Protein Unfolding

Experimental Guidelines

Adrian Velazquez-Campoy

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 101004806

MOSBRI
Molecular-Scale Biophysics Research Infrastructure

▶ How to quantitate protein stability? T_m? $[D]_m$?

Stability ΔG Difference in Gibbs energy between the native state and the unfolded state

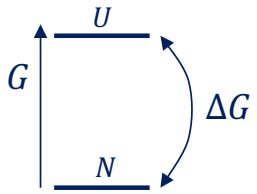
We might determine equilibrium constants by measuring equilibrium concentrations ($\Delta G = -RT \ln K_{eq} = -RT \ln[U]/[N]$)
But, populations of conformational states are not comparable
(e.g., what if ΔG = 5 kcal/mol)

We must "stress" the protein in order to populate other less stable conformational states…
Temperature or denaturant trigger evolution through a series of equilibrium states, and, later on, final extrapolation will provide the sought information

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 101004806

MOSBRI
Molecular-Scale Biophysics Research Infrastructure

Quantitate stability? ⟶ **Energy required to destabilize the molecular tridimensional structure**



Experimental techniques:

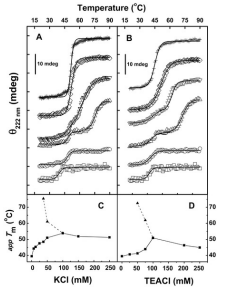
- **Spectroscopy (UV, F, CD, NMR, FTIR)**
- **Calorimetry (DSC)**

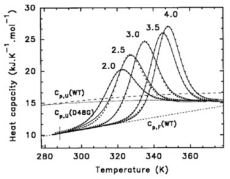
- **Property with different values for different states**
- **Signal proportional to the unfolding progress**
- **Local or global information**

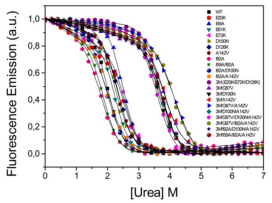
$$K, \Delta G$$

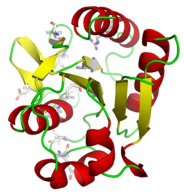
“extrinsic” variables
T, pH, μ, [s]

“intrinsic” variables
mutations









- **Information on inter- and intramolecular interactions involved in (un)folding**
- **Information on functional groups involved in (un)folding**
- **Information on physiological and pathological mechanisms**

This project has received funding from the European Union’s Horizon 2020 research and innovation programme under grant agreement No 101004806

MOSBRI
Molecular-Scale Biophysics Research Infrastructure

Setting experiment parameters

- Experimental technique?
- Solution composition
- Sample preparation
- Sample concentration
- Scanning rate

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 101004806

MOSBRI
Molecular-Scale Biophysics Research Infrastructure

Experimental conditions

- Physiological conditions?
- Thermodynamic stability
- Kinetic stability
- Aggregation/precipitation
- Physiological/technological relevance

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 101004806

MOSBRI
Molecular-Scale Biophysics Research Infrastructure

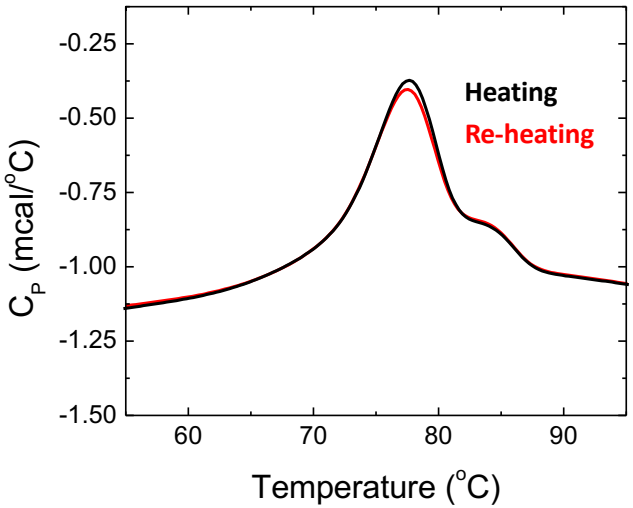
Control experiments

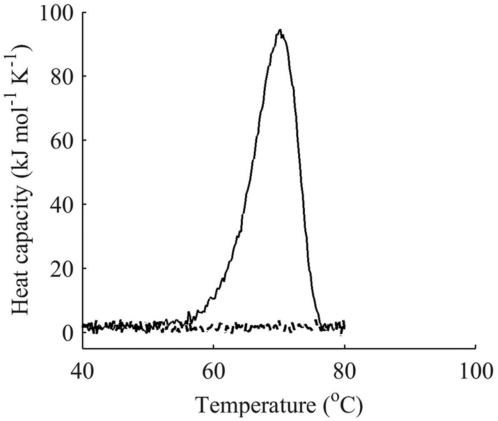
- Experimental condition scanning
  pH, ionic strength, co-solutes…
- Water/buffer test (clean, contaminants?)
- Reversibility test (equilibrium?)
- Scanning rate test (equilibrium?)
- Concentration test (association, aggregation?)

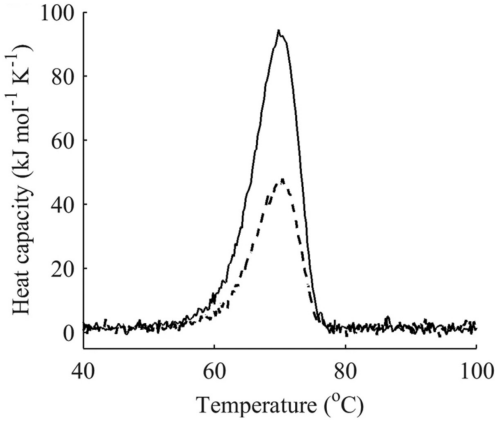
This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 101004806

MOSBRI
Molecular-Scale Biophysics Research Infrastructure

Unfolding reversibility test





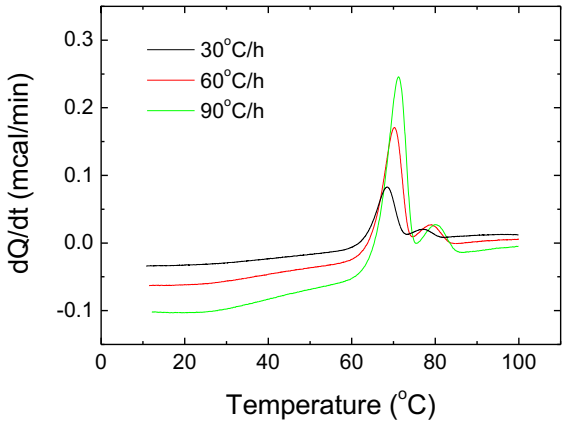


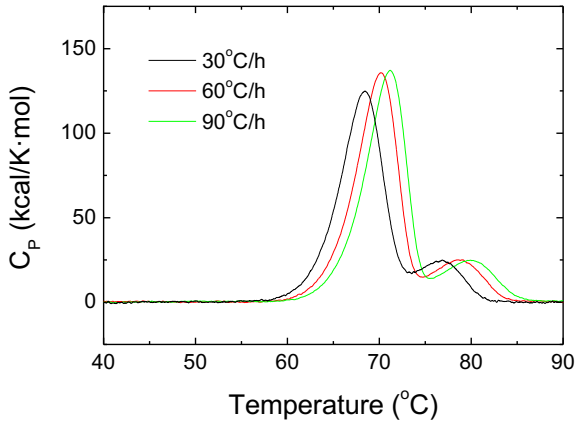
This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 101004806

MOSBRI
Molecular-Scale Biophysics Research Infrastructure

Scanning rate

$$C_P = \frac{dQ}{dt}\frac{1}{v}$$





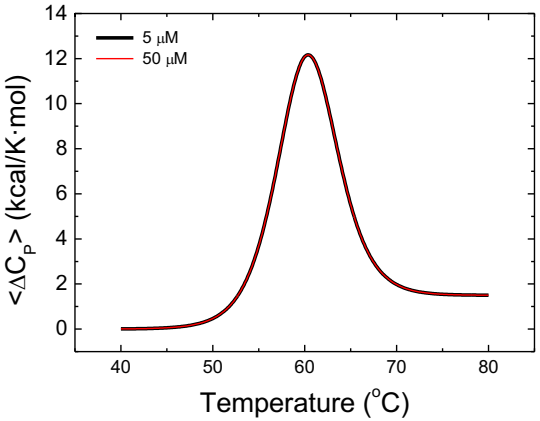
- **Standard scanning rate: 1°C/min**
- **Potential kinetic coupling with additional processes**

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 101004806

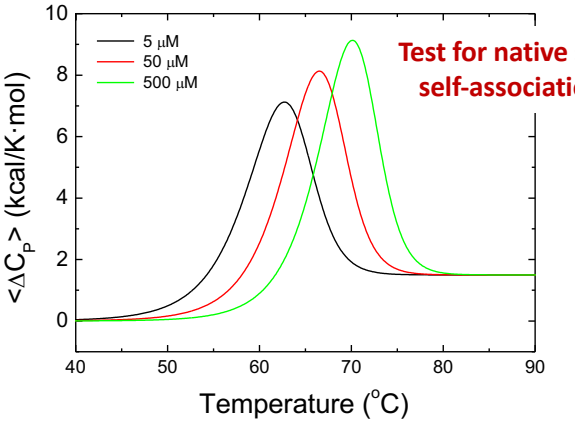
MOSBRI
Molecular-Scale Biophysics Research Infrastructure

Protein concentration

$N \leftrightarrow U$



$N_2 \leftrightarrow 2U$



- **Equilibrium equations are more complex (unfolding coupled to association/dissociation)**
- **Caveats: protein availability, solubility and/or aggregation**
- **Test for self-association or aggregation in the native/unfolded state**

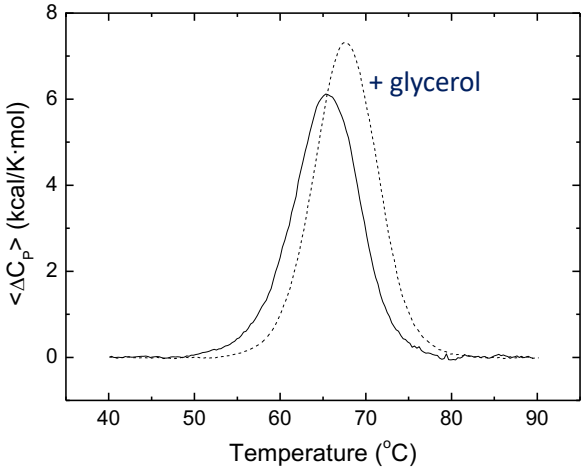
This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 101004806

MOSBRI Molecular-Scale Biophysics Research Infrastructure

Presence of co-solutes and co-solvents

$$\frac{\partial \ln K}{\partial \ln[X]} = \Delta n_X$$

$$\frac{\partial \ln K}{\partial [osmolality]} = -\frac{\Delta n_w}{55.6}$$



DMSO for solubilizing hydrophobic compounds
Glycerol, salts for solubilizing proteins

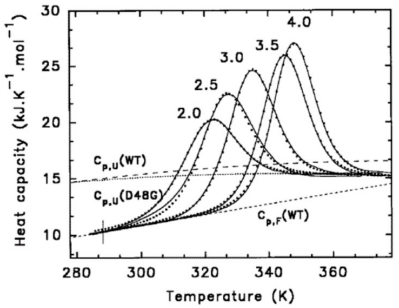
This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 101004806

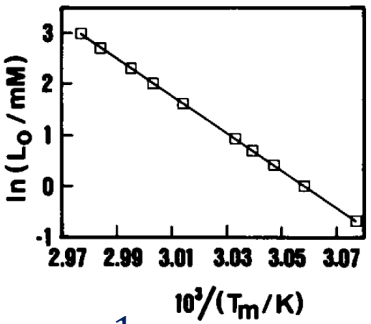
MOSBRI
Molecular-Scale Biophysics Research Infrastructure

There are Good's buffers, but no bad buffers

Not really true… (?)

$$\frac{\partial \ln K}{\partial \ln[X]} = \Delta n_X \qquad \frac{\partial \log K}{\partial pH} = -\Delta n_H$$





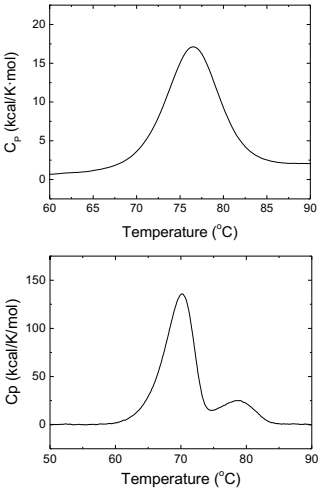
$$\frac{\partial \frac{1}{T_m}}{\partial pH} \approx -2.303 \frac{R}{\Delta H(T_m)} \Delta n_H \qquad \frac{\partial \frac{1}{T_m}}{\partial \ln[X]} \approx \frac{R}{\Delta H(T_m)} \Delta n_X$$

A buffer with pKa ≃ pH and low ionization enthalpy is preferable

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 101004806

MOSBRI Molecular-Scale Biophysics Research Infrastructure

Additional information + Ockham's Razor

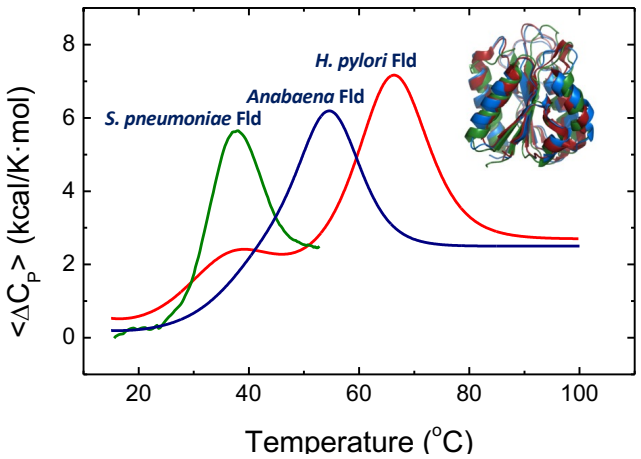


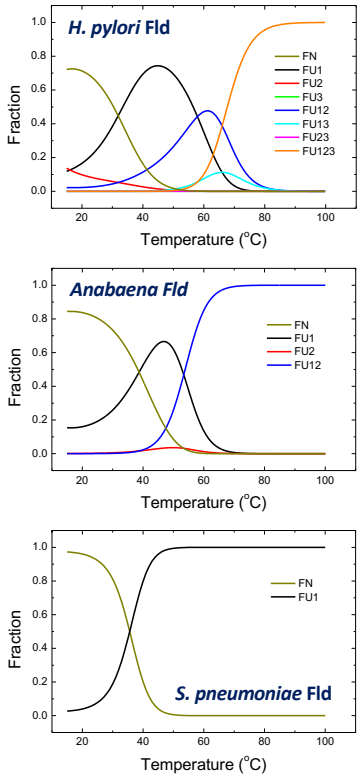
- Consider known information
- Revise thermogram
- Look for unusual features (?)
- Start with simplest compatible model
- Increase model complexity until needed
- Assumptions/constraints of model
- Values of the estimated parameters

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 101004806

MOSBRI Molecular-Scale Biophysics Research Infrastructure

Stability & folding cooperativity





Rodriguez-Cardenas et al. *PLoS ONE* 2016 **11** e0161020
Cremades et al. *Biochemistry* 2008 **47** 627-639
Irun et al. *Journal of Molecular Biology* 2001 **306** 877-888

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 101004806

MOSBRI Molecular-Scale Biophysics Research Infrastructure

▶ Stability & folding cooperativity

Structural similarity does not result in stability or folding cooperativity

- **Similar proteins may exhibit different structural stability**
- **Similar proteins may exhibit different conformational landscape and (un)folding cooperativity**

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 101004806

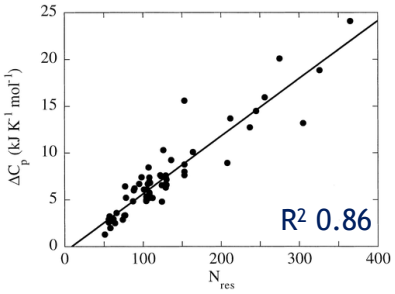
MOSBRI
Molecular-Scale Biophysics Research Infrastructure

Structural parameterization of protein stability

$$\Delta C_P \approx 13.9\, N_{res} \quad (\text{cal K}^{-1}\,\text{mol}^{-1})$$

$$\Delta C_P \approx 0.146\, \Delta ASA_{total} \quad (\text{cal Å}^{-2})$$

$$\Delta C_P \approx 0.45\, \Delta ASA_{ap} - 0.26\, \Delta ASA_{pol}$$

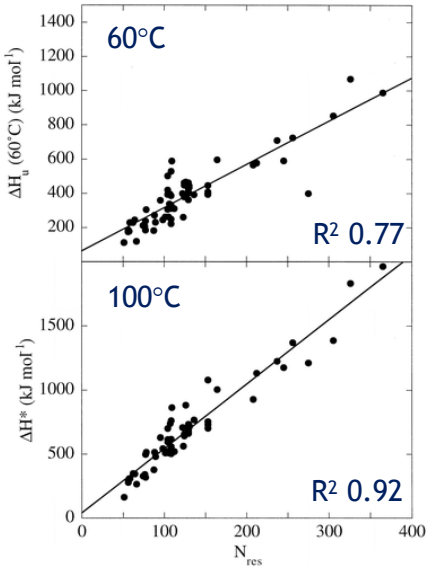


$$\Delta H(60°\text{C}) \approx 700\, N_{res} \quad (\text{cal mol}^{-1})$$

$$\Delta H(60°\text{C}) \approx 7.2\, \Delta ASA_{total} \quad (\text{cal Å}^{-2})$$

$$\Delta H(60°\text{C}) \approx -8.44\, \Delta ASA_{ap} + 31.4\, \Delta ASA_{pol}$$

$$\Delta H(100°\text{C}) \approx 1260\, N_{res} \quad (\text{cal mol}^{-1})$$



Freire. *Pure & Applied Chemistry*1997 **69** 2253-2261
Robertson & Murphy. *Chemical Reviews* 1997 **97** 1251-1268
Xie & Freire. *Proteins* 1994 **19** 291-301
Murphy & Freire. *Advances in Protein Chemistry* 1992 **43** 313-361

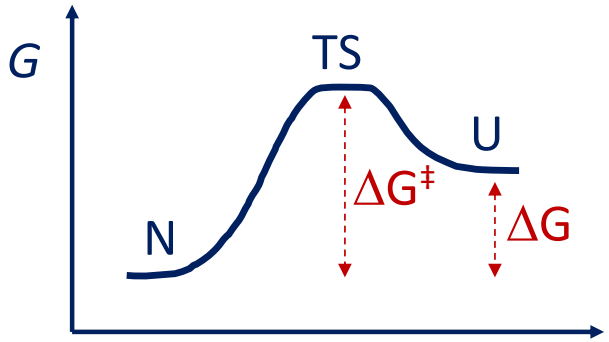
This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 101004806

MOSBRI Molecular-Scale Biophysics Research Infrastructure

Unfolding equilibrium

↓

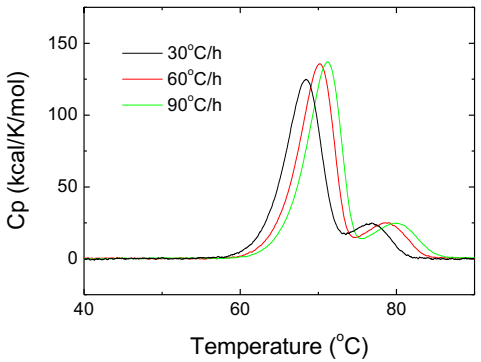
No kinetic information
No irreversible processes



This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 101004806

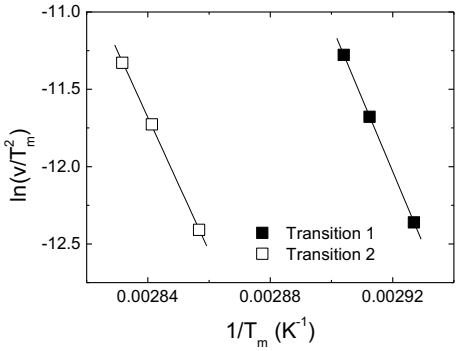
MOSBRI
Molecular-Scale Biophysics Research Infrastructure

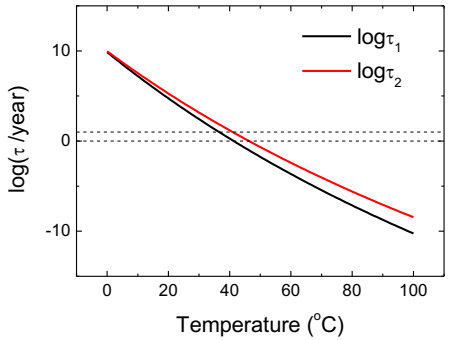
Irreversible unfolding & kinetic stability coupling



$$N \xrightarrow{k} F \qquad k = Ae^{-E_a/RT}$$

$$\frac{v}{T_m^2} = \frac{AR}{E_a} e^{-E_a/RT_m}$$



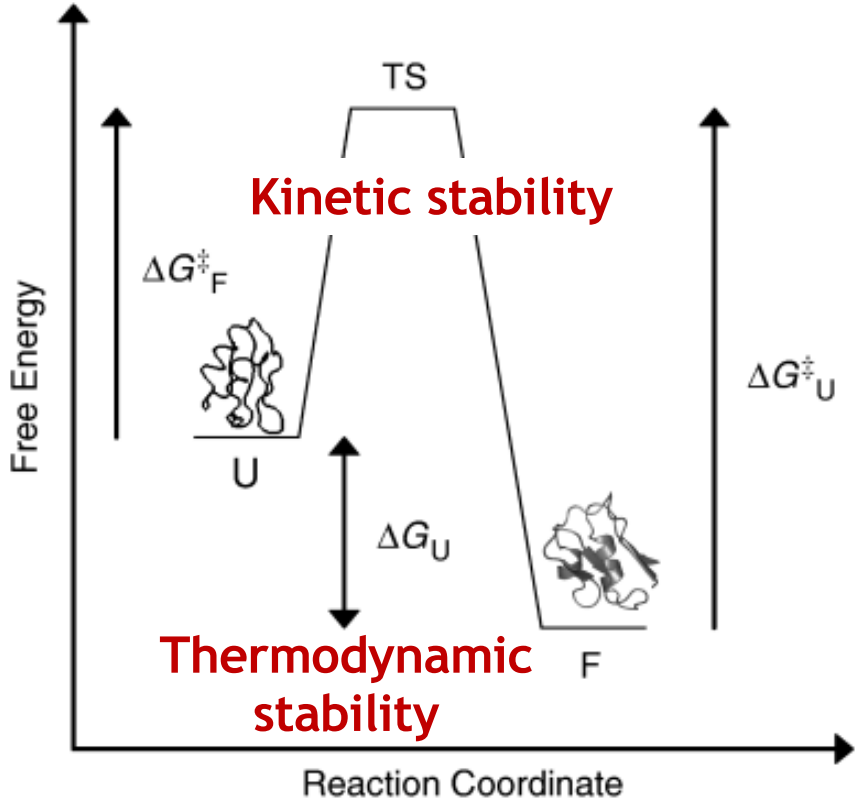


Sanchez-Ruiz. *Biophysical Journal* 1992 **61** 921-935

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 101004806

MOSBRI Molecular-Scale Biophysics Research Infrastructure

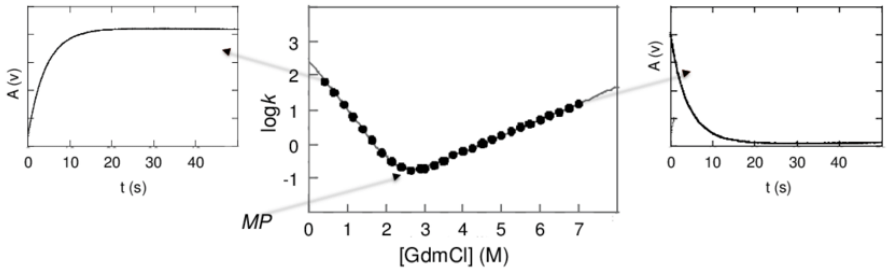
Stability: kinetic study

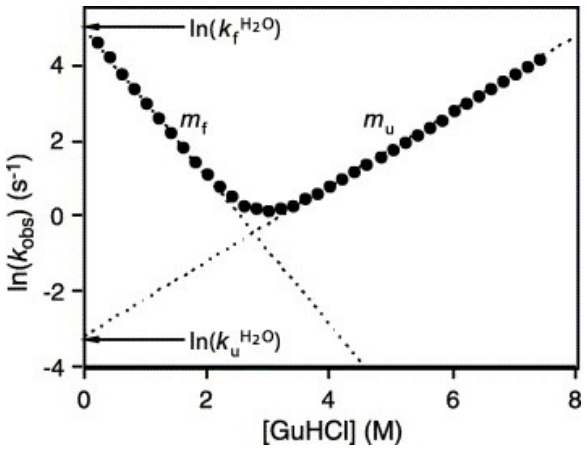


This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 101004806

MOSBRI
Molecular-Scale Biophysics Research Infrastructure

Stability: kinetic study



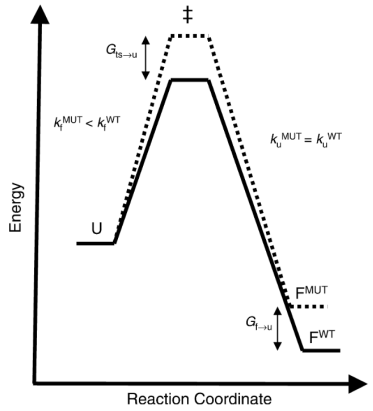


$$k_{obs} = k_F + k_U$$

$$\ln k_{F,U} = \ln k_{F,U}^{w} + m_{F,U}[D]$$

$$K = \frac{k_U}{k_F} \qquad K^w = \frac{k_U^w}{k_F^w}$$

$$m = m_U - m_F$$



This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 101004806

MOSBRI Molecular-Scale Biophysics Research Infrastructure

Advanced experimental design

- Several thermally-induced transitions
- Oligomer unfolding assay
- Ligand-induced stabilization (TSA)
- Lattice-like molecule assay
- Membrane transition assay
- Kinetically coupled transitions
- Irreversible transitions
- Association/dissociation coupling
- Absolute heat capacity determination

…

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 101004806

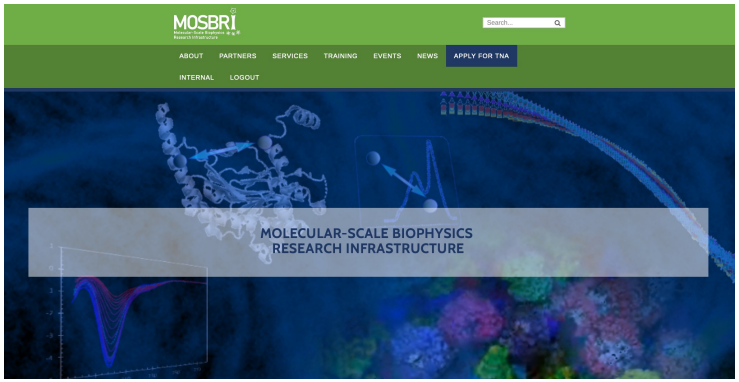
MOSBRI
Molecular-Scale Biophysics Research Infrastructure

▶ Protein stability depends on:

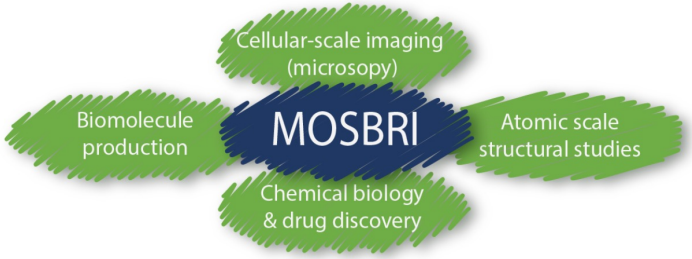
- Sequence (net charge, charge distribution, hydropathy profile, mutations, natural variability…)
- Post-translational modifications (deletion, phosphorylation, hydroxylation, acetylation, methylation…)
- External conditions (T, P, pH, μ…) and excipients/solutes
- Chemical modifications (deamidation, oxidation, proteolysis and hydrolysis, β-elimination, racemization…)
- Interacting molecules (ligands, osmolytes…)
- Presence of surfaces and interfaces/interphases

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 101004806

MOSBRI
Molecular-Scale Biophysics Research Infrastructure

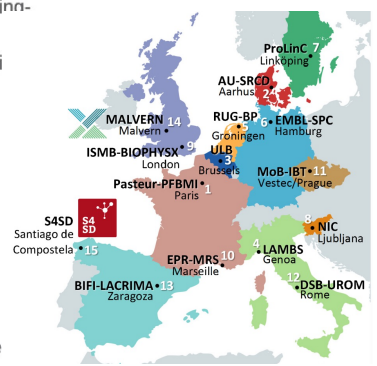


H2020-INFRAIA-02-2020, 101004806
Institut Pasteur (www.mosbri.eu)
5 M€ / 2021-2025



The **MOlecular-Scale Biophysics Research Infrastructure (MOSBRI)** enables ambitious integrative multi-technological studies of biological systems at the crucial intermediate level between *atomic-resolution structural descriptions and cellular-scale observations*.

Its consortium of 2 companies and 13 academic centres of excellence from 11 countries gathers a wide complementary panel of cutting-edge instrumentation and expertise, leveraging barriers that currently hinder the optimal exploitation of molecular-scale biophysical approaches in the fields of biomedicine, biotechnology, biomaterials and beyond. **MOSBRI** provides European academic and industri researchers with a one-stop shop Trans-National Access to the latest technological developments in advanced spectroscopies, hydrodynamics, thermodynamics, real-time kinetics and single molecule approaches.



It will play a major role in standardization and policy-making in the field by:

- carrying out Joint Research Activities to develop innovative methodologies
- designing robust quality control guidelines and FAIR-compatible archiving formats and databases
- engaging with instrumentation, pharma, biotech and CRO SMEs

Networking activities will multiply the impact of **MOSBRI**, by efficiently sharing and disseminating theoretical and practical knowledge through training events in Europe, contributing to:

- the emergence of a highly qualified new generation of scientists
- outreach to scientific communities currently unaware of the full potential of the integrated use of molecular-scale biophysics tools

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 101004806

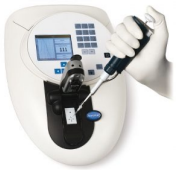
MOSBRI Molecular-Scale Biophysics Research Infrastructure

Chirascan (Applied Photophysics)

Cary Eclipse (Agilent)

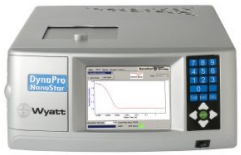
Cary 100 (Agilent)

DynaPro NanoStar (Wyatt)



Nanovue (GE Healthcare)

Cary 3500 (Agilent)



DynaPro Plate Reader III (Wyatt)

DS-11 FX (Denovix)

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 101004806

MOSBRI
Molecular-Scale Biophysics Research Infrastructure

Auto-PEAQ-DSC & VP-DSC (MicroCal – Malvern-Panalytical)

FluoDia T70 (PTI)

Mx3005p (Agilent)

CLARIOstar & FLUOstar (BMG Labtech)

MicroTime 200 (PicoQuant)

Tycho NT.6 (NanoTemper)

SpectraMax iD5 (Molecular Devices)

CFX Plus 96 & 384 (BioRad)

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 101004806

MOSBRI
Molecular-Scale Biophysics Research Infrastructure

Auto-iTC200 & VP-ITC (MicroCal – Malvern-Panalytical)

Monolith NT.155Pico (NanoTemper)

Biacore T200 (GE Healthcare)

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 101004806

MOSBRI
Molecular-Scale Biophysics Research Infrastructure