High pressure SAXS studies

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EMBL
Outlook

• Thermodynamics of protein unfolding by pressure
• High pressure SAXS: Technique
• Pressure induced protein unfolding
• Pressure effects on the protein-protein interaction
• Pressure effects on other soft matter systems
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Why Pressure?

- Important thermodynamic quantity
- Only affects volumetric properties (Le Chatelier)
- "biomimetic": deep sea animals
- Effect on protein stability
- Technological relevance (food industry)
Which pressures induce protein unfolding?

- The (pressure-) unfolded state has a smaller volume than the folded state (~1% effect).
- **Pressure 1 – 7 kbar**: effect on **non-covalent** bonds: Changes of the **tertiary** structure.
- **Pressure > 10 kbar**: effect on **covalent** bonds: changes of the **primary** and **secondary** structures.
Hawley theory (S.A. Hawley 1971)

Description of unfolding for a two-state system (folded ↔ unfolded) in terms of the Gibbs free energy change $\Delta G(p, T)$:

\[
\Delta G(p, T) = \Delta G_0 - \Delta S_0 (T - T_0) - \Delta C_p \left[ T \left( \log \frac{T}{T_0} - 1 \right) + T_0 \right] + \Delta V_0 (p - p_0) + \frac{\Delta \kappa}{2} (p - p_0)^2 + \Delta \alpha (p - p_0)(T - T_0)
\]

(Expansion in $p, T$ up to second order)
Hawley theory

$\Delta G(p, T)$: elliptical shaped phase diagram

- Heat-denaturation
- Cold-denaturation
- Pressure-denaturation
- (Stress-denaturation)
Heat-denaturation

- Simultaneous change of total energy and volume
- Entropy driven
- Highly unfolded state (lacking nearly residual secondary order)
- Random coil – like state
- Aggregation-prone
Cold-denaturation / Stress-denaturation

Cold
• Energy driven
• Highly dependent on the water structure
• Not as unfolded as heat
• Often beyond the freezing point of water

Stress (negative pressure)
• Hardly studied (complicated experiments, computer studies)
• Methods: Berthelot tubes, ultrasound
Pressure-denaturation: Mechanisms

- pressure induced decrease of the protein volume only
- depends highly on the solvent (water)
- reversible

- Collapse of internatal cavities
- Electrostriction
- Hydrophobic interaction: Water-mediated interactions become favored
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Methods to study high pressure effects

High pressure environment (in situ)

• Fluorescence (Trp residues)
• NMR
• FT-IR spectroscopy (secondary structure)
• Circular dicroism (secondary structure)
• **SAXS** & SANS (tertiary structure)
• Optical microscopy (cell structures)
High pressure SAXS: HP sample cell

High pressure sample cells

- Pressure range: 1 bar – 4....7 kbar
- X-ray windows: two flat diamonds
- Pressurizing medium: water

High pressure SAXS

Experimental set-up

• 1) HP sample cell
• 2) Pressure sensor
• 3) (Manual/motorized) spindle pump

BW4, DORIS III (2010)

I22, Diamond (2017)
HP windows

- Diamond windows for sealing
  - High flux (synchrotron)
  - Higher photon energy due to X-ray absorption
  - Pseudo-Kossel lines

HP buffer

• Stand buffer samples:
  • \[ \text{HPO}_4^{2-} + \text{H}_2\text{O} \rightleftharpoons \text{PO}_4^{3-} + \text{H}_3\text{O}^+ \]
  ⇒ number of charges increases

• HP buffer
  • \[ \text{TrisH}^+ + \text{H}_2\text{O} \rightleftharpoons \text{Tris} + \text{H}_3\text{O}^+ \]
  ⇒ number of charges constant

Pressure favors charged state
 ⇒ change in pH for standard buffers
 ⇒ use special buffer
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Staphyloccocal nuclease (SNase)

- 149 amino acids, $M_w = 16.8$ kDa
- Globular protein
- No disulfide bonds -> destabilized
- Standard protein for high pressure studies
- Several mutants available
HP SAXS – SNase

High pressure effects

- Decrease of $I(0)$
  $\Rightarrow$ reduced contrast as water gets compressed

- Increase of radius of gyration
  $\Rightarrow$ unfolding

SNase – \( p \) vs \( T \)

- \( p \) - unfolding at \( p \sim 2 – 3 \) kbar
- Two-state folding
- Pressure-induced unfolded state is more compact than the heat induced one

SNase - $p$ - $T$ phase diagrams

Experimental phase diagram of WT Snase and destabilized mutant.

G. Panick et al., Biochem. 38, 4157 (1999).

Effect of crowding (self and Ficol crowding)

SNase kinetics – \( p \) – jump

Rapid pressure increase / release

\( \Rightarrow \) Unfolding / Folding reaction

\( \Rightarrow \) Activation volumina (hydration) of the transition state

- Two-state kinetics
- Positive activation volume

\( \Rightarrow \) dehydration, transition state close to native state

SNase - (de-)stabilization by cosolvents

Cosolvents can change the pressure – effect

- Kosmotropic: stabilizing
- Chaotropic: destabilizing

Urea destabilizes,
TMAO stabilizes

Roughly: Pressure + additional perturbations are additive

SNase mutants

Single AA exchange in hydrophobic core of a hyperstable SNase variant

- Change in protein stability
- Large + charged: destabilize
- Change of charged state: change of stability

Other HP SAXS studies

**T4 lysozyme mutants**
- Penetration of water into hydrophobic core

**Urate oxidase**
- $p$-induced dissociation of tetramers

**Ankyrin repeat domain**
- Two-state folding also for non-globular proteins

Other HP SAXS studies – recent

**Microtubuli**

- Dissociation of microtubuli

• **tRNA**: P-induced changes are small compared to temperature (C. Schuubb et al., 2015)

• **RNAse A**: $p$ and $T$ produce different denaturate states (T.M. Ryan et al., 2016)

• **Immunoglobulin G**: stable up to 5 kbar (N. König et al., 2017)

• **Calmodulin**: ligand-dependent stabilization against pressures (S. Cinar et al., 2018)

• **Alcohol dehydrogenase**: Dimer dissociation by pressure (K. Julius et al., 2018)

⇒ Recently more studies but still more to be explored: *terra incognita*

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Concentrated protein solutions

- Thermodynamics of protein solutions (temperature density phase diagram) fabrication of crystals
- “biomimetic”: cytosol \((c_p > 300 \text{ mg/ml})\)
- Investigating the protein-protein interaction

Temperature – density phase diagram


Scheme of a cell’s interior

Lysozyme

- 129 amino acids, \( M_w = 14.3 \text{ kDa} \)
- from hen egg white \( \Rightarrow \) cheap, high purity, large amounts
- breaks glycopeptide chains of bacterial cell walls
- present in various secrets such as saliva
- often used “model protein”

solutions used
- diluted: \( c_p = 10 \text{ mg/ml} \)
- concentrated: \( c_p = 100; 200 \text{ mg/ml} \)
- pressure insensitive buffer (bisTris 25 mM) at pH 7
Concentrated solutions

For concentrated solutions of proteins

\[ I(q) \sim N P(q) S(q) \]

Additional interparticle interference

\[ S(q) = 1 + 4\pi n \int_0^\infty (g(r) - 1) r^2 \frac{\sin(qr)}{qr} \, dr \]

\( g(r) \): radial pair distribution function

- Probability of finding a particle at a distance \( r \) from another one
- Depends on interparticle interactions

Information on

- Interparticle distance
- Interparticle interaction
SAXS – Structure factor $S(q)$

Properties:

- $S(0) = n k_B T \kappa_T$
- $S(q) = 1$ for large $q$

$\kappa_T$: compressibility

$n$: number density

$k_B$: Boltzmann constant

$T$: absolute temperature

Depends on:

- Type of interparticle interaction
- Strength of interaction

hard sphere for different particle numbers $\eta$
Modeling the interaction potential

- form factor of an ellipsoid of revolution

- DLVO potential

\[
V_{DLVO}(r) = \frac{e^2 Z_{eff}^2}{4\pi\varepsilon_0\varepsilon_r (1 + 0.5 \frac{\sigma}{\lambda_D})^2} \exp\left(-\frac{r - \sigma}{\lambda_D}\right) \frac{\exp\left(-\frac{r - \sigma}{d}\right)}{r} - J\sigma \frac{\exp\left(-\frac{r - \sigma}{d}\right)}{r}
\]

- use of Mean Spherical Approximation (MSA) to determine \(S(q)\)
- \(Z_{eff}\) and \(d\) kept constant; \(p\) dependence of \(\varepsilon_r\)
- free parameter for refinement is \(J\)

\(e\): elementary charge, \(\varepsilon_0\): vacuum permittivity, \(\sigma\): diameter, \(Z_{eff}\): effective charge, \(\varepsilon_r\): permittivity, \(\lambda_D\): Debye screening length, \(J\): attraction strength, \(d\): range of attraction

Concentrated solutions & the structure factor $S(q)$

Nonlinear shift of the correlation peak with increasing pressure

- $p < 2.0$ kbar: shift to higher $q$
- $p > 2.0$ kbar: shift to smaller $q$

Attraction strength $J$ & interaction potential $V(r)$

Minimum at $J = 2.0$ kbar ($c_p = 100$ mg/ml)

Pressure-induced change of repulsion barrier

$\sigma = 2.99$ nm, $d = 0.27$ nm, $Z_{eff} = 8$


Effect on protein concentration and salt added

\( S_{\text{eff}}(q) \) for 10 wt.% at \( T = 25^\circ C \) with 100 mM NaCl

Minimum present for different
- Protein concentrations
- Temperatures
- NaCl amount

Reentrant liquid-liquid phase separation

- 1 phase -> 2 phases
  (low & high concentrated)


Results of the SAXS measurements

Measurements on lysozyme in buffer solution

• nonlinear pressure dependence of the interaction potentials

• present for different temperatures, concentrations, and NaCl

• no unfolding within the pressure range studied
  (form factor, FTIR spectroscopy)

• no change of the proteins’ effective charge as pressure-insensitive buffer is used

➢ influence of water


Properties of water

- transport properties of water (diffusion coefficient, viscosity) have extrema at 2 kbar

⇒ collapse of the second hydration shell of water starts at 2 kbar
⇒ change of the water moderated interaction ("effective screening")

To study this effect in more detail the influence of osmolytes was investigated!

Osmolytes: Small biologically relevant molecules

Different small molecules affect proteins

• kosmotropes: stabilizing
• chaotropes: destabilizing

Biologically relevant are:

**Urea** \( ((\text{NH}_2)_2\text{CO}) \)
- degradation product in organisms
- destabilizes proteins

**TMAO** (Trimethylamine N-oxide, \( (\text{CH}_3)_3\text{NO} \))
- stabilizes proteins => counteracts urea
- TMAO concentration in fishes increase with sea depth

2:1 mixtures (urea:TMAO) are present in deep sea organisms

SAXS on lysozyme in osmolyte solutions

SAXS curves show **different pressure dependence** for the two cosolvents

- TMAO: strong effect
- urea: like in buffer

⇒ change of the interaction

Different pressure dependence

**TMAO**: strong influence
- minimum at 2.5 kbar
- steep decay
- weak nonlinear effect

**urea**: as in buffer solution

**urea/TMAO mixtures**: compensation
- 4:1 & 2:1 as buffer solution
- 1:1 TMAO dominates

Interpretation

• collapse of the 2. hydration shell in buffer and urea
• no collapse for TMAO in solution
• the effects are cancelled in case of mixture

urea
• fits perfectly into the water network

TMAO
• enhances the hydrogen bonding (“water structure maker”)
• 2. hydration shell is located more outwards ) contrary to pressure


TMAO's singular role

- Various co-solvents have been tested
- Only TMAO induces a higher attractivity and strong shift of the minimum to larger pressures

⇒ The role of TMAO on the protein interaction is singular

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High pressure lipids & membranes

Structural transitions in lipids and membranes by pressure

⇒ Complex pressure – temperature phase diagrams

Can be perfectly probed by high pressure SAXS

Mixtures of model: DMPC / DHPC (3.2 : 1) at 61.9 °C:
- Decrease of Bragg reflection
- Increase of broad peak
⇒ Lamellar to nematic transition

Pressure jump: time-resolved SAXS
⇒ Kinetics of the phase transition

Nanoparticles

- Gold NP coated with PEG-ligands
  - Stable, low dispersity, functionalization
  - Applications in medicine, catalysis, sensors, ...

- Diluted:
  - Only the form factor of the gold core
  - Radius: 6.15 nm, polydispersity: 7 %

- Concentrated:
  - Structure factor
  - Pressure induced changes

Nanoparticles

- Compression of the ligand shell
- Decrease of the second virial coefficient $b_2$

⇒ Collapse of the ligand shell
⇒ Transition from repulsive to attractive by pressure

Pressure-induced supercrystal formation

Addition of high salt concentrations

⇒ Formation of fcc lattice
⇒ is reversible
⇒ salt type and concentration change crystallization
⇒ lattice constant depends on ligand length, not on the salt

Summary

• Pressure induces structural transitions in biological and soft matter
• HP SAXS allows to probe these transitions
• Pressure effect on structure and interactions
• Alternative / different effects in comparison to e.g. $T$
• More studies are nowadays performed but still a lot to be explored
• Pressure effect on macromolecules still *terra incognita*
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