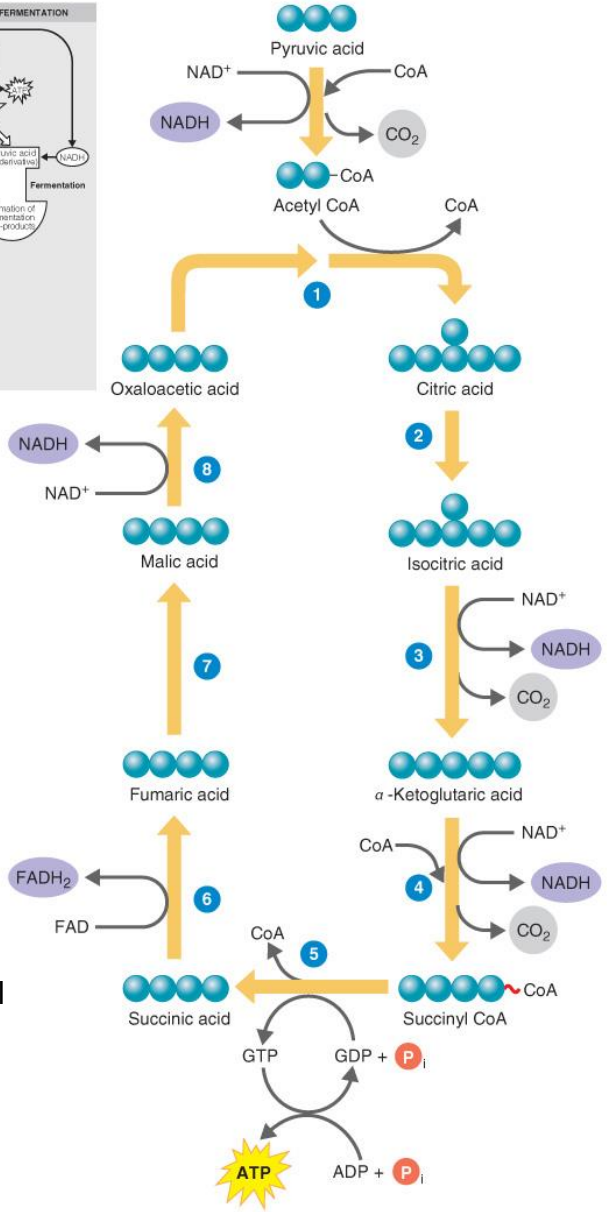
8 An enzyme relocates the remaining P of 3-phosphoglyceric acid to form 2-phosphoglyceric acid in preparation for the next step.

9 By the loss of a water molecule, 2-phosphoglyceric acid is converted to phosphoenolpyruvic acid (PEP). In the process, the phosphate bond is upgraded to a high-energy bond.

10 This high-energy P is transferred from PEP to ADP, forming ATP. For each initial glucose molecule, the result of this step is two molecules of ATP and two molecules of a three-carbon compound called pyruvic acid.

The Krebs cycle.





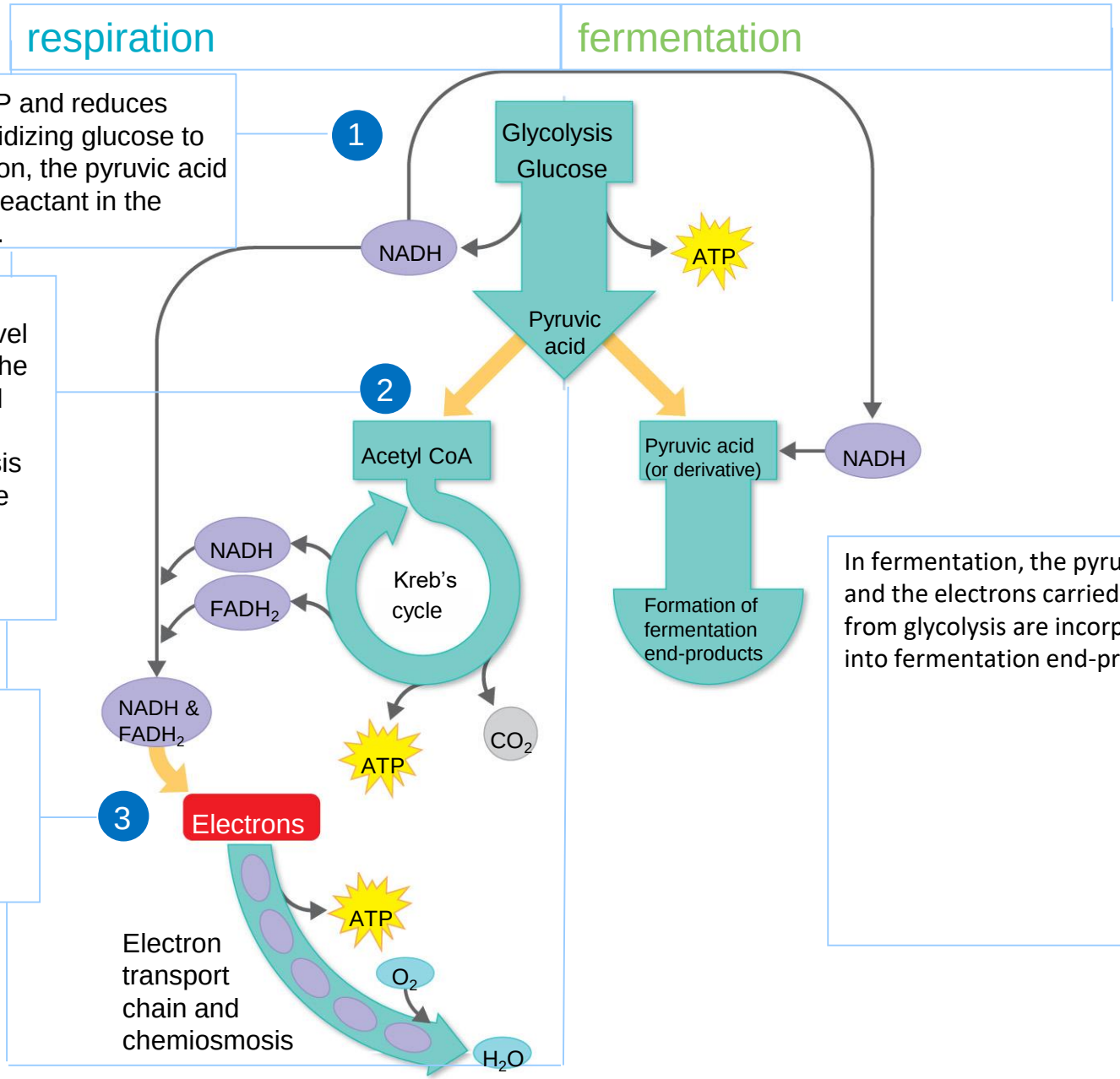
1 A turn of the cycle begins as enzymes strip off the CoA portion from acetyl CoA and combine the remaining two-carbon acetyl group with oxaloacetic acid. Adding the acetyl group produces the six-carbon molecule citric acid.

2 – 4 Oxidations generate NADH. Step 2 is a rearrangement. Steps 3 and 4 combine oxidations and decarboxylations to dispose of two carbon atoms that came from oxaloacetic acid. The carbons are released as CO_2, and the oxidations generate NADH from NAD^+. During the second oxidation (step 4), CoA is added into the cycle, forming the compound succinyl CoA.

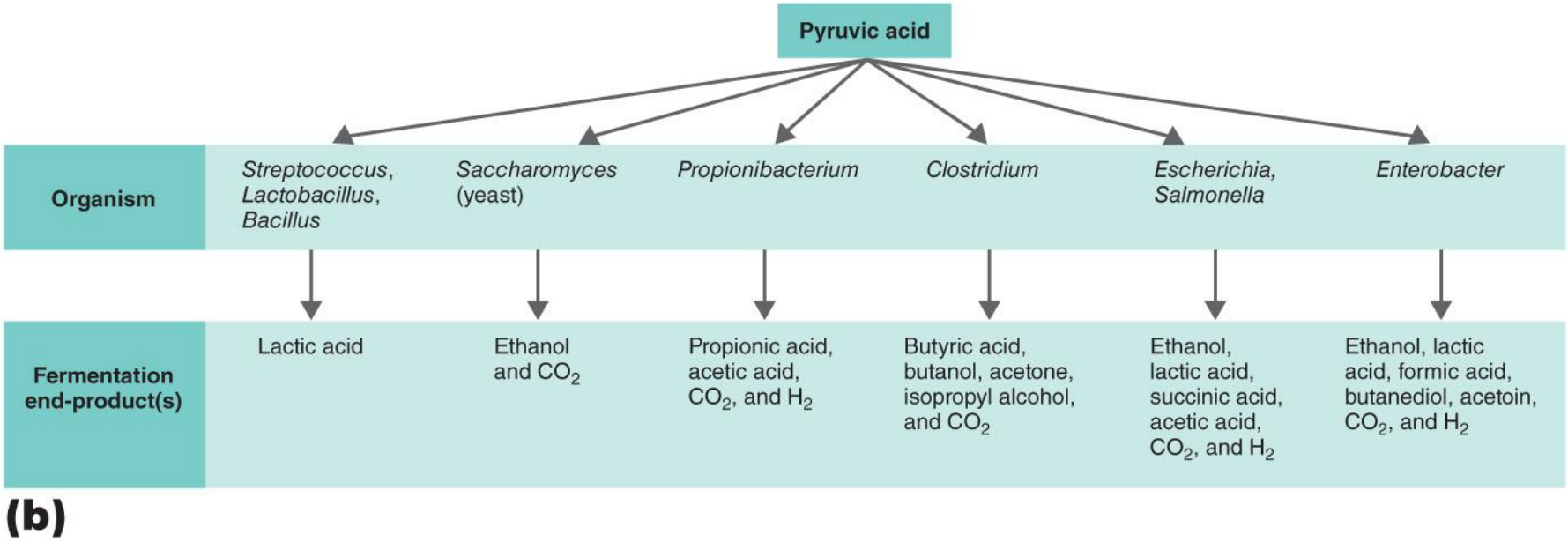
6 – 8 Enzymes rearrange chemical bonds, producing three different molecules before regenerating oxaloacetic acid. In step 6, an oxidation produces $FADH_2$. In step 8, a final oxidation generates NADH and converts malic acid to oxaloacetic acid, which is ready to enter another round of the Krebs cycle.

5 ATP is produced by substrate-level phosphorylation. CoA is removed from succinyl CoA, leaving succinic acid.

An Overview of Respiration and Fermentation.



Fermentation and by-products

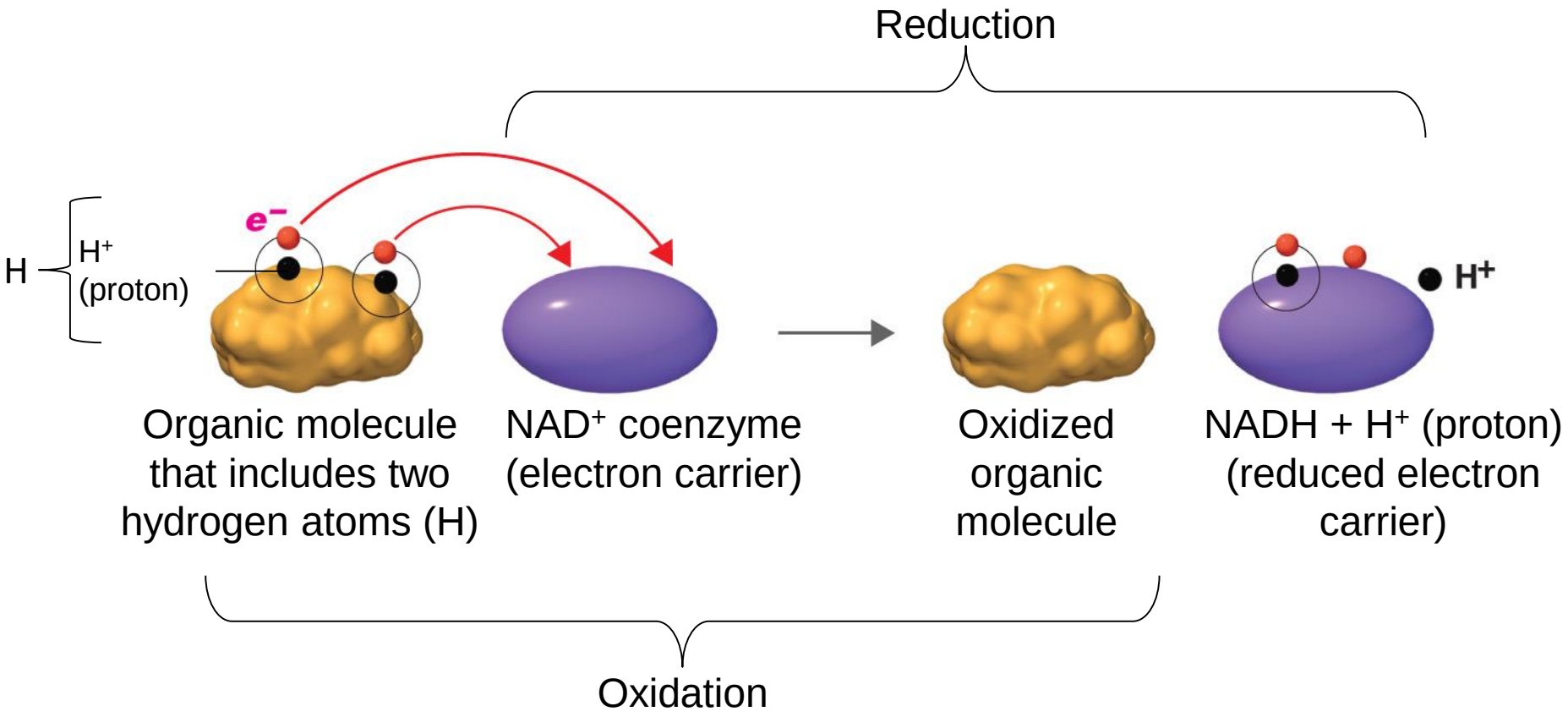


(b)

Redox balance

- In order to keep the metabolism running each reduction reaction has to be coupled with an oxidation reaction
- Although chemically very similar, the redox cofactors NADH and NADPH serve distinct biochemical functions
  - The electrons of NADH are transferred primarily to oxygen, driving oxidative phosphorylation of ADP to ATP or other terminal electron acceptors
  - NADPH, in contrast, drives anabolic reduction reactions
- Enzymes (transhydrogenases) that can transfer electrons from NADPH to NAD^+ and from NADH to $NADP^+$ are not generally found in organisms
  - NADPH and NADH reactions must be balanced!

Representative biological oxidation



Thermodynamic feasibility of a biochemical reaction

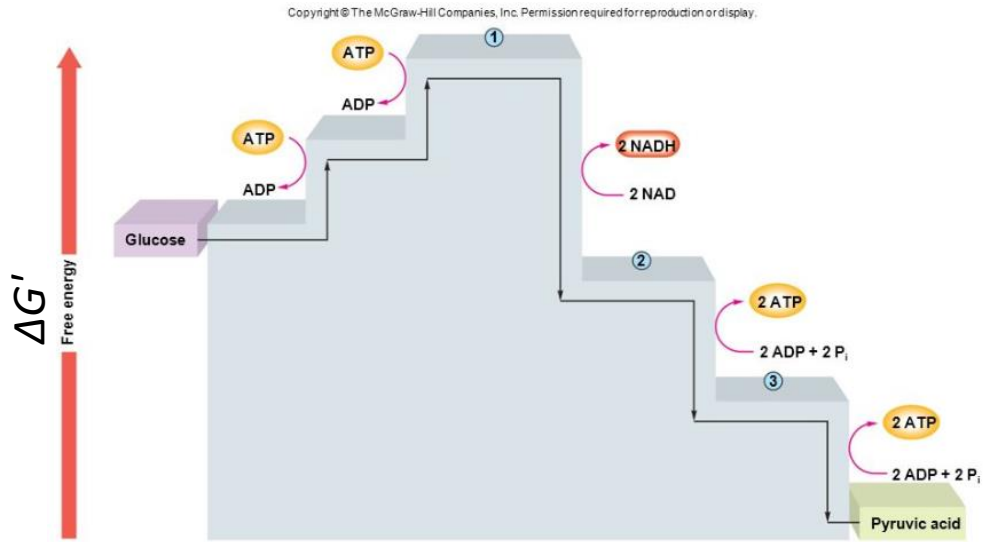
- Chemical reactions can be described as feasible if they occur without an external source of energy: $\Delta G' < 0$
- In the case of biochemical systems, unfeasible reactions may still occur if they are coupled to reactions that are feasible
  - if they are coupled to reactions that are feasible (consumption of ATP)
  - or by adjusting concentrations of substrates and products

- ΔG' informs if reaction takes place but not how quickly the reaction goes

Thermodynamic feasibility of a biochemical reaction

- if they are coupled to reactions that are feasible (consumption of ATP)

Example: glycolysis



Thermodynamic feasibility of a biochemical reaction

- or by adjusting concentrations of substrates and products

- Thermodynamic feasibility of reaction $A \rightarrow B$

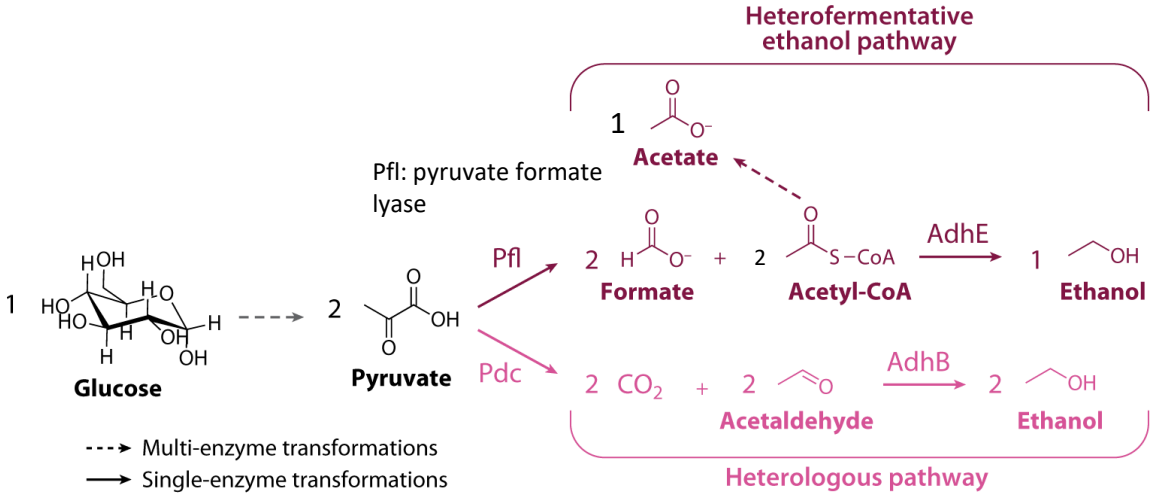
  $-\Delta G' = \Delta G'^{\circ} + RT \ln([B] / [A]) < 0$

- If product B is efficiently removed or turned over reaction will be become feasible

Carbon efficiency

- Most products are derived from a carbon source, typically from glucose
- In order to increase carbon efficiency most of the carbon utilized should be converted into the product
  - Minimizing of by-product formation (typical metabolic by-products are formate, acetic acid, ethanol)
  - With a running TCA cycle, metabolites are completely oxidized to CO_2
- Measure for carbon efficiency
  - mole C product / mole C substrate

Carbon efficiency



1 glucose -> 1 ethanol

1 glucose -> 2 ethanol

Pdc: Pyruvate decarboxylase

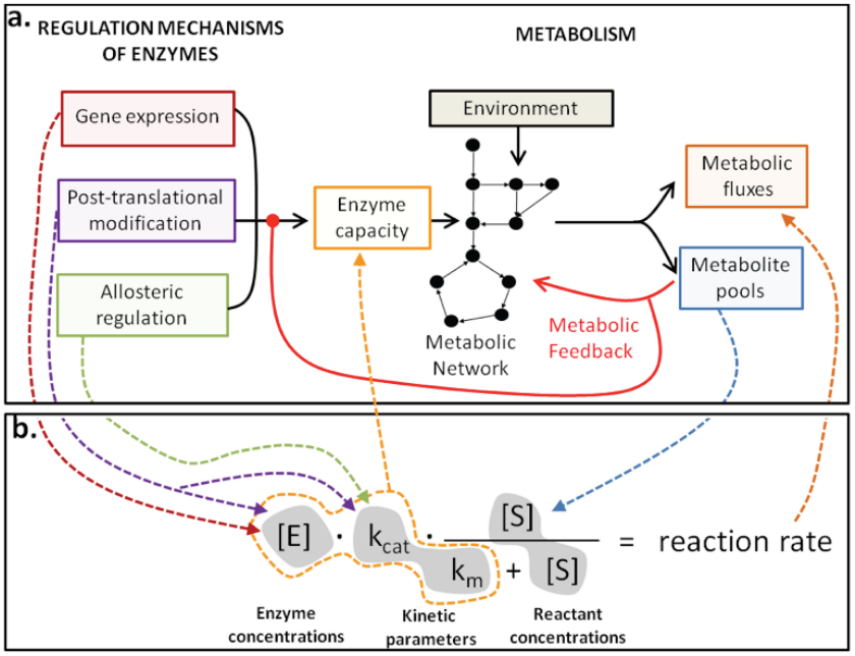
Zymomonas mobilis
Pyruvate decarboxylase alcohol dehydrogenase II

- Computing can be applied to identify the energetically most favorable pathway

Liu and Khosla, 2010, Annual Rev Genet 44:53-69

Metabolic fluxes depend on many parameters

- *In vivo* enzyme activity & amount is modulated by various regulatory processes
- Metabolite concentrations are a function of:
  - thermodynamics
  - reaction kinetics
- Flux through a metabolic pathway is a global network property that depends on both

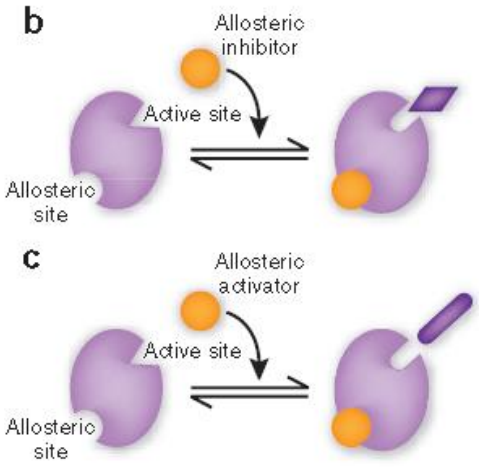


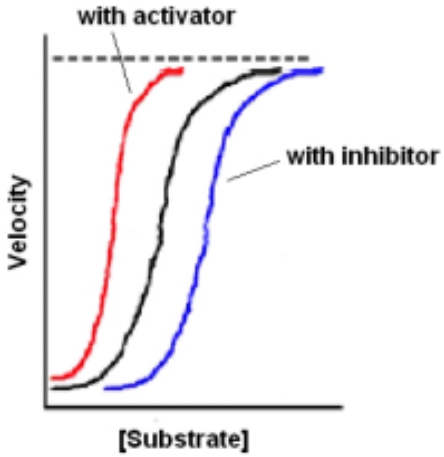
Mechanisms of metabolic regulation

- Modulation of activity of enzymes
  - Inhibition
  - Feedback inhibition and activation
  - Phosphorylation or other post-translational modifications
- Concentration of enzymes
  - Synthesis rate
  - Protein degradation
  - Irreversible inhibition

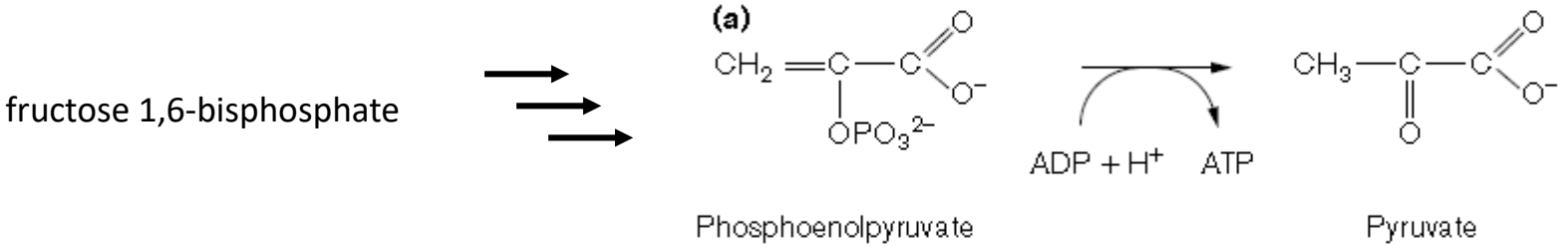
Allosteric regulation

- Allostery is defined as the regulation of protein function, structure and/or flexibility through the binding of a ligand or another protein (= effector) at a site distant from the active site (allosteric site).
- Allosteric regulation can increase or decrease reaction rates





Allosteric regulation, pyruvate kinase (mammalian)



- Its own substrate PEP and fructose 1,6-bisphosphate, an intermediate in glycolysis, enhance enzymatic activity
  - Thus, glycolysis is driven to operate faster when more substrate is present
- ATP is a negative allosteric inhibitor
  - Thus, ensures that catabolic and anabolic reactions are balanced

How to increase flux through a pathway if a step is controlled by allosteric enzyme?

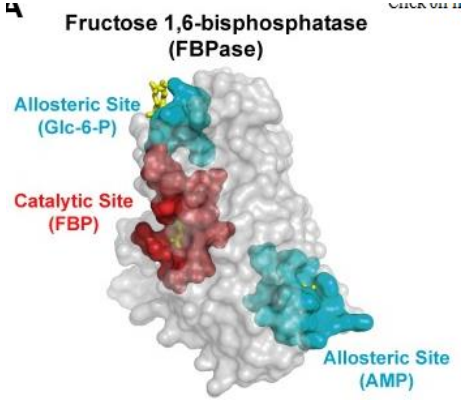
- Overexpressed endogenous enzyme is subjected to same type of regulation
- Use heterologous enzyme which is regulated differently
  - heterologous expression of Pyruvate kinase from *B. stearothermophilus* in *E. coli* led to an increase in specific glucose consumption in contrast to the overexpressed endogenous enzyme

Emmerling et al., 1999, Metabolic Engineering

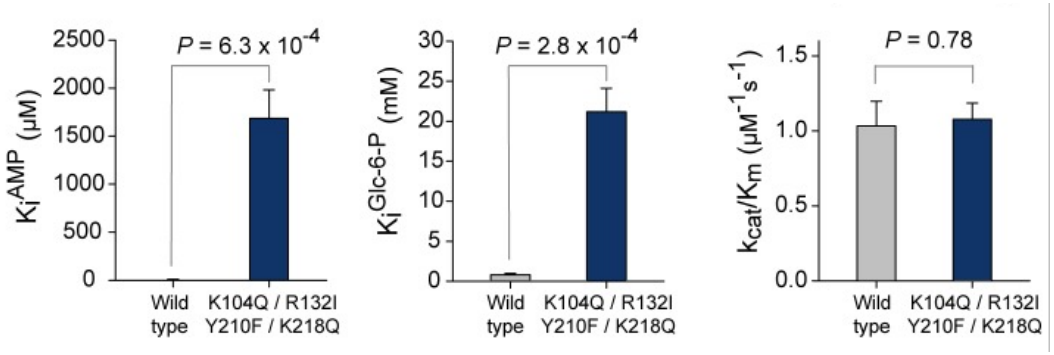
- Mutations in allosteric site of *E. coli* FBPase

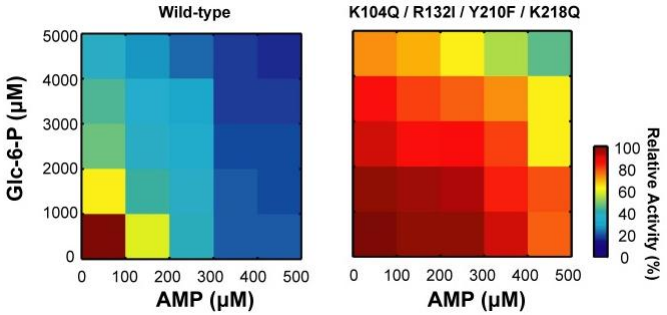
Mutations in allosteric site of *E. coli* FBPase abolish regulation

D-fructose 6-phosphate + phosphate <=> fructose-1,6-bisphosphate



AMP and Glc-6-P are allosteric inhibitors of the reaction





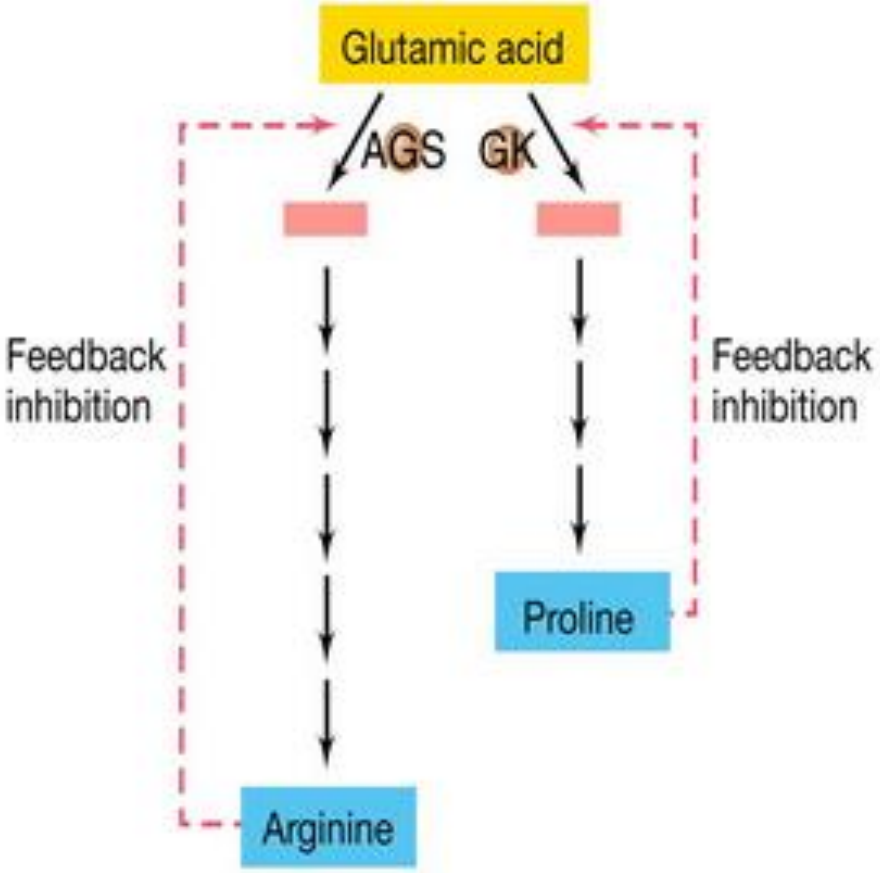
Enzyme performance not affected!!

Yang et al., 2012, PLoS Comput Biol. 2012 July; 8(7):

Feedback inhibition

- **Feedback inhibition** occurs when an end product synthesized after a chain of anabolic pathways becomes an inhibitor that binds at allosteric site of the first enzyme that made this end product
  - Thus the enzyme no longer can bind the substrate at its active site.
  - The metabolic pathway is then switch off and can no longer produce the end products

Feedback inhibition in amino acid biosynthesis



Feedback inhibition in a branched biosynthetic pathway.

A key intermediate in each pathway is shown in pink. The enzymes being inhibited are:

- N-acetyl glutamate synthase (AGS)
- γ-glutamyl kinase (GK)

- At the same time expression of the enzyme can be under feedback repression