ESRF	**Experiment title:** Towards "in cell" structural biology: studying bacteriorhodopsin at work inside intact Halobacterium Salinarum cells.	**Experiment number**: SC-3400
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Report:

Aim of the present experiment was to study the structural dynamics of bacteriorhodopsin (BR) *in vivo* with time-resolved wide-angle X-ray scattering (TR-WAXS) [1], and compare the results with those obtained on BR *in vitro*. BR is a proton pumping protein contained in the purple membrane (PM) patches of the *Halobacterium Salinarum* (HS) cells, a unicellular organism of the class of halophilic archea [2]. This class of bacteria live in high temperature saturated salt brines that are exposed to bright sunlight. BR converts the energy of green light (500-650 nm) into a proton gradient, which in turn is used for ATP production.

At difference with previous TR-WAXS investigations on BR [3], during the present experimental session we have mainly investigated the following two kind of samples containing BR: ***suspensions of packed HS intact cells*** and **concentrated solutions of PM fragments**. One of the relevant difference between these two samples is that BR contained in PM fragments does not experience the high salt gradient typical of its physiological environment. BR was photoexcited with circularly polarized nanosecond laser pulses at 532 nm (EKSPLA laser). Samples have been loaded in a custom Mylar sample holder that we have specifically designed and used for TR-WAXS measurements on cell suspensions [4]. The green laser pulses hit the sample holder bottom surface, while X-ray pulses (at 90 degrees from the laser) probed the photoexcited sample volume. Visible light at 532 nm is mainly absorbed by BR molecules and triggers a series of conformational changes leading to the transfer of a proton from the inside of the HS cell to the outside.

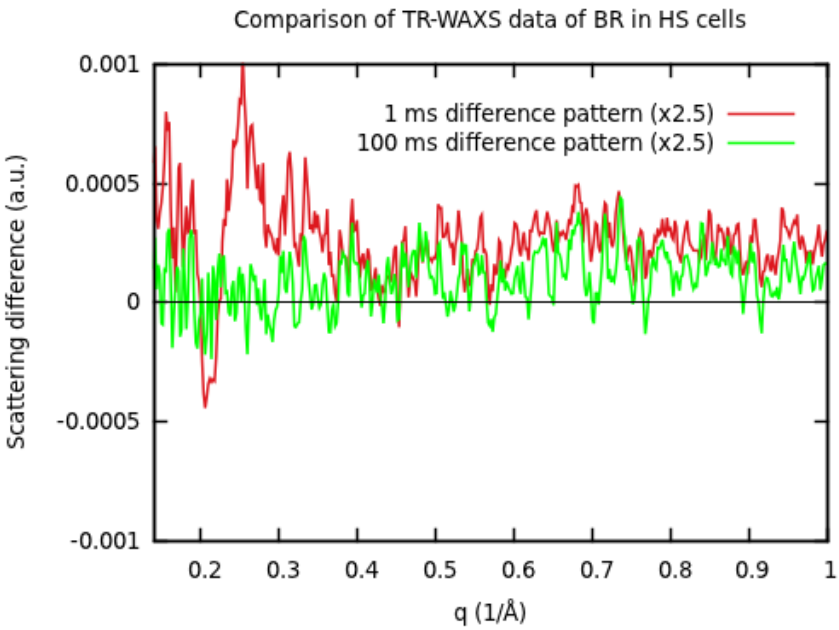
Three main experimental issues arose while measuring TR-WAXS data on intact HS cells:

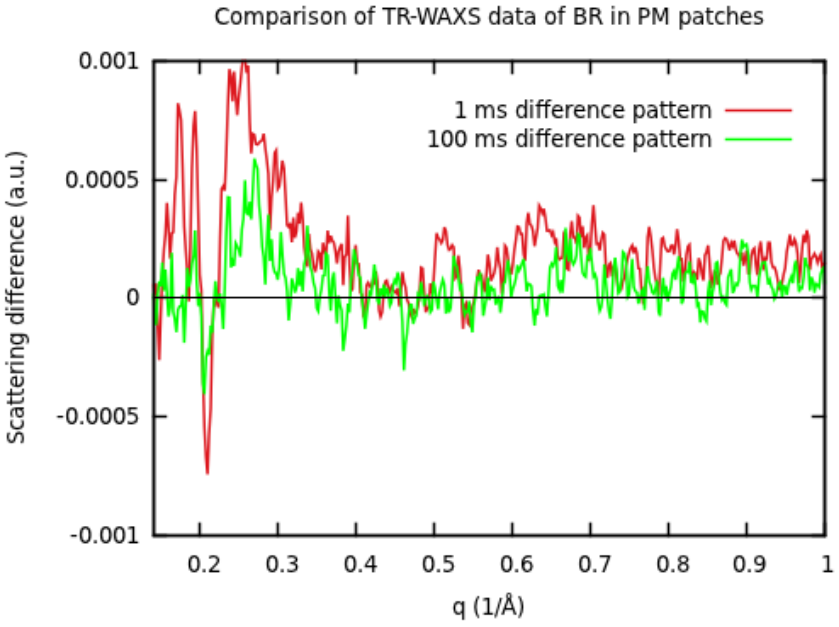
1) light scattering reduces the penetration depth of the photoexciting laser beam;
2) the reaction kinetics of BR depends strongly on laser energy/repetition rate;
3) samples were contaminated with salt microcrystal impurities.

Because of experimental issue 1, we had to optimize the vertical position of the sample (relatively to the X-ray beam vertical position) so that X-ray pulses hit the sample at 200 μm from its bottom surface. The position of the sample holder bottom edge was carefully determined with a vertical scan of the X-rays transmission before each new acquisition.

In view of experimental issue 2, during the first two days we have also performed a laser energy titration and several test measurements at different laser repetition rates (10 Hz, 5 Hz, and 2 Hz) in order to find the best experimental conditions to measure reliably the BR photocycle in an amount of time compatible with the typical available beam time. Note that during data acquisition the sample holder was translated back and forth so that each successive X-ray/laser pulse hit a fresh portion of the sample (the effective repetition rate of the experiment was higher than the laser repetition rate).

Experimental issue 3 arose because of the high salt concentration of the samples (this last issue holds for the concentrated solutions of PM patches as well). HS cells have been dissolved in a 4 M basic salt solution, which mimics the typical environment where this kind of cells live. Moreover, about 150 mM of phosphate buffer was added to all the samples in order to minimize the effect of X-ray radiation damage (water radiolysis is expected to induce significant pH changes in unbuffered solutions). This last precaution turned out to be a bad idea since part of the phosphate buffer (added to a solution already close to salt saturation) precipitated and gave rise to phosphate microcrystals, which were clearly visible in CCD images as Bragg peaks. In view of the strong heterogeneity characterizing these kind of highly concentrated samples, the angular distribution of these phosphate microcrystals was not isotropic and differ from one region of the sample to another, thus complicating significantly the data reduction and averaging.





In spite of these experimental issues, we have been able to collect TR-WAXS data both on PM fragments and HS cells. Although the signal-to-noise ratio of the data is low, a difference in the kinetics of the two samples is clearly seen by comparing the TR-WAXS difference patterns measured after 1 and 100 ms from photoexcitation (see the two figures on the right). While BR in HS cells has completed its photocycle already within 100 ms (top panel), a difference signal is observed at the same time delay in the case of the solution of PM patches.

The 4-bunch mode at which the ESRF synchrotron was running during our experiment is not ideal for measuring kinetics at long time delays. Indeed, a uniform (or 7/8+1) mode would have allowed us to have a much higher number of X-ray photons per chopper opening time, thus speeding up the acquisition time.

Bibliography

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