ESRF	**Experiment title:** Time-resolved X-ray footprinting of RNA polymerase-DNA interactions and of multiprotein complexes	**Experiment number**: **SC- 2450**
Beamline: ID10C	**Date of experiment**: from: 12 March 2008 to 16 March 2008 8 am	**Date of report**: 27-02-2009
Shifts: 12	**Local contact(s)**: Federico Zontone	*Received at ESRF:*

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Report: We have setup the technique of time-resolved X-ray footprinting at the ID10 beamline at the ESRF to study the dynamics of biological macromolecular interactions. This is the report for the beamtime we obtained in March 2008. During these four days we carried out experiments on two ongoing projects. The first is on the study of the structural intermediates in the formation of a transcriptionally active complex by RNA polymerase by the use of time-resolved DNA X-ray footprinting. This project is a collaboration of two groups, one at the LBPA, CNRS/Ecole Normale Supérieure de Cachan (France) and the second at the Max-Planck-Institute of Biochemistry (Germany). The second project is on the study of the multi-protein complexes that are formed during the complement immune system response by the use of time-resolved X-ray protein footprinting. This project is a collaboration of two groups, the one at the LBPA, CNRS/Ecole Normale Supérieure de Cachan (France) and the Laboratoire Analyse et Modélisation pour la Biologie et l'Environnement (UMR CNRS 8587) Université d'Evry-Val-d'Essonne in Evry (France).

Summary of the experimental approach: The hydroxyl radicals produced from the radiolysis of water during irradiation of a DNA or protein sample with an X-ray beam can be used to probe the solvent accessible surface of biological macromolecules(*1*). The main advantage of using an X-ray beam from a synchrotron light source is the high flux of photons allowing for microsecond exposure times and thus permitting a high time resolution of the experiment. The abstraction of a proton from the backbone sugar of polynucletides DNA or RNA by the hydroxyl radical results in the cleavage of the chain that can be detected and quantitated subsequently in the laboratory. Only those sites on the polynucleotide that are accessible to the solvent, and thus not specifically bound by the protein, will be cut. In case of protein footprinting instead, the oxidation products on the amino acid side chains are detected by mass spectrometry of the peptides obtained by cleaving the sample with a proteolytic enzyme following after x-ray exposure(*2*). A specifically modified stopped flow apparatus is used in order to control the exposure time in the microsecond timescale and to mix the samples in the millisecond to minute timescale.

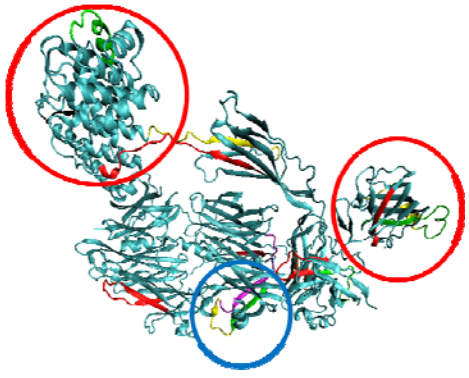
Project 1. We originally setup X-ray footprinting at ID10 to study the structural kinetics of the process of promoter recognition by *Escherichia coli* RNA polymerase (RNAP). This work resulted in a first publication in 2005 in PNAS(*3*). In that work we described the dynamics and the structure of the intermediates in the

pathway to the transcriptionally active complex on the T7A1 promoter. For the first time we identified the structural rearrangements taking place in the process of promoter recognition and open complex formation in addition to the dynamics of the equilibria formed between the different intermediates in the pathway. We have since continued this work with an improved experimental setup to explore this process in greater detail.

Aims: Having described this process on the wild type promoter DNA sequence at 37° C the next aim was to characterize the influence of DNA sequence and temperature on this process. We decided to study a mutant promoter where one of the two key sequences recognized by the enzyme has been changed to its consensus form, in addition we also studied the pathway at a decreased temperature, 20°C, where DNA strand separation becomes energetically unfavorable. In order to characterize the mechanism of DNA melting by RNA polymerase we have coupled time-resolved X-ray footprinting with time-resolved permanganate reactivity in order to measure the increased accessibility of the thymine bases as the two strands of DNA become distorted and separated.

Achievements: During this beamtime we were able to repeat our previous experiments (carried out in February and July 2007) to confirm the results we had obtained and to complete some of the datasets, especially those of the 20°C experiments. We have since completed the data analysis and redacted a manuscript that is currently under review in Nucleic Acids Research (a copy can be made available upon request). The improved experimental setup (mainly thanks to a new stopped-flow apparatus) has resulted in an increased time resolution and reproducibility of the experiments compared to our previous study. Our results clearly show that at 37°C DNA melting takes place prior to the rate-limiting step and burial of downstream DNA within the jaws of the enzyme, contrary to the established model. At 20°C instead burial of downstream DNA is necessary in order for DNA melting to occur, this step is dependent on a rate-limiting protein conformational change. In addition the comparison of the results obtained on the wild type and the mutant T7A1 promoters has permitted us to observe the accumulation of an off-pathway structural intermediate at 37°C resulting from the unstable protein-DNA contacts formed within the -10 region of the wild type promoter.

Project2. In the last couple of years we began a collaboration with the group of Régis Daniel of the Laboratoire Analyse et Modélisation pour la Biologie et l'Environnement (UMR CNRS 8587) Université d'Evry-Val-d'Essonne in Evry (France) in order to carry out time-resolved footprinting experiments on large protein complexes. The laboratory of Dr. Daniel has been studying the formation and activation of the C1q-C1r-protease complex (~790 KDa), a necessary step in the complement immune response system(*4*, *5*). Protein footprinting is particularly useful for this study since this large multi-protein complex is very difficult to crystallize, due to its size and flexibility. In addition we been studying the formation of the C3b-H complex involved in the same regulatory cascade leading to discrimination between microbes and host. While the structure of the C3b protein is known (see figure), H is a highly flexible array of 20 homologous short consensus repeats (SCR) domains. The aim of this project is to characterize the conformational changes upon formation of the C3b-H complex leading to the inactivation of the C3b protein.



Achievements: The laboratory of Régis Daniel has setup a protocol for the quantitative analysis of the modified peptides by nano-LCMS/MS on a Dionex 3000 chromatography system coupled to a LTQ Orbitrap XL mass spectrometer. This improved protocol allows for a quantification of both the increase in oxidized peptide and the decrease of its unoxidized equivalent. For the analysis of C3b, H and the C3b-H complex we have obtained between 71% and 81% coverage by the identified peptides. **Figure of the C3b protein.** The red circles show the peptides that become protected from oxidation upon formation of the C3b-H complex, in blue the peptides that become more solvent accessible upon formation of the complex, indicating a protein conformational change unmasking a possible site for proteolysis.

1. B. Sclavi, S. Woodson, M. Sullivan, M. Chance, M. Brenowitz, *Methods Enzymol.* **295**, 379 (1998).
2. K. Takamoto, M. R. Chance, *Annu.Rev.Biophys.Biomol.Struct.* **35**, 251 (2006).
3. B. Sclavi *et al.*, *Proc Natl Acad Sci U S A* **102**, 4706 (2005).
4. B. Tissot, R. Daniel, C. Place, *Eur J Biochem* **270**, 4714 (Dec, 2003).
5. B. Tissot *et al.*, *Biochemistry* **44**, 2602 (Feb, 2005).