ESRF	**Experiment title:** Activ site's structure of giant hemoglobin from earthworm studied by X-ray absorption spectroscopy	**Experiment number:** 08-01-186
Beamline: BM08	**Date of experiment:** from: 17-11-'99 to: 20-11-'99	**Date of report:** 25-02-2000
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Report: Amoung all the known hemoglobins, the annelid's ones presents several interesting peculiarity: they are extra-cellular macro molecules which results insensitive to the regulative molecules known to act in vertebrates (such as DPG) while a structural and functional role for calcium ions was suggested. A single protein molecula (mass = 3.6 MD) carries about 144 Fe-heme containing subunit (divided into four classes of chains named a,b,c,d) as well as a number of linker chains that are unable to bind oxygen but seems involved into the assembly of the macro molecules.

We studied in solution, at iron k edge, pH 7.2 (tris-HCl buffer 0.15 M) and in presence of 10 mM calcium, the activ site structure of an heartworm hemoglobin bound to different ligands: an oxygen molecule, a carbonmonoxyde or in the deoxygenated state. All the XANES data were collected in fluorescence mode. Dealing with sample which could give rise to dissociation equilibria, great attention was devoted to the characterization and purification of the sample. For this matter we induced dissociation of the whole protein followed by further purification on column. After this treatment we collected the eluted fraction corresponding to the main peak reported in fig 1. Such an eluted fraction should correspond to the bigger subunit of the molecula that mainteins intact cooperativity and oxygen affinity. in the presence of 10 mM calcium ions because. In fig 2 we compared the spectrum of purified whole hemoglobin with that of the eluted sample in the Fe^{+2} oxygenated state. The data showed that, concerning the activ site structure, the eluted fraction is

equivalent to the whole molecula, while larger differences result from a comparison between the heartworm and an ordinary human hemoglobin. The measurements were performed on O_2 and CO derivatives. In the case of the oxy-derivative (see fig. 3) the position and intensity of the main peak change and a meaningful decrease in the absorption in the range 7160-7175 eV (spin state sensitive region) was detected. Even larger was the structural differences found in the CO-derivatives suggesting modification of the iron's first neighboring as well as stresses on the activ site due to the different ligand bonding geometry. Lastly we tried to collect the spectrum of the deoxy-derivative obtained by addition of different concentration of sodium ditionite to the hemoglobin solution. In fig. 4 we report the spectrum of human deoxy hemoglobin (solid line) and two spectra of the heartworm hemoglobin. The dashed line spectrum refers to the lower concentration of ditionite and is characterised by a red shift at the edge of 1.7 eV while, as a reference, the red shift in vertebrates hemoglobin is 2.5 eV. Since this could be explained by an incomplete reduction of the worm hemoglobin, we doubled the concentration of ditionite used obtaining the dashed-dotted line spectrum. Unfortunatly the relevant modification detected at high concentration of ditionite could hardly be assigned to a complete reduction, while it seems more probable that a pH shift towards acid values, induced by ditionite, could have denaturated the protein.

Fig. 1

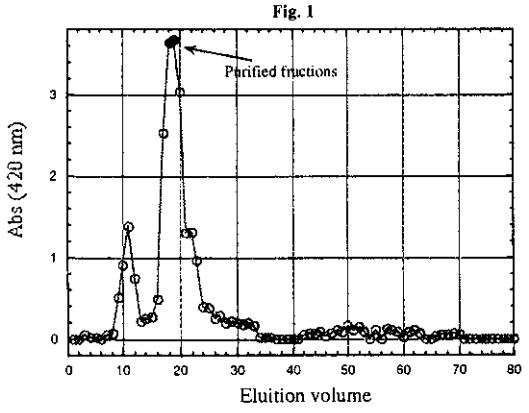

FIG. 2

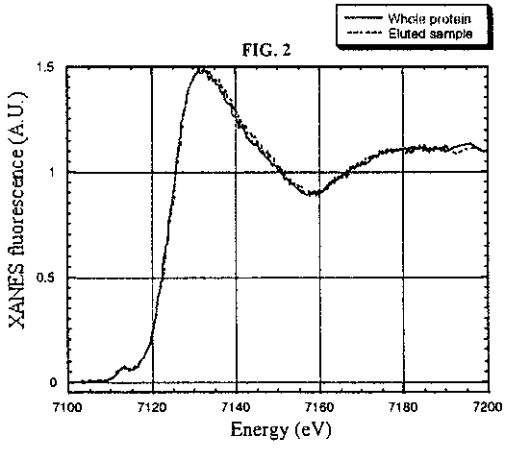

Fig. 3

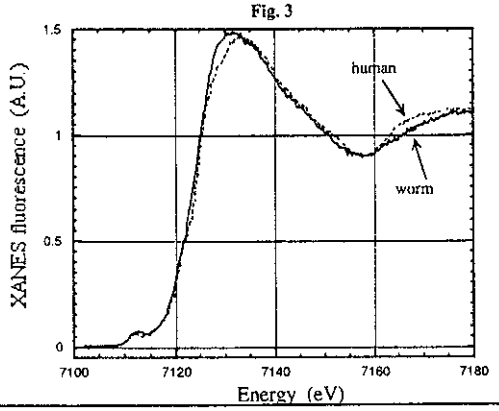

Fig. 4