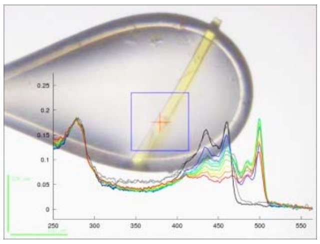

Kinetic crystallography of a glycosyltransferase

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ESRF

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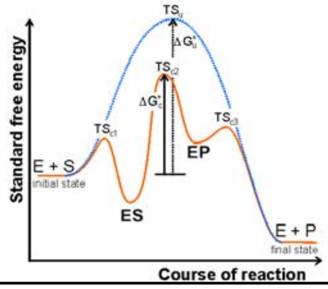
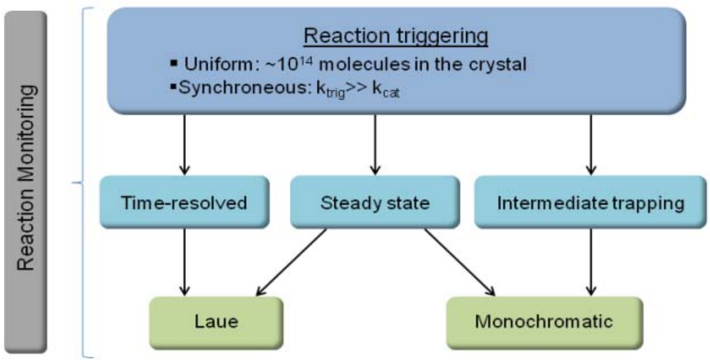
1. Principles of kinetic crystallography

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**: $E + S \rightleftharpoons ES \rightleftharpoons EP \rightleftharpoons E + P$



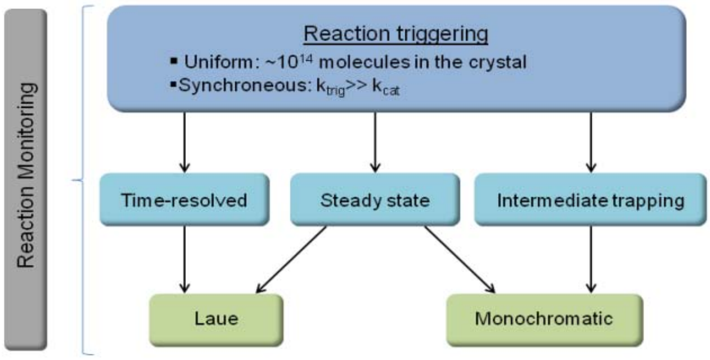
Possible types of experiments



- **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



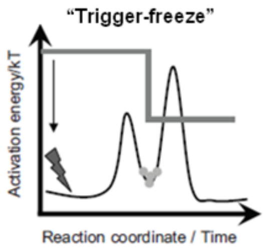
• **EQUILIBRIUM:** Initiate reaction at room temperature until equilibrium is reached (easiest)

• **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

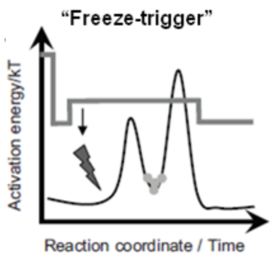
= **FREEZE** the reaction

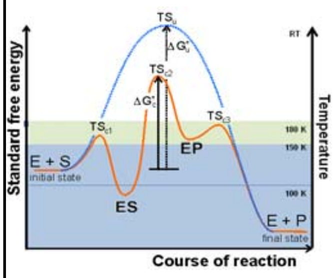
• Solve X-ray structures

•Requirement: applicable to reactions needs to be slow enough compared to the speed of flash-cooling (t > ~10 seconds)

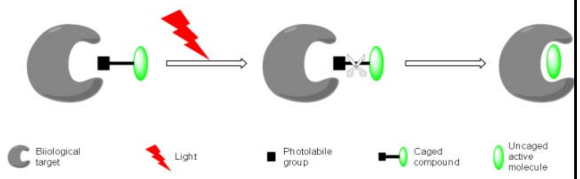
• Tricks can be used to slow down reactions: mutation, temperature, pH

‘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**



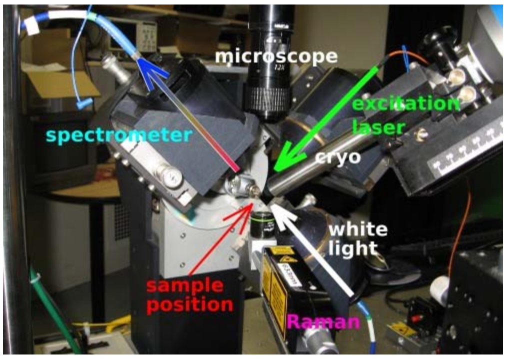
Use *in crystallo* spectroscopy to monitor reactions (when possible)

- **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

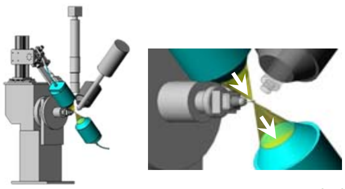
A **microspectrophotometer** consists of
- a goniometer
- 4 objectives
- a video microscope
- a cryostream



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

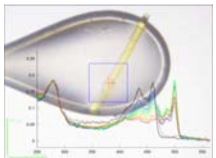
Off-line setup: different modes of operation

Absorption mode

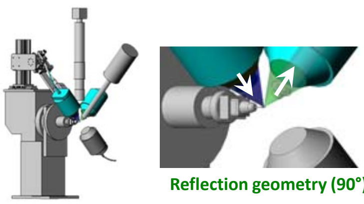


Transmission geometry (0°)

Series of absorption spectra

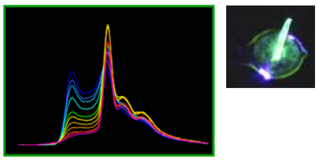


Fluorescence mode

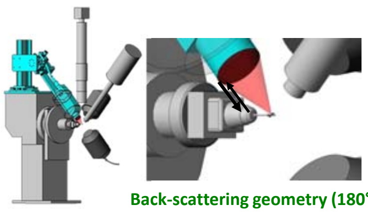


Reflection geometry (90°)

Series of fluorescence emissions spectra

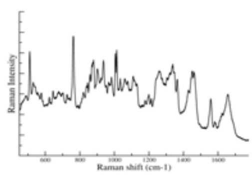


Raman mode

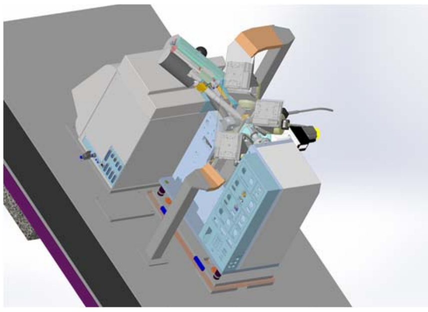


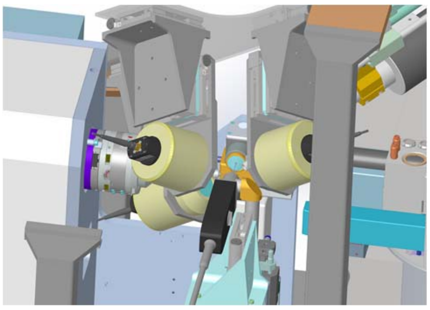
Back-scattering geometry (180°)

Raman spectrum



Future automated setup





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

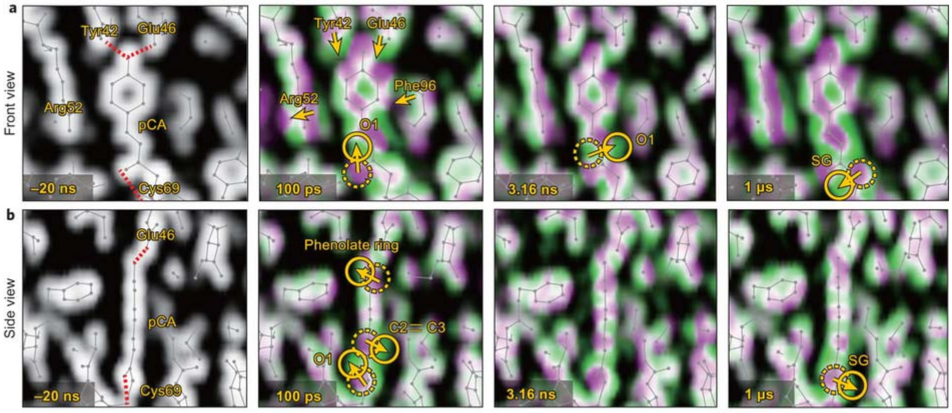
2. Examples of kinetic crystallography experiments (coloured proteins)

2.1 A time-resolved diffraction experiment

Jung *et al. Nature Chemistry* 2013 'Volume-conserving *trans–cis* isomerization pathways in photoactive yellow protein visualized by picosecond X-ray crystallography'

- 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

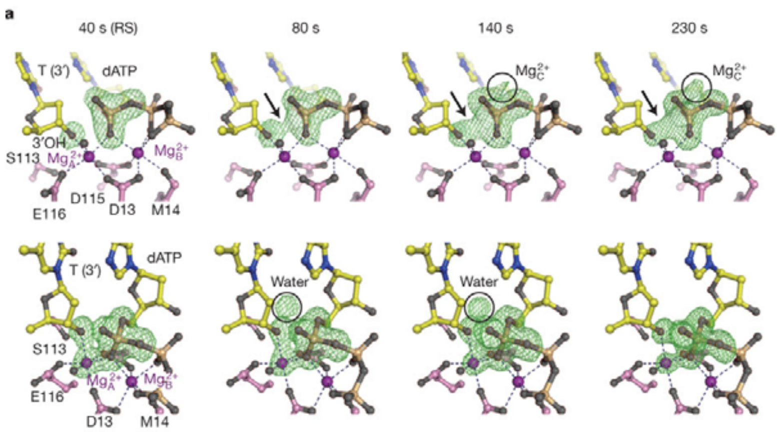
Performed at ESRF and APS



2.2 A (trivial) 'trigger-freeze' trapping experiment

Nakamura *et al. Nature* 2012 'Watching DNA polymerase η make a phosphodiester bond'

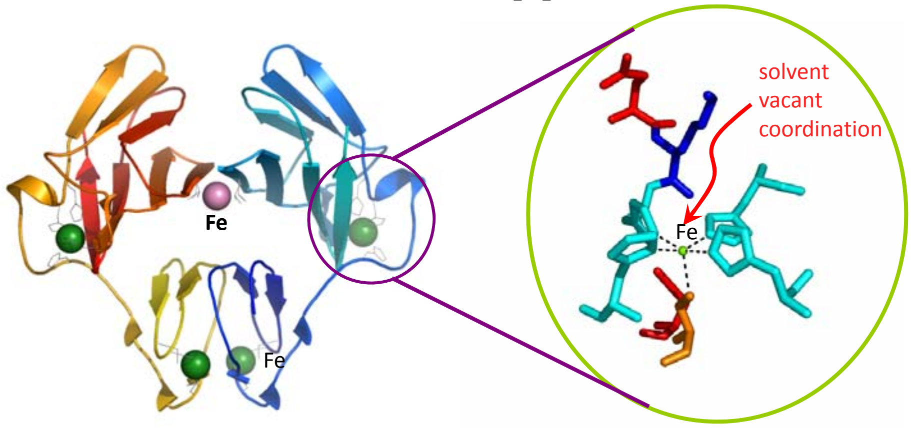
- Native polymerase co-crystallized with DNA and dATP without Mg^{2+}
- Soaking with Mg^{2+} = **TRIGGER**
- Reaction 20-100 times slower in crystals than in solution (reduced thermal motion)
- **FREEZING** after 40 to 300 s



2.3 An elaborated 'trigger-freeze' trapping experiment

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

$$O_2^{\bullet-} + 2H^+ + SOR(Fe^{2+}) \rightarrow H_2O_2 + SOR(Fe^{3+})$$



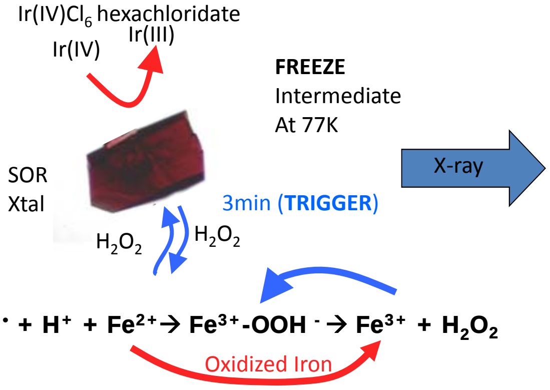
$$O_2^{\bullet-} + H^+ + Fe^{2+} \rightarrow Fe^{3+}\text{-}OOH^- \xrightarrow{+H^+} Fe^{3+} + H_2O_2$$

Trap the proposed "hydroperoxo" Intermediate *in crystallo ?*

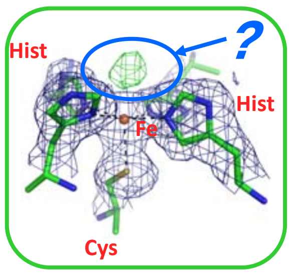
Puzzling crystallographic data

Experiment:

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

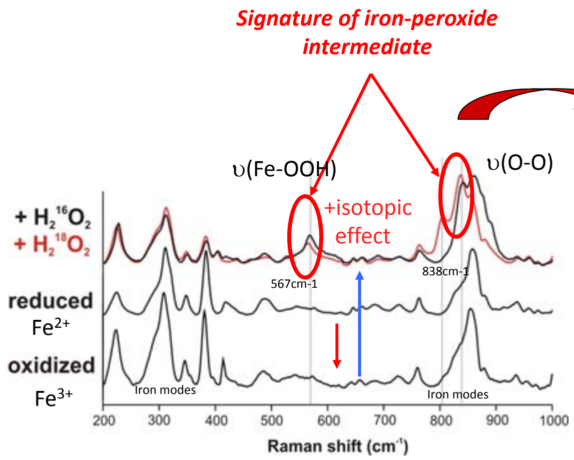


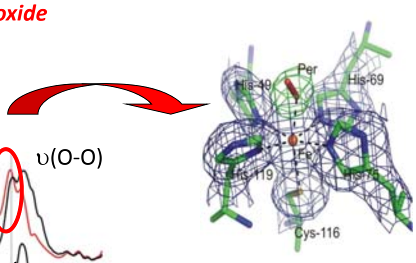
$$O_2^{\bullet-} + H^+ + Fe^{2+} \rightarrow Fe^{3+}\text{-}OOH^- \rightarrow Fe^{3+} + H_2O_2$$



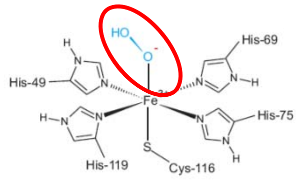
- Limited crystallographic resolution (1.95 Å) (owing to H_2O_2 soaking)
- Unexplained electron density peaks, relevant intermediate species?

In crystallo Raman Spectroscopy of SOR





Pre-resonant Raman spectra @ 785nm

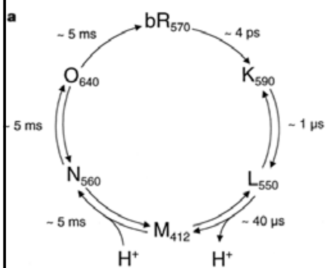


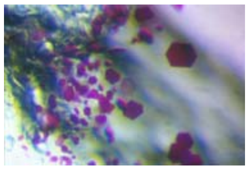
End-on Iron-peroxide intermediate

Katona *et al.*, *Science* (2007)

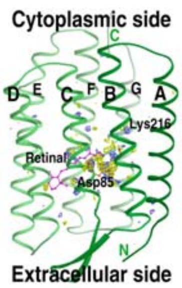
2.4 A 'freeze-trigger' trapping experiment

Structural stud of the photocycle of bacteriorhodopsin



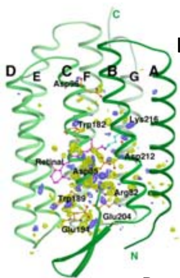


- Crystals flash-cooled in liq. N2 (**FREEZE**)
- T = 110 K, green light illumination + X-ray = steady-state
- T = 170 K, green + red light (**TRIGGER**) -> 100 K = Freeze-trigger
- Build-up of intermediates verified by absorption spectra



K-state structure

Edman *et al.*, *Nature* 1999



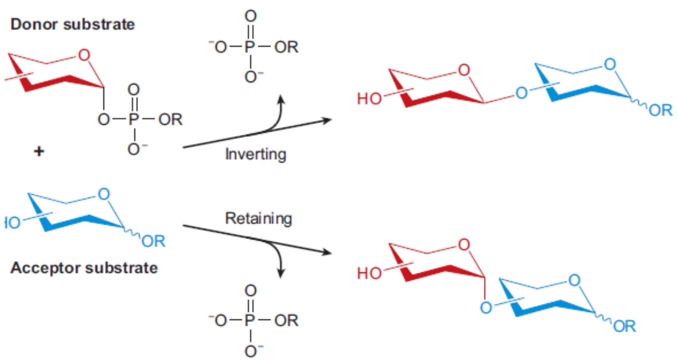
L-state structure

Royant *et al.*, *Nature* 2000

3. Studies of a human glycosyltransferase by kinetic crystallography approaches

3.1 Glycosyltransferases

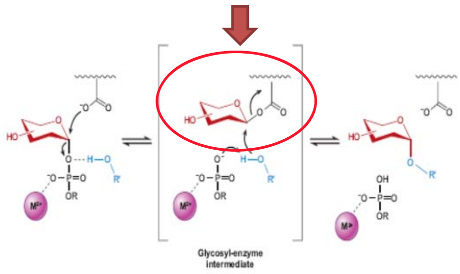
- Glycosyltransferases (GTs) catalyses the transfer of a sugar residue from a donor to a wide range of specific molecules
- Two mechanisms of transfer:



Mechanism of retaining glycosyltransferases?

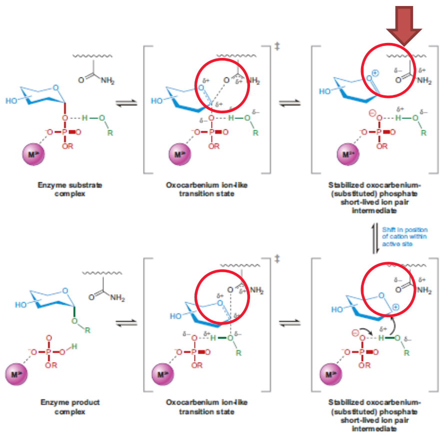
Mechanism 1

Covalent intermediate



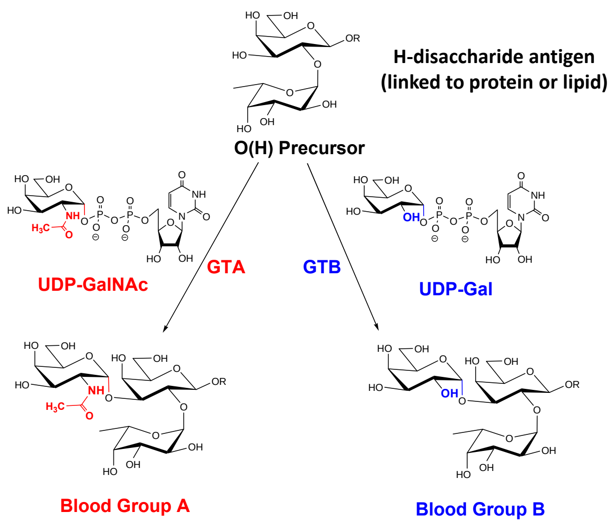
Mechanism 2

Oxocarbenium intermediate



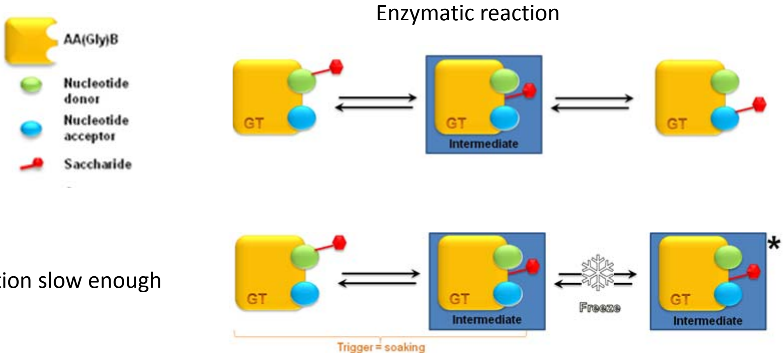
Human blood Group synthases GTA and GTB

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



3.2 TRIGGER-FREEZE approach with substrate acceptor

Enzymatic reaction



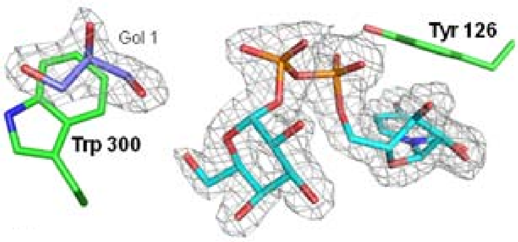
If reaction slow enough

Experiment:

TRIGGER : soaking with (green) = UDP-Gal or UDP-GalNAc in presence of (blue) = H2O or glycerol

FREEZE after a few minutes to several hours

TRIGGER-FREEZE approach with UDP-Gal

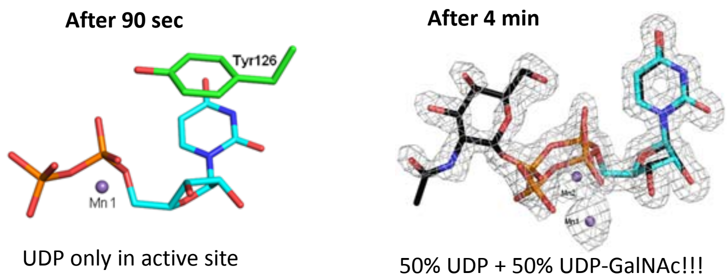


UDP-Gal present at 100% after 3 hours of soaking
at 75% after 6 hours of soaking

After overnight soaking, crystals do not diffract any more
-> reaction too slow for our experimental conditions

TRIGGER-FREEZE approach with UDP-GalNAc

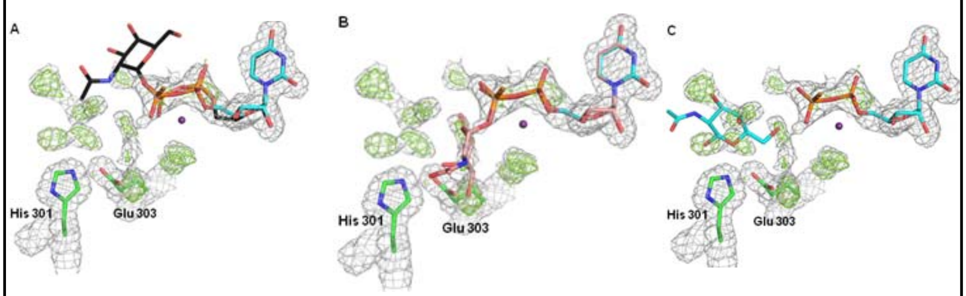
No structure ever of a GT in complex with UDP-GalNAc alone, presumably because the reaction is too fast



UDP only in active site

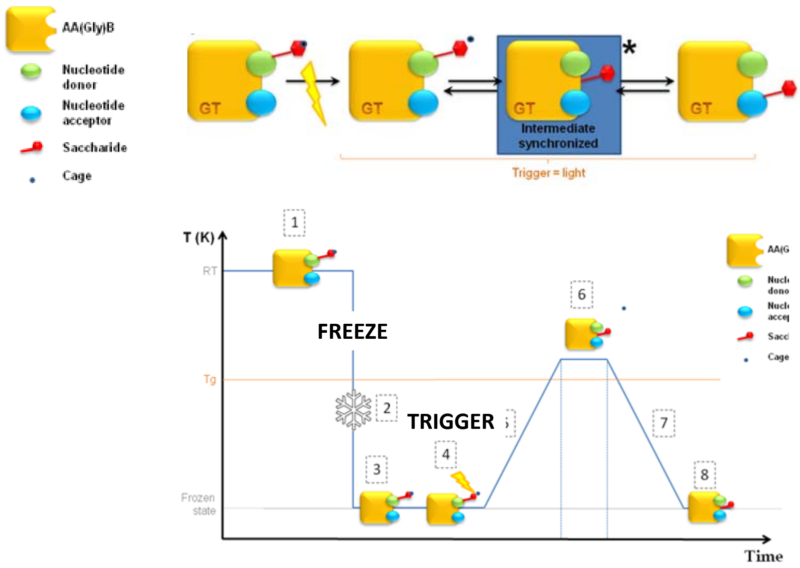
50% UDP + 50% UDP-GalNAc!!!

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

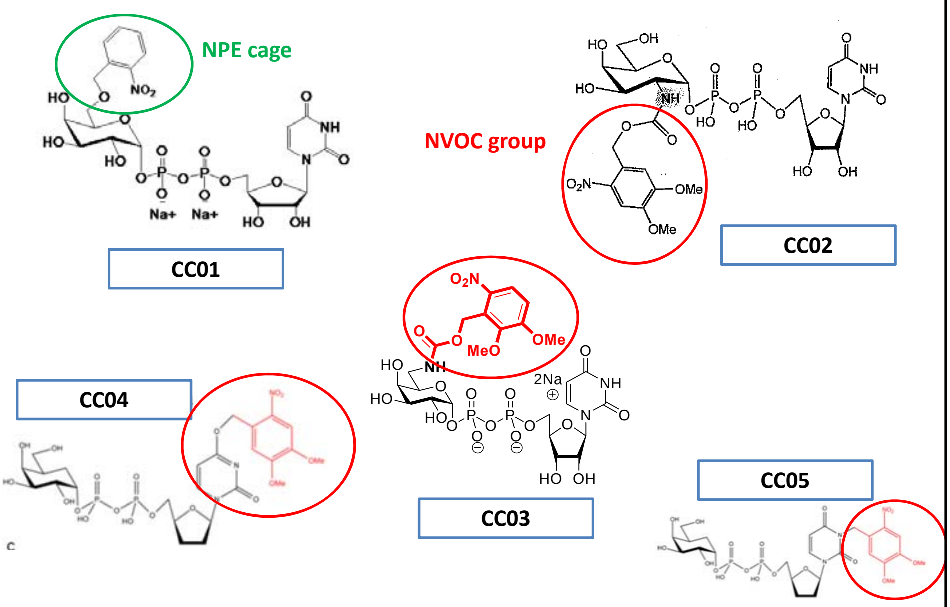


- Three structures can be tentatively modelled
- For longer times, UDP in active site
- Fast reaction, which must be slowed down by substrate inhibition

3.3 Principle of the caged compound approach FREEZE - TRIGGER

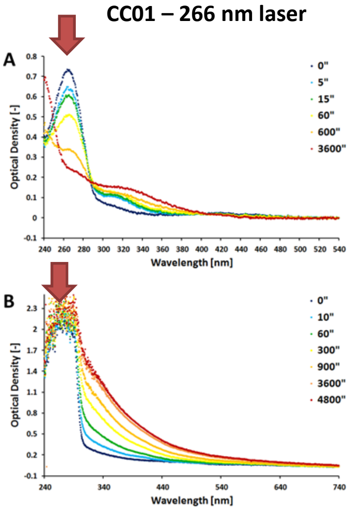


Caged compounds used in the study



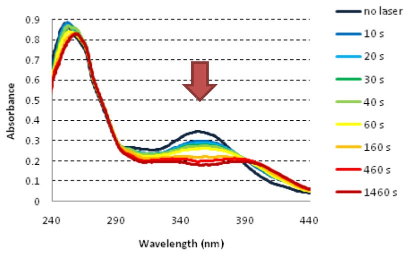
Photolysis at 100 K monitored by UV-vis absorption

CC01 – 266 nm laser

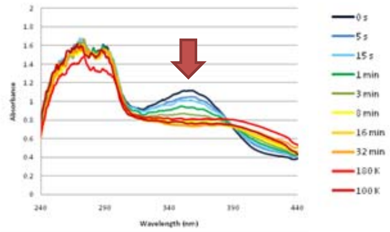


CC01 – 355 nm laser

In solution

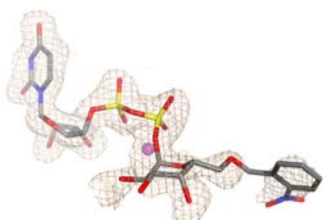


In crystals

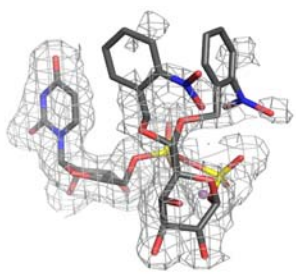


Structure of the cage in CC01 bound to the enzyme

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



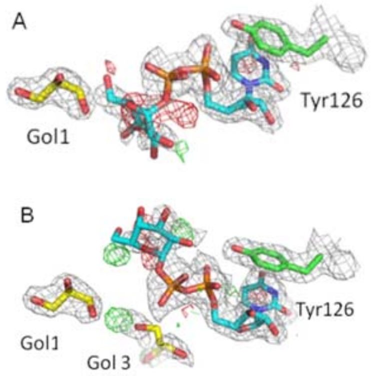
Space group $P2_12_12_1$



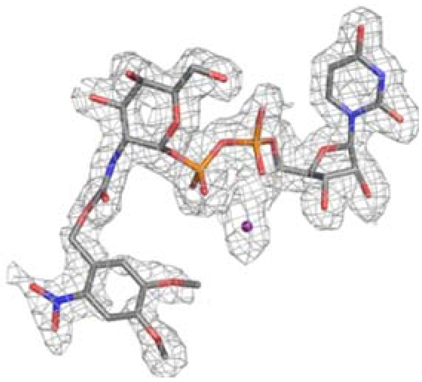
Space group $C222_1$

Structure of the cage in CC02 bound to the enzyme

- Obtained in presence and absence of glycerol as cryoprotectant



With glycerol


Without glycerol

Summary of caged compound results

CC	Caging group	Inhibition effect	Photolysis efficiency	Localization in structure	Conformation of sugar
CC01	NPE	✓	✓	✓	Tucked-under or solvent A
CC02	NVOC	✓	✓	✓	Solvent B
CC03	NVOC	✓	*nd*	✗	?
CC04	NVOC	✓	*nd*	✗	Tucked-under
CC05	NVOC	✗	*nd*	✗	✗

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Christelle Breton
Anne Imberty
Serge Pérez

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