EXPERIMENT MX-886 on ID14-2 (23-02-2009/24-02-2009)

Abstract:

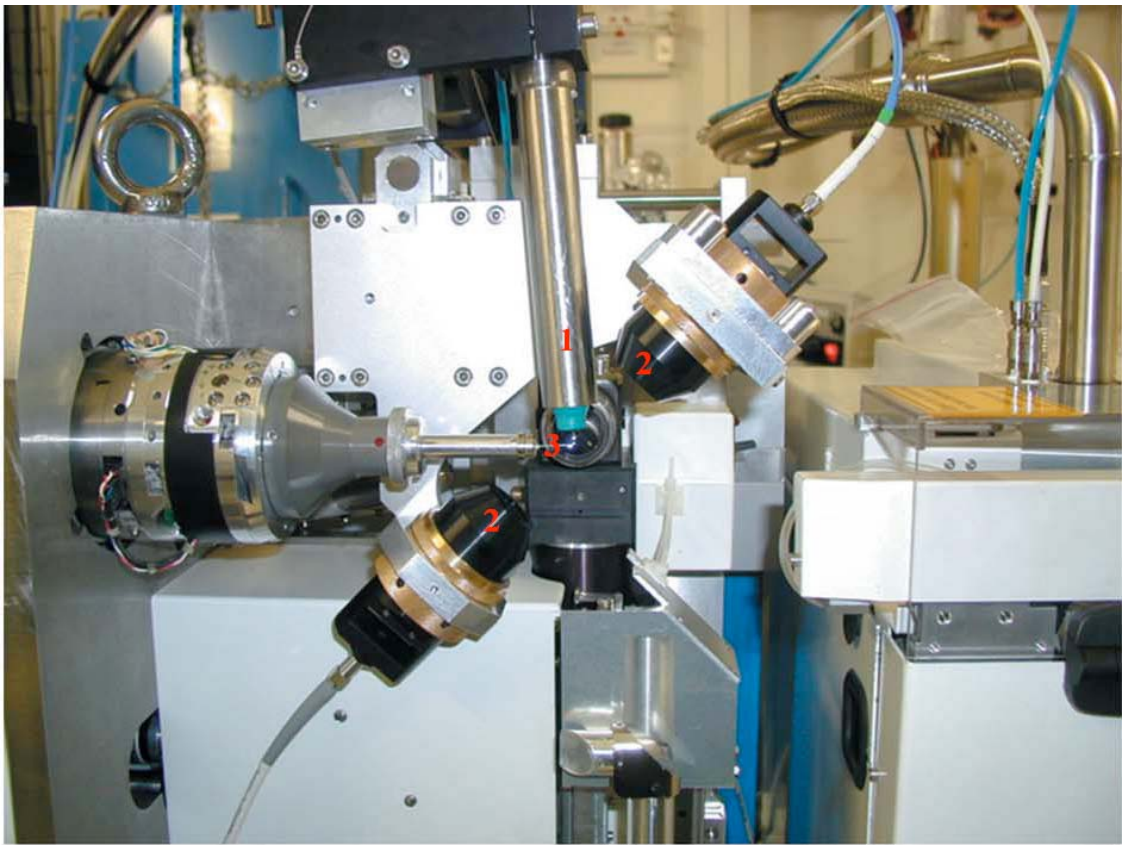
In this experiment we have analysed the photoreduction induced by X-ray at 13 keV of four different samples at 100 K: Sperm Whale Myoglobin (SWMb), Horse skeletal muscle Myoglobin (HMb), Neuroglobin (Ngb), EryK Cytochrome P450 (EryK). Crystals have been frozen and mounted in cryo-loops under liquid N_2.
We have recorded optical spectra before, during and after X-ray exposure on beamline ID14-2.

Background:

The use of radiation produced by third-generation synchrotron sources produces high magnitude X-ray intensities that cause radiation damage of crystalline biological samples. Cryo-cooling of the sample slows diffusion of the free radicals created by the ionizing effects of X-rays, preserving the quality of the sample and increasing the amount of data that can be collected. Therefore, crystals are usually kept at cryogenic temperature, typically 100 K, for data collection.
However, even at cryogenic temperatures, radiation damage does not stop. Metal sites seem particularly susceptible to radiation damage and metalloproteins can be reduced during X-ray irradiation.
Metal cofactors can absorb free electrons thereby changing their oxidation states, which can affect their conformation and coordination, respectively, and thereby the three-dimensional structures of the ligating proteins. This makes the structural characterization of defined redox states a not trivial task because electrons liberated in the sample by X-rays during crystallographic data collection can alter the redox state of the active site.
It is possible to have an on-line monitoring system to identify the species being characterized crystallographically because X-rays serve not only as a probe but are in fact also a pump. For many metallo- and particularly hemoproteins this is relatively straightforward because they have unique UV-Vis absorption spectra that are sensitive with respect to changes of the heme group and its surroundings. Their spectra can be monitored in crystals by microspectrophotometry.
Our aim is to measure photoreduction induced by X-rays, in four different hemoproteins in order to understand if there are differences in photoreduction rate between hexacoordinated (neuroglobin) and pentacoordinated systems (sperm whale, horse myoglobins and EryK CYP450).

Experiment set-up:

Instrumentation:



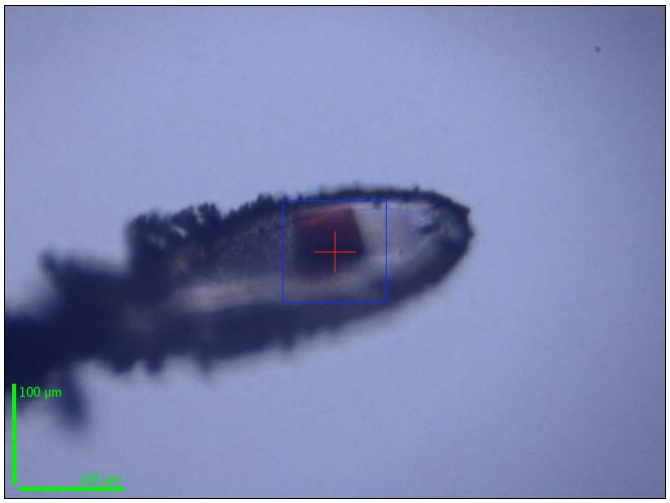
This image represents the design of the online microspectrophotometer installed on ID14-2. In the image are visible:

1) Nitrogen Cryostrem
2) On-line microspectrophotometer
3) Loop mounted crystal

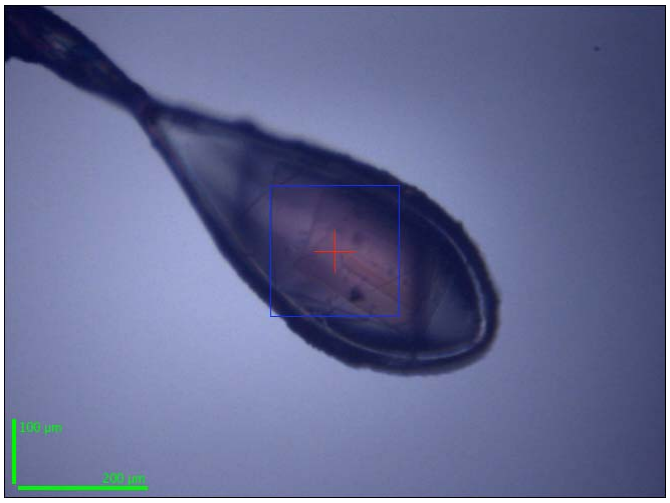
The on-line microspectrophotometer is equipped with a combine deuterium-halogen lamp (DH-2000, Oceans Optics) and a HR4000 spectrophotometer (Oceans Optics). It allows recording absorption spectra from 250 to 850 nm. The focus of the spectrophotometer (nominal spot diameter of 25 μm) is smaller than that the X-ray beam, ensuring that only X-ray irradiated material contributes to the recorded spectra.

Samples:

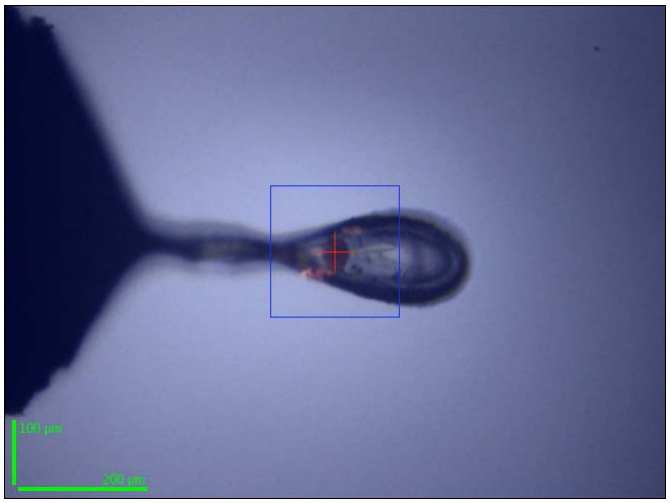
Crystals of **Neuroglobin** grew in a 1:1 mixture of protein (10 mg/ml) and reservoir solution (1.6 M ammonium sulfate, 0.1 M MES, pH 6.5, 10% v/v dioxane), at 20°C in the hanging-drop method, and their size was 75 x 75 x 75 μm. Crystals were transferred into a cryoprotectant solution containing 26% glycerol, 0.1 M MES, pH 6.5 and 1.6 M ammonium sulphate. After a 30 s soak, the crystals were flash-cooled in liquid nitrogen.



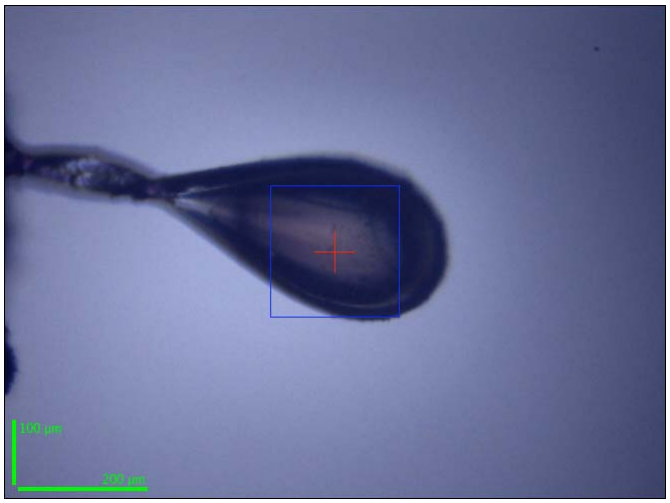
Crystals of **horse skeletal muscle Myoglobin** grew in a 1:1 mixture of protein (9 mg/ml) and reservoir solution (2.7 M ammonium sulphate, 0.1 M Tris, HCl, pH 8.0), at 20°C in the hanging-drop method, and their size was 300 x 200 x 1.5 µm.
Crystals were transferred into a cryoprotectant solution containing 25% glycerol, 0.05 M potassium phosphate buffer, pH 6.5 and 2.5 M ammonium sulphate. After a 30 s soak, the crystals were flash-cooled in liquid nitrogen.



Crystals of **sperm whale Myoglobin** grew in a 1:1 mixture of protein (10 mg/ml) and reservoir solution (2.4 M ammonium sulphate, 0.05 M potassium phosphate buffer, pH 6.5), at 20°C in the hanging-drop method, and their size was 38 x 38 x 12 µm.
Crystals were transferred into a cryoprotectant solution containing 25% glycerol, 0.05 M potassium phosphate buffer, pH 6.5 and 2.5 M ammonium sulphate. After a 30 s soak, the crystals were flash-cooled in liquid nitrogen.



EryK CYP450 grew in a 1:1 mixture of protein (10 mg/ml) and reservoir solution (48% ammonium sulphate, 0.1 M Bis Tris buffer, pH 6.0), at 20°C in the hanging-drop method, and their size was 38 x 38 x 12 μm.
Crystals were transferred into a cryoprotectant solution containing 25% glycerol, 0.1 M Bis Tris buffer, pH 6.0 and 48% ammonium sulphate. After a 30 s soak, the crystals were flash-cooled in liquid nitrogen.



Data collection:

We have mounted all the crystals in the ferric form and we have recorded optical spectra before, during and after X-ray exposure, as shown in figure 1. The orientation of the crystal with respect to the UV-Vis source has been optimized to maximize the signal from the metal centre with minimal baseline absorbance. The crystal has been kept fixed in this position with respect to the UV-Vis and X-ray beams during irradiation.
We have followed the $Fe^{3+} \rightarrow Fe^{2+}$ reduction of the heme iron in all samples, induced by X-ray flux, at cryogenics temperatures (100 K). Figure 1 represents absorption spectra of photoreduction of met-

SWMb (a), met-HMb (b), met-Ngb (c) and met-EryK (d).
All the crystal have been irradiated at 13.3 KeV.

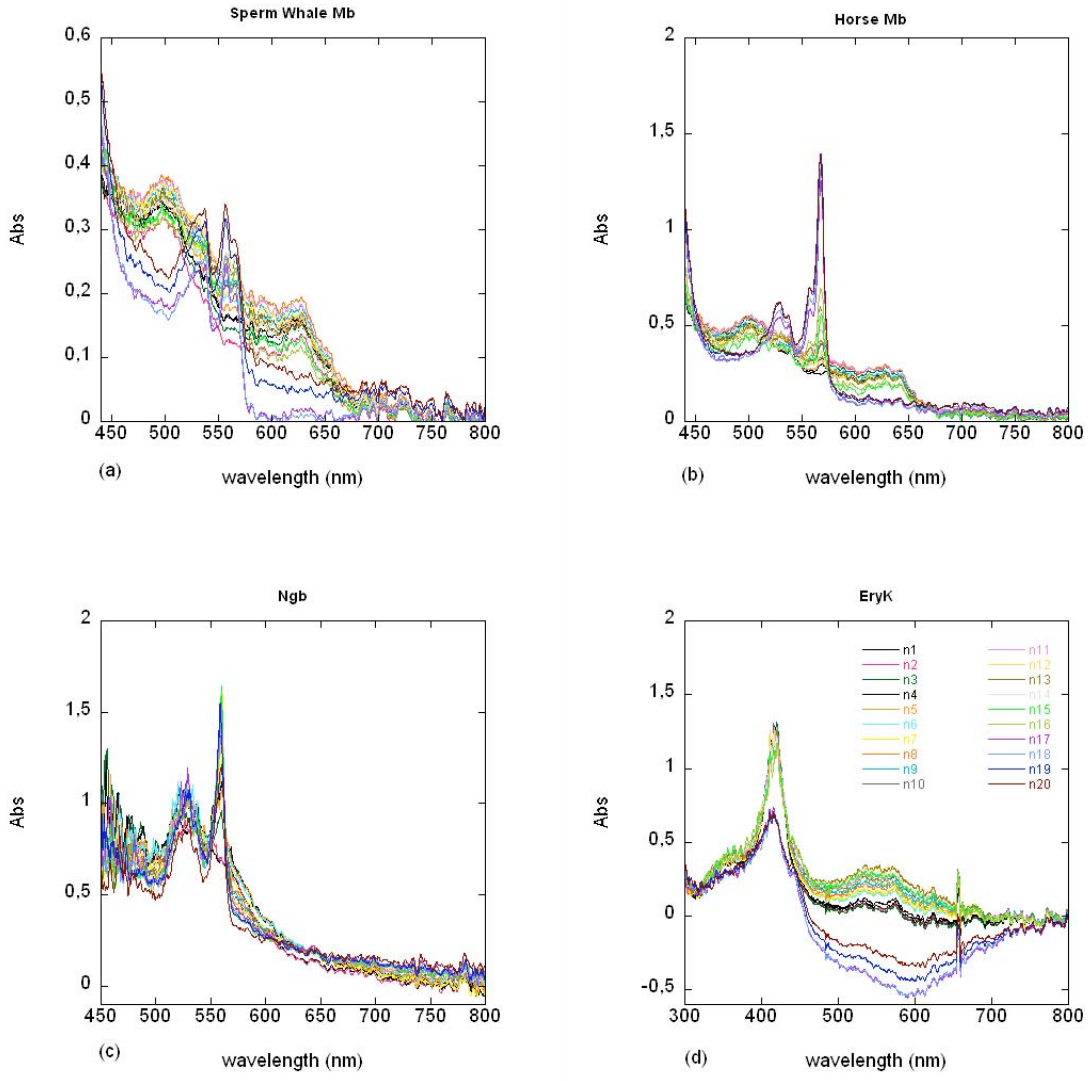


Figure 1. Absorption spectra of samples that we have acquired before, during and after irradiation at 13 keV: SWMb (a), HMb (b), Ngb (c) and EryK (d).

SWMb has been invested with a flux of $1.52*10^{11}$ photons/s with a beam size of 0.2 x 0.2 mm, necessary to irradiate all the crystal. The black line in Figure 1a corresponds to SWMb sample before

illumination and it has the typical aspect of Myoglobin at pH = 6.5. During X-ray exposure photoreduction of crystal took place: two new double peaks at 530/537 nm and 557/567 nm arise; the purple line (n17) represents the spectrum of the ferrous form of sperm whale Myoglobin which has a water molecule at the sixth ligand position of the heme iron. Given the crystal temperature this is a thermally trapped non-equilibrium state in which a water molecule is still bound to the iron. The brown line (n20) represents the mean of all spectra after X-ray illumination; it has the same form of the last spectrum (blue line, n19) during X-ray illumination, indicating that the spectrum is unchanged upon switching off X-ray illumination.

Figure 1b shows photoreduction of HMb; the sample has been invested with a flux of $1.51*10^{11}$ photons/s and the beam size that has been fixed is (0.2 x 0.2) mm, necessary to irradiate all the crystal. The black line corresponds to HMb before illumination and it has the typical aspect of Myoglobin at pH = 8.0. Figure 1 b shows the changes in the UV-Vis absorption spectrum of ferric horse Myoglobin upon X-ray irradiation; the black line represents the typical aspect of a ferric Myoglobin at pH = 8.0 and during irradiation two new double peaks at 528/537 nm and 557/567 nm appear.

Ngb has been invested with a flux of $3.37*10^{10}$ photons/s and the beam size that has been fixed is (0.1 x 0.1) mm, necessary to irradiate all the crystal. The black line in Figure 1c corresponds to Ngb sample before illumination and it has the typical aspect of met-Ngb. Figure 1c shows absorption spectra of photoreduction of met-Ngb crystals kept at 100 K that have been taken before, during and after X-ray irradiation. The black line corresponds to Ngb crystal recorded before X-ray illumination and it has the typical aspect of met-Ngb; during irradiation two new peaks at 528 nm and 558 nm arise, corresponding to the reduced ferrous form.

EryK has been invested with a flux of $1.15*10^{11}$ photons/s and the beam size that has been fixed is (0.2 x 0.2) mm, necessary to irradiate all the crystal. The black line corresponds to EryK sample before illumination and it has the typical aspect of met-EryK.

The influence of background on absorbance measurements is very strong, we therefore subtracted the contribution of the cryoprotectant solution to the absorption spectra of samples that we have acquired before, during and after irradiation at 13 keV. Figure 1 (a b d) represents absorption spectra of SWMb, HMb and EryK, after the reduction of background contribution; Ngb absorption spectra have been not corrected because the background contribution was marginal.

For the acquisition of spectra of Myoglobins and Neuroglobin we have used the halogen lamp of the microspectrophotometer that gives the possibility to visualize a range of wavelength between 400 and 850 nm, while for the acquisition of EryK spectra we have used the halogen lamp plus the deuterium lamp to have a wider range of wavelength (250 – 850) nm and to analysed also Soret peack.

In Figure 2 we report the time coarse of photoreduction for each sample. Figure 2a represents time course of the 558 nm peak of sperm whale, horse Myoglobin and Neuroglobin; figure 6b represents time course of the 415 nm peak of EryK.

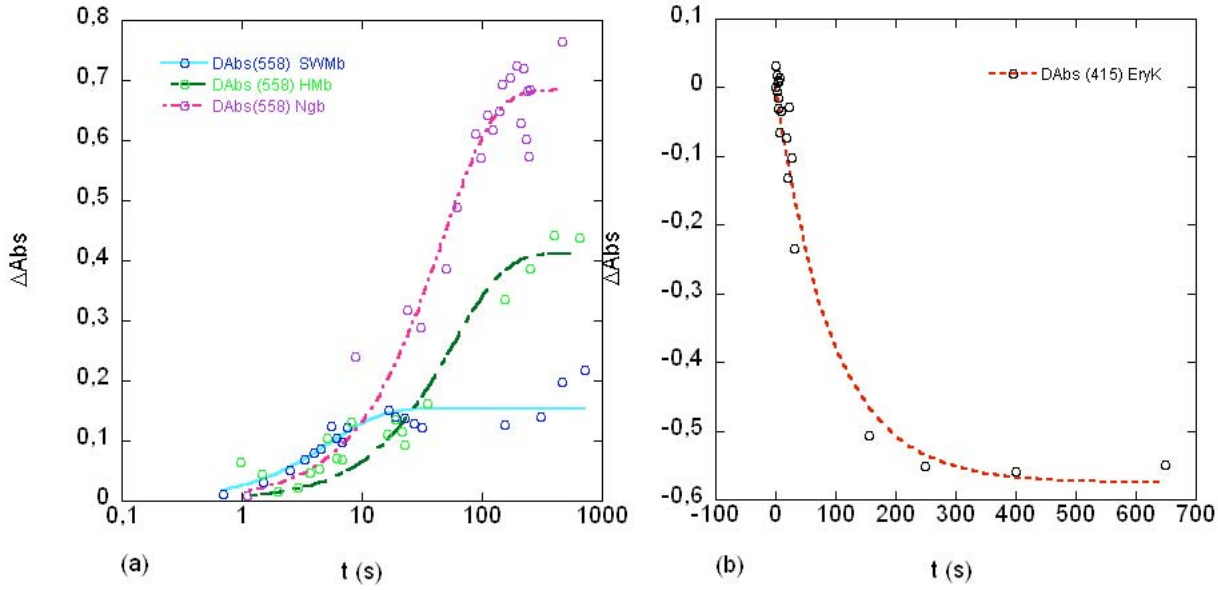


Figure 2. (a) Time course of the 558 nm peak of sperm whale Myoglobn (blu circle) and its relative exponential fit (marine line), horse Myoglobin (green circle) and its relative exponential fit (green dashes), and Neuroglobin (violet circle) and its relative exponential fit (pink dashes); figure 2b represents time course of the 415 nm peak of EryK and its relative exponential fit (red dashes).

Each spectrum has been fitted with an exponential function. In table 1 we report the results of the exponential fit for different samples; the parameter k represent the rate constant of the photoreduction process.

Sample	**a (u.a.)**	**k (s^{-1})**
SWMb	0.154 ± 0.009	0.18 ± 0.03
HMb	0.41 ± 0.02	0.018 ± 0.003
Ngb	0.68 ± 0.02	0.022 ± 0.003
EryK	-0.57 ± 0.03	0.011 ± 0.002

Table 1. Parameters obtained by exponential fit of time course for different samples.

Conclusion:

From the observation of the rate contant k it is possible to notice that the photoreduction of Neuroglobin, that is an hexacoordinate system, is comparable with horse Myoglobin and EryK photoreduction, within experimental error. From these preliminary results it seem that the photoreduction of sperm whale Myoglobin is ten times faster than the other hemoproteins analized.