Biological Chemistry Laboratory
Biology 3515/Chemistry 3515
Spring 2023

Lecture 19

Electrophoresis and Thiol-disulfide Chemistry

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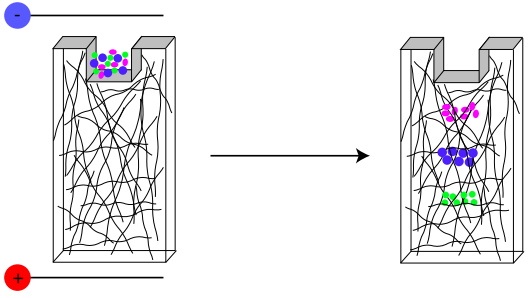
Electrophoresis Through a Gel



- Rate of migration through the gel depends on:
  - Strength of the electric field.
  - Net charge of the protein.
  - Size and shape of the protein.
  - Density of the gel matrix
- Proteins with different mobilities migrate as “bands” in the gel.

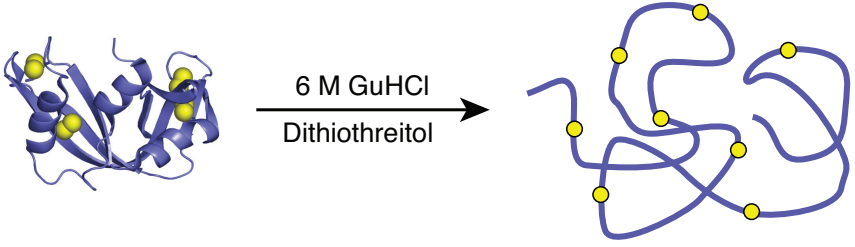
Two Major Variants of Gel Electrophoresis for Proteins

1. Non-denaturing (“native”) electrophoresis.
   - Carried out in the absence of denaturants, though sometimes relatively low or high pH values are used.
   - Protein migrates through the gel on the basis of its intrinsic net charge, size and shape, and the sieving effect of the gel.
2. SDS gel electrophoresis
   - Proteins are denatured in the presence of sodium dodecyl sulfate (SDS), a detergent that disrupts protein structures and complexes.
   - Mobilities reflect molecular weights of polypeptide chains.
   - Very useful for analyzing complex samples and macromolecular complexes composed of multiple polypeptides (*e.g.,* viruses, organelles, membranes).
   - By far the most commonly used form of protein electrophoresis.

Outline of Experiment 5

- Day 1:
  1. Preparation of modified RNase A
- Day 2:
  1. Non-denaturing gel electrophoresis of native and modified RNase A
  2. Trypsin treatment of RNase A forms
- Day 3:
  1. SDS gel electrophoresis of trypsin-treated RNase A samples
  2. Image capture of non-denaturing gel
- Day 3+1 (first day of experiment 6):
  1. Image capture and quantitation of SDS gel

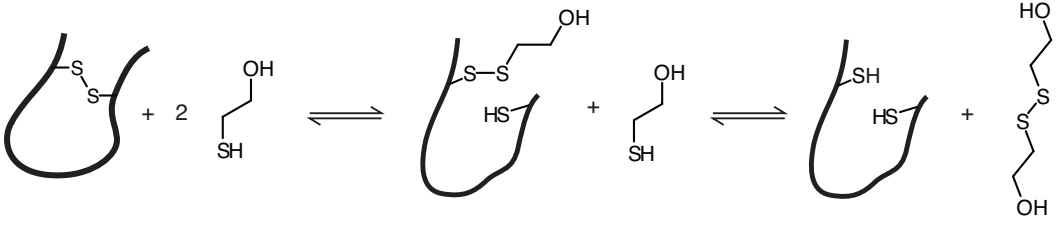
Unfolding RNAse A by Reducing its Disulfides



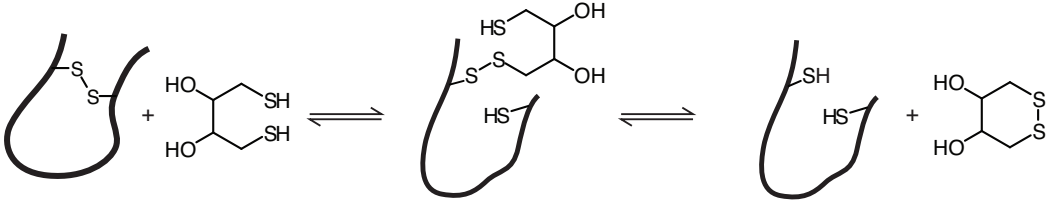
- Dithiothreitol reduces protein disulfides.
- GuHCl accelerates the reaction by unfolding the native conformation and exposing the disulfides.
- Without disulfides, the folded protein conformation is unstable.
- Unfolded protein is a broad ensemble of rapidly interconverting conformations.

Reduction of Protein Disulfides by Thiol-Disulfide Exchange

- By 2-mercaptoethanol (β-mercaptoethanol, BME)

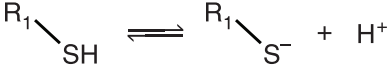


- With dithiothreitol (DTT, Cleland's reagent)



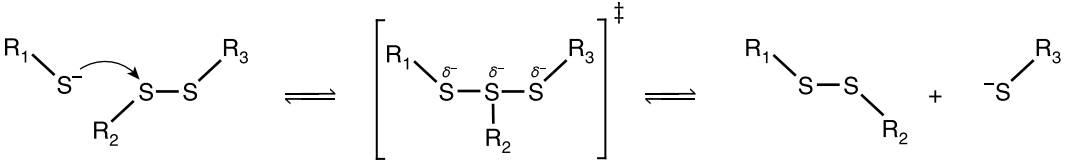
Thiol-disulfide Exchange Chemistry

- Reactive species is the ionized thiol group, a thiolate:



  - Un-ionized thiol is not very reactive.
  - Ionized Cys is the most reactive of all amino-acid side chains.

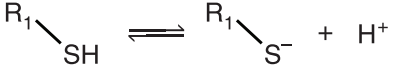
- Exchange reaction:



  - Reaction is an SN2 nucleophilic substitution.
  - For the ionized thiolate, the second-order rate constant is about $20\,s^{-1}M^{-1}$

Clicker Question #1

What is the pK_a of a thiol?



A) 5–6

B) 6–7

C) 7–8

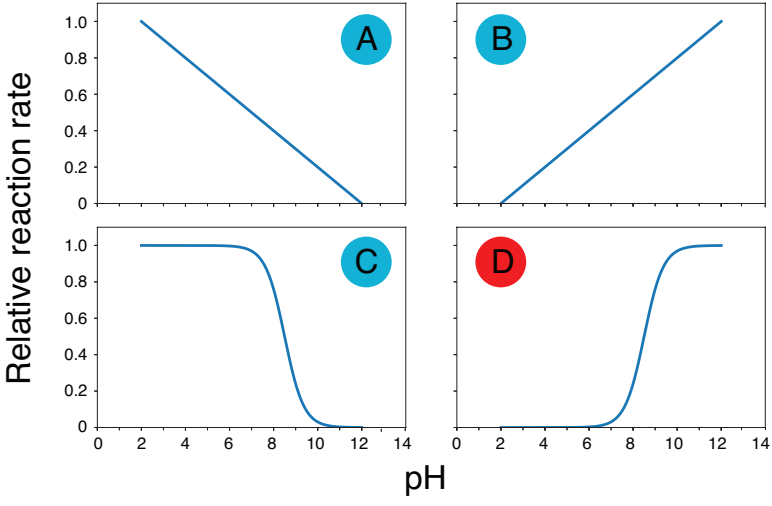
D) 8–9

E) 9–10

All answers count for now.

Clicker Question #2

How does the reaction rate change with pH?



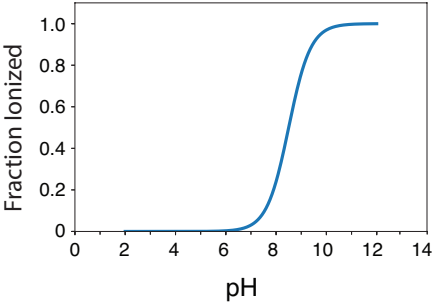
All answers count for now.

Why Does the Reaction Rate vs. pH Curve Have the Shape it Does?

- The reaction rate is proportional to the fraction of molecules in which the thiol is ionized.

$$f_{\text{ion}} = \frac{[\text{R}{-}\text{S}^-]}{[\text{R}{-}\text{S}^-] + [\text{R}{-}\text{SH}]}$$

- The fraction ionized increases as the pH increases.

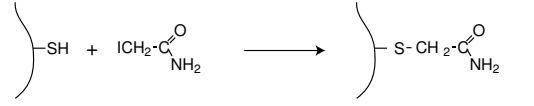


Reformation of Disulfides Can be Prevented by Alkylating the Cys Thiols

- Reaction with iodoacetic acid

Protein thiol | Iodoacetic acid | Carboxymethylated protein thiol

- Reaction with iodoacetamide



Protein thiol | Iodoacetamide | Carboxyamidomethylated protein thiol

- Reactions are essentially irreversible.
- Thiolate is the reactive species, and the rate increases with pH.

Three Forms of RNase A for Electrophoresis Experiment

1. Native RNAse A (N). Compact, net positive charge.
2. Reduced and carboxyamidomethylated (RCAM). Less compact than native, same net charge as native.
3. Reduced and carboxymethylated (RCM). Less compact than native, decreased positive charge.

How will they behave upon electrophoresis?

Clicker Question #3

Which form of RNAse A will migrate most rapidly through a non-denaturing gel?

A) Native RNAse A (N). Compact, net positive charge.

B) Reduced and carboxyamidomethylated (RCAM). Less compact than native, same net charge as native.

C) Reduced and carboxymethylated (RCM). Less compact than native, decreased positive charge.

All answers count for now.

Modification of RNase A: Reduction Reaction

	Amount	Final conc.
GuHCl	1.14 g	6 M
1 M Tris-HCl, pH 8	0.2 mL	0.1 M
0.1 M EDTA	0.2 mL	0.01 M
0.1 M DTT	0.2 mL	0.01 M
4 mg/mL RNAse A	0.5 mL	1 mg/mL

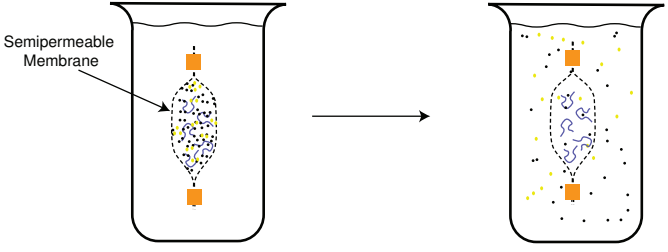
- Dissolve GuHCl in Tris, EDTA and DTT solutions.
- Add RNAse A and mix well.
- Incubate reduction solution for 30 min at room temperature.
- Divide reaction into two tubes.

Modification of RNase A: Alkylation Reactions

- After 30 min incubation of reduction reactions:
  - Add 150 μL of 0.3 M iodoacetamide solution to the “RCAM” tube.
  - Add 150 μL of 0.3 M Na-iodoacetate solution to the “RCM” tube.
- Mix well and incubate for 30 min at room temperature.
- Add 100 μL of 1 M HCl.

Modification of RNase A: Dialysis

- Before trying to electrophorese the samples, we have to get rid of all of the excess reagents, which will interfere with electrophoresis (especially the GuHCl)
- Dialysis is a simple method for separating very large molecules (*e.g.,* proteins and nucleic acids) from small ones.



- The tricky part: Keeping unfolded proteins soluble.

Keeping the Unfolded Proteins Soluble

- The problem:
  - When globular proteins unfold, non-polar groups are exposed to water, generally reducing their solubilities.
  - GuHCl greatly increases the solubility of the unfolded proteins.
  - But, when GuHCl is dialyzed away, proteins become insoluble.
- The solution:
  - RCM and RCAM RNAse A have have much greater solubility at low pH ($\approx$ pH 2).
  - Use 0.01 M HCl as dialysate (solution outside of bag).
  - AND, protonate the tris before beginning dialysis by adding HCl.