Cryopreservation of Biological Samples: From Art to Science (?)

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Macromolecular Diffraction Facility at CHESS

Outline

- Cryocrystallography basics
- Why do we add cryoprotectants?
- **Ultrafast cooling** – **without cryoprotectants**
- **Ultraslow cooling** – **without cryoprotectants!**
- Mechanisms of radiation damage
- New opportunities for temperature controlled studies of structure and dynamics

Cryocrystallography

Cooling protein crystals (and other biological samples) to T~100 K reduces radiation damage and extends sample lifetimes by ~10^2.

But cooling can cause:

- formation of crystalline ice
- disruption of structure and order
- degradation of diffraction properties

Temperature dependent measurements between 150 K and 250 are difficult.

Why does flash cooling create disorder?

- Macromolecular crystals are 20-90% water.
- **Slow cooling**: water transforms to **hexagonal ice** with a 9% specific volume increase.
- **Flash cooling** below $T_g \approx 150$ K: water supercools into an **amorphous** phase.
- **Pure water:** vitrification only with cooling rates of **10^6 K/s** for drops <10 μm.
- **Cryoprotectants allow vitrification with smaller cooling rates.**

Proteins as cryoprotectants

- Proteins also reduce cooling rates required to achieve vitrification (Sartor, Mayer et al., 1992).

Cooling rates for vitrification of hydrated lysozyme powders:

23-38% water (1st to 2nd hydration shell):	<4 K/s
38-41%:	<24 K/s

⇒ **Penetrating cryoprotectants necessary to prevent internal crystallization only in samples with high solvent content or large solvent channels?**

What happens to water during flash cooling?

- Large specific volume change at 0°C eliminated.

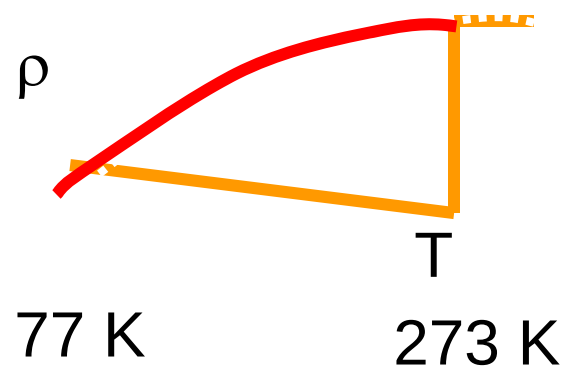
Common assumption:

For amorphous ice, $\rho(T \sim 100\ K) \approx 1\ g/cm^3$

Experiment:

For **amorphous ice**, $\rho(77\ K) \approx 0.94\ g/cm^3$

For **hexagonal ice**, $\rho(77\ K) \approx 0.93\ g/cm^3$



∴ **Flash cooling and slow cooling produce comparable increases in water's low temperature specific volume.**

- **Protein crystals are composite materials consisting of interpenetrating water and protein structures.**

- Protein crystal unit cell volumes shrink by ~3-6% between 293 and 100 K, while solvent expands.

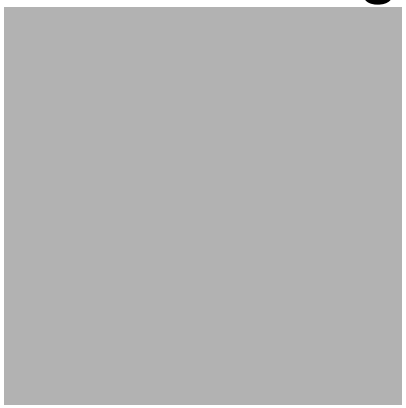
- **Differential contraction of water and protein lattice during cooling causes water to be expelled from some regions and to accumulate in others**

  ⇒ **variation in protein lattice constants.**

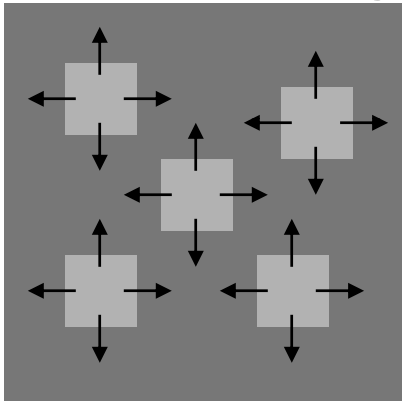
  ⇒ **plastic failure of lattice.**

  ⇒ **broadened mosaic.**

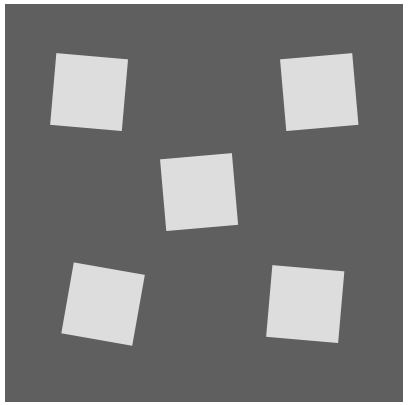
Before flash cooling:



During flash cooling:

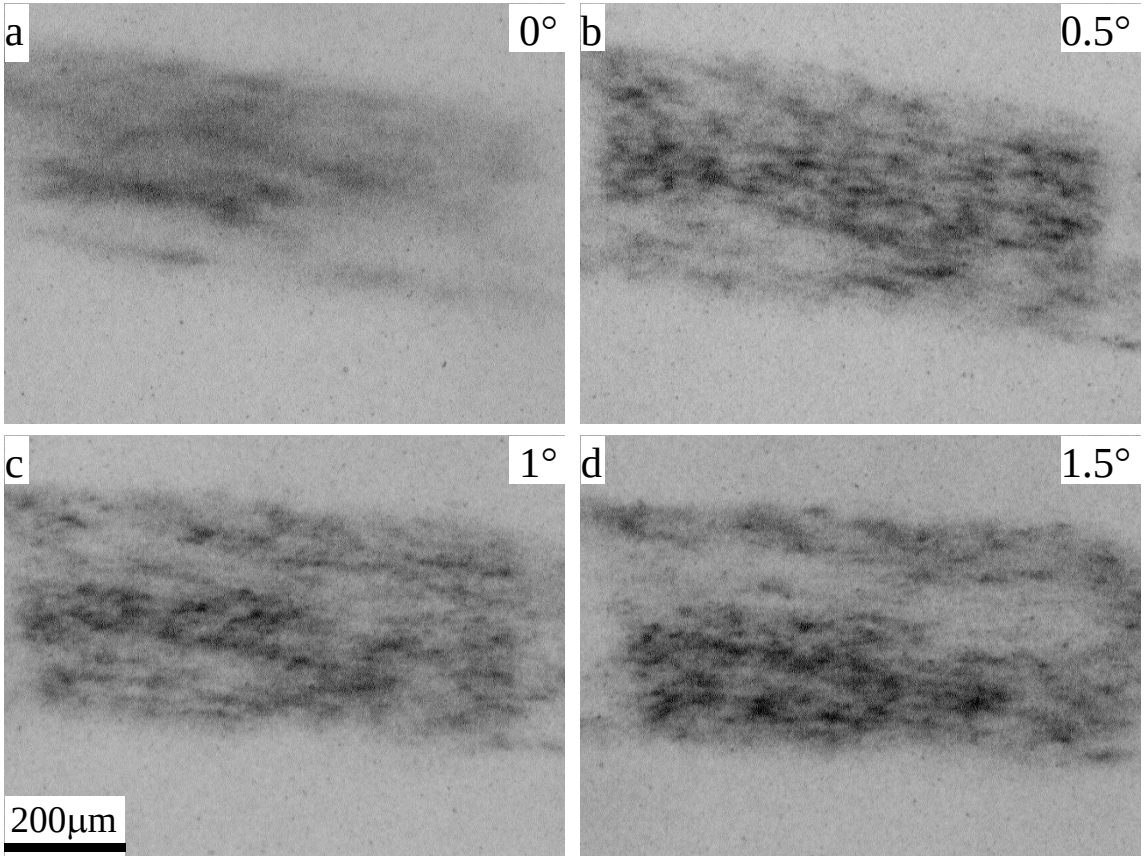


After flash cooling:



- Darker shading = larger water concentration

Rocking image sequence for a triclinic lysozyme crystal:



Fast versus Slow Cooling

All physico-chemical properties of protein crystals and their constituents are temperature dependent

E.g., cell volume, salt solubility, pH, protein conformation.

Very Fast Cooling

- no time for crystallization, relaxation and redistribution; only specific volumes change.

Very Slow Cooling (assume no ice formation):

- sample remains in quasi-equilibrium as sample constituents relax and redistribute, so low T state is homogeneous

Conventional Flash Cooling

Teng and Moffat, 1998:

N_2 stream:	~400 K/s;
Liquid nitrogen:	~400 K/s;
Liquid Propane:	~1000 K/s

Cooling times:

To below 220 K:	~ 0.2 s
To T_g=150 K:	~ 0.4 s

We are in neither the slow nor fast cooling limit, but in some messy intermediate regime!

How can we cool faster?

- **Decrease sample volume V**
  Cooling rate ~ $V^{-1/2}$ ~ $L^{-3/2}$
  1 mm to 20 μm should give a factor of **~350**

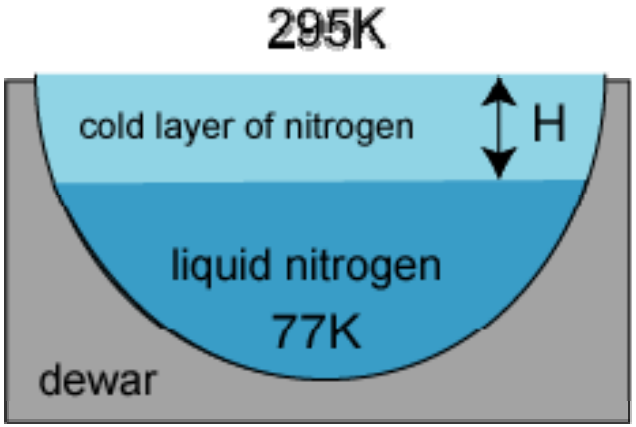
- **Plunge in liquid rather than gas stream**
  ~ factor of **20**

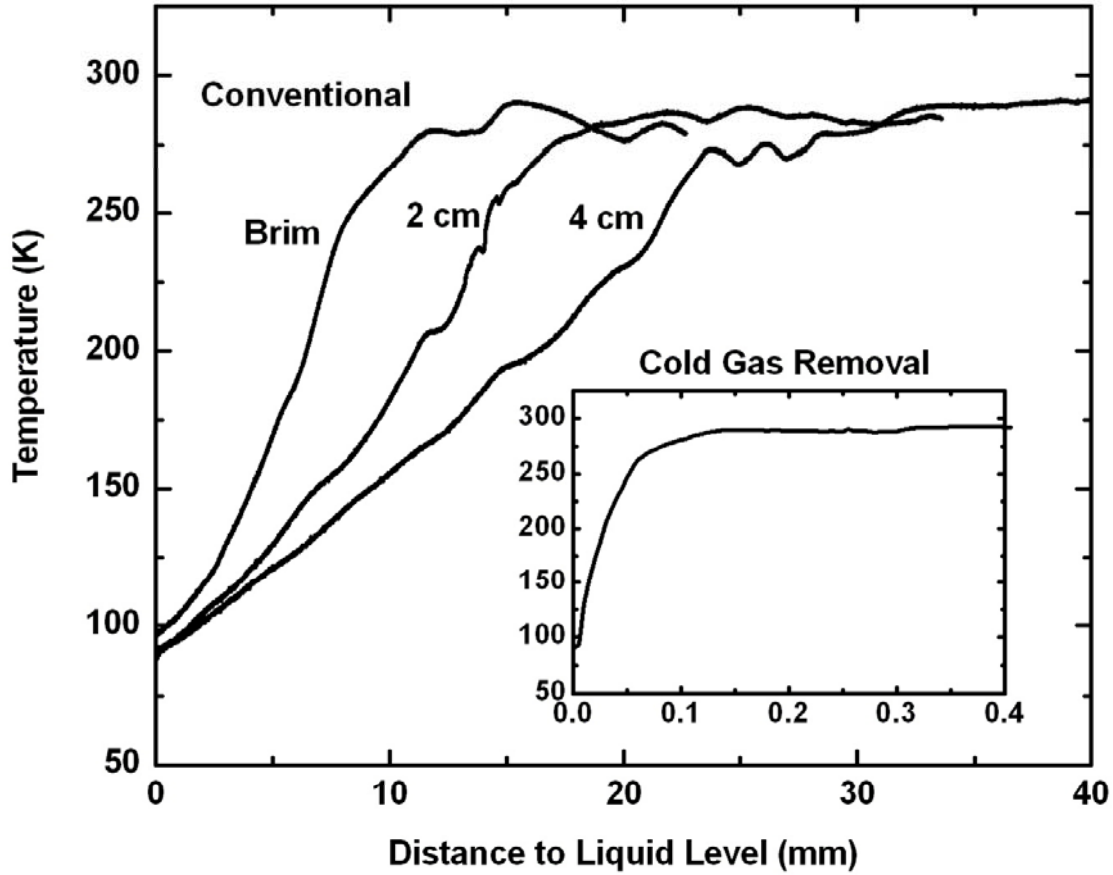
- **Plunge in liquid propane instead of nitrogen**
  ~ factor of **2-4**

- **Increase plunge velocity in liquid**
  ~ factor of **2**

But: Comparable cooling rates and diffraction outcomes with gas stream and liquid plunge cooling observed.

WHY? A cold gas layer forms above the liquid cryogen

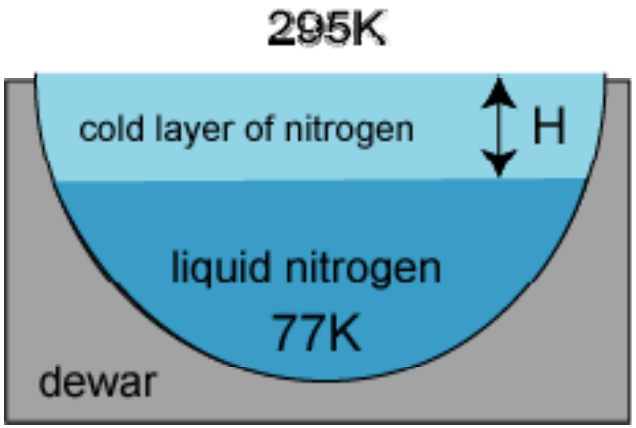




For volumes < 0.1 µl, cooling below T_g occurs in the gas layer, **before the crystal reaches the liquid cryogen!**

Ultra-Fast Cooling ("Hyperquenching")

Remove the cold gas layer by blowing or sucking.



With gas layer:

dT/dt < **10^3 K/s** (L<500 μm)

Remove the gas layer:

dT/dt = **2 x 10^4 K/s** (L~80 μm)

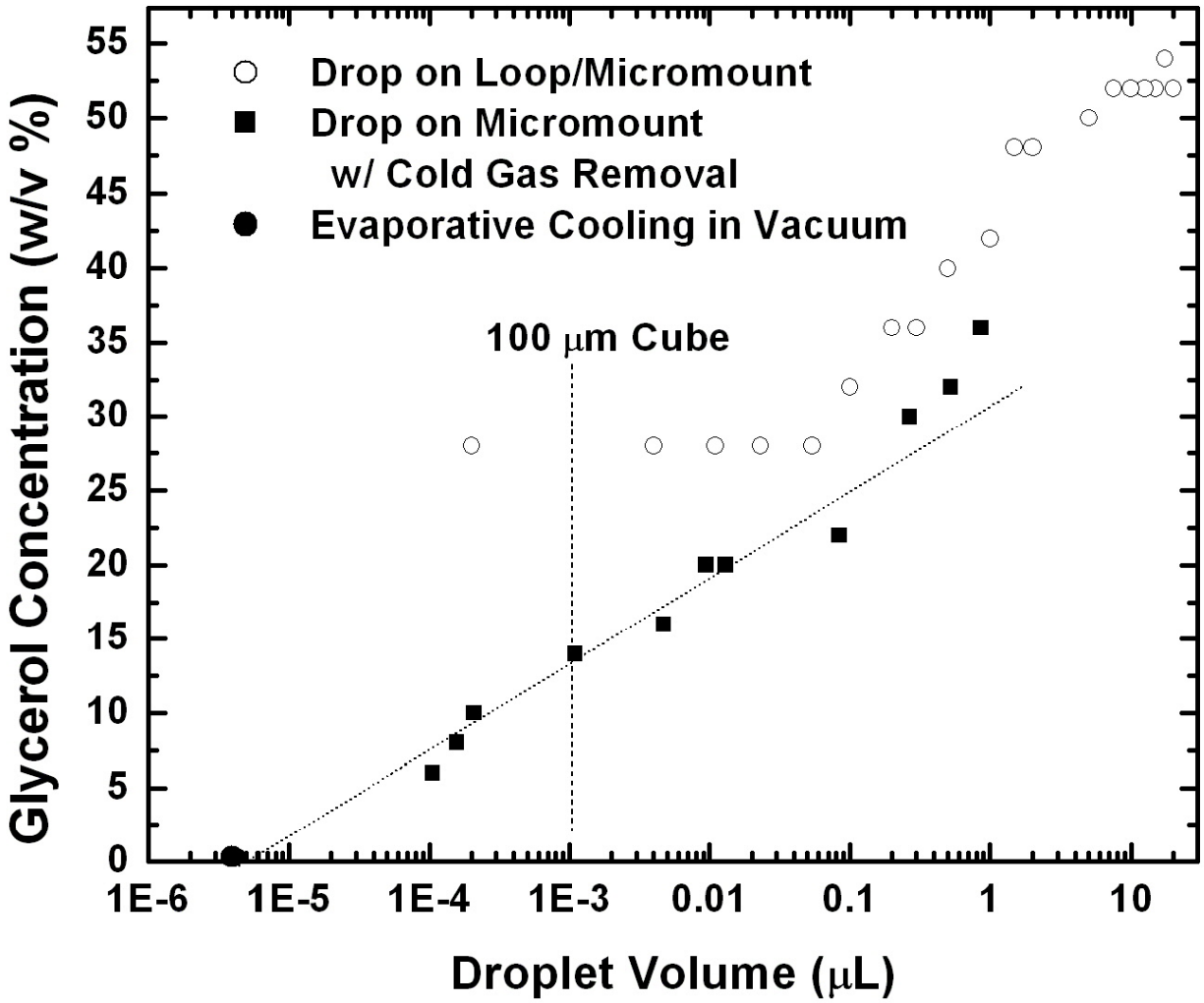
~10^5 K/s (L~20 μm)

Faster cooling ⇒
Less cryoprotectant required to vitrify solvent.

100 μm: 13% w/v

50 μm: 8% w/v

10 μm: 3% w/v



Ultra-Fast Cooling

- 20-1000 times faster than flash cooling – as fast as state-of-the-art cryo-em cooling methods.

- **Successful cooling of all proteins (so far) to T=100 K without penetrating cryoprotectants**

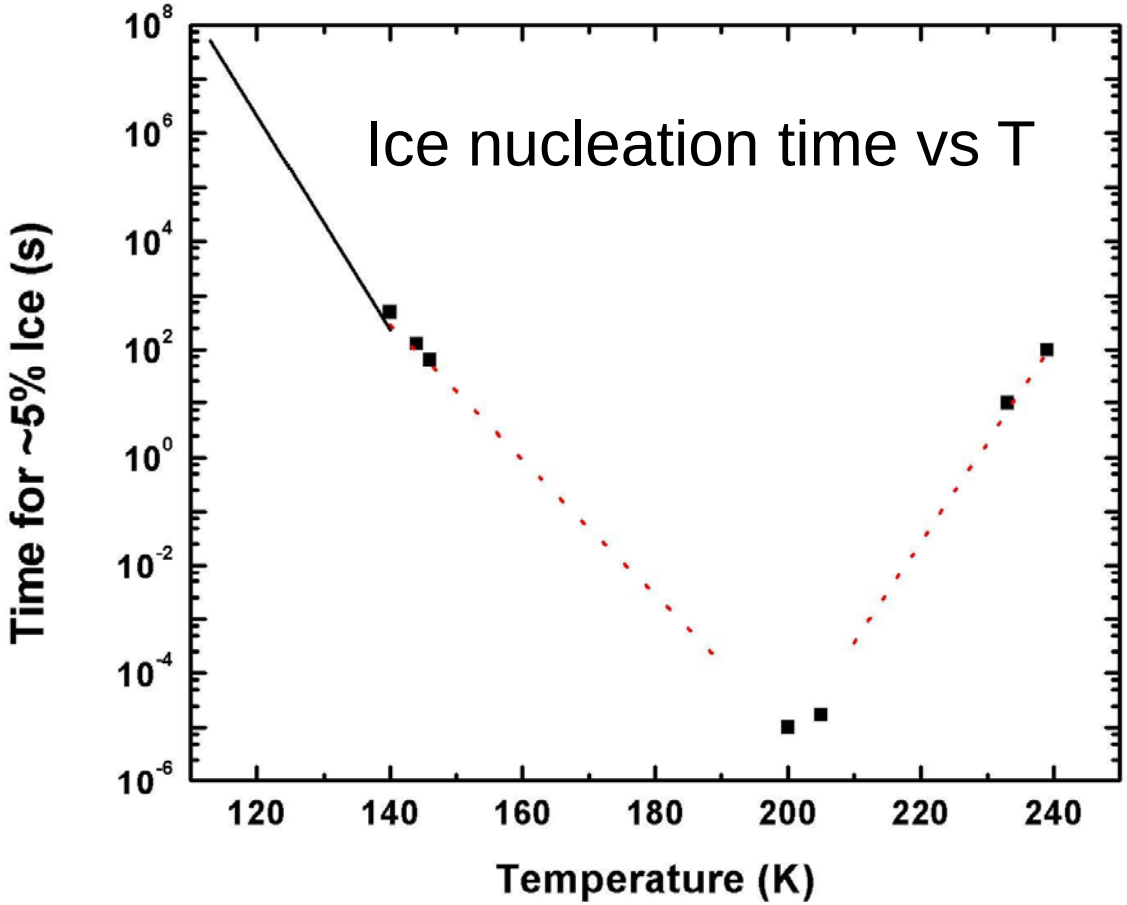
- More reliable and consistent cooling
- Improved crystal order and diffraction (?)
- More accurate trapping of room-temperature structure (?)

What about Ultra-Slow Cooling?

- sample remains in quasi-equilibrium as sample constituents relax and redistribute; low T state is homogeneous

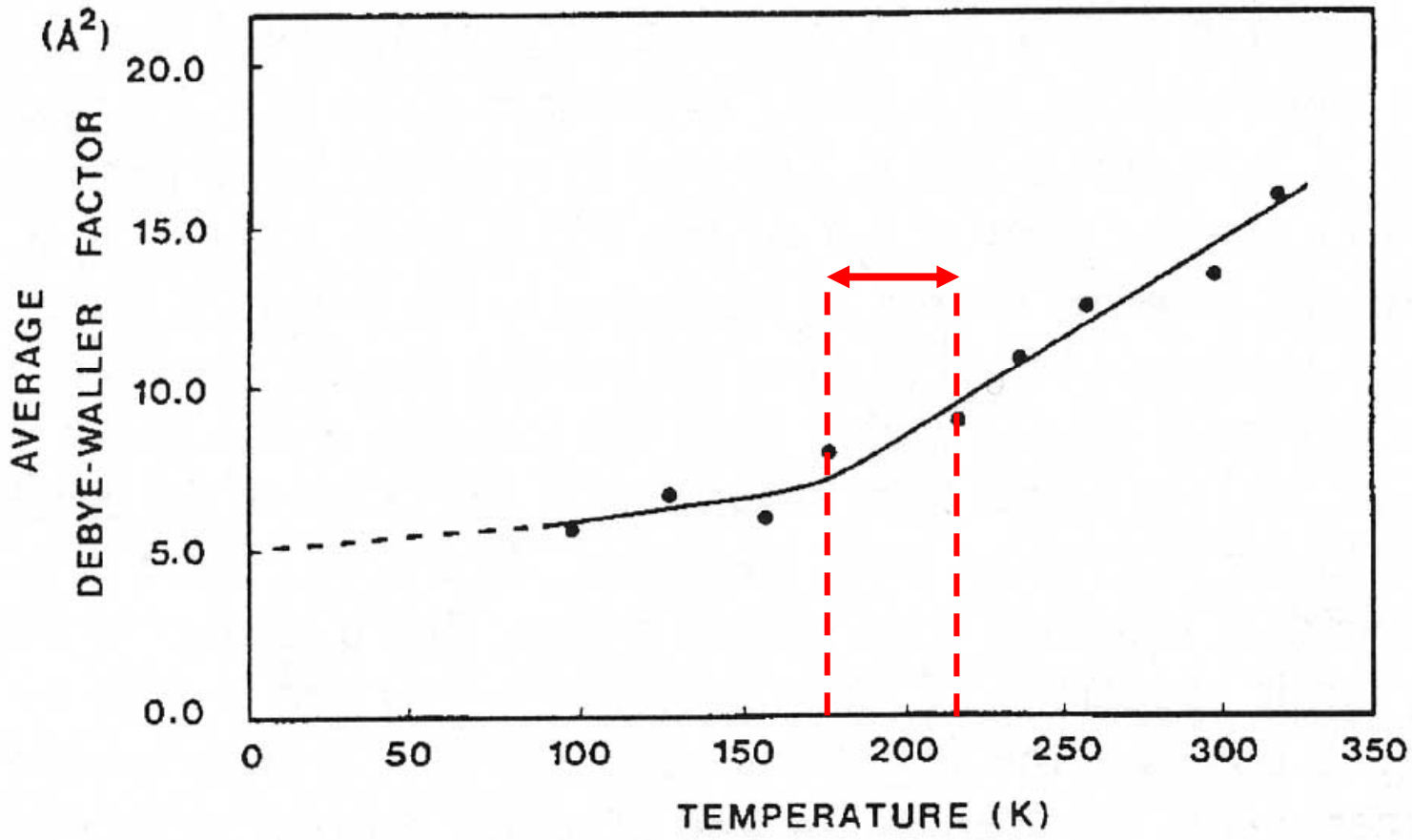
Time for ice nucleation ~10^{-5} s at T=200 K

-need cooling rates of 10^7 K/s



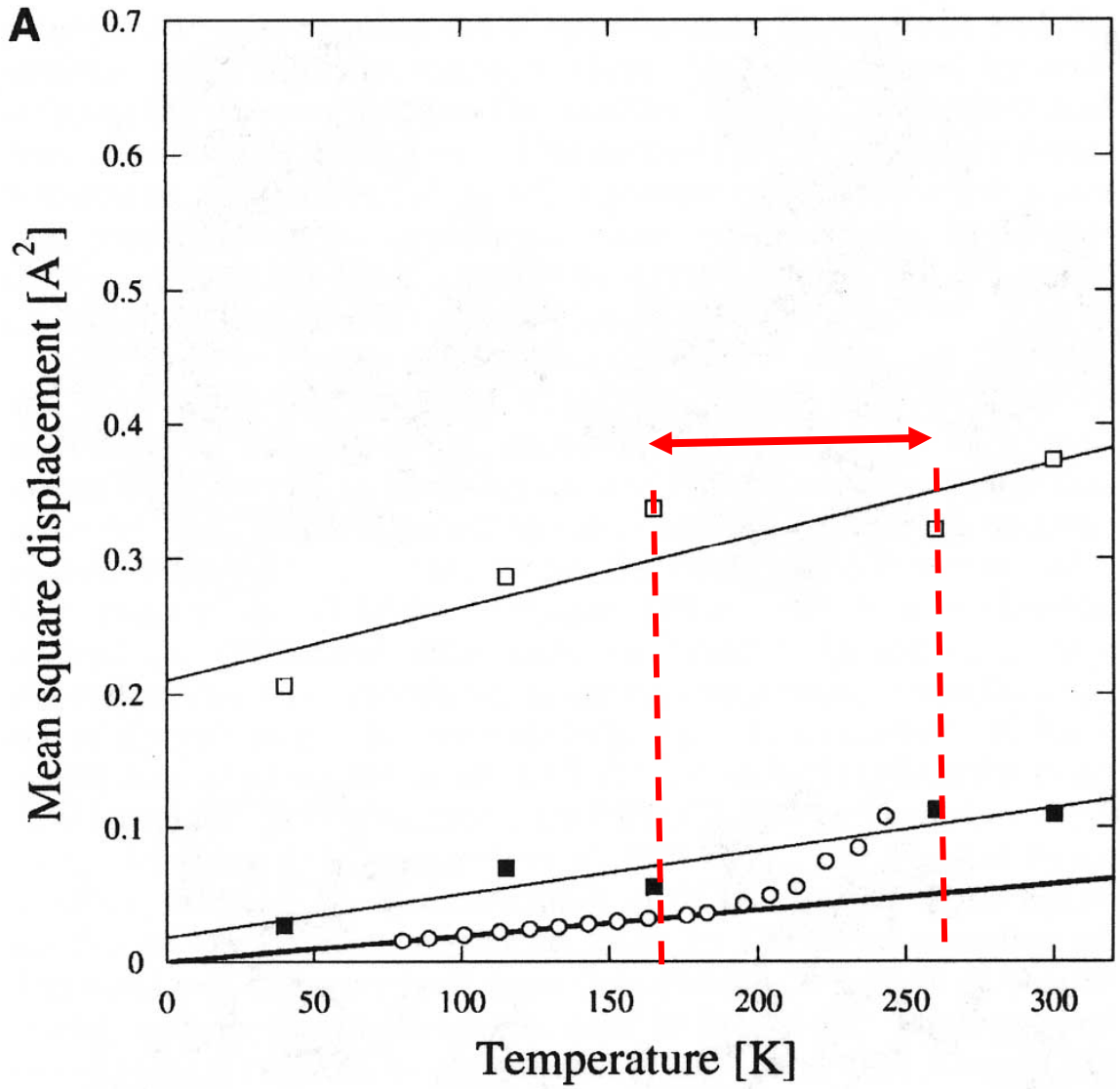
Ice formation prevents structural studies at 180 K < T <220 K

Tilton, Dewan and Petsko, 1992:



Ice formation prevents structural studies at 180 K < T <220 K

Chung and Parak, 2001:

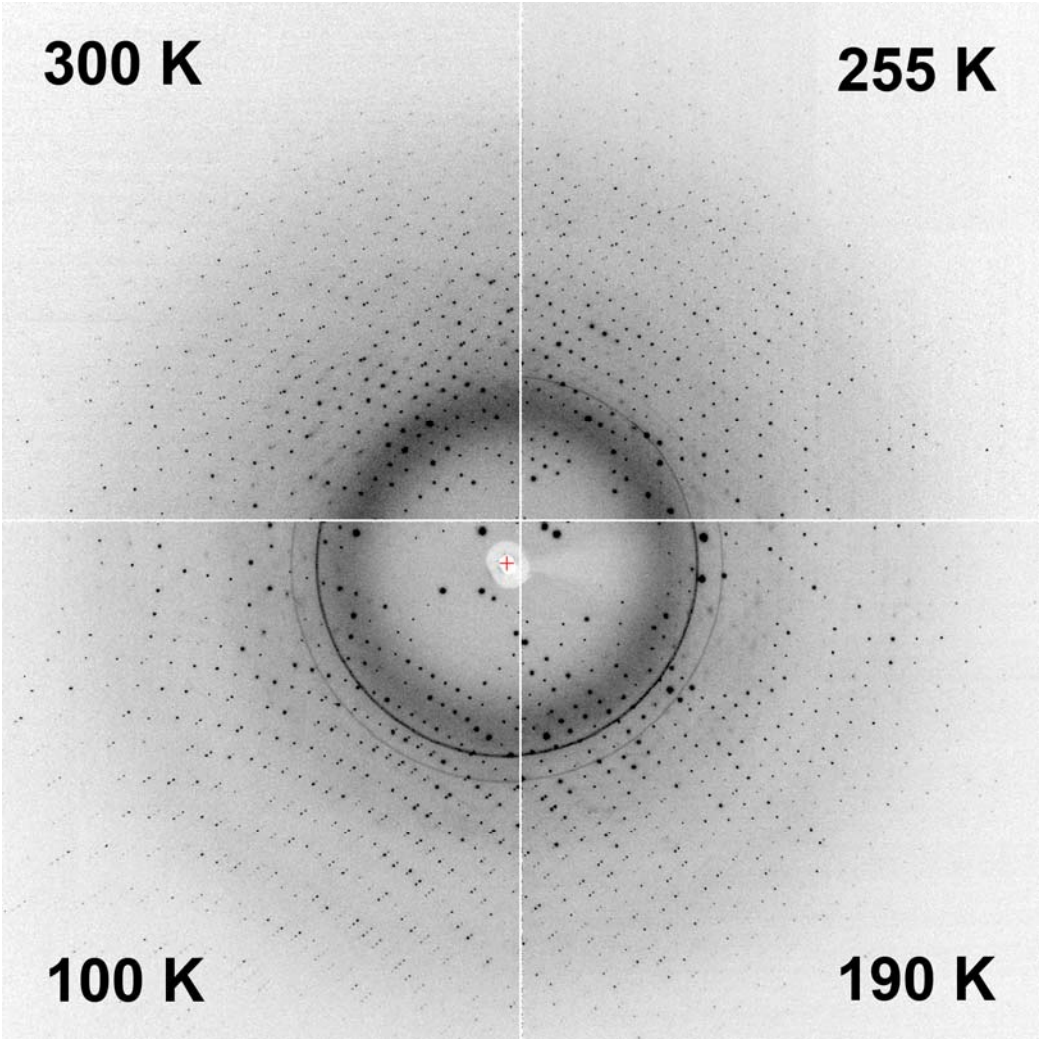


Data collected on cooling from T=300 K to T=100 K at 0.1 K/s

Thaumatin

56% solvent

No penetrating cryoprotectants



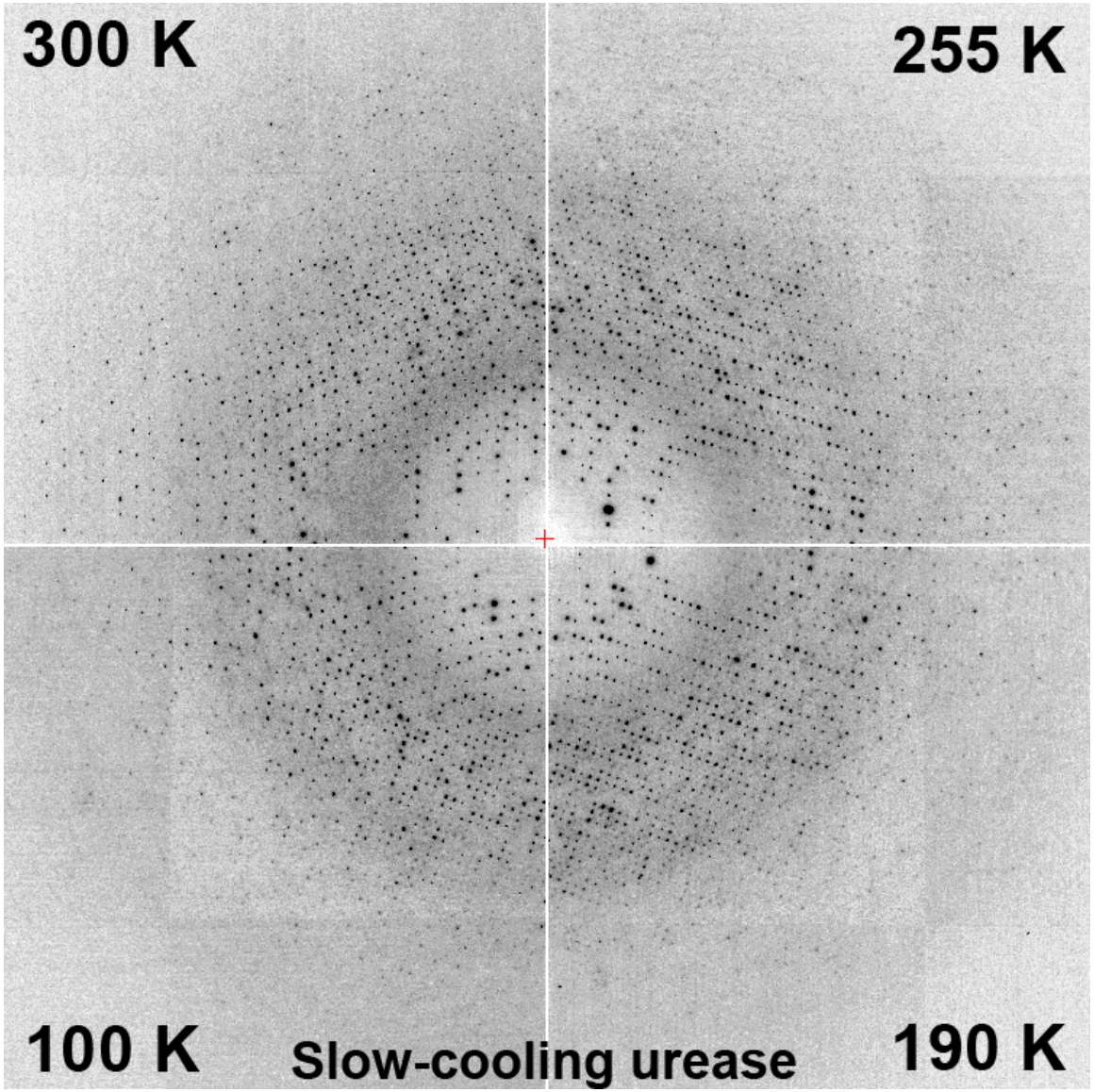
Cooling at 0.1 K/s

Urease

48% solvent

4.9 x10^6 Å^2 cell

No penetrating cryoprotectants

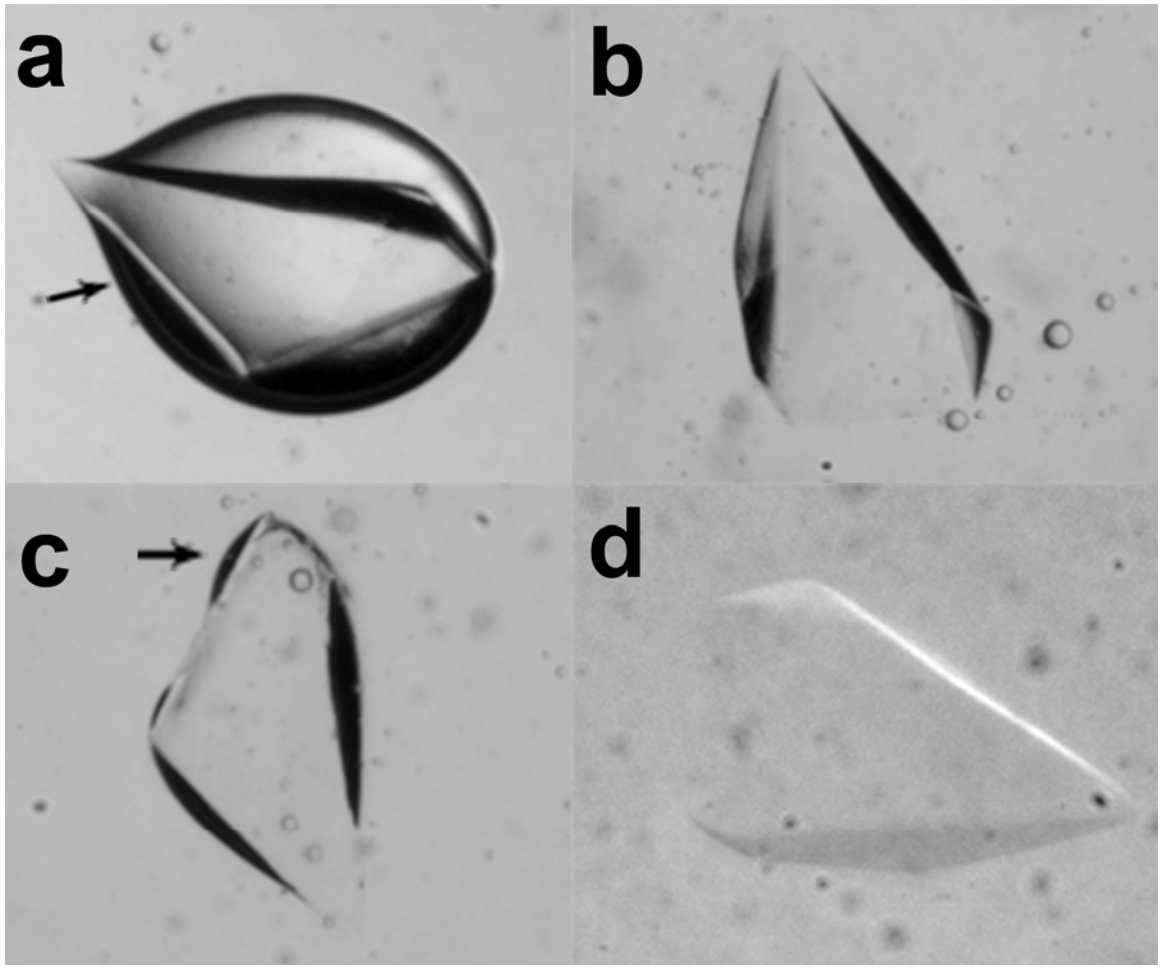


No formation of crystalline ice inside the crystal during cooling at 0.1 K/s

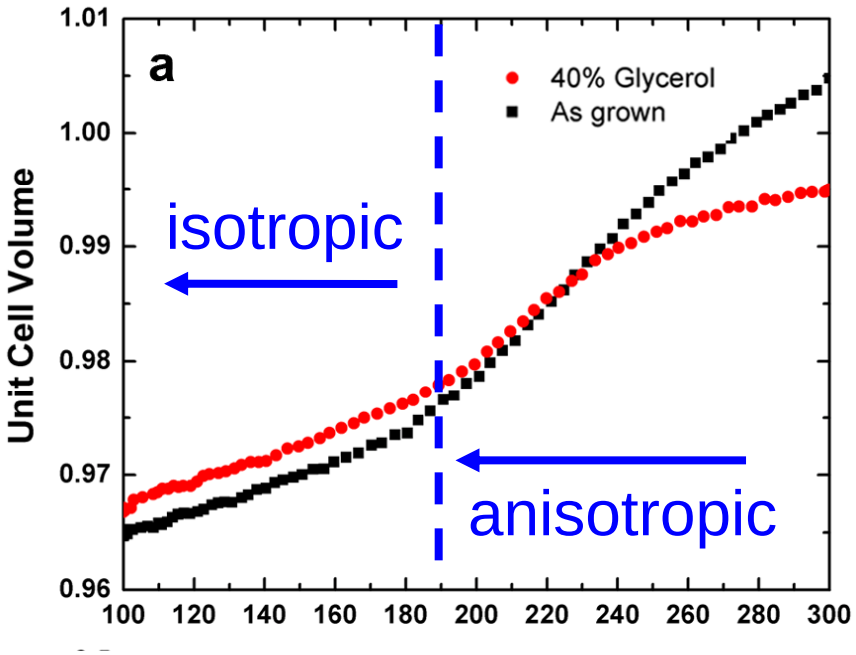
$\Rightarrow$ **Crystalline protein is as effective in suppressing ice nucleation as a 70% glycerol solution**

Key:

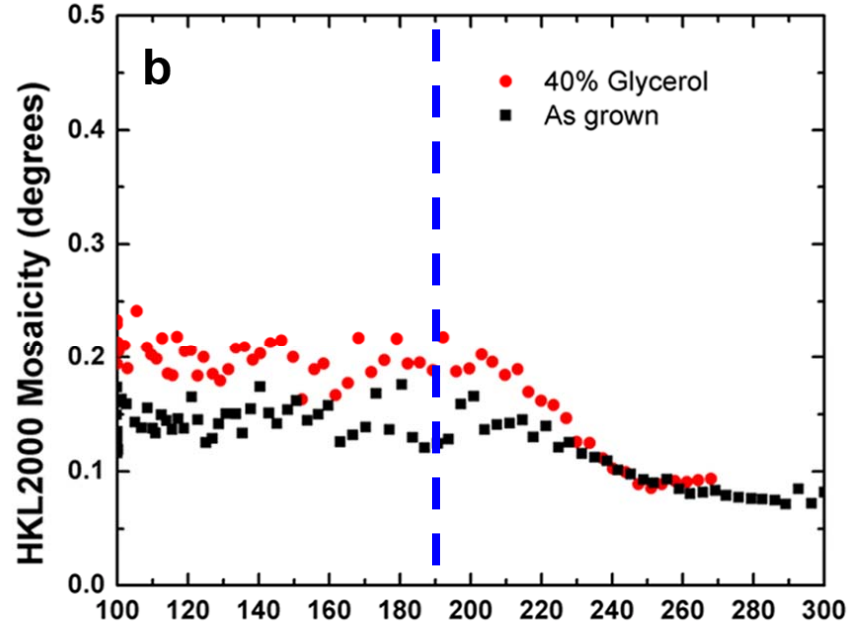
Completely remove all surrounding solvent

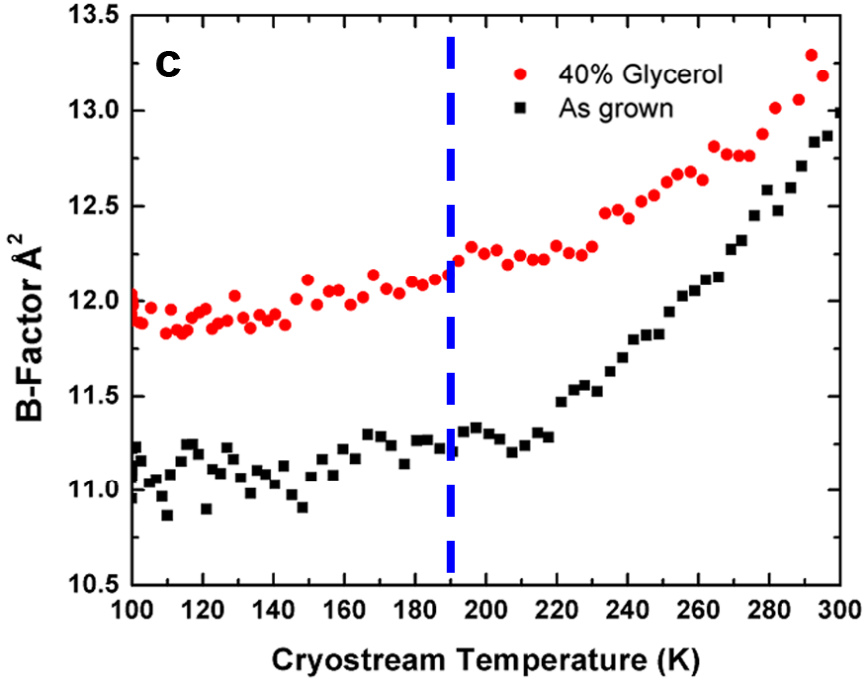


Data collection at **all** T between 300 K and 100 K



Thaumatin cooled at 0.1 K/s



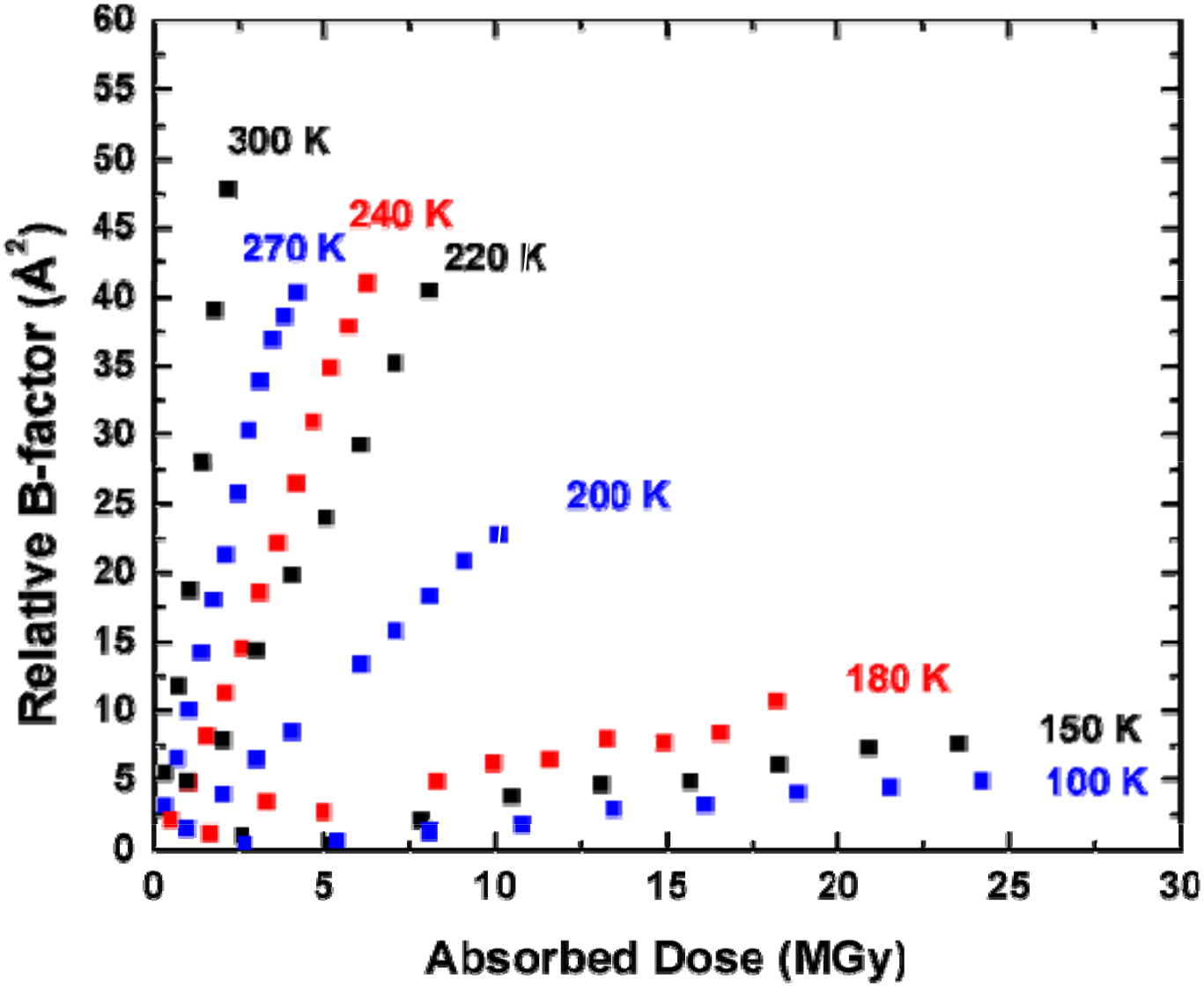


Slow Cooling & Variable Temperature Data Collection: A General Method?

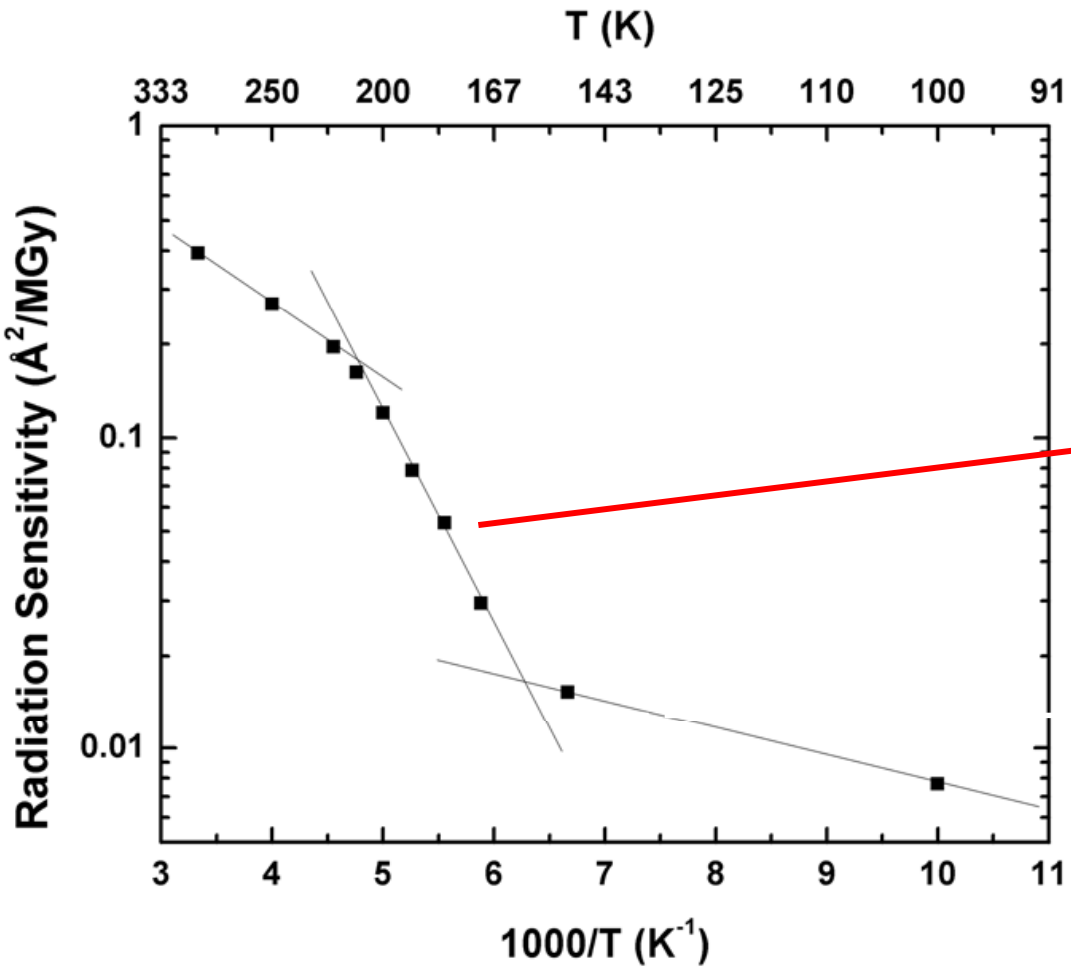
Protein	Lowest Mosaicity T= 100 K	Cryoprotective Mother Liquor Components
Insulin	**0.06°**	12.5 % (w/v) ethylene glycol
Urease	0.45°	1.6 M Li_2SO_4
Thaumatin	**0.11°**	1.5 M Na-K-Tartrate
Lysozyme	0.4°	1.2 M NaCl
Trypsin	0.9°	25 % (w/v) PEG 8000 0.2 M ammonium sulfate

Temperature Dependence of Radiation Damage

Thaumatin, no penetrating cryoprotectants



Activation Energies and Mechanisms of Radiation Damage

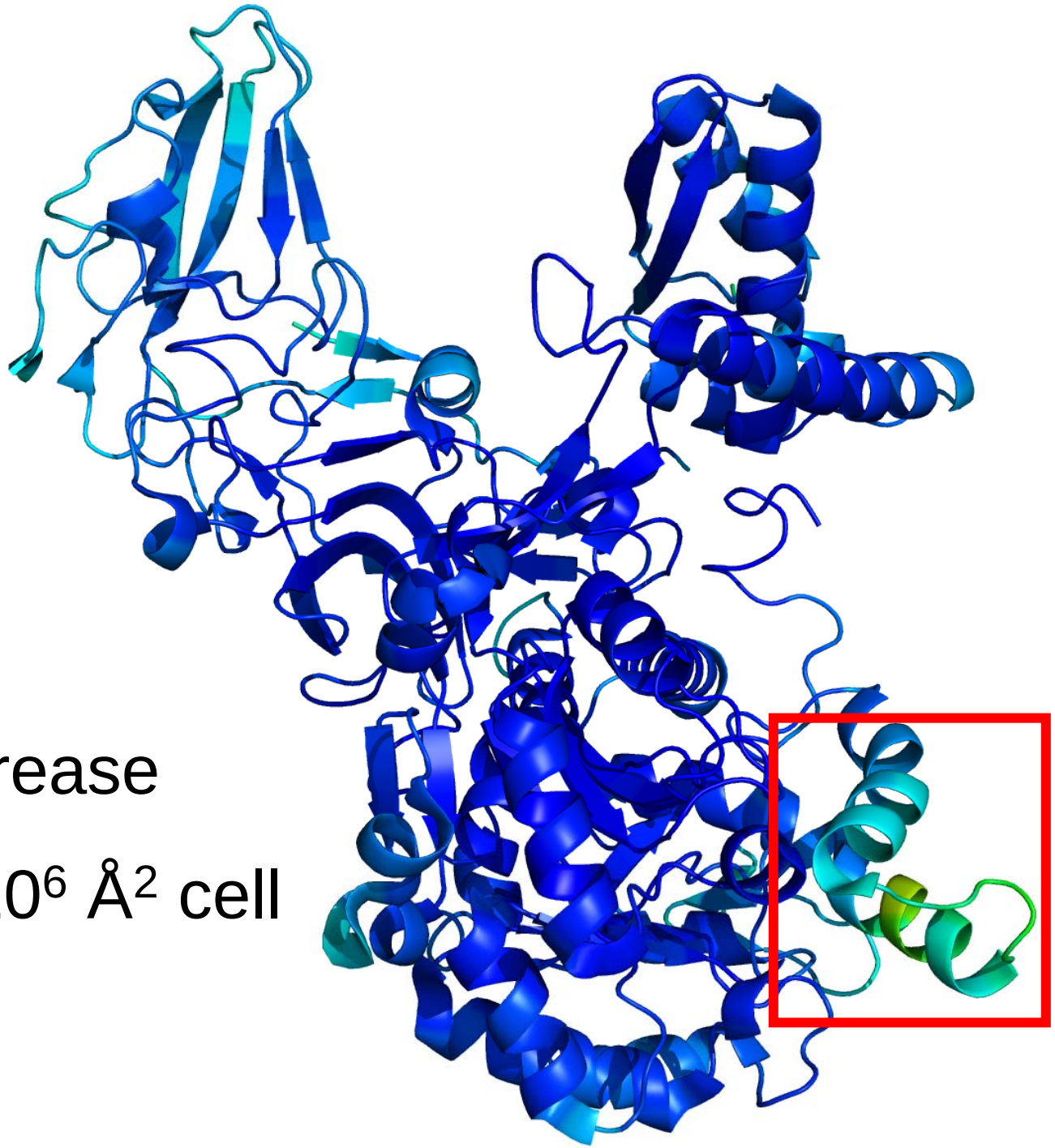


Radiation sensitivity drops by a factor of 10 between 225 K and 160 K with

E_a~3.7 kcal/mol

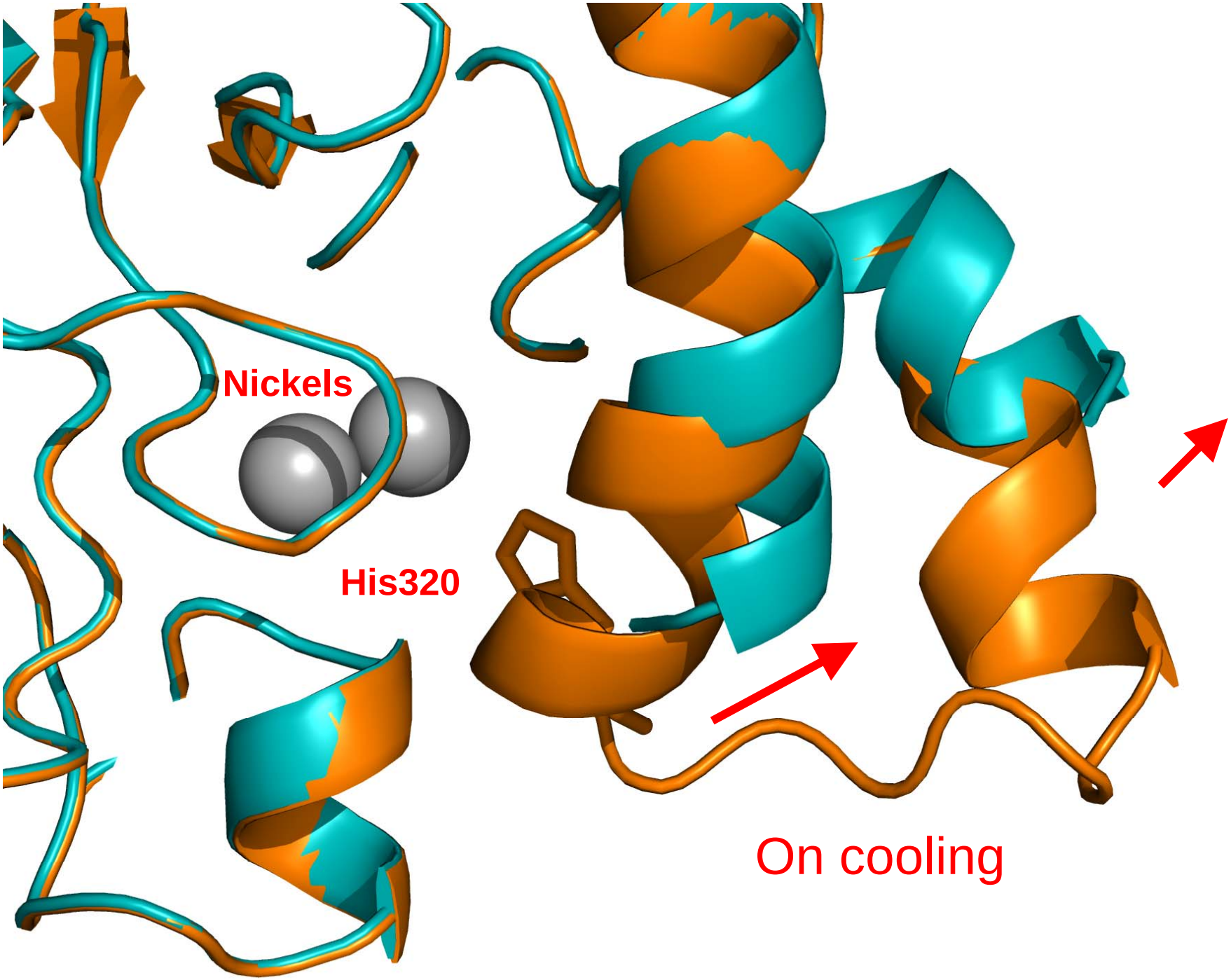
NMR data for diffusion of lysozyme hydration water:

E_a~3.5 - 4.1 kcal/mol

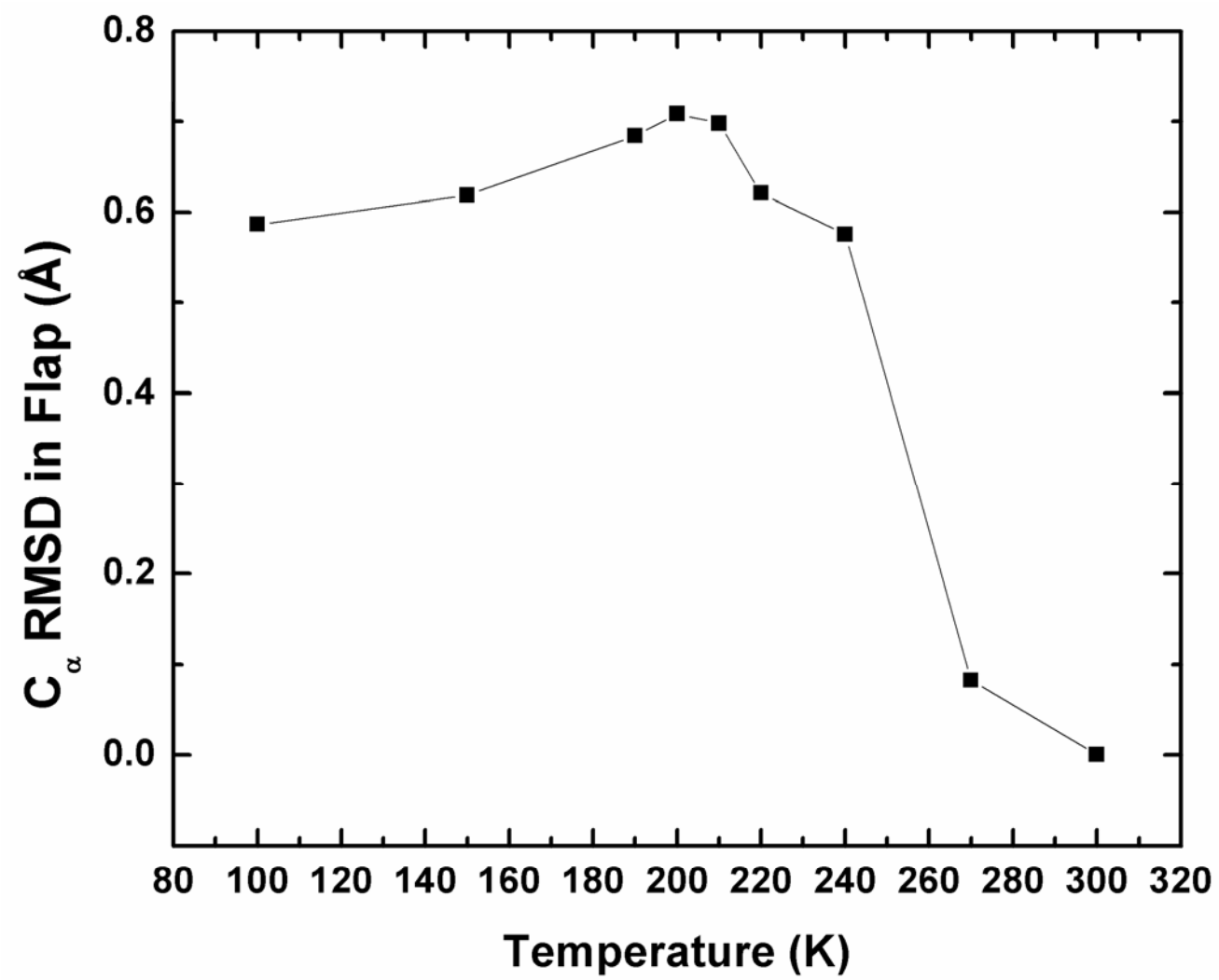


Urease

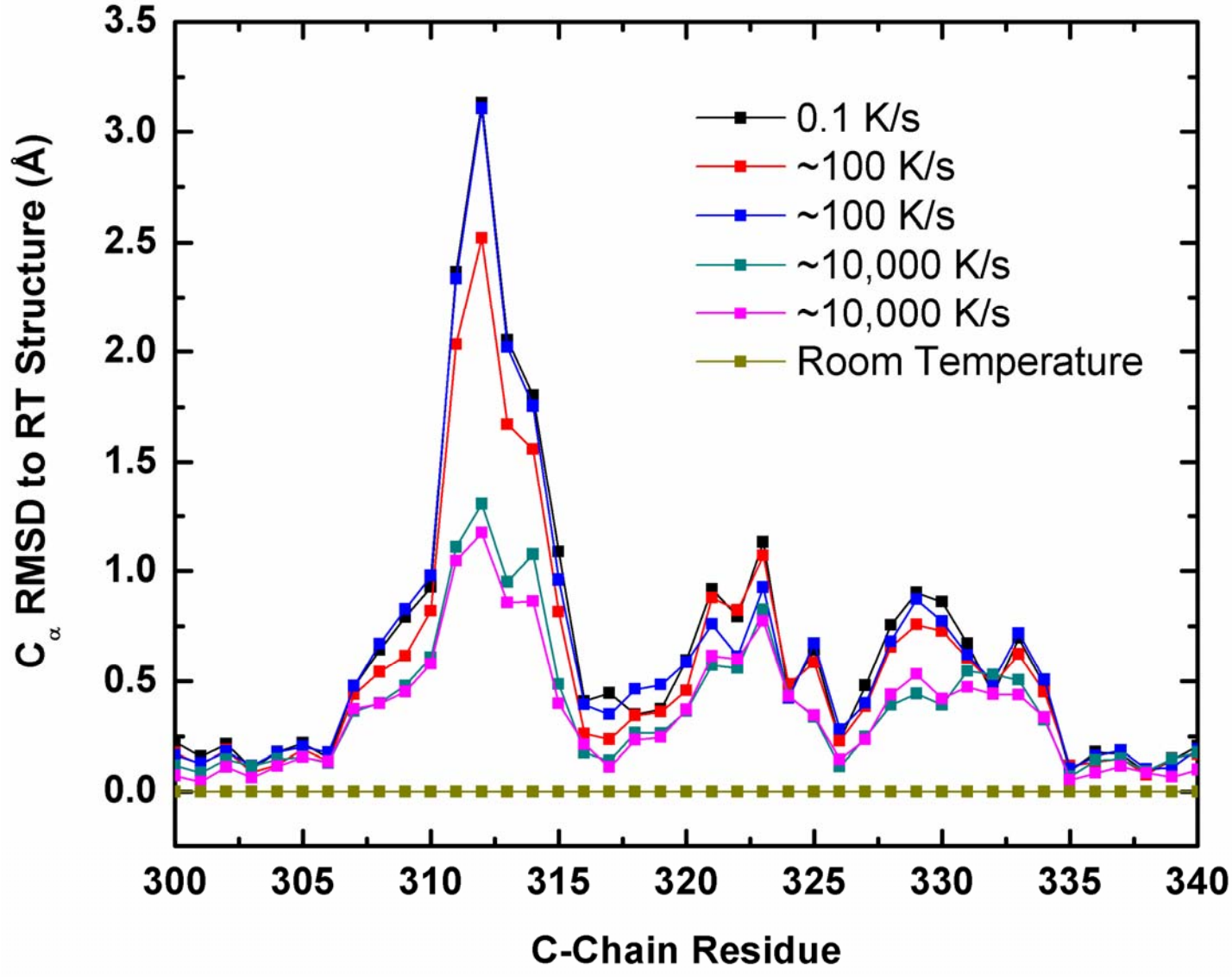
4.9 x10^6 Å^2 cell


Nickels
His320
On cooling

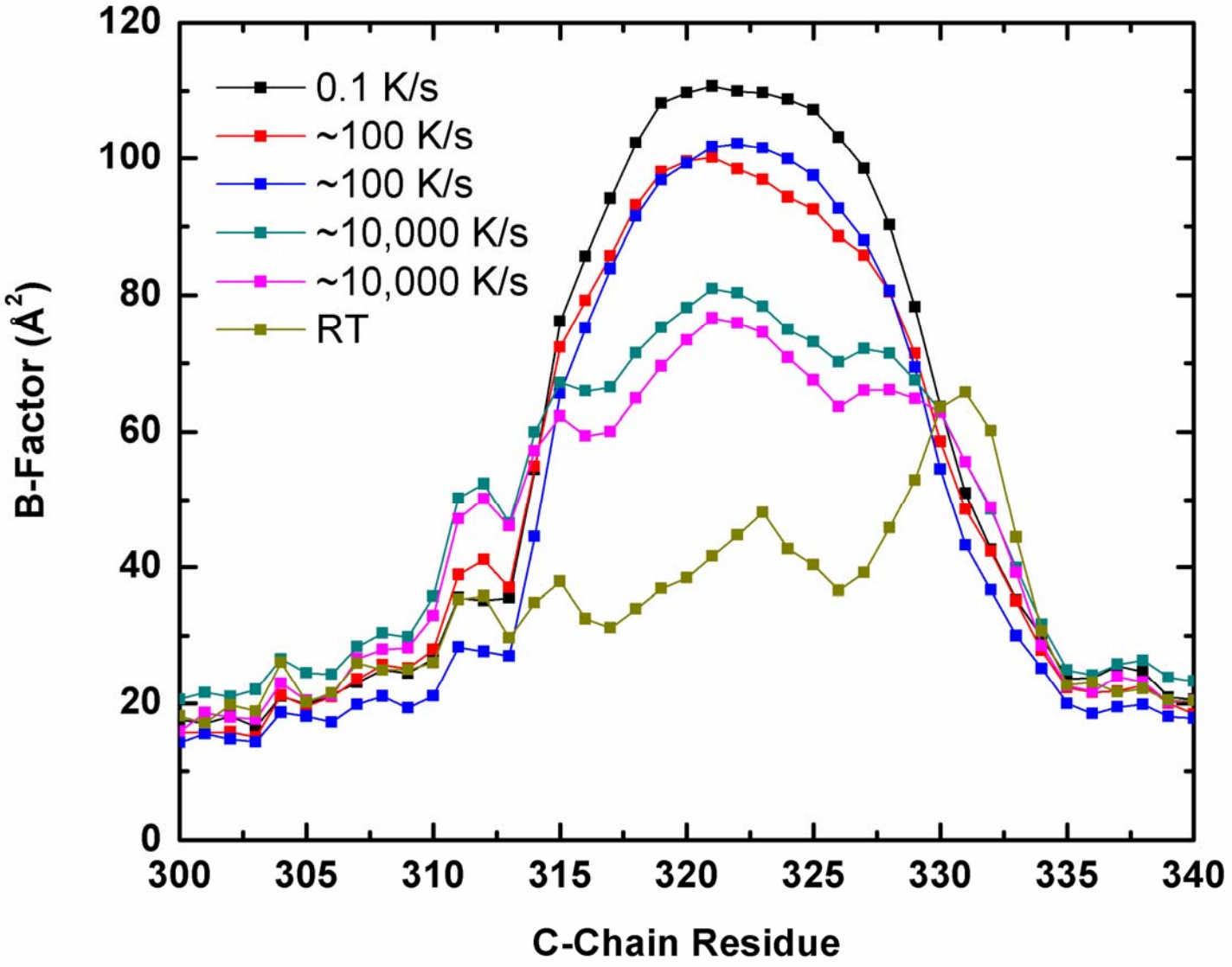
Structure versus temperature



Structure versus cooling rate



Structure versus cooling rate



Summary

Differential contraction of solvent and protein creates disorder on cooling.

Ultra-fast cooling using cold-gas removal

- allows most / all samples to be cooled without cryoprotectants.
- can more accurately capture the room temperature structure.

Ultra-slow cooling using external solvent removal

- allows many samples to be cooled without cryoprotectants.
- can yield improved low-temperature sample order.
- allows structural studies at arbitrary temperature; conformational states and energy landscapes.

Broader Implications

- **Cryopreservation: Art $\rightarrow$ Science**
- Long term storage of protein solutions
- Cryopreservation of cells (sperm, egg, stem, …) and tissues