ESRF	**Experiment title:** Temperature dependent XANES of photolysed YQR myoglobin	**Experiment number**: CH 1405
Beamline: ID 26	**Date of experiment**: from: 27 November 2002 to: 03 December 2002	**Date of report**: 31 January 03
Shifts: 18	**Local contact(s)**: Thomas Neisius	*Received at ESRF:*

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Report:

The experiment CH1405 performed at ID 26 was a partial success. We have been able to characterize at least two different Mb mutants with one of the photolysis protocols we would study, clarifying the feasibility of the entire experiment provided some specific conditions are fulfilled. Our major problem was the temperature control of the sample that must be extremely precise and reproducible to permit us to use different photolysis protocols, which is the key element to the success of these measurements. We tested two different setups.

We began the experiment using the Helium cryojet and with this setup we were able to collect good spectra of our sample in the resting condition (CO bound one). During the first 9 shifts in the initial configuration, we also established that under x-ray illumination: a) the temperature on the sample was below 180K. In fact, the protein remained CO bound for hours in the cryo stream avoiding auto-oxidation and showed very little radiation damaging induced by prolonged x-ray exposure. b) the minimum temperature achieved (under x-ray illumination) was far from 20 K, the minimum temperature required to trap the protein in some of the photoexcited intermediate states under investigation. We spent a considerable amount of effort in performing our experiment in this configuration, carefully aligning the cryojet stream, x-ray beam and laser beam and reducing the distance between the nozzle and the sample to the minimum possible. We also tried to diminish ice condensation (which interferes with photolysis) by building a small kapton chamber to contain the sample holder and cryojet nozzle, leaving only two small holes for laser beams. This configuration suffered from two difficulties, 1) condensation of ice during the long illumination and measurement times of these experiments and 2) the unknown sample temperature. In temperature derivate

spectroscopy, it is necessary to accurately control the temperature of the sample. With the cryojet, the sample temperature is too sensitive to the alignment.

We reported these difficulties to the local contact and we all agreed that a different setup was necessary. With the help of all the beam line staff we switched to a cold finger cryostat (kindly borrowed from Kenneth Larsson, ID08). We mounted a second temperature sensor directly to our sample holder, giving us an accurate temperature reading of the sample when the sample was not under illumination. In addition, it was necessary to build a cold shield (allowing us to reach 15 K) and to use a conducting window material (sapphire, kindly provided by Kenneth Larson) to minimize the heating the sample during x-ray and laser illumination. We also tested this configuration quite carefully, using various photolysis protocols. We still had sample warming due to the x-ray absorption, making it impossible to measure the low temperature photoproduct spectrum. However, there are other intermediate states that can be populated using various illumination protocols and investigated at higher temperatures. By illuminating the sample with laser light while cooling at 0.5 K/min from 220 K to 160 K and then rapidly cooling to 15 K, we were able to trap the CO ligand outside of the heme pocket. This photolysis protocol has been used previously ([1,2]). Below180 K (glassy transition temperature for Myoglobin in glycerol), CO is forced to remain in the solvent matrix and cannot rebind to the protein iron atom till the temperature is above 180 K. At 15 K, we were able to acquire the spectrum of a fully photolyzed species. This was possible because the warming effects of the x-ray beam were insufficient to reach the temperature of rebinding (180K). In this condition we could at least do one of the prefigured experiments; in fact we were able to follow the heme relaxation process and distinguish it from the rebinding process. In figure 1A, we report the spectra of the CO bound species and of the photolyzed one collected at 15K after the pumping protocol. In figure 1B there are the difference spectra obtained subtracting two spectra collected at consecutive temperatures in the formal range 15K-160K. If there was not the warming effect due to the x-ray beam we should not be able to see any rebinding till temperature was higher than 180 K. The rebinding process instead, started to appear between 120-140 K. From our measurements we were able to estimate the amount of heating from the x-ray beam ranges from 70 K at low temperature to 50 ±10 K at higher temperatures. As a concluding remark, we would like to underline that we tried in both setups to reduce the intensity of x-ray beam using different attenuators without being able to eliminate the heating effect. ID26 is in fact a very high performance beam line but with too high photon flux for this type of experiments. The best way to decrease the amount of sample heating is to defocus the x-ray beam, distributing the energy over a larger area of the sample while not sacrificing signal.

In conclusion, we were able to show the feasibility of using the XANES technique coupled to accurate and specific photolysis protocols to investigate different dynamic processes in myoglobin. However, sample heating from the x-ray beam has to be minimized to allow investigation of the low temperature intermediate states. We wish to continue this project, applying for new beam time as we consider this approach a very powerful and novel method to study the relationship between dynamic and function in proteins. We are also really motivated by the partial success of being able to distinguish spectroscopically two main dynamic processes occurring after photolysis of CO in both our mutants. For investigation of the

low temperature intermediates, we strongly recommend access to a beam line such as BM30B. This beam line also developed a quick EXAFS technique necessary for the experiment, but has the great advantage of being able to focus and defocus the x-ray beam at the sample, to reduce the warming effect. Under these conditions, measurement of the low temperature photolyzed species has already been demonstrated [3,4] and we strongly believe that the feasibility of the experiment would be significantly improved.

We would like to thank all the staff of ID 26 and in particular we wish to acknowledge Bernard Gorges, of the Sample Environmental Group, for his helpfulness and resourcefulness and Kenneth Larsson for loaning us his equipment and his advice.

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FIG 1

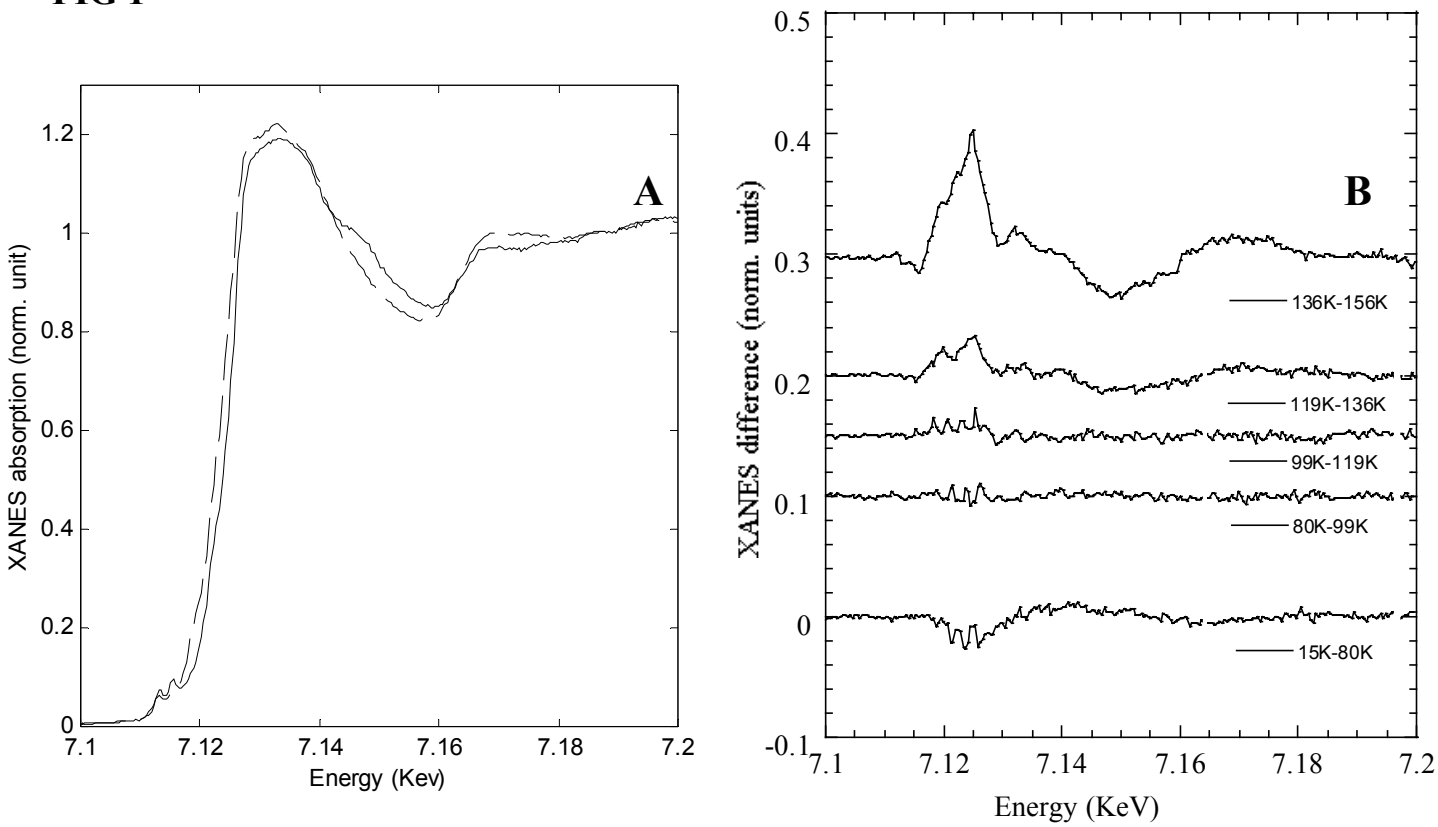

Fig 1: Panel A: solid line Mb-YQR CO bound species. Dashed line Mb – YQR photolyzed species.
Panel B: difference spectra between photolyzed species acquired starting from 15 K and increasing temperature by consecutive steps of 20 K. Not all the temperatures acquired are reported. Two different dynamic process are evident, first a relaxation process that ends at 119 K followed by a rebinding process.