Prometheus Protocol PR-P-003

Folding Kinetics of Lysozyme

Lysozyme is an enzyme that prevents bacterial infections by attacking peptidoglycan, a component of certain bacterial cell walls. Peptidoglycan is composed of the repeating amino sugars N-acetylglucosamine (NAG) and N-acetylmuramic acid (NAM), which are crosslinked by peptide bridges. Lysozyme hydrolyzes the bond between NAG and NAM, increasing the bacteria's permeability and causing the bacteria to burst. It is widely distributed in plants and animals. The majority of the lysozyme used in research is purified from hen egg whites.

chemical unfolding | guanidine hydrochloride | unfolding kinetics | refolding kinetics | ΔG | c_{50}

A1. Target/Fluorescent Molecule

Lysozyme
uniprot.org/uniprot/B8YK79

A2. Molecule Class/Organism

Glycoside hydrolases
Gallus gallus (Chicken)

A3. Sequence/Formula

KVFGRCELAA AMKRHGLDNY RGYSLGNWVC AAKFESNFNT QATNRNTDGS TDYGILQINS RWWCNDGRTP GSRNLCNIPC
SALLSSDITA SVNCAKKIVS DGNGMNAWVA WRNRCKGTDV QAWIRGCRL

A4. Purification Strategy/Source

Sigma-Aldrich GmbH
L6876

A5. Stock Concentration/Stock Buffer

32 µg lyophilized powder

A6. Molecular Weight/Extinction Coefficient

14.3 kDa
37,970 $M^{-1}cm^{-1}$ (ε_{280})

A7. Dilution Buffer

50 mM Tris-HCl, pH 7.8, 150 mM NaCl, 10 mM $MgCl_2$, 0.05% TWEEN® 20

D1. nanoDSF System/Capillaries

Prometheus NT.48 (NanoTemper Technologies GmbH)
Standard Capillaries Prometheus NT.48 nanoDSF Grade (PR-C002, NanoTemper Technologies GmbH)

or

Prometheus NT.Plex (NanoTemper Technologies GmbH)
Standard 24-Capillary Chips Prometheus NT.Plex nanoDSF Grade (PR-AC002, NanoTemper Technologies GmbH)

D2. nanoDSF Software

PR.TimeControl v1.0.2 (NanoTemper Technologies GmbH)
nanotempertech.com/prometheus-software

D3. nanoDSF Experiment

1. Dissolve 764 mg of guanidine hydrochloride (Gua-HCl, MW 95.53) in 1 mL of dilution buffer to obtain an 8 M solution. Heat the solution until all Gua-HCl is dissolved.

Unfolding

2. Prepare 12 small PCR tubes according to the following table:

Tube	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
8 M Gua-HCl (µL):	140	130	120	110	100	90	80	70	65	60	55	50
Dilution buffer (µL):	10	20	30	40	50	60	70	80	85	90	95	100

3. Resuspend 32 µg lysozyme in 32 µL dilution buffer to obtain a 1 mg/mL solution.
4. Prepare 7 new tubes with 2 µL of lysozyme solution in each.
5. Start a new session of the *PR.TimeControl* software.
6. Go to 'Measurement Scan' and prepare a run with the following settings:
    a. Only capillary 1 selected
    b. Isothermal
    c. 25°C
    d. 10 min
    e. 100% excitation power

The following steps should happen **as fast as possible**:

7. Add 30 µL of the solution from tube **1** of step 2 to one of the lysozyme vials from step 4 to obtain 32 µL of a **7 M** GuaHCl solution (this will start the **unfolding** process). Quickly mix 4 – 5 times by pipetting.[1]
8. Load one capillary from this solution and place it on position 1 of the Prometheus capillary tray.
9. Place the magnetic lid to fix the capillary.
10. Start the measurement.
11. Repeat these steps for tubes **2 – 7** to obtain unfolding curves for **6.5 M**, **6 M**, **5.5 M**, **5 M**, **4.5 M**, **4 M** Gua-HCl.

[1] When using the Prometheus NT.Plex, GuaHCl solution can be added to multiple tubes at once using a multichannel pipette. The solutions can then be loaded into a capillary chip and all unfolding/refolding kinetics can be recorded simultaneously.

Refolding

12. Resuspend new 32 µg lysozyme in 32 µL of the 8 M Guanidine hydrochloride solution from step 1 to obtain a 1 mg/mL solution and **incubate** for **10 minutes**.
13. Prepare 5 new tubes with 2 µL of lysozyme solution in each.
14. Go to 'Measurement Scan' and prepare a run with the following settings:
    a. Only capillary 1 selected
    b. Isothermal
    c. 25°C
    d. 10 min
    e. 100% excitation power

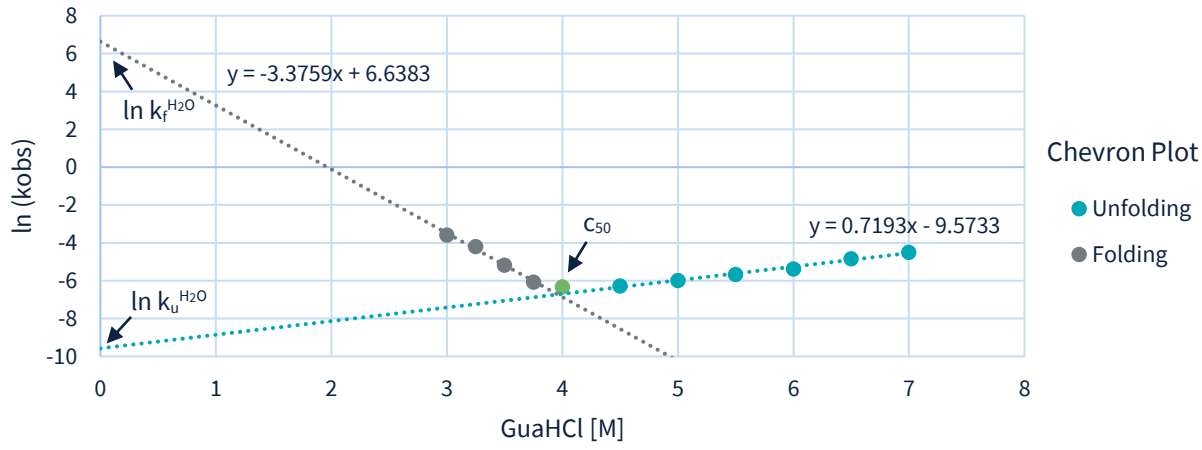
The following steps should happen **as fast as possible**:

15. Add 30 µL of the solution from tube **8** of step 2 to one of the lysozyme vials from step 13 obtain 32 µL of a **4 M** GuaHCl solution (this will start the **refolding** process). Quickly mix 4 – 5 times by pipetting.
16. Load one capillary from this solution and place it on position 1 of the Prometheus capillary tray.
17. Place the magnetic lid to fix the capillary.
18. Start the measurement.

Repeat these steps for tube **9 – 11** to obtain refolding curves for **3.75 M**, **3.5 M**, **3.25 M**, **3 M** Gua-HCl.

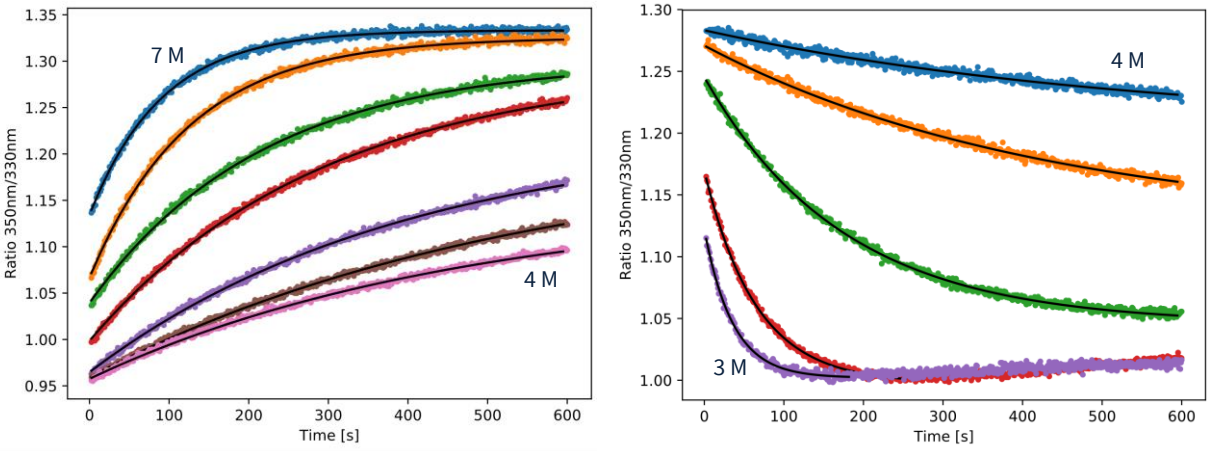
D4. nanoDSF Results

$k_f^{H_2O} = 762\ s^{-1}$ | $k_u^{H_2O} = 5.3 \cdot 10^{-5}\ s^{-1}$ | $\Delta G = RT \cdot [\ln(k_f^{H_2O}) - \ln(k_u^{H_2O})] = 40.8$ kJ/mol | $c_{50} \sim 4$ M



The denaturation midpoint c_{50} is defined as the denaturant concentration at which both folded and unfolded states are equally populated at equilibrium.

Gua-HCl (M)	7	6.5	6	5.5	5	4.5	4	3.75	3.5	3.25	3
k_{obs} (10^{-3} s^{-1})	11.1	8.0	4.6	3.4	2.5	1.9	1.8	2.3	5.7	15.1[2]	27.7[1]



D5. Reference Results/Supporting Results

ΔG = 37.2 kJ/mol | c_{50} = 4.2 M	Absorbance measurements Ahmad et al., Biochem J 287 (1992) 481-485
k_f^{H2O} = 447 s^{-1} | k_u^{H2O} = 6.2 · 10^{-7} s^{-1}	Tryptophan fluorescence Kiefhaber et al., PNAS 92 (1995) 9029-9033

E. Contributors

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[2] Under strongly native conditions, lysozyme refolds on parallel pathways, resulting in double-exponential refolding kinetics (Kiefhaber et al., PNAS 92 (1995) 9029-9033).

[3] NanoTemper Technologies GmbH, München, Germany | nanotempertech.com