Techniques in protein biochemistry and thermodynamics

BIOS 10016

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Objectives

- Know all definitions
- Describe the importance of assays and differentiate between direct and indirect methods
- Calculate specific activity, percent yield and purification level
- Describe how assays and PAGE can be used to assess protein purity.
- Describe the principles of PAGE and SDS-PAGE
- Describe the theory, principles, conditions required and the results of the different purification procedures discussed:
  - Size-exclusion chromatography
  - Ion exchange chromatography
  - Affinity purification
- **EXAM I ENDS HERE**
- Know all definitions
- Describe the first two laws of thermodynamics
- Assess if a reaction is endothermic, exothermic or isothermic
- Describe the standard state and biological standard state conditions
- Assess if and describe why a reaction is spontaneous or not
- Assess if the entropy of a system increases or decreases
- Differentiate between ΔG° and $\Delta G^{\circ\prime}$
- Calculate the equilibrium constant from ΔG° and vice versa
- Calculate ΔG from initial concentrations of substrates and products

Laboratory techniques

Protein purification

Requires:

- A source (natural or genetically engineered)
- A method to assess purity (activity assays, gel-based)

Direct activity assay

Test used to measure the enzymatic activity of a specific enzyme by directly detecting the change in a substrate or the formation of a product as the reaction occurs.

activity $\frac{\text{amount}}{\text{time}}$

standard enzyme activity, U $\frac{\mu}{\text{min}}$

specific activity $\frac{\text{enzyme activity (Units)}}{\text{mg of total protein}}$, a measure of purity (as mg of total protein decreases, activity increases, considering the amount of protein of interest remains the same)

Percent yield (how much activity is left after performing a certain step relative to original sample):

$$\text{Percent yield} = \frac{\text{remaining total activity}}{\text{initial total activity}} \times 100\% \tag{1}$$

Purification level (how much purer the sample is after a certain step relative to original sample):

$$\text{Purification level} = \frac{\text{specific activity}}{\text{initial specific activity}} \times 100\% \tag{2}$$

***P*oly*A*crylamide *G*el *E*lectrophoresis (PAGE)**

- Matrix is **polyacrylamide** that has holes and pores, changing how quickly the material can pass through in response to electric field. Bigger molecules move much slower
- Separates proteins based on **charge and size**
- **Cathode** (negative) attracts **positive** ions
- **Anode** (positive) attracts **negative** ions
- Proteins migrate towards electrode of opposite charge

*S*odium *D*odecyl *S*ulfate-PAGE (SDS-PAGE)

- SDS (negatively charged detergent) binds tightly to proteins (1.4 g SSDS/1 g protein)
- All proteins become very negatively charged and separation depends only on size
- 3° and 4° structure disrupted (denatured)
- Often reducing conditions used:
  - DTT
  - β-Mercaptoethanol (β-ME)
- $\implies$ disulfide bonds broken
- Protein mobility $\propto \dfrac{1}{\log(\text{molecular weight})}$
- Smears indicate impurity; isolated proteins indicate purity

Chromatography

Gel filtration/Size exclusion column chromatography

- Separated based on size
- Presence of a gel matrix with holes and gel beads attached
- Proteins elute (come out) in order of decreasing molecular weight, shown by a peak in the graph
- Larger molecules elute first (excluded from pores) because they interact less with the gel beads
- Smaller molecules elute later (enter pores) because they interact more with the gel beads
- Time of elution $\propto \dfrac{1}{\log(\text{molecular weight})}$

Ion exchange chromatography

Anion exchange chromatography The **resin** is **positively charged**, the **protein** to be isolated is **negatively charged**.

Cation exchange chromatography The **resin** is **negatively charged**, the **protein** to be isolated is **positively charged**.

- pH > pI $\implies$ negative proteins
- pH < pI $\implies$ positive proteins
- More charges $\rightarrow$ higher affinity

- Once charged molecules are stuck to the resin, elution is done by changing pH or salt concentration in the buffer
- Four steps:
  - Equilibration
  - Sample application and wash
  - Elution
  - Regeneration

Affinity purification

A **ligand** is used to isolate the protein (can be a substrate, inhibitor, or antibody that specifically binds to the target protein)

Hexahistidine tag: HHHHH - N or C terminus

binds Ni_2^+ or Co_2^+

1. Column has Ni_2^+ and Co_2^+ attached. pH should be around 8.0 so that histidine is not positively charged.
2. Wash the protein-bound column to get rid of undesired proteins
3. Elute the protein by lowering the pH (protonating the histidine) or adding imidazole to disrupt electrostatic interaction
4. Imidazole binds, protein comes off

End of Exam I

Thermodynamics

Key terms

energy is the capacity to do work or to transfer heat

work is organized motion that results in a specific physical change through the displacement or movement of an object

heat is energy transferred as the result of a temperature difference

enthalpy (H) total heat content of the system or surroundings

entropy a measure of the number of microstates in a system or the surroundings

Δ (delta) means "change in"

Gibbs free energy (G) a measure of spontaneity; $\Delta G = \Delta H - T\Delta S$

The laws of thermodynamics

First law

Energy cannot be created nor destroyed, but it can be changed from one form to another

$$\Delta H_{\text{system}} = -\Delta H_{\text{surroundings}}$$

Enthalpy (H) is related to internal energy of the system (U). In biological systems, $\Delta H = \Delta U$

exothermic system releases heat to the surroundings $\Delta H_{\text{system}} < 0$

endothermic system absorbs heat from the surroundings $\Delta H_{\text{system}} > 0$

isothermic no exchange of heat with the surroundings $\Delta H_{\text{system}} = 0$

Second law

All spontaneous processes result in an overall increase of entropy in the universe

$$\Delta S_{\text{system}} + \Delta S_{\text{surroundings}} > 0$$

Spontaneous reactions occur automatically if left alone (no intervention required) and are energetically favourable

$$\mathrm{C_6H_{12}O_{6(s)}} + 6\,\mathrm{O_{2(g)}} \rightarrow 6\,\mathrm{CO_{2(g)}} + 6\,\mathrm{H_2O_{(g)}} \quad \Delta H = -2800\mathrm{kJ/mol}$$

Entropy is a measure of **randomness**, and every spontaneous process results in a positive entropy change in the universe. How does this reaction result in increased entropy?

1. Number of molecules increases (from 7 to 12)
2. Heat released
3. Solid to gas

How living systems counteract entropy

- Living systems are very ordered!
- To live we must increase the disorder of the surroundings
- Ingest complex molecules and expel small molecules and heat
- Organisms with $\Delta S_{\text{univ}} = 0$ are dead
- THE SUN MAINTAINS ORDER! pop off queen

Describing spontaneous processes

Gibbs free energy

The entropic and enthalpic components of a reaction can be combined into a general description of spontaneity.

$$\Delta G_{\text{system}} = \Delta H - T\Delta S \tag{3}$$

conceptually:

free energy of the system = entropy of surroundings − temperature × entropy of the system

exergonic spontaneous, negative ΔG

endergonic not spontaneous, positive ΔG

equilibrium at $\Delta G = 0$

Standard free energy change (ΔG°) tells us the direction of a reaction at **standard state**:

- 1M initial concentration
- pressure = 1 atm
- temperature can vary, generally 0°C or 25°C

In biological systems, we make an exception for $[H^+]$

H^+ = 1M, meaning the pH is 0

- Under biological standard conditions, pH = 7, $[H^+] = 1 \times 10^7$
- ΔG°' = **biological standard free energy change**

Calculating the ΔG away from equilibrium

$$\mathrm{aA} + \mathrm{bB} \rightleftharpoons \mathrm{cC} + \mathrm{dD}$$

If $\Delta G < 0$, the forward reaction is spontaneous.
If $\Delta G = 0$, the reaction is at equilibrium.
If $\Delta G < 0$, the reverse reaction is spontaneous.

Spontaneity depends on the reactant and product concentrations. You can change the temperature and concentration to favor certain reactions (forward and back reaction).

When doing problems with temperature, always convert into Kelvin! (°C + 273)

Enzymes

Combustion of glucose

$$\mathrm{C_6H_{12}O_{6(s)}} + 6\,\mathrm{O_{2(g)}} \rightarrow 6\,\mathrm{CO_{2(g)}} + 6\,\mathrm{H_2O_{(g)}}$$

Activation barrier must first be overcome before a reaction can happen (sugar will not be combusting on its own)

Enzymes...

- are catalyst proteins (speed up a reaction)
- end in -ase
- catalyze 1000s of specific reactions in the human body
- will always return to original conformation
- accelerate reactions by lowering activation energy
- are low in concentration relative to the substrates
- bind substrates with high specificity
- are highly regulated by inhibitors or modifications
- do not change the $\mathrm{K_{eq}}$ and $\Delta\mathrm{G}^\circ$ for the reaction; only affects the barrier
- function depends on protein structure