ESRF	Experiment **title: Conformations of the active site in partly unfolded carbonmonoxymyoglobin by XANES spectroscopy**	**Experiment number:** LS467
Beamline: BM29	**Date of Experiment:** from: 20/11/96 to: 29/11/96	**Date of Report:** 28/02/98
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Guanidine hydrochloride (Gdn-HCl) is the most commonly used denaturant for proteins and its effects on myoglobin unfolding have been widely studied (Pace et al. 1979). In particular, it has been assumed for a long time that increasing Gdn-HCl concentration destabilises the protein structure up to a complete co-operative unfolding at critical concentration C_m (Pace, 1986). Recently Hagihara and co-workers (Hagihara et al. 1993) have found that Gdn-HCl, at low concentrations, refolds acid-unfolded apomyoglobin and cytrochrome c at pH=2, stabilising the molten globule state. These opposite effects of Gdn-HCl on protein stability have been discussed recently (Myers et al. 1995) and have been related to possible changes in protein accessible surface area. Furthermore a particular behaviour of the 0.5 MGdn-HCI concentration has been pointed out by optical spectroscopy measurements (Natali et al. 1998)

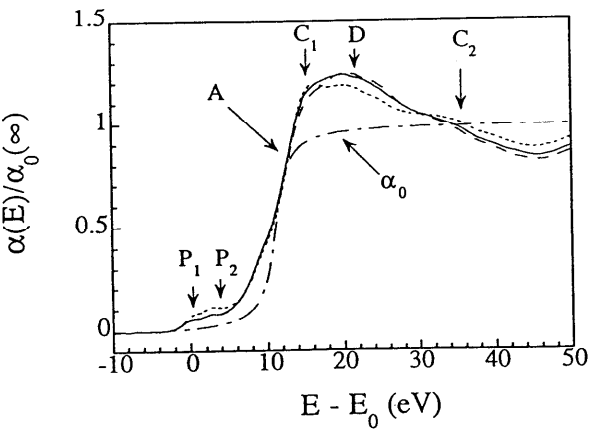
In this work we have explored the conformational landscape of MbCO in native and partly denatured states induced by different concentration of Gdn-HCI. In particular following characteristic absorption peaks related to the conformational parameter Fe-CO

bonding angle, we have investigated the protein dynamics using XANES technique to identify changes in the photoproduct at low temperature and to link small structural rearrangements of the active site with different rebinding processes.

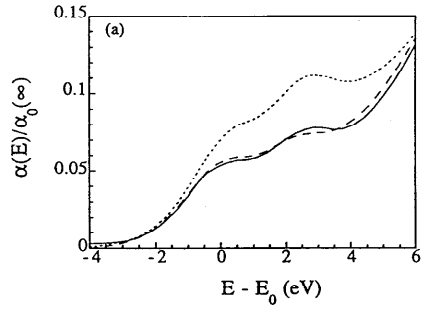
The acquired Fe K-edge spectra of native and partly denatured (with 0.5 M and 1.4 M Gdn-HCl) horse MbCO at 25 K are reported in Fig, 1

It has been pointed out, through previous XANES simulations, that P_1, C_1 and C_2 peaks, are close related to axial scattering of the photoelectron, while the P_2, A and D peaks depend on heme-scattering (Bianconi et al. 1985).

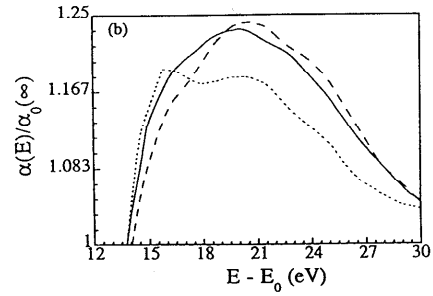
As there is no variation in the position of the A peak under denaturing conditions, it means that no energy shift of the maximum of the derivative occurs indicating that, for all different sample the Fe-Np distance (where Np is referred to pyrrolic nitrogens) doesn't change. The largest spectral variations, corresponding to the pre-edge and edge regions, are magnified in panels :1a and lb respectively. According to previous results (Hagihara et al. 1993; Goto et al. 1990; Mayr et al. 1993) the opposite effects of Gdn-HCl on protein stability are clearly visible.



Fe K-edge XANES spectra of native horse MbCO (solid line), 0.5 M Gdn-HCl MbCO (dashed curve) and 1.4 M Gdn-HCl MbCO (dotted curve) measured at 25 K; the zero energy corresponds to 7112.5 eV; the atomic absorption coefficient α_0 is also shown.The peak of interest are labeled.

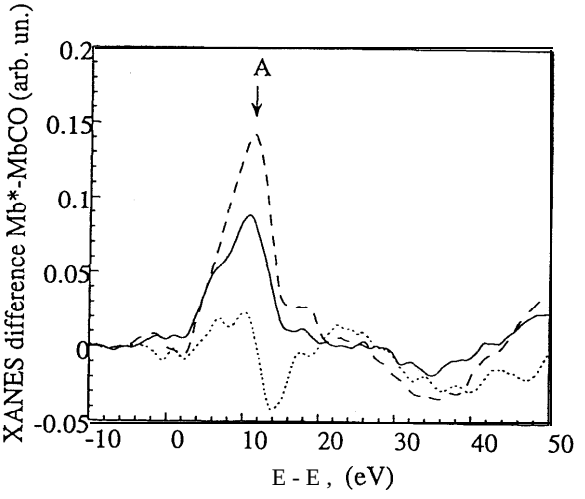


Panel a: zoom of pre-edge region (P_1 and P_2 peaks)



Panel b: zoom of C_1 peak

By extrapolation from theoretical curve of previous MS simulation (Della Longa et a]. 1994), the angles between the normal to the heme and the CO dipole have been estimated: for the native state we have estimated a tilting angle about 27 while for CH and CL samples we have calculated respectively 14 and 33, Therefore, the low denaturant concentration (0.5 M Gdn-HCl) induces an active site conformation with a tilting angle increased with respect to the native one; while the high denaturant concentration induces, on the contrary, the protein conformation with a binding geometry with the CO dipole close to the normal to the heme plane. To investigate if this structural rearrangement is associated to a kinetic effect, we have studied the XANES of photoproducts Mb* obtained at 25K under continues illumination for 1 hour. The XANES spectra have been recorded in the native and photolised state at the same temperature. In figure 2, we have reported the spectra differences Mb*-MbCO for all the samples (where Mb* corresponds to the photolised states). The results confirm that there is a different behaviour with respect to native state, between the low and the high denaturing condition. In particular, all the samples show a red-shift of the edge and, consequently, all the spectra differences Mb*-MbCO have a maximum in correspondence of the edge energy of the MbCO state (Della Longa et al . 1994): the sample with 0.5M Gdn-HCl has the maximum value while the 1.4M sample has the minimum one. This fact suggests that there is a structural rearrangement with a lengthening of the Fe-Np distance but, even that, as the same illumination and acquisition time has been used for all the samples, the increasing of the intensity of the maximum of the spectra difference Mb*-MbCO, indicates a stronger Fe-CO binding and a slower recombination rate.



Fe K-edge XANES spectra difference (Mb*-MbCO) for native MbCO (solid line), 0.5 M Gdn-HCl MbCO (dashed curve) and 1.4 M Gdn-HCl MbCO (dotted curve)

REFERENCES

Bianconi, A., A. Congiu Castellano, P. J. Durham, and S. S. Hasnain. 1985. The CO bond angle of carboxymyoglobin determined by angular-resolved XANES spectroscopy. *Nature. 3* 18: 685-686.

Della Longa, S., I. Ascone, A. Fontaine, A. Congiu Castellano, and A. Bianconi. 1994. Intermediates states in ligand photodissociation of carboxymyoglobin studied by dispersive x-ray absorption. *Eur. Biophys. J* . 23: 361-368.

Goto, Y, L. Calciano, and A. L. Fink . 1990. Acid induced folding of proteins. *Proc. Natl. Acad. Sci. USA. 87: 573-577.*

Hagihara, Y., S. Aimoto, A. L. Fink, and Y. Goto. 1993. Guanidine Hydrochloride-induced folding of proteins. *J. Mol. Biol. 23* 1: 180- 184.

Mayr, L.M., and F. X. Schmid. 1993. Stabilisation of a protein by guanidinium chloride. *Biochemistry. 32: 7994-7998.*

Myers, J. K., C. N. Pace, and J. M. Scholtz. 1995. Denaturant m values and heat capacity changes: relation to changes in accessible surface areas of protein unfolding. Protein *Science. 4:* 2138-2148.

Natali, F., L. Moretti, F. Boffi, A. Bianconi, S. Della Longa, A. Congiu Castellano. 1998. Light induced states in MbCO denatured with guanidine hydrochloride. Eur. Biophys. J. 27: 1 -7

Pace, C. N., and K. E. Vanderburg. 1979. Determining globular protein stability: guanidine hydrochloride denaturation of myoglobin. *Biochemistry.* 18, n. 2: 288-292.

Pace, C.N. 1986. Determination and analysis of urea and guanidine hydrochloride denaturation curves. *Methods. Enzymol.* 13 1: 266-289.