J. Synchrotron Rad. (1999). **6**, 392–393

Fe-heme structure and dynamics in Thr72 → Ile mutant *Scapharca inaequivalvis* hemoglobin by X-ray absorption spectroscopy

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The Fe site structure in the recombinant wild-type and Thr72 → Ile mutant of the cooperative homodimeric hemoglobin (HbI) of the mollusc *Scapharca inaequivalvis*, has been investigated by measuring the Fe K-edge X-ray Absorption Near Edge Structure (XANES) spectra of their deoxy and carbonmonoxy derivatives, and the cryogenic photoproducts of the carbonmonoxy derivatives at T = 12K. The overall data are interpreted as indicating that the T structure of the Fe-heme site in the mutant deoxy-HbI has been destabilized and converted towards an R-like, "high affinity" structure, as previously suggested (Gambacurta *et al.*, 1995).

Keywords: hemoproteins; synchrotron radiation; photolysis; *Scapharca*.

1. Introduction

The homodimeric hemoglobin from the bivalve mollusc *Scapharca inaequivalvis* has been the subject of numerous investigations because of the marked co-operativity in oxygen binding (Chiancone *et al.*, 1981, Ikeida-Saito *et al.*, 1983) that in vertebrates has been observed only in tetrameric hemoglobin. The X-ray structure of both the unliganded and liganded forms has been solved at high resolution (Royer *et al.*, 1990; Royer, 1994). The two hemoglobin subunit closely resemble the myoglobin fold, whereas the quaternary structure is very different from that observed in vertebrate hemoglobins. The subunit interface is formed by the heme-carrying helices E and F, which are external in mammalian hemoglobins, so that the two heme groups are in direct structural communication across this interface via a hydrogen-bond network, which involves a cluster of water molecules. The same study indicates that Phe97 (a residue in hydrogen-bond contact with the Nδ1 of the proximal histidine) is extruded towards the subunit interface upon ligand binding, and makes a close hydrophobic interaction with Thr72, suggesting that Phe97 plays a critical role in modulating oxygen affinity.

The Thr72 → Ile mutant has been recently expressed with the aim to alter the subunit communication mechanism, by increasing the hydrophobic character of the interface. This mutant exhibits enhanced oxygen affinity, and markedly reduced cooperativity (Gambacurta *et al.*, 1995).

We have attempted a XANES study of the recombinant wild type and Thr72 → Ile mutant HbI, in order to elucidate the subtle structural differences at the Fe-heme site between the two proteins. The XANES data are interpreted by assuming that the Fe-heme geometry in the deoxy-form of mutant HbI has been converted in a more relaxed, 'high affinity' form. This fact can give relevant contributions to the observed differences in oxygen binding parameters for the mutant.

2. Experimental

S. inaequivalvis HbI cDNA was expressed in *Escherichia coli* strain JM105 and the oxygenated holoprotein was purified as previously described (Gambacurta *et al.*, 1993). Thr72 → Ile mutant was obtained by site directed mutagenesis on the HbI cDNA. Expression was performed in *E. coli* strain HB101 and the HbI mutant was purified as previously described (Gambacurta *et al.*, 1995). The CO derivatives of the wild-type and mutant protein were obtained by equilibrating the oxy derivatives (in 0.1 M phosphate buffer, pH 7.0) under 1 atm of CO. Deoxy derivatives were obtained by addition of few grains of dithionite to the oxygenated protein solutions, properly equilibrated under nitrogen gas. Protein concentration was 5mM in heme.

Fe K-edge x-ray absorption spectra were collected in fluorescence mode at the beam line D21 of the LURE synchrotron facility by using an energy-resolving array detector made by 7 Ge elements of very high purity from CANBERRA industries. The energy resolution at the Fe K_α fluorescence (6400 eV) was 170 eV. A Si(311) double crystal used as channel-cut was adopted as monochromator, and the spectral resolution at the Fe K-edge was about 1 eV. Harmonic contamination was rejected by using a total reflection mirror after the monochromator. The spectra of the CO and deoxy derivatives (4 frames of about 601 experimental points with 0.2 eV energy step) have been acquired at 150K and have a total signal averaging of 32 s/point. The fluorescence counts jumped from 70 counts/s/element before the edge (7100 eV), to about 850 counts/s/element above the edge (7250 eV), giving a total count jump of about 175000 with a statistical noise to count ratio of 10^{-2} before the edge and 3 10^{-3} after the edge. In all experimental spectra, the energy is aligned at the absorption threshold of metallic Fe foil.

For the photolysis experiments the CO derivative samples were cooled at T = 12K by using a liquid helium cryostat (model 22C Cryodine Cryocooler by CT Cryogenics, temperature controller model 80S by Lake Shore Cryotronics, and silicon diode temperature sensor DT-470-SD by Lake Shore). Photolysis was then achieved by prolonged illumination (white light) from a fibre optic illuminator for about two hours. During this period, distinct spectral changes in the XANES spectra at E = 7124 eV were observed to reach a steady state. After this period, XANES data were collected mantaining the sample under illumination.

3. Results and discussion

The Fe K-edge XANES spectra of recombinant wild type deoxy-HbI (dashed curve) and Thr72 → Ile mutant (solid curve) deoxy-HbI in solution are shown in Fig.1. The absorption edge of Thr72 → Ile deoxy-HbI is blue-shifted (about +1 eV) towards the spectrum of the oxy form. The spectra of the oxy form of wild type and mutant HbI are both very similar to human oxy-Hb in the R-state, (data not shown).

The Fe K-edge XANES spectra of the the CO-form (thin solid curve), its cryogenic photoproduct at T = 12K (thick solid curve), and the deoxy-form (dashed curve) of wild type HbI (lower curves) and mutant HbI (upper curves) are shown in Fig. 2. According to the overall shape of the XANES spectra, including the intensity of peaks C and C2, the Fe site geometry in HbI-CO resembles that of sperm whale and horse MbCO (having an average Fe-C-O angle of about 150 degrees; Royer, 1994), while the Fe-CO geometry in Thr72 → Ile HbI-CO is more similar to human HbCO (having an average Fe-C-O angle of about 170 degrees, Baldwin, 1980; Pin *et al.*, 1994 and references therein).

The XANES spectra of the cryogenic photoproducts have been acquired under continuous illumination by a white light source at 12K. At such a low temperature, the protein matrix remains frozen in the CO form. The Fe-heme structures of the HbI* photoproducts are similar; however, while in the case of HbI* the data show an incomplete structural relaxation of the Fe-heme towards its deoxy-like (T) form, the relaxation in Thr72 → Ile HbI* is almost complete towards the Fe-heme structure of Thr72 → Ile deoxy-HbI. These results lead to explain the observed blue-shift of Thr72 → Ile deoxy-HbI towards the spectrum of the oxy form in agreement through the suggestion that the structure of the Fe-heme site in the mutant deoxy form has been destabilized and converted from a T-like to a R-like, "high affinity" structure (Gambacurta *et al.*, 1995; Falconi *et al.*, 1998) The XANES data are in agreement with i) the relaxation of the tension at the heme iron in the mutant, as indicated by the more axial shape of the EPR spectrum of the nitrosylated derivative (Gambacurta *et al.*, 1995); ii) the visible CD spectrum of the unliganded mutant, suggesting a releasing of the constraint of the proximal histidine, which is instead present in the wild type protein (Gambacurta *et al.*, 1995); and iii) the shift to lower energy of the 'conformation sensitive' band III of the deoxy mutant with respect to the wild type, and a less asymmetric Soret band, which suggest a more planar heme structure in the mutant (Falconi *et al.*, 1998).

Taken together, all these evidences suggest that no tertiary restraint affects the Fe-heme dynamics of mutant HbI; hence the observed dramatic enhancement in oxygen affinity of this hemoprotein, and the decreasing of cooperativity, can be explained, at the Fe site level, by a reduction (suppression) of the proximal work that contributes to the T → R transition.

This work was supported by grant of INFM, Italy and by EC's TMR programs and grants from MURST and CNR, Italy.

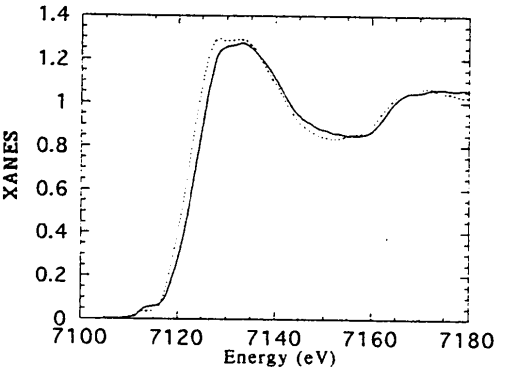

Figure 1

Fe K-edge XANES spectra of recombinant wild type deoxy-HbI (dashed curve) and Thr72 → Ile mutant (solid curve) deoxy-HbI in phosphate buffer, pH 7, T=150K.

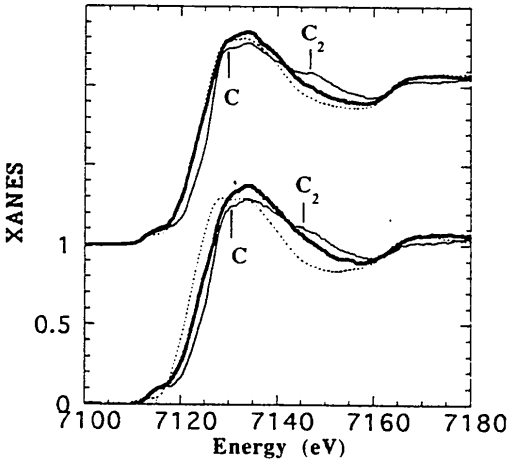

Figure2

Fe K-edge XANES spectra of the CO-form (thin solid curve), its cryogenic photoproduct at T = 12K (thick solid curve), and the deoxy-form (dashed curve) of wild type HbI (lower frame) and mutant HbI (upper frame)

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(Received 10 August 1998; accepted 1 December 1998)