Experiment SC-3145 / May 2011

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Aim of the experiment:

For this experiment, our aim was to take advantage of the temporal and spatial resolution that can be achieved with the Time-Resolved Wide-Angle X-Ray Scattering (TR-WAXS) setup at beamline ID-09B to better understand the gating dynamics of *Gloeobacter violaceus* Ligand-Gated Ion Channel (GLIC), a bacterial homologue belonging to the Cys-loop receptors family.

It has been shown that GLIC is a proton-gated ion channel[1]. At neutral pH, the homopentameric protein remains "closed" and impairs the flow of ions through the membrane into which it is embedded. As the pH is lowered, it starts to "open" and to be permeant to cations (Na/K).
A high-resolution structure of the protein in its "open" conformation has been solved and described[2], but we are still lacking an atomic representation of its "closed" form. Also lacking, despite our efforts in modelling it[3], is a clear representation of its gating mechanism, that is a structural representation of the sequence of events that leads the protein from its closed to open conformation, in reaction to an increase in proton-concentration.

Andersson and coworkers have recently shown that it was possible to follow, in solution, the conformational dynamics of a membrane protein[4], at intermediate temporal (sub-microsecond to millisecond) and spatial (at least at the level of the secondary structure) resolutions, when there is a way to trigger the reaction with light.

In the case of GLIC, the change in conformation can be triggered by a rapid change in pH. Abbruzzetti and coworkers have described a chemical compound that would be eager to perform this role: the 1-(2-nitrophenyl)ethyl sulfate (NPE or caged sulfate). After laser photolysis, this photoactivatable compound is able to quantitatively release protons in water at neutral pH with rates in the 10^7-10^8 s^{-1} range[5].

The basic principle of our experiment was then to expose a solution of GLIC with the caged sulfate, buffered at neutral pH, to a laser flash, and to successively record WAXS spectrum at variable time delays. The laser would create a pH jump in the sample and trigger the conformational change, that we would then be able to monitor with X-rays. The setup at ID-09B would allow the two incoming pulses to cross in the same sample volume, with tunable delays. Another buffer (acetate) was present in the solution to ensure a final pH in the 4-5 range.

In the following, we shortly describe the outcome of this experiment, and explain why it did not bring the results originally expected. We also propose solutions to the problems encountered, in the perspective of performing a new experiment.

Summary of the experiment:

. Preliminary:

In a previous experiment performed on the SWING beamline at SOLEIL (data not published), we addressed whether or not the protein is still structurally sensitive to a pH change when solubilized in detergents, and if it is, whether the technique of SAXS allows us to detect it. To do so, we recorded SAXS profiles of the protein at two pH, acidic and neutral. At both pH, a set of SAXS profiles were recorded just after the sample eluted through a pre-equilibrated column. The contribution of the buffer to the scattering was recorded at the end of the elution, and substracted to the sample profile. The presence of free micelles or protein-bound detergents was followed along the elution through recording of the refractometric index (RI).

The RI (data not shown) was negligible everywhere but at the elution peak, giving indication that all detergents were either bound to the protein, or free in solution (i.e. not in micelles). We are then confident that the substraction of the buffer contribution to the scattering is correct.

The average (and normalized with respect to the value of the intensity at q=0) profiles are displayed on Fig.1, as well as the difference between the profile obtained at acidic pH and the one obtained at neutral pH, weighted by the standard deviation at neutral pH.
This way to weight the difference was chosen for two reasons: it is q-independant and it allows to locate one profile on the distribution of the other.

Altogether, this data clearly shows that the scattering of the "acidic" sample is different from the "neutral" one. Whether this difference is due to a conformational change of all the proteins in the sample, or only the reflect of a partial displacement of the equilibrium between two conformations still remains unclear.

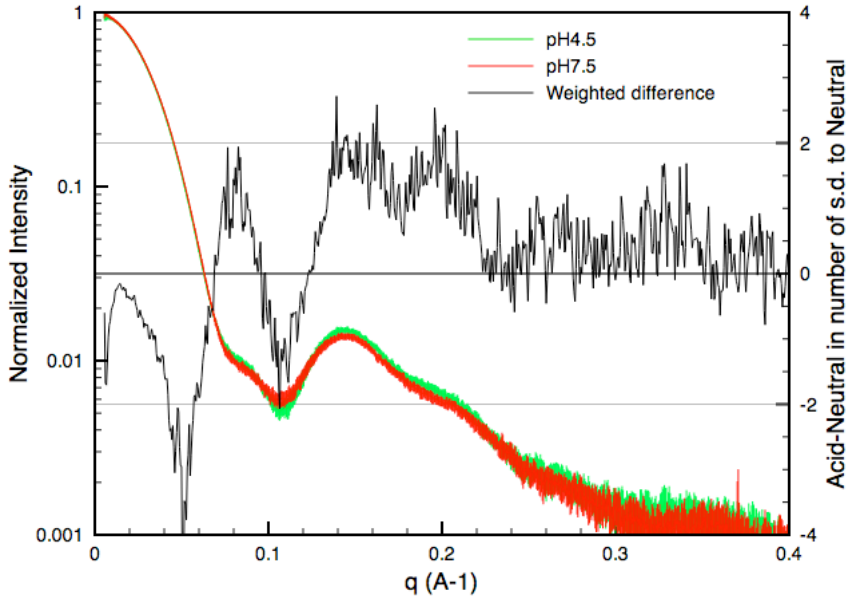

Figure 1: Experimental SAXS profiles averaged around the elution peaks, at neutral (red) and acidic (green) pH. The difference between both profiles is shown as the neutral one substracted to the acidic one, weighted by the standard deviation at neutral pH.

. Preparation:

The first step in the experiment SC-3145 consisted in producing a sufficient quantity of purified GLIC. Despite a well-optimized expression/purification protocol, we had to face two problems: the low production yield of GLIC, and the presence of a contaminant (maltoporin) even after stringent purification.

Considering that recording WAXS profiles necessitated quite high concentration of protein (in order to maximize the signal-to-noise ratio), that the caged sulfate photolysis was irreversible (meaning that the samples were not reusable for further measurements), and the timing constraint for the experiment, the low-production yield forced us to concentrate our efforts for more than two months on the protein's production only, at a much higher cadence (and cost) than usual.

In addition, the presence of the contaminant lead us and our collaborators to design and test a construct knocking-out the production of maltoporin, in a limited period of time.

Both problems were overcome, at the expense of additional tests that we would have liked to perform prior to the experiment, such as possible undesired effects of the caged sulfate on GLIC's function in cells, and in solution.

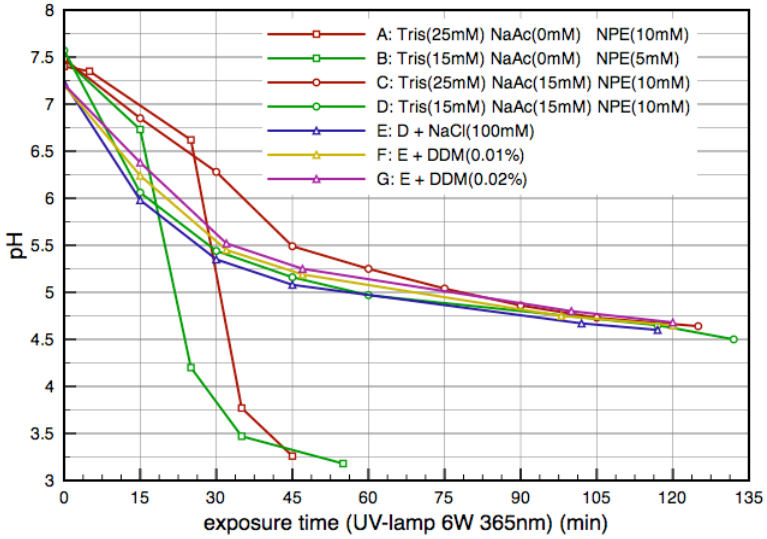

Figure 2: pH jump monitoring in various buffer conditions.
Each solution was first set to a pH of approx. 7.5 then exposed to a UV-lamp (6 W-365 nm) in a quartz cell, and the pH was followed in time using a small diameter pH-meter. All solutions contain 10 mM NPE, except solution B (5 mM). Solutions A and B only contain Tris buffer (25 mM and 15 mM, resp.), while all other solutions contain both Tris and Na-Acetate buffer. Solutions D,E,F,G contain 15 mM of each buffer. Solution E,F,G contain 100 mM NaCl. Solution F and G contain DDM detergent at a concentration of one and two CMC, respectively.

Nevertheless, we were able to test the ability of the caged sulfate (NPE) to generate a pH jump from approx. 7 to approx. 4.5 in a buffered solution similar to the one we would use during the experiment (see Fig.2). From this experiment, we see that the presence of the second buffer (Na-Acetate) indeed prevented the pH to go below the target pH value of 4.5 (see curves C,D,E,F,G versus A,B), and that Tris buffer, if in higher concentration, would slow down the pH-jump. Also interesting in this experiment is the observation that the presence of salt (NaCl) and detergent (DDM) had almost no impact on the pH-jump. The protein was not present in the solution (too costly), but we tested on-site during the experiment the generation of the same pH jump using pH-paper.

. Actual experiment:

At our arrival on the beamline, the UV-laser was set up, and all the optics aligned by the local team. This step is crucial since parameters such as laser-Xray overlap, time-delay, laser power, synchronisation, etc. are of fundamental importance for this kind of study, and the experience of the local team on these aspects was of great value to us.

The only point that was let to our care was the sample preparation and positioning on the beamline. We took great benefit of these four days of experiment to start optimizing the sample holding. We realized the sample should not be sealed in a capillary, but rather be circulated through it (see Fig.3) thus allowing to keep the same quartz capillary for many measurements, and avoiding possible non-desired scattering differences due to the varying position of the capillary with respect to the beamline(s). Not to mention that aligning a capillary can be time-consuming.

Once the sample-filled quartz capillary is aligned, a typical data collection procedure would consist in taking dozens of WAXS snapshots along the capillary (the translation step size and speed having been first optimized). The cumulated signal on the CCD detector is then stored (as a "off" image) while the capillary is moved back to its initial position. Then the same translation procedure is re-iterated and 6 "off" images are collected. A 7th iteration is performed, but this time every WAXS snapshot is preceded by a UV-laser flash at a given time delay, and the "on" image is stored (Since the photolysis is irreversible, it was not possible to collect more than one "on" image per sample).

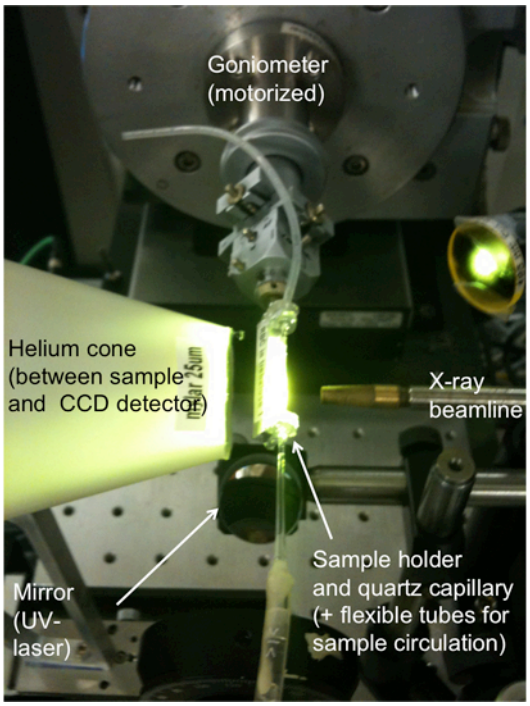

Figure 3: Sample environment for data collection.
The sample holder was attached to the goniometer head, a capillary inserted through its pinholes and glued at both ends. Flexible tubes are tightly attached at both ends of the capillary. The capillary is then aligned perpendicularly to both the X-ray and UV-laser beamlines (the UV-laser ray being directed vertically with a mirror, while the X-ray is directed horizontally) with the goniometer. A Helium-filled cone is positioned between the capillary and the CCD detector (in order to minimize the contamining scattering from the air). A syringe (not present on the picture) is inserted at one end of the flexible tube for sample circulation. Note that it would be possible to motorize it.

In our case, considering that it takes a few microseconds for the protein to change its protonation state, we decided that it was not necessary to probe time delays below this limit, although the beamline could afford it. One positive consequence is that we could use X-ray multi-pulses, hence rising the photon count on the CCD, without using too much protein material.

A few other parameters were optimized, such as the laser power (finally set to 350 µJ), or the concentration of the caged proton (finally set to 10 mM), but to our great surprise, we were only able to detect even little changes in the scattering signal after flash photolysis, for time delays ranging from 50 µs to 10 ms.

As an illustration, we present on Fig.4 the results obtained for a time delay of 50 µs on our purest protein sample. As we see on this figure, if there is a difference between both profiles, it is below one standard deviation of the "Off" profile for most of the profile. The difference can then hardly be attributed to the protein conformational change (even though the difference measured at Q ~ 0.1 $Å^{-1}$ is qualitatively consistent with what we had measured in SAXS). It seems unlikely that the difference would be greater if we increased the statistics (that is, averaged over more measurements).

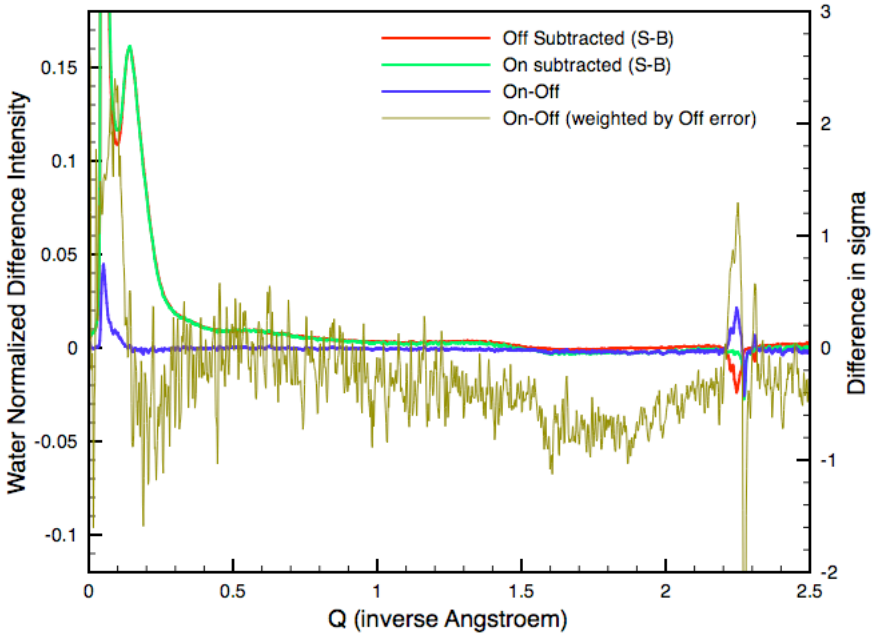

Figure 4: Profiles obtained before (red) and after (green) flash photolysis, for a time delay of 50 microseconds. The "Off subtracted" profile (in red) is the difference between the normalized average of 6 images collected on the protein sample and the buffer sample before photolysis. The "On subtracted" profile (in green) is the difference between the image collected on the protein sample and the buffer sample 50 µs after flash photolysis. The "On-Off" profile (in blue) is the absolute difference of the two former profiles, while the "On-Off (weigthed by Off error)" profile (in dark yellow) is the difference weighted by the standard deviation computed on the 6 "Off" images.

This result suggests that despite the pH jump, the Closed-to-Open equilibrium is not dramatically displaced toward the "Open" state at acidic pH. Or if it is, much less than during our previous SAXS experiment.
"Something" in the solution displaces the equilibrium toward one state or the other, at all explored pH.

. Control experiments after the on-site measurements:

Even though we did not know that at the time of the experiment, it is well known in the neurobiologist community that caged-compounds can act as inhibitors on channel receptors (even though it is not true for all caged-compounds on all receptors). To test the hypothesis of our caged sulfate interfering with GLIC, before and/or after photolysis, we were helped by Marie Prevost from the group of Pierre-Jean Corringer at Institut Pasteur. These experiments were conducted in July 2011.
In brief, what was done is the following: two solutions of NPE were prepared, at both pH. Each solution was then split into two, one being kept, the other being exposed to UV light for a sufficient period of time. The pH of all solutions was then reset to its initial value, providing us with two samples at neutral pH, one before and one after photolysis, and two samples at acidic pH, one before and one after photolysis.

As depicted and summarized in Fig.5, this control experiment shows that the caged sulfate and its products of photolysis inhibit to a significant extent the activation of GLIC by protons.

This finding suggest that during the TR-WAXS experiment, the caged-proton, while necessary to generate a pH jump that would displace the equilibrium from the Closed to the Open conformation, prevented such a displacement to occur, at least to a certain extent.

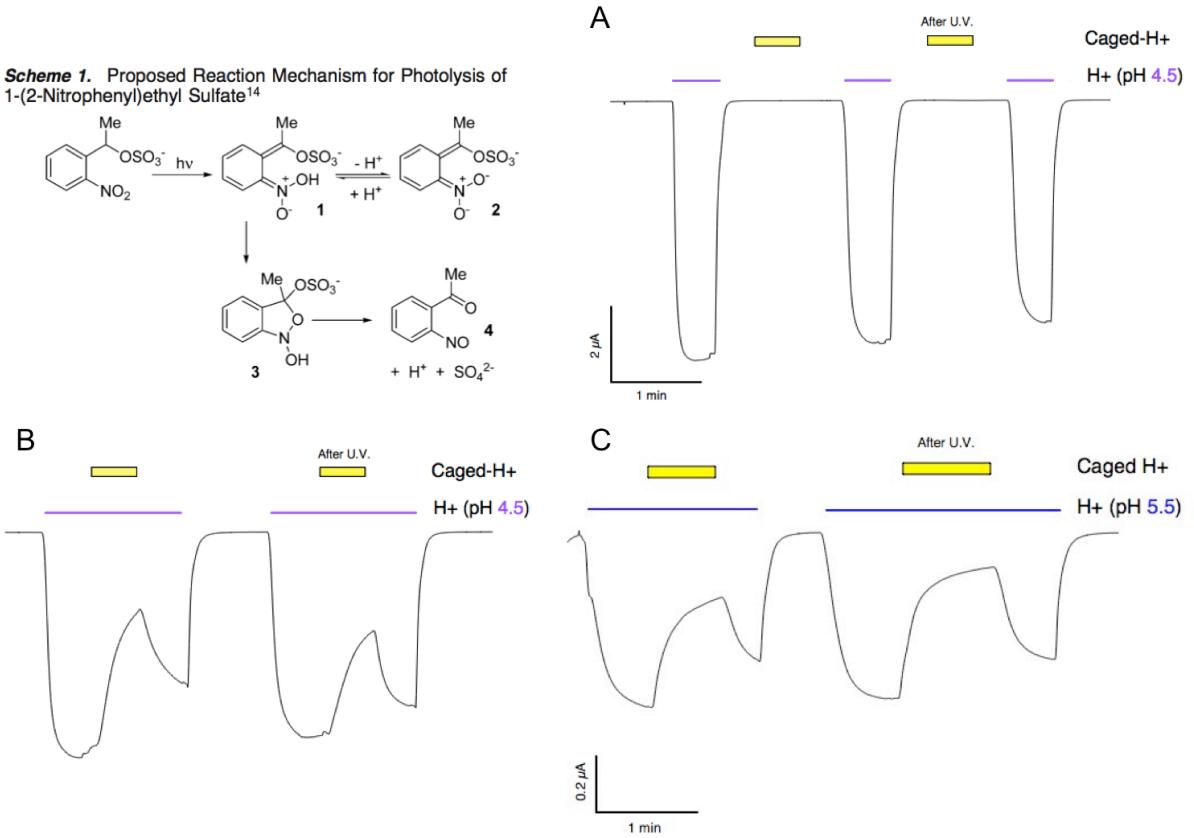

Figure 5: Effect of the caged sulfate on the function of GLIC, before and/or after photolysis.
In the upper-left inset, the mechanism for photolysis of NPE as proposed in [5] is depicted. The caged-sulfate is first photolyzed in the compound **1** which is in an acid-base equilibrium with **2** (pKa ~ 3.7), this step releases protons in the solution. **1** is degraded in the transient species **3** which is then rapidly degraded in **4**, releasing sulfate and more protons. **A B C** show recording of current through Xenopus oocytes with overexpressed GLIC, under application of protons and/or pH-equilibrated solution of NPE before and after photolysis.
A- After a control application where a strong current is elicited by a solution at pH 4.5, a solution of NPE buffered at neutral pH is applied on the oocyte, eliciting no current. Another control application of acidic solution is then applied, eliciting again a strong current (maybe less pronounced though). A solution of photolyzed NPE buffered at neutral pH is applied in turn, not eliciting any current. A final control application of acidic solution is applied, creating again a strong current (maybe somewhat less pronounced).
B- These currents were recorded on the same oocyte as **A**. Application of a pH 4.5 solution elicits a strong current, strongly reduced as soon as we apply a solution of caged sulfate buffered at the same pH. The current is recovered as soon as the application of the caged sulfate is stopped. The procedure is re-iterated with a photolyzed solution of caged sulfate, leading qualitatively to the same observation. The solution of photolyzed caged sulfate used for this measurement went down to a pH below 3 during UV-exposure. This pH being below the pKa of compound **1**, it is highly probable that this compound is at least partially degraded in **4**. In addition, the photolysis might have been only partial, hence there probably is a mixture of the caged sulfate and all photolysis products in the post-photolysis solution applied. It is then ambiguous to attribute a role to each compound in the inhibition observed.
C- To try to resolve the ambiguity mentioned above, we re-buffered at pH 5.5 the neutral pH-buffered solution containing the caged sulfate. Indeed, the UV-exposed solution did not go below a pH of 5 during UV-exposure, hence limiting the degradation of **1** in **4**. The relative amount of **2** with respect to all other photolyzed product is then likely to be higher than in the solution tested in **B**. Both solutions re-buffered at pH 5.5 were then used as in **B**, and it appears clearly that even if the caged-sulfate (which clearly inhibits the current) has not been totally photolyzed, there is an additional inhibitory effect after photolysis, probably due to **2**, and maybe to other photolysis products.

Conclusion / Perspectives:

From SAXS measurements, we have shown that it was possible to observe a difference in the scattering of GLIC when solubilized in detergent at two different pH. Since it had been previously shown in real cells that protons were able to activate the protein, we are confident that it is possible, with such techniques, to retrieve structural informations about the different conformations explored by the protein in its gating equilibrium.

This led us to go one step further and take advantage of the technologies developped at beamline ID-09B to retrieve time-resolved structural information of the gating dynamics of the protein. Unfortunately, and despite our efforts, we realized after the experiment was made that the photo-activable compound used to trigger the conformational change had a side-effect on the protein, displacing its closed-to-open equilibrium toward the closed state. This reduced even more the fraction of proteins that underwent gating in our samples, and prevented us from recording significant differences in the scattering patterns.

Nevertheless, a recent paper[6] describes for the first time the activation of another prokaryotic LGIC, ELIC, by a family of agonists, among which the neurotransmitter GABA. Switching from GLIC to ELIC to study the gating dynamics of LGIC would present many advantages.

First, ELIC has an opening probability that is certainly much higher than GLIC. In other terms, the fraction of activated versus closed ELIC is likely to be more important than in GLIC, hence creating an even more important difference in the scattering patterns; this assertion could be easily checked by SAXS.

Second, it is known (private communication) that the production yield of ELIC is much higher than GLIC, rendering the production step much faster, cheaper, and yielding much more material for the TR-WAXS experiment.

Another important point is that caged-GABA is available commercially, and we could, if needed, have collaborators helping us in synthetizing caged-compound of a more efficient derivative of GABA (e.g. cysteamine).

We would then be able to check potential inhibitory effects of the caged-compound or its photolysis products with electrophysiological techniques (thanks to our collaborators at Institut Pasteur), in order to ensure that the conformational change is not inhibited during the TR-WAXS measurements. For more confidence, we would actually screen every chemical compound present in the buffer for such an undesired effect.

Finally, it would be possible to build a flow-cell that would facilitate the circulation of protein and buffer samples during the experiment.

We have already engaged preliminary studies of ELIC. We are confident that our ongoing effort in optimizing the production/purification protocol of the protein will soon be fruitful, thus allowing us to measure the SAXS spectrum of the closed and open (with cysteamine) forms of ELIC at SWING beamline (coll. with J.Perez) during the fall of this year. We also have plans to record spectrum of ELIC with N-terminal attached gold nanoparticules, providing an original way to retrieve structural informations on the conformational change of the protein, in solution.

References:

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