

7.88 Lecture Notes - 9
7.24/7.88J/5.48J
The Protein Folding Problem

- **Fluorescence spectroscopy**
- **Denaturation and Denaturing agents**
- **Denatured State as a random coil (First Approx.)**
- **Renaturation/Refolding Protocols**
- **Detection of partially folded Intermediates**

So far we have examined the experimental evidence for three cases

- Ribonuclease S
- Heptad Repeat protein -Tropomyosin ; Coil <> helix transition in aqueous solution:
- S-peptide coil >helix transition in solution

However, as we noted earlier the 22,000 + proteins in the protein database are primarily soluble globular proteins; And probably more than half to 3/4 of the proteins encoded by genomes are in this class:

So need to understand how sequence drives fold, or to be A probably the majority of proteins encoded

Methods for the study of the conformation of folding intermediates

A. Fluorescence Spectroscopy

J. R. Lakowicz Ch 23 pp518-567

Many molecules that absorb light in the UV or visible spectra, also re-emit photons, at longer wavelength: Fluorescence

Phe Tyr and Tryp absorb in the region of:

Amino Acid	Abs max (nm)	Molar absorbance M⁻¹ cm⁻¹
Phe	257.4	197
Tyr	274.6	1420
Tryp	279.8	5600

Absorbance $A = \log_{10}(I_0/I)$:

I_0 is transmittance by reference solution, I is transmittance by sample.

Molar absorbance is $= A/bc$, where b is path length in centimeters, and c is concentration in moles/liter

280mu. Phe peak is about 255 but 1/20 that of tryptophan peak at 280.

Absorption in this region of the spectrum is due to changes in electronic energy levels, levels of orbital electrons.

Photon absorption occurs in 10^{-15} seconds, so short that nucleus doesn't change much in time interval; so goes up in energy level almost instantaneously, but lifetime is much longer, 10^{-8} seconds.

Return to ground state usually multi-step:

- small losses of energy in atomic collisions \gg Goes back to lowest vibrational energy level state of excited electronic state (faster than emission);

If molecule doesn't blow up, then after short period of time,

- returns to lowest energy level emitting a photon; since excited state has lost a little energy, then photon emitted will be lower energy, longer wavelength than photon absorbed;

Lambda max for fluorescence:

Phe	282	0.04
Tyr	303	0.21
Tryp	340	0.20

Emission occurs on nanosecond time scale; Process occurs like radioactive decay $I = I_0 e^{(-t/g)}$, where t is time after end of light, g is average lifetime of fluorescent state; Because emission is a property of valence electrons, sensitive to environment;

Fluorescence; tryptophane: in water 350; buried in proteins 330.

Native fluorescent spectrum

Denatured fluorescent spectrum:

B. Denaturation and the Denatured State

These studies were carried on purified chains refolding in vitro; of course in cells chains released from ribosomes, but some 20 years passed before it was possible to study protein folding intermediates in vivo:

Now let's consider studying this problem for a complete polypeptide chain of a globular protein in the general case, for example globin with its 7 helices; to examine the folding pathway for the globin chain in the test tube, we would need unfolded molecules;

We also need to reach the unfolded or partially unfolded state to recover active forms of incorrectly or misfolded molecules; Eli Lilly manufactures human insulin by expressing the cloned gene in *E. coli*. When they harvest the cells the insulin chains accumulate as an insoluble mass called an "inclusion body". In order to obtain biologically active insulin the chains must be solubilized and then refolded in vitro.

It turns out that maintaining an unfolded state of biologically evolved polypeptide chains is not trivial

In order to investigate how chains reach their native state, need access to some defined unfolded –denatured state.

In order to recover correctly folded forms of incorrectly folded or misfolded proteins, also need denatured state

Practical;

To maintain proteins active, need to understand processes of denaturation.

To recover proteins from denatured states, need some knowledge thereof. IN fact general result of cloning gene into expression vector, is generation of non native aggregated state called an inclusion body:

Obtaining native protein requires successfully refolding in vitro;

- Requires access to soluble state
- Method to study basis of stability of native conformation.
- To study folding pathway, need defined unfolded starting state.

Denaturation; Process of the loss of nature structure: associated with unusual phase transition associated with structure loss: upon mild treatment:

The denatured state is usually identified by the

- Loss of biological functions
 - Loss of catalytic activity
 - Loss of ligand binding properties
 - Loss of antibody recognition: and
- Change in solution properties
 - Increased viscosity
 - Aggregates large enough to scatter light
 - Change in spectroscopic properties.

Under conditions in which Covalent modification not detectable - The term denatured refers to an operation: it's the description of a state in terms of having passed through operation.

Denaturing Agents:

Globular proteins can be denatured with a wide variety of agents:

- Heat
 - Denatured chains usually partially structured and aggregated (treatment with additional denaturing agents causes further changes).
 - Often not reversible on cooling outside very narrow set of conditions.
 - Covalent side reactions as temperature increases.

Tropomyosin is an exception!

- pH
 - Ionization of side chains breaking ion pairs, salt bridges
 - Denatured chains highly charged:
 - Increased charge density leads generalized electrostatic repulsion;
 - Unfolded chain is structured
- Organic solvents
 - Benzene
 - Phenol
 - Trichloroethylene
 - Acetone
 - Ethanol
 - Chloroform
 - Acetonitrile
 - Often neither native nor denatured state soluble in these solvents
 - Difficult to compare conformations between immiscible solvents
 - Some induce alpha-helicity, like TFE
- Detergents
 - sodium dodecyl sulfate
 - Enormously important for analysis; denatured state binds 1.4gm/gram of detergent - denatured state is highly structured:
 - Often is micellar form that is strongly denaturing; so detergent must be above cmc, critical micelle concentration;
 - Difficult to remove detergents rapidly and efficiently

- Certain inorganic salts
 - Lithium bromide
 - Sodium bromide
 - potassium thiocyanate (KSCN)
 - However, not general ionic strength, NaCl, KCl don't do it.
 - Ammonium sulfate is stabilizing
- High Pressure
 - Useful for multimeric proteins; dissociation reaction
- Organic solutes
 - Urea
 $\text{NH}_2 - \text{C}=\text{O} - \text{NH}_2$
 - Not an electrolyte: effects sensitive to pH and ionic strength
 - Guanidinium Hydrochloride
 $\text{NH}_2 - \text{C} = (\text{NH}_2 + \text{Cl}^-) - \text{NH}_2$
 - Strong electrolyte. So denaturation always coupled with high ionic strength

Experimental work on protein unfolding as a pre-condition for establishing an unfolding refolding equilibrium is dominated by two reagents;

C. What is the State of these Denatured Molecules?

In both of these solvents -urea and GuHCl - a wide variety of proteins are denatured to species that approximate very closely to random coils. For example, for a true random coil, whose end to end to distance or radius of gyration is described by:

Sensitive measure of molecular volume is viscosity; unfolded chains occupy larger volume than folded, and so interfere with fluid flow, increasing viscosity; Viscosity is a measure of work done to produce a given rate of liquid flow; represents resistance to liquid flow;

Add solute, viscosity generally increases.

When protein denatures, interaction –binding- surface with solvent increases sharply, >> viscosity increases;

Intrinsic viscosities of native and GuHCl (5.0 -7.5M) denatured reduced chains(b-MeTh)
 –25

Protein	MW	Native	Denatured/Reduced
RNase	13,700	3.3	16.6 9.4
Lysozyme	14,300	2.7	17.1 6.5
Hemoglobin	64,500	3.6	18.9
beta lactoglobulin	36,800	3.4	22.8 19.8
tropomyosin	76,000	45	33

For a truly random coil, intrinsic viscosity is directly related to molecular weight through an equation of the general form

[Have to extrapolate to zero shear to make sure molecules aren't orienting in solution.]

$$\eta_{sp}/c = An^b$$

Where M_0 is the mean molecular weight of the amino acid residues;
 n = number of residues

Finding of a straight line relating log of η_{sp}/c to log of An^b Determination of viscosity versus molecular weight after denaturation is good evidence for a random coil conformation in the resulting chains

C. Tanford, K. Kawahara, and S. Laoponje (1967)
 Proteins as Random Coils. I. Intrinsic viscosities and sedimentation coefficients in concentrated guanidine hydrochloride JACS 89 729.

For GuHCl Tanford found $A = 77.3$, $b = 0.666$

If plot logarithmically log $\{\eta_{sp}/c\} M$ versus log n get linear relationship

Here is data from Charles Tanford on the linear variation of intrinsic viscosity as a function of chain length for polypeptide chains in high concentrations (5 - 7.5 M GuHCl) at 25°C.

$$\log[\eta_{sp}/c]M^0$$

$$\log n$$

A similar result is obtained with the same proteins in 8M urea, with very small differences in parameters, $A = 76$, $b = 0.655$.

One care need to take with urea, at neutral and alkaline pH decomposes slowly to form cyanate ion. This reacts with free amino groups to form carbamyl derivatives.

D. An Equilibrium Denaturation Experiment

Native protein in buffer: dilute to increasing concentrations of urea: examine properties; for example enzyme activity and fluorescence quenching of tryptophanes

Incubate until no further changes:

Cautions: urea decomposes to reactive cyanates; watch out for metals contamination: need to re-crystallize ultra-pure stuff;

In folded state, prolyl peptide bonds stabilized by folded structure; in unfolded state, isomerization proceeds in solution; very slow; at equilibrium cis trans prolyl peptide bonds different than in first few minutes after unfolding:

The general nature of the denaturation process, somewhat independent of method is that it is usually highly cooperative, showing a steep change in the state with the variable. Looks like sloppy phase transition;

That is, the molecules are either native or denatured, with limited evidence of intermediates. Thus in the transition state have mixture of native and denatured. Like phase change.

The Mechanism of Urea and Guanidinium Denaturation:

Large literature on action of GdHCl on proteins, but still no mechanism. This is flip side of the very limited ability to assess what is holding the protein together - high concentrations necessary Organic solutes -

Strong electrolyte. So denaturation always coupled with high ionic strength for denaturation, for example 6M, these reagents are occupying roughly half the solvent volume. General notion is that they are acting changing the structure of water.

Two studies that give some notion of what is going on:

Hibbard and Tulinsky Biochemistry 17, 5460-5468. Equilibrated crystal of alpha-chymotrypsin with 2MGuHCl or with 3M urea and examined diffraction pattern.

Obviously this is below denaturation transition, but approaching it.

GDHCl disrupted primarily surface of molecules in crystal. Solvent becomes better solvent for surface chains.

Urea appeared to diffuse into molecule; perhaps solvates non-polar groups otherwise buried. non-polar M.

What about proteins lacking disulfide bonds:

E. Criteria for existence of partially folded intermediates

During equilibrium unfolding and refolding experiments:

Protein diluted into various concentrations of denaturant: incubated until no change in signal; (determine empirically)

Renaturation: Fully denatured protein diluted back to increasing concentrations of buffer; samples it until no further change in signal;

In reading for Thursday you will see that time this sample has incubated in denaturant is a critical variable in refolding kinetics”

1. Complex Equilibrium denaturation

Here is equilibrium denaturation/renaturation experiment:

If curve has a break in slope, normally interpreted as due to presence of a partially folded intermediate at intermediate denaturant concentrations: This is data for

In the middle of the transition at least three species populated to some extent, U, I, and N

2. Non coincidence of signals

Here is denaturation curve for a protein, in which experimenters following two different signals: fluorescence and circular dichroism;

Fluorescence groups being exposed to solvent at lower GdHCl concentration than overall backbone unfolding:

Suggests that partially folded species formed, with region around tryptophan disordered, but most of protein intact:

3. Occasionally see third class of curves

Here is one for RNase T from Pace et al RNase T: fluorescence increases, then decreases; Can have other kinds of curves

Now of the utmost importance to distinguish kinetic experiments - initiate reaction follow course, under conditions that back reaction not significant, and equilibrium experiment, in which ignore pathway, examine only distribution of end states:

In a kinetic experiment evidence for more than two states would come from presence of more than one kinetic phase:

- If only one transition, $U \gg N$, then expect single rate process:
- If in studying kinetics observe complex kinetics, for example:

- Initial fast rate followed by slow rate followed by much slower rate, evidence that more than one process going on, must be at least one other species present;

Let's review point about intermediates:

If only two states, then fluorescence either 2 (native) or 1 (quenched) (tryp 59)

Lets say monitor some other signal, CD alpha helicity: value is 2 or 5; Then both curves will of necessity have same shape; all values simply sum of two populations:

If partially folded intermediate, its properties could differ from N and U: Its tryp could be buried, as in N ($f=2$), but its helices only partially formed - say CD value three:

Then CD curve would differ from fluorescence curve:

Then as proceed through folding transition, these values follow distribution of partitioning;

Part C