EXPERIMENTAL REPORT

MX-805 ID14-2 (05-12-2008/07-12-2008)

Characterization of photolytic intermediates of carbonmonoxy Neuroglobin

Partecipants:

Chiara Ardiccioni, Beatrice Vallone
Dipartimento di Scienze Biochimiche
Università di Roma "La Sapienza"
P.le Aldo Moro, 5
00185 - Roma
Italy

Alessandro Arcovito
Istituto di Biochimica
e Biochimica Clinica
Università Cattolica del Sacro Cuore
Largo Francesco Vito 1
00168 Roma Italy

Dominique Bourgeois, Martin Weik
LCCP IBS
41 Rue Jules Horowitz
38027 GRENOBLE Cedex 1
FRANCE

Here we describe two experimental sessions, one at ID14-2 and one at the Cryobench , since they are connected and complementary.

EXPERIMENT MX-805 on ID14-2 (05-12-2008/07-12-2008)

In this experiment we have analyzed the photoreduction of Ngb, recording optical spectra before and during X-ray exposure.
We also attempted the determination of the structure of the photolytic intermediate of NgbCO (NgbCO*).

Experimental set-up:

1) Loop mounted crystal
2) On-line microspectrophotometer
3) Helium-jet

The first 24 hours were dedicated to setting up the beamline: Helium-jet installation and microspectrophotometer installation and alignment were carried out. This work has been possible due to the strategic collaboration of Peter Van Der Linden, from the Experimental Support Group and Martin Weik from the IBS.

Data collection:
We have mounted Ngb crystals in the ferric form and we have recorded optical spectra with the on-line microspec before and during X-ray exposure, as shown in figure 1. We have followed the photoreduction of protein caused by X-ray flux. Figure 1 represents the photoreduction of met-Ngb crystals kept at 30 K.

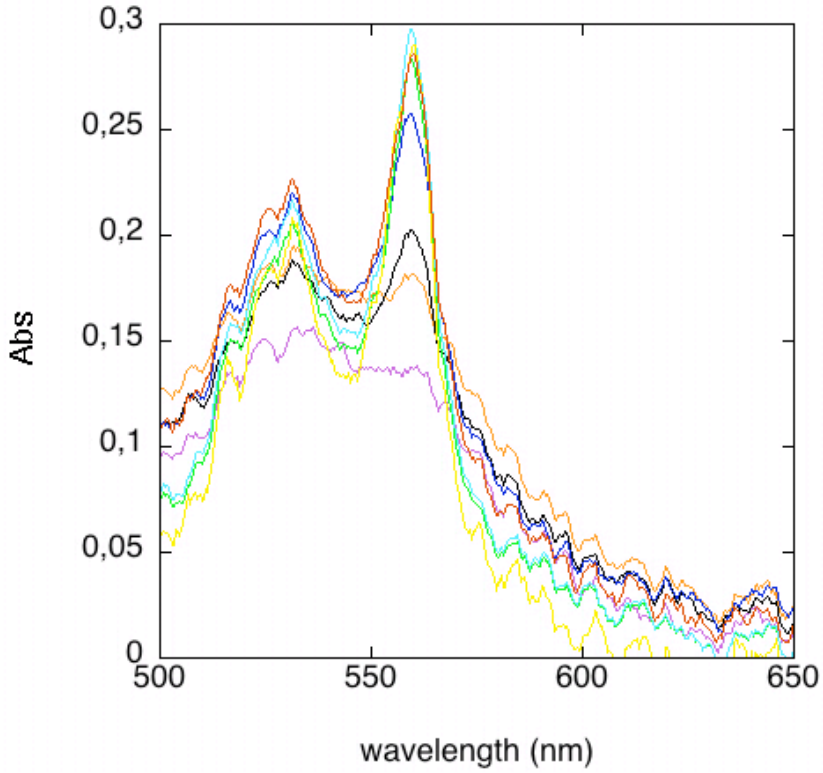

Figure 1 *Absorption spectra of photoreduction of met-Ngb crystals kept at 30 K that have been taken before and after X-ray irradiation at 13.3 KeV with a flux of 7.3 X 10^{-9} photons s^{-1} and focused beam. The violet line corresponds to Ngb crystal recorded beforeX-ray illumination and it has the typical aspect of met-Ngb [2].*

Figure 2 shows the time course of the 508.5 nm peack. The data have been fitted with an exponential function. The half time of the reaction is about 25 s so that in the time window reported a full reduction of Ngb is achieved (Fe^{3+} → Fe^{2+}).

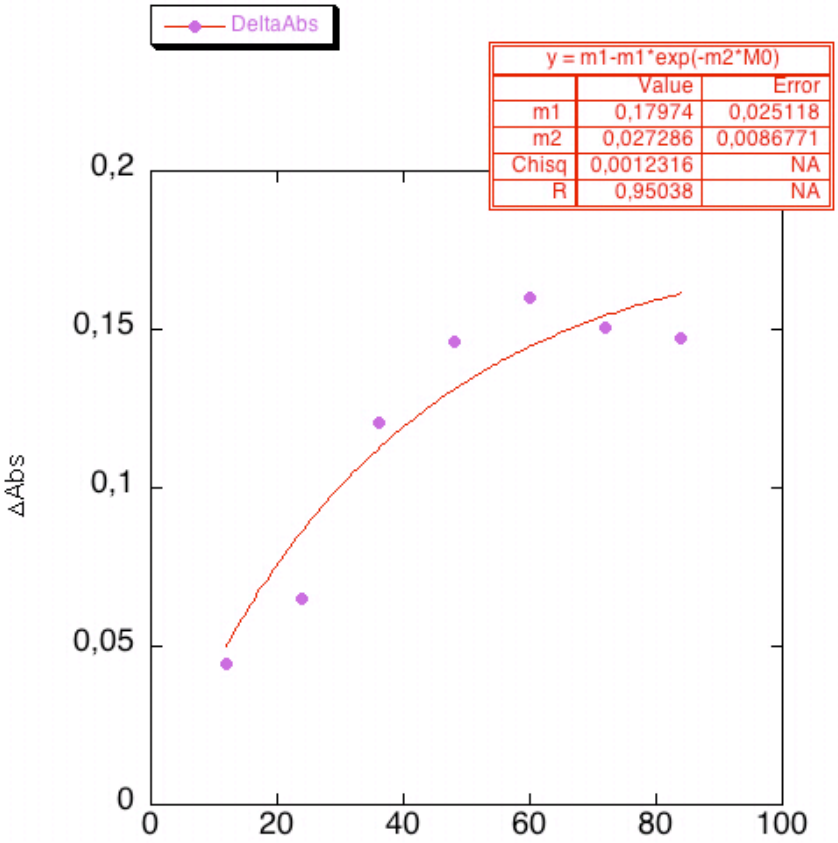

Figure 2 *Time course of the 508.5 nm peack. The data have been fitted with an exponential function (red line).*

In this part of the experiment the formation of ice on the crystal surface has been much more detrimental than during off-line spectrophotometric measurements.
This was probably due to the geometry of the system and to the rotation of the sample during data collection that increased gas turbulences, enhanced by the need to change manually the temperature adjusting the needle valve of the He-jet. It was very difficult to reach 15K and it was impossible to carry out data collection at this temperature.
Consequently we could not attempt data collection in conditions where the photolytic intermediate NgbCO* is trapped.
Data statistics from collection carried out at 30K show that crystals diffract to 1.8 Å resolution (see table below), allowing the determination of the structure with the detail necessary to detect CO docked within the protein matrix, as expected in the photolytic intermediate NgbCO*.
In conjunction with the data reporting the experimental session at the Cryobench, we could nevertheless assess the feasibility of the determination of the structure of NgbCO*, provided that the performance of the He-stream is improved, eliminating the severe icing effect.

Statistics of data obtained for Ngb at ID14-2 in ESRF

Resolution = 40 - 1.80 Å (1.83 – 1.80 Å)*

Space group = H32

χ^2 = 1.584 (1.207)

R_{fac} = 0.043 (0.124)

R_{fac}^2 = 0.050 (0.116)

Completeness = 94.3 % (99.1 %)

Mosaicity = 0.506

Unit cell parameters (Å): a = 88.335 b = 88.335 c = 113.54

* Data into parenthesis refer to the last resolution shell.

CRYOBENCH (03-12-2008/05-12-2008)

The first part of experiment consists of the acquisition of absorption spectra of Neuroglobin (Ngb) crystals with the off-line microspectrophotometer at the cryobench of ESRF [1].
Crystals of Ngb were prepared in different oxidation and ligation states: ferric (Fe^{3+}), ferrous (Fe^{2+}) and ferrous CO-bound, frozen and mounted in cryo-loops under liquid N_2.

Experiment set-up:

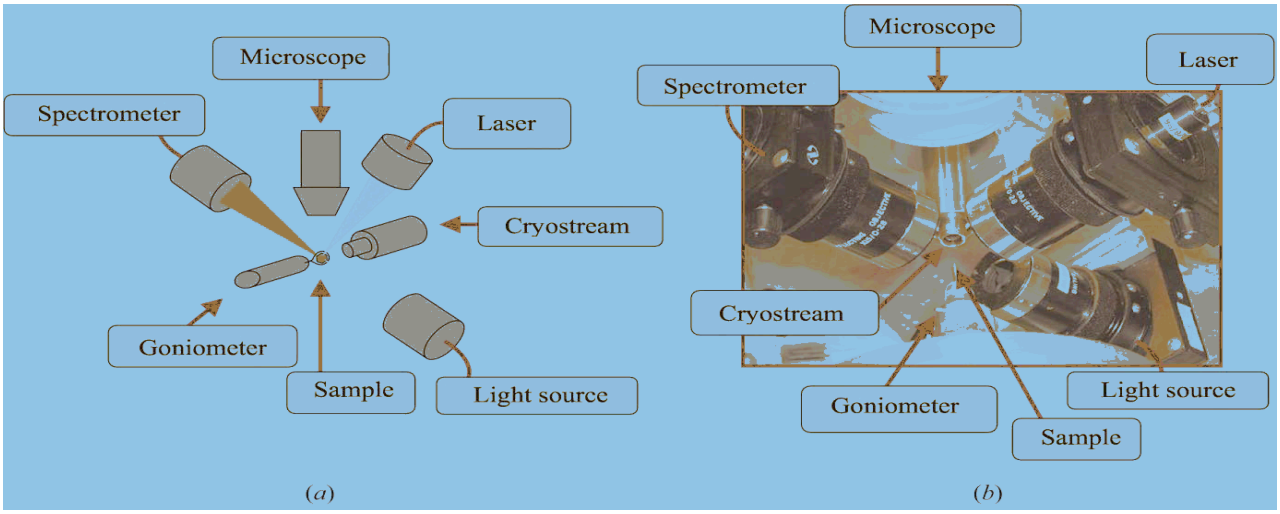

Figure from Bourgeois et al., J. Appl. Cryst., (2002), 35, 319-326.

Instead of the standard setup with a nitrogen stream, we have used a helium-jet to achieve temperatures as low as 15K, keeping in mind that this is the nominal temperature of the instrument controller. The actual temperature of the sample was not measured, but it is likely to be higher, although in the same range.

Data collection:
The first samples analyzed were Ngb crystals in the ferric form (fig. 3).
Subsequently spectra from Ngb crystals in the ferrous form (reduced with Na dithionite) were collected. The absorption spectra of these two crystal forms have the typical aspect of met-Ngb and ferrous Ngb, respectively [2].

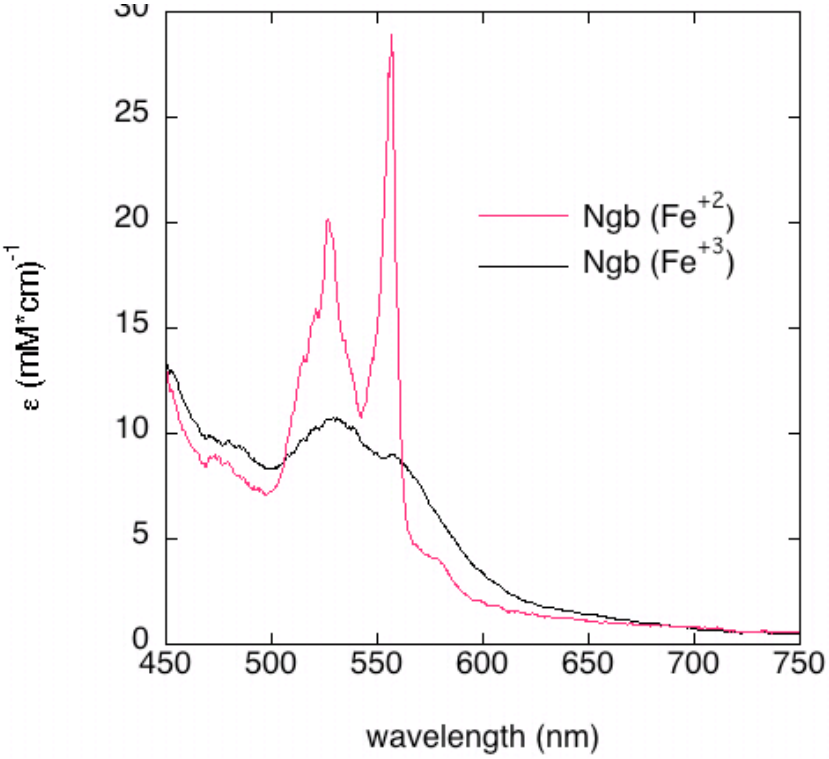

Figure 3 *Absorption spectra of Neuroglobin crystals at 15 K. The black line corresponds to the ferric form and the purple line to the ferrous form of Ngb, that was obtained soaking crystals with the reducing agent Na dithionite.*

Figure 4 shows absorption spectra of NgbCO crystals at two different values of temperature, 15K and 30K (nominal temperature of the Helium jet).

A photolytic intermediate of NgbCO (NgbCO*) was observed by FTIR [3], this state can be trapped only at temperatures lower than 40K, since at higher temperatures geminate rebinding does not allow it to be populated.

As far as spectrophotometric measurements are concerned, the NgbCO* should correspond to a deoxy pentacoordinated species, which is very different from the deoxy hexacoordinated species shown in figures 1 and 3, that is also the spectrum observed in solution.

Indeed Figure 4 shows a spectrum at 15K that corresponds to a deoxy pentacoordinated state, which so far has never been observed for Ngb in crystals, indicating that the even the lamp probe of the microspectrophotometer is sufficient to achieve full photolysis and consequently we were able to trap the, NgbCO* species at 15K.

Consistently, at temperatures higher than 30K, geminate rebinding becomes dominant, leading to a spectrum that corresponds to the NgbCO spectrum as reported in the literature.

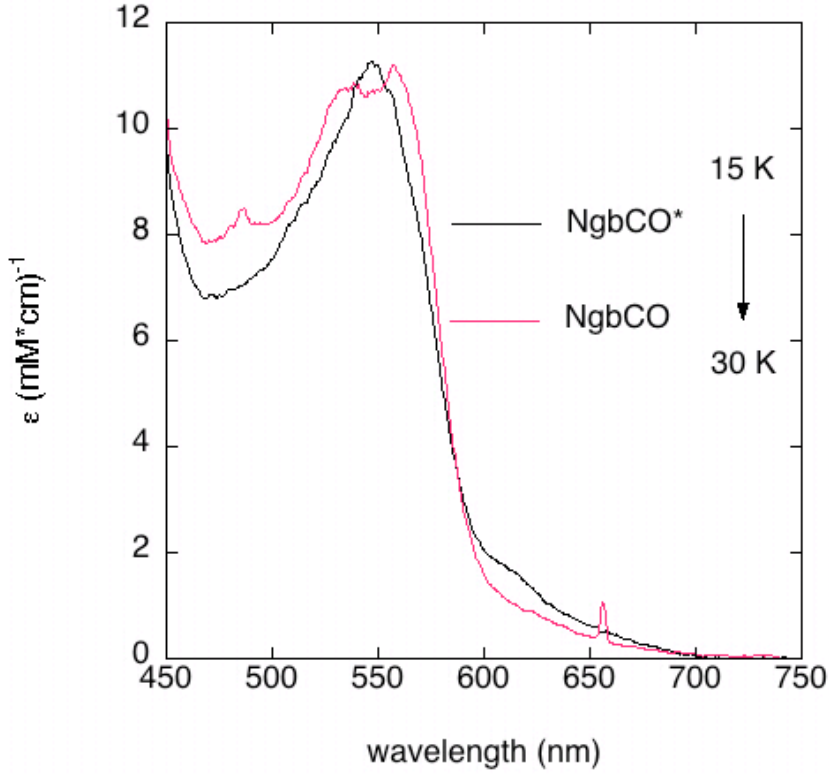

Figure 4 *Absorption spectra of NgbCO crystals; at low temperature (15 K) is possible to trap photoyitic intermediate NgbCO* (black line) under illumination of the probing lamp of the microspectrophotometer. Increasing the temperature to 30 K is sufficient to obtain the CO bound species (purple line).*

In order to confirm that the spectum reported in Figure 4 corresponds to NgbCO* we tested its reversibility, by cycling the temperature induced transition from 15K to 30K and *vice versa.* The transition from photolitic intermediate NgbCO* at 15 K to Ngb CO-bound at 30 K was clearly followed, with full reversibility, after several cycles (Figure 5).

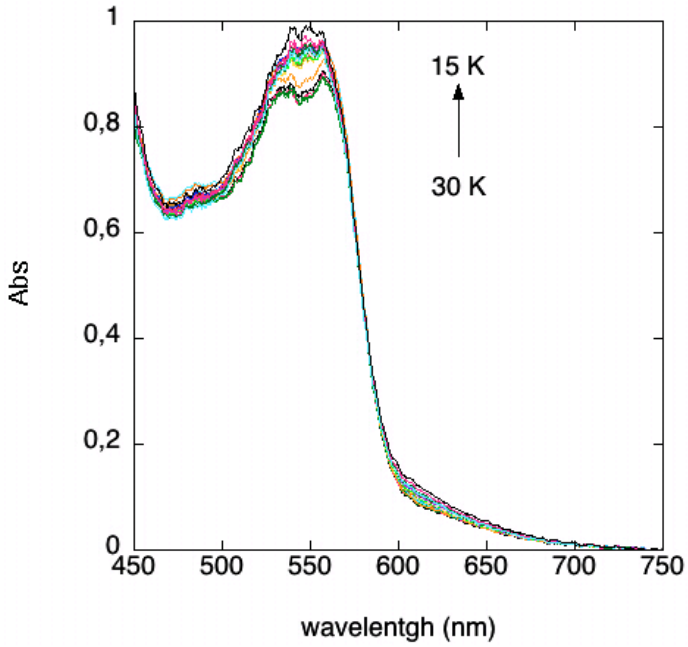

Figure 5 *Ngb-CO absorption spectra collected lowering the temperature from 30 K to 15 K. There is a transition from Ngb CO-bound species to the photolitic intermediate NgbCO*. The process is completely reversible.*

The temperature was varied manually, rotating the 'needle valve' of the Helium-jet because the computerized controller was not available. This posed a problem, because the change of temperature is connected with a brutal change of helium flux, enhancing the formation of ice on the crystals. Crystal icing and mechanical microfluctuation due to gas flux led to a variation of the focus and optic length, thus affecting the quality of the spectra.

Conclusion

The experimental session was very successful, since we obtained the data we were aiming to: spectra of different species of Ngb in crystals and isolation and characterization of the temperature stability of the NgbCO* photolytic intermediate.
The optical spectra of Ngb, always correspond to an hexacoordinated form, since in the absence of a gaseous ligand such as CO, O_2 or NO the proximal histidine occupies the sixth coordination position. For the first time a pentacoordinated state was trapped in the crystal (the NgbCO* species). This result is interesting *per se* and demonstrates the feasibility of the determination of the 3D structure of this photolytic intermediate.

References:

[1] Bourgeois et al., "A microspectrophotometer for absorption and fluorescence studies of protein crystals " J. Appl. Cryst., (2002), 35, 319-326.
[2] Sylvia Dewilde et al., “Biochemical Characterization and Ligand Binding Properties of Neuroglobin, a Novel Member of the Globin Family” J Biol Chem. (2001), 42, 38949-55.
[3] Kriegl JM et al., “Ligand binding and protein dynamics in neuroglobin.” Proc Natl Acad Sci U S A (2002),99, 7992-7997.