Biological Chemistry Laboratory
Biology 3515/Chemistry 3515
Spring 2017

Lecture 22:

Quiz 2 Answers, Discontinuous Stacking Gels and Analysis of the RNAseA Digestion Experiment

4 April 2017

©David P. Goldenberg
University of Utah
goldenberg@biology.utah.edu

From Quiz 2, Problem 1a

Data from an enzyme kinetics experiment:

[S] (μM)	Δ A/min
0	0.0
50	0.020
100	0.028
250	0.036
500	0.040
750	0.042
1000	0.043

Estimate K_m and V_{max}.

- K_m is equal to [S] when $V = V_{max}/2$
  K_m **is not equal to** $V_{max}/2$!
- V_{max} corresponds to $\Delta A/\min \approx 0.045$.
- $K_m \approx 60\,\mu M$
- For V_{max}, $\Delta A/\min$ has to be converted to μM/min by dividing by the extinction coefficient and cuvette path length.

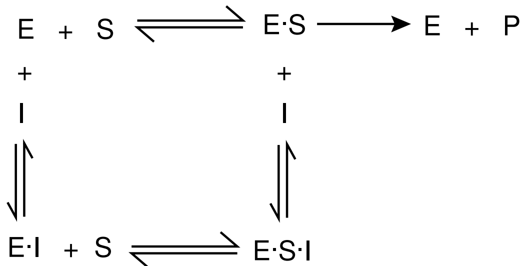
From Quiz 2, Problem 2a

- β-lactamases hydrolyze penicillin and other β-lactam antibiotics and can make bacteria resistant to these antibiotics.
- Want an inhibitor of β-lactamase to make bacteria susceptible to antibiotics again.
- Want inhibitor to be effective at both high and low concentrations of β-lactamase substrate.
- Should inhibitor bind to free enzyme, enzyme-substrate complex or both?

From Quiz 2, Problem 2a

Noncompetitive Inhibition:

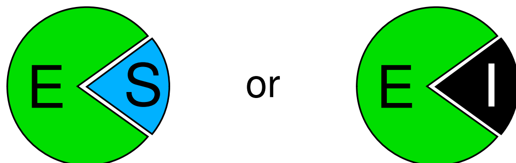
- Inhibitor binds equally to free enzyme and enzyme-substrate complex



- Inhibitor effectively lowers concentration of both E and E·S
- Reduces apparent V_{max} and lowers velocity at all substrate concentrations.s

From Quiz 2, Problem 2b

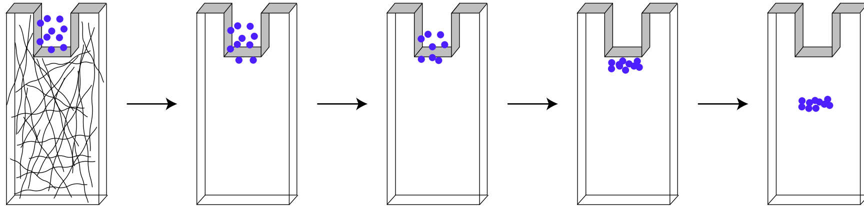
- The chemists have made a compound designed to mimic the substrate, but does not undergo catalytic reaction.
- What type of inhibitor is this most likely to be?
- Competitive inhibition:



- Only effective at lower substrate concentrations.

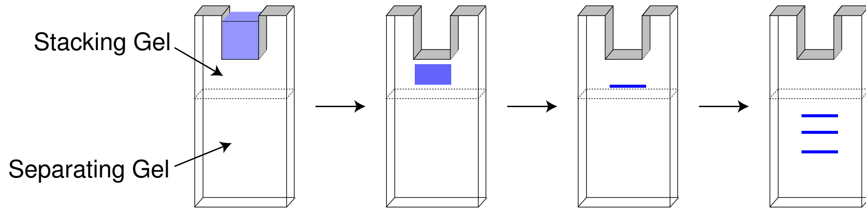
Resolution and Sensitivity Depend on Narrow Protein Bands

- Natural “stacking” of protein molecules as they enter a gel:



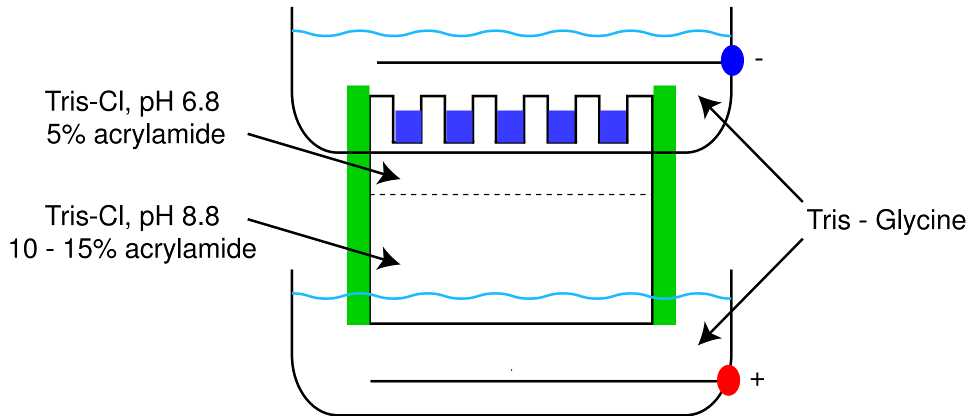
- Final protein bands are often much narrower than the original sample applied in the well.

Stacking and Separation in a Discontinuous Gel

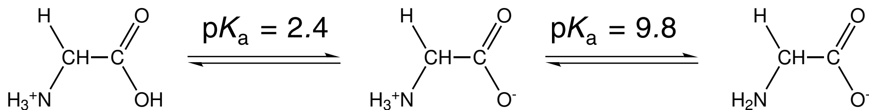


How does it work?

Buffer Compositions Control Stacking and Separation

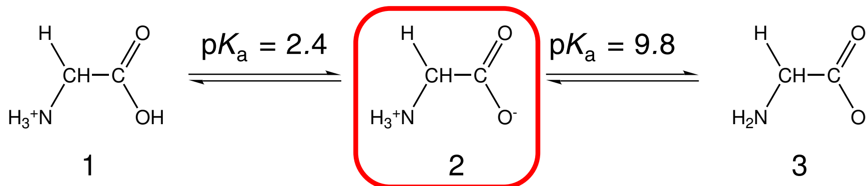


Glycine Ionization Equilibria



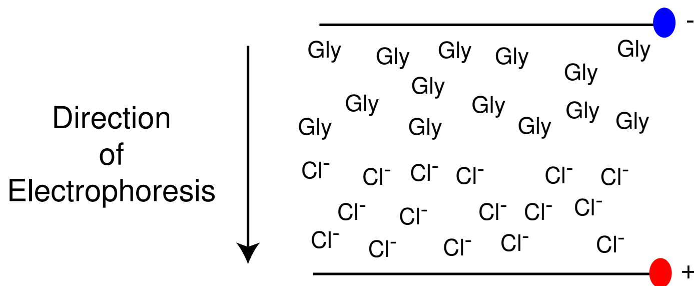
Clicker Question #1

Which form of glycine will predominate in the stacking gel (pH 6.8)?



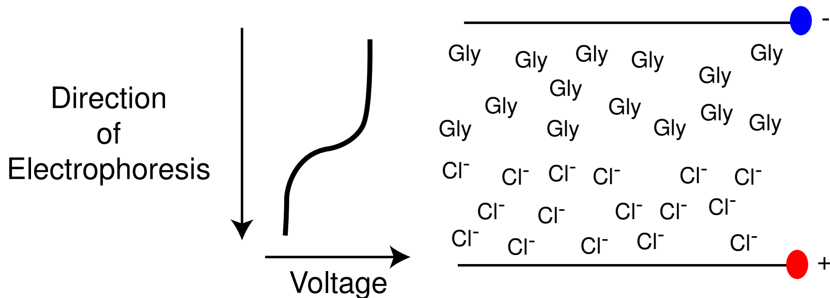
- ≈ 0.1% of glycine is negatively charged.
- Average electrophoretic mobility is very low.

Formation of an Ion Front



- Cl^- has much higher mobility than Gly:
  Electrical resistance (R) is lower in the Cl^- region.
- The two regions are in series electrically:
  Electric current (I) must be the same in both.
- Ohm's law: $V = I \cdot R$:
  The voltage is higher in the Gly region.

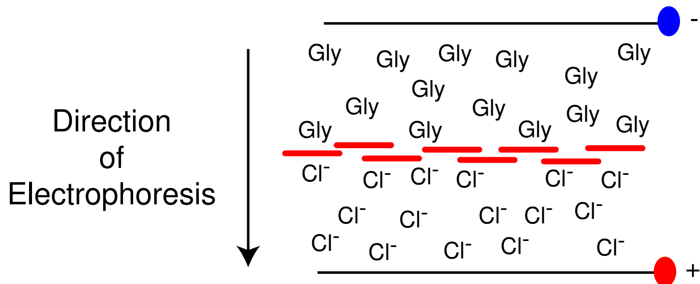
Voltage Gradient Sharpens the Ion Boundary



- If a Gly molecule diffuses ahead into Cl^- region, it experiences a lower voltage and slows down.
- If a Cl^- ion diffuses back into the Gly region, it experiences a higher voltage and speeds up until it reaches the boundary.
- Ion boundary and voltage gradient become progressively sharper.

What Happens to the Protein?

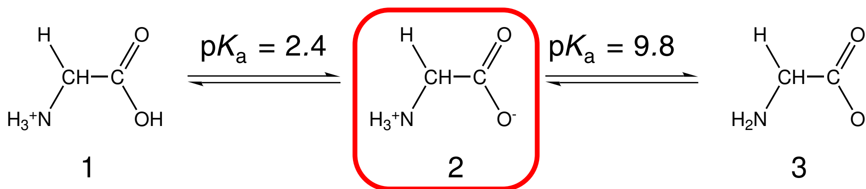
- SDS-protein complexes have mobilities between those of Gly and Cl^-
- Proteins are trapped between fast Cl^- ions and slow glycine



- Proteins form a very tight band.

Clicker Question #2

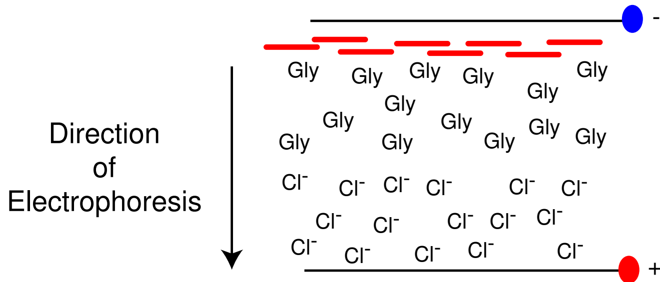
Which form of glycine will predominate in the separating gel (pH 8.8)?



- ≈ 10% of glycine is negatively charged.
- Average glycine electrophoretic mobility much higher than at pH 6.8.

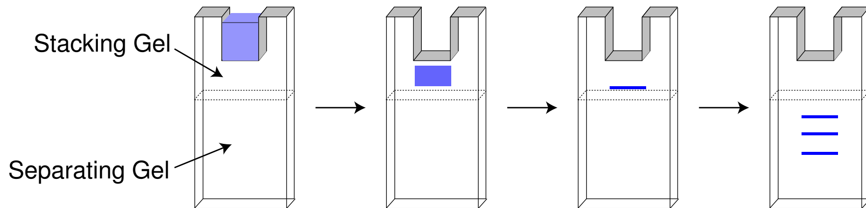
In Separating Gel:

- Gly mobility increases, becomes greater than SDS-protein mobility, but still slower than Cl^-.

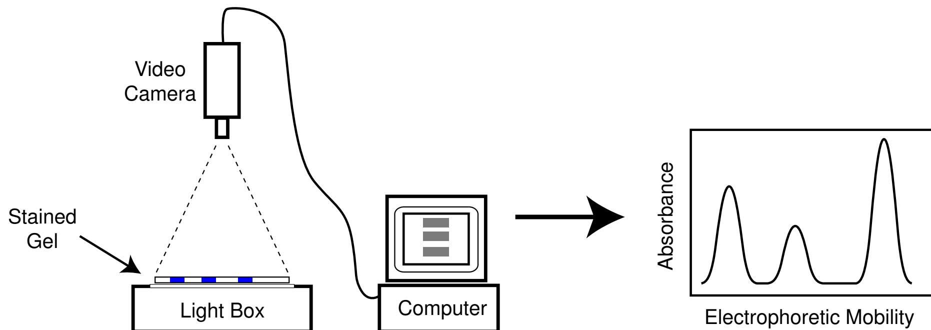


- SDS-protein complexes are no longer trapped in a sharp voltage gradient.
- SDS-protein complexes are separated on basis of molecular weight.

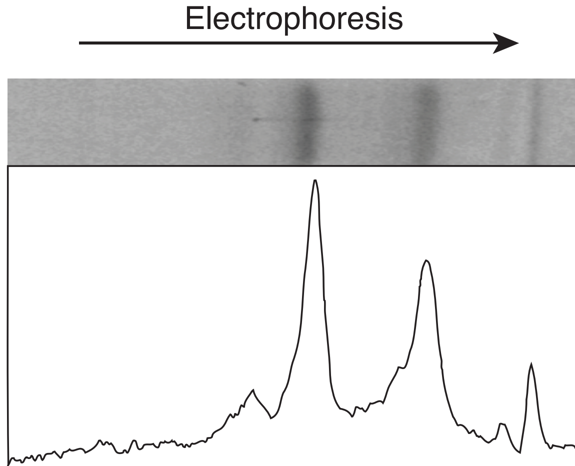
Stacking and Separation in a Discontinuous Gel



Video Densitometry of Gels

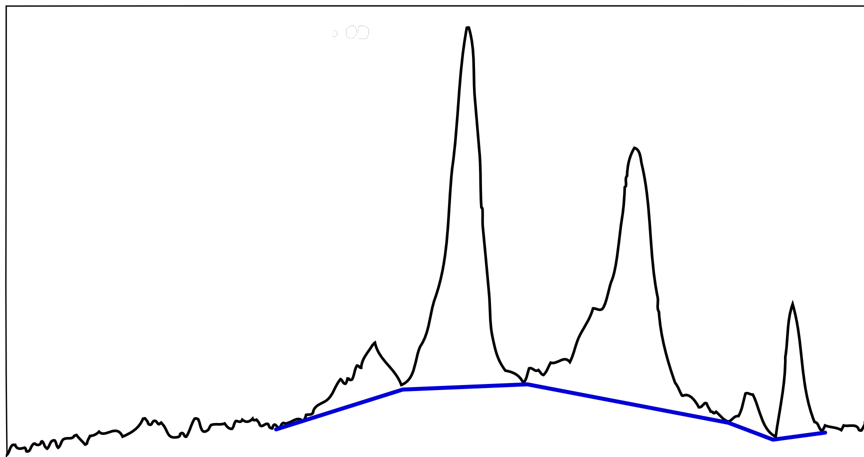


Scans of SDS Gel Lanes



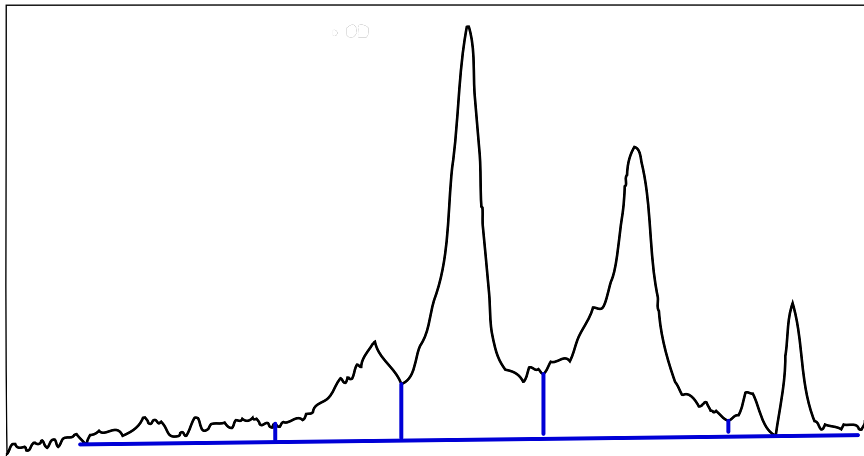
- Integrals of peaks are proportional to protein concentrations.
- Have to define limits of individual peaks and baseline.

One Way to Draw Baselines



- Implies that there is a broad distribution of stain below the peaks that is unrelated to protein concentration.

Another (Correct) Way to Draw Baselines



Kinetic Analysis

- Michaelis-Menten Equation: $V = \dfrac{[\mathrm{E}]k_{\mathrm{cat}}[\mathrm{S}]}{K_{\mathrm{m}} + [\mathrm{S}]}$

- If $[\mathrm{S}] \ll K_{\mathrm{m}}$:
  (We will assume this as a simplification, without much evidence.)

  $$V = \frac{[\mathrm{E}]k_{\mathrm{cat}}[\mathrm{S}]}{K_{\mathrm{m}}}$$

- A first-order rate expression: $\dfrac{d[\mathrm{S}]}{dt} = -V = -k_{app}[\mathrm{S}]$

  where:

  $$k_{app} = \frac{[\mathrm{E}]k_{\mathrm{cat}}}{K_{\mathrm{m}}}$$

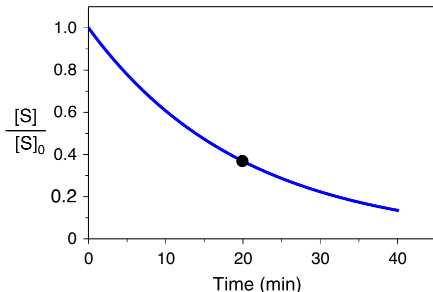
The Time Course for Digestion

- First-order rate expression: $\frac{d[\mathrm{S}]}{dt} = -k_{app}[\mathrm{S}]$

- After integration:

$$\frac{[\mathrm{S}]}{[\mathrm{S}]_0} = e^{-k_{app} \cdot t}$$

$[\mathrm{S}]_0 =$ initial substrate concentration



- Take logarithms and solve for k_{app}

$$k_{app} = -\frac{\ln\left([\mathrm{S}]/[\mathrm{S}]_0\right)}{t}$$

Estimating $k_{\mathrm{cat}}/K_{\mathrm{m}}$ from a Single Time Point

- From integrated rate expression:

$$\ln\left([\mathrm{S}]/[\mathrm{S}]_0\right) = -k_{app} \cdot t$$

$$k_{app} = -\frac{\ln\left([\mathrm{S}]/[\mathrm{S}]_0\right)}{t}$$

- Calculate $k_{\mathrm{cat}}/K_{\mathrm{m}}$ from k_{app} and [E]:

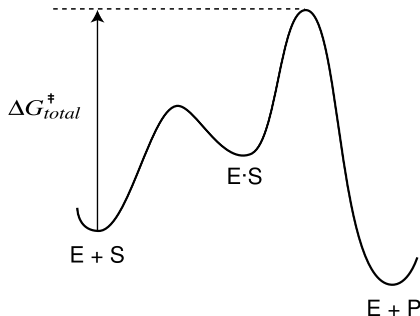
$$k_{app} = \frac{[\mathrm{E}]k_{\mathrm{cat}}}{K_{\mathrm{m}}}$$

$$k_{cat}/K_m = k_{app}/[\mathrm{E}]$$

The Significance of k_{cat}/K_m

- If $K_d \approx K_m$:

$$\Delta G^{\ddagger}_{total} = C - RT \ln\left(\frac{k_{cat}}{K_m}\right)$$



- Catalytic efficiency is favored by a large value of k_{cat} and a small value of K_m.
- How does k_{cat}/K_m compare for a protein and a synthetic substrate?

Methods for Detecting Proteins in Gels

- To detect all proteins:
  - Staining with coomassie blue:
    Dye binds to proteins and excess is washed away
    Sensitivity: - $\approx$ 0.1–1 μg/band
  - “Silver staining”:
    Based on reduction of silver ions to metallic silver in vicinity of protein.
    Much more sensitive than coomassie blue; as little as 10 ng/band
    Messy, finicky and sensitive to artifacts.
  - Staining with fluorescent dyes
    Dye fluorescence is enhanced when bound to proteins
    Much more sensitive than coomassie blue
  - Other dyes and metal ions.
- To detect specific protein classes:
  - Specific dyes for phosphoproteins
  - Specific dyes for glycoproteins

Steinberg, T. H. (2009). Protein gel staining methods: An introduction and overview. *Methods Enzymol.*, 463, 541–563. http://dx.doi.org/10.1016/S0076-6879(09)63031-7