Kinetic crystallography of a glycosyltransferase

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1. Principles of kinetic crystallography
What is kinetic protein crystallography?

- X-ray protein crystallography -> structure determination of proteins that are, in principle, in a resting state

- Proteins are often active in the crystalline state (reaction rate potentially affected)

- **Kinetic crystallography** = structure determination of **unstable species**:
  - Reaction intermediate states (unstable in time)
  - X-ray sensitive states (unstable in X-ray dose)

- Example of **enzymatic reaction**:

  \[ E + S \rightarrow ES \rightarrow EP \rightarrow E + P \]

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### Possible types of experiments

- **TRIGGERING**: by diffusion of substrate or irradiation with visible light/X-rays

- **SYNCHRONISATION**: the reaction needs to be initiated in all molecules at the same time – potential significant problem in soaking experiments

- **HOMOGENEITY**: the same proportion of molecules needs to be activated throughout the crystal – potential problem in crystals with high optical density in irradiation experiments

A crystal structure is the average of billions of molecules
Possible types of experiments

- **EQUILIBRIUM**: Initiate reaction at room temperature until equilibrium is reached (easiest)
- **TIME-RESOLVED**: genuine ‘live’ crystallography - very demanding on crystal properties: diffraction quality, robustness, repeatability of reaction
- **STEADY-STATE**: initiate reaction continuously and collect
- **INTERMEDIATE TRAPPING**: ‘Trigger-freeze’ and ‘Freeze-trigger’

‘Trigger-freeze’ trapping approach

- Initiate the reaction in the protein crystal at room temperature, for instance by soaking with substrate
  - **TRIGGER** the reaction
- Flash-cool crystals at different time points
  - **FREEZE** the reaction
- Solve X-ray structures
- Requirement: applicable to reactions needs to be slow enough compared to the speed of flash-cooling (t > ~10 seconds)
- Tricks can be used to slow down reactions: mutation, temperature, pH
‘Freeze-trigger approach’

- Block the whole system by flash-cooling the crystal at cryogenic temperature
  = FREEZE the reaction

- Initiate the reaction (by light, sometimes with X-rays)
  = TRIGGER the reaction

- Apply temperature profile to provide the system with some added free energy which will allow the reaction to go further

- Application to naturally photosensitive proteins, or proteins that are made artificially photosensitive = caged compounds

Use in crystallo spectroscopy to monitor reactions (when possible)

- **Goal**: focus and collect light on a ~10-100 μm diameter spot

- **How**: Magnifying objectives, optical fibers, precision translation stages, video camera, lasers

- For protein micro-samples (crystal / nL solution)

- Low and room temperature experiments (dehumidifier)

- **One thing to keep in mind**: Crystals are extremely concentrated in chromophores -> potential artefacts to look at:
  - Saturation of absorption peaks (+ loss of signal)
  - Apparent shift of fluorescence peaks
  - Difficulty of optimizing Raman signal
Experimental setup of the Cryobench at the ESRF

- Located next to beamline ID29

A **microspectrophotometer** consists of:
- a goniometer
- 4 objectives
- a video microscope
- a cryostream

> All point at the sample = **mimic of the structural biology beamline setup**

**Off-line setup**: different modes of operation

- **Absorption mode**
  - Transmission geometry (0°)

- **Fluorescence mode**
  - Reflection geometry (90°)

- **Raman mode**
  - Back-scattering geometry (180°)
Future automated setup

- Minidiffractionometer MD2-M
- Sample Changer SC3
- Objectives (x3) + Raman

2. Examples of kinetic crystallography experiments (coloured proteins)
2.1 A time-resolved diffraction experiment


- Photoactive yellow protein = small cytosolic photoreceptor thought to be responsible for the negative phototactic response of certain bacteria
- The reaction is repeatable
- Laser pulse = 35 or 100 ps
- Delay between laser and X-ray pulses = 0 to 20 ns

Performed at ESRF and APS

2.2 A (trivial) ‘trigger-freeze’ trapping experiment

Nakamura et al. Nature 2012 ‘Watching DNA polymerase η make a phosphodiester bond’

- Native polymerase co-crystallized with DNA and dATP without Mg^{2+}
- Soaking with Mg^{2+} = TRIGGER
- Reaction 20-100 times slower in crystals than in solution (reduced thermal motion)
- FREEZING after 40 to 300 s
2.3 An elaborated ‘trigger-freeze’ trapping experiment

Superoxide Reductase (SOR) converts the toxic superoxide ion into hydrogen peroxide

\[ O_2^{-•} + 2H^+ + SOR(Fe^{2+}) \rightarrow H_2O_2 + SOR(Fe^{3+}) \]

\[ O_2^{-•} + H^+ + Fe^{2+} \rightarrow Fe^{3+} \cdot OOH \cdot \rightarrow Fe^{3+} + H_2O_2 \]

**Trapping the proposed “hydroperoxo” Intermediate in crystallo?**

Puzzling crystallographic data

- **Experiment:**
  - Use of law of mass action
  - Point-mutant slowing down the reaction

Ir(IV)Cl₆ hexachloridate

Ir(IV)

Ir(III)

**FREEZE**

Intermediate

At 77K

SOR

Xtal

\[ O_2^{-•} + H^+ + Fe^{2+} \rightarrow Fe^{3+} \cdot OOH \cdot \rightarrow Fe^{3+} + H_2O_2 \]

- Limited crystallographic resolution (1.95 Å) (owing to H₂O₂ soaking)
- Unexplained electron density peaks, relevant intermediate species?
**In crystallo** Raman Spectroscopy of SOR

**Signature of iron-peroxide intermediate**

Pre-resonant Raman spectra @ 785nm

End-on Iron-peroxide intermediate

*Katona et al., Science (2007)*

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### 2.4 A ‘freeze-trigger’ trapping experiment

**Structural study of the photocycle of bacteriorhodopsin**

- Crystals flash-cooled in liq. N2 (**FREEZE**)
- T = 110 K, green light illumination + X-ray = steady-state
- T = 170 K, green + red light (**TRIGGER**) -> 100 K = Freeze-trigger
- Build-up of intermediates verified by absorption spectra

**K-state structure**

**L-state structure**

*Edman et al., Nature 1999*

*Royant et al., Nature 2000*
3. Studies of a human glycosyltransferase by kinetic crystallography approaches

3.1 Glycosyltransferases

- Glycosyltransferases (GTs) catalyses the transfer of a sugar residue from a donor to a wide range of specific molecules
- Two mechanisms of transfer:
Mechanism of retaining glycosyltransferases?

**Mechanism 1**

Covalent intermediate

**Mechanism 2**

Oxocarbenium intermediate

Human blood Group synthases GTA and GTB

- ABO is the most important blood group system in transfusion medicine

UDP-GalNAc GTA

H-disaccharide antigen (linked to protein or lipid)

O(H) Precursor

UDP-Gal GTB

Blood Group A

Blood Group B
3.2 TRIGGER-FREEZE approach with substrate acceptor

Enzymatic reaction

If reaction slow enough

Experiment:

TRIGGER : soaking with = UDP-Gal or UDP-GalNAc in presence of = H2O or glycerol

FREEZE after a few minutes to several hours

TRIGGER-FREEZE approach with UDP-Gal

UDP-Gal present at 100% after 3 hours of soaking
at 75% after 6 hours of soaking

After overnight soaking, crystals do not diffract any more
-> reaction too slow for our experimental conditions
TRIGGER-FREEZE approach with UDP-GalNAc

No structure ever of a GT in complex with UDP-GalNAc alone, presumably because the reaction is too fast.

After 90 sec

After 4 min

UDP only in active site

50% UDP + 50% UDP-GalNAc!!

After 24 min of soaking with UDP-GalNAc: mixture of various states

- Three structures can be tentatively modelled
- For longer times, UDP in active site
- Fast reaction, which must be slowed down by substrate inhibition
3.3 Principle of the caged compound approach
FREEZE - TRIGGER

Caged compounds used in the study

NPE cage

NVOC group

CC01

CC02

CC03

CC04

CC05
Photolysis at 100 K monitored by UV-vis absorption

In solution

In crystals

Structure of the cage in CC01 bound to the enzyme

- Obtained in presence of glycerol as cryoprotectant
- Two space groups
- At least three conformations – cage not well-resolved

Space group $P2_12_12_1$

Space group $C22_1$
Structure of the cage in CC02 bound to the enzyme

- Obtained in presence and absence of glycerol as cryoprotectant

Summary of caged compound results

<table>
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<th>CC</th>
<th>Caging group</th>
<th>Inhibition effect</th>
<th>Photolysis efficiency</th>
<th>Localization in structure</th>
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