XAS study of the active site of a bacterial heme-sensor

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Abstract. Denitrifying bacteria control NO and NO₂ cytosolic levels by regulating the expression of denitrification gene clusters via REDOX signalling of specific transcriptional factors that may act as NO sensors in vivo. A protein belonging to the subclass DNR (dissimilative nitrate respiration regulator) from Pseudomonas aeruginosa has been recently suggested to be a heme containing protein. Very recently the three dimensional structure of the apo-form of DNR (in the absence of heme) has been determined by X-Ray crystallography, whereas the holo-form (in the presence of heme) has not yet been crystallized. We have investigated the heme local structure in solution of ferric and ferrous holo-DNR by XAS. The Fe K-edge XANES spectrum of the ferric adduct displays typical features of a low-spin hexacoordinate Fe-heme complex, having two histidines ligated. After chemical reduction, relevant changes of the XANES fingerprints suggest a repositioning of the heme inside the hydrophobic core of the protein in agreement with previously reported structural and spectroscopic evidence. Partial release of the axial ligands leaves the Fe(II)heme available, and very reactive, to bind exogenous ligands like NO, thus supporting its role as the cofactor involved in NO sensing activity.

1. Introduction

Denitrifying bacteria keep the steady-state concentration of nitrite and nitric oxide (NO) below cytotoxic levels by controlling the expression of denitrification gene clusters via REDOX signalling of specific transcriptional regulators [1]. The NO dependence of the transcriptional activity of promoters regulated by these protein factors, has indicated that they may act as NO sensors in vivo. A homodimeric protein belonging to the subclass DNR (dissimilative nitrate respiration regulator) from Pseudomonas aeruginosa has been recently suggested to be a heme containing protein [2]. There is a great clinical interest in the regulation of denitrification, as in P. aeruginosa it is strictly related to
virulence [3]. Very recently the three dimensional structure of the apo-form of DNR (in the absence of heme) has been determined by X-Ray crystallography [4], whereas the holo-form (in the presence of heme) has not yet been crystallized. In the present work, we have investigated in solution the ferric and ferrous holo-DNR by XANES in order to elucidate the Fe-heme environment and test its possible role as a cofactor in NO sensing activity. In fact XANES spectra can be successfully used as fingerprints of the axial ligation of hemes in solution as previously shown [5, 6].

2. Methods
The Fe K-edge XANES spectra of ferric and ferrous holo-DNR have been collected in fluorescence mode at ESRF-BM30B, Grenoble, by using a 30-elements ultra-pure Ge detector. The spectra were calibrated by assigning the first inflection point of the Fe foil spectrum to 7112. The energy stability of each spectrum was carefully assessed by checking the position of a glitch in the $I_0$ at 7220 eV.

3. Results
In Fig. 1, the Fe K-edge XANES spectrum of the formally “ferric” holo-DNR is shown. Its derivative spectrum is shown in the inset. The spectrum displays some rather broad features (1-7 in the absolute spectrum and α-δ in the derivative spectrum), all typical of hexa-coordinated hemes and is reminiscent of the spectra reported for Fe(III)TPP(Imid)$_2$ and neuroglobin, both having a bis-imidazole Fe-heme [5, 7].

![Figure 1. Fe K-edge XANES spectrum of ferric DNR. In the inset, the derivative spectrum at the edge is displayed. DNR was 1.6 mM, in 32 mM phosphate buffer, pH 7.4, 240 mM NaCl, gly 20%](image)

In order to have deeper insight on the axial coordination and homogeneity level of heme-iron in DNR, we have compared in Figure 2 the derivative XANES spectrum of the formally ferric holo-DNR (dotted curves) with different coordination models available to us (solid curves, see the caption of Figure 2 for details on the models chosen). As shown in this Figure, the highest similarities concerning the features α-δ are obtained with the spectrum of formally ferric neuroglobin (Ngb), where a bis-histidine axial coordination of the heme occurs. In neuroglobin, the ferric heme is rapidly photoreduced under X-ray irradiation, resulting in a red-shift of the XANES edge with respect to that of Fe(III)TPP(Imid)$_2$, a model compound for bis-His coordination [7]. In the absence of further available models for axial coordination (for example from tyrosine) the heme iron in formally ferric DNR is assigned to a low-spin hexacoordinated species, very likely a bis-histidine photo-reduced one.
**Figure 2.** The derivative spectrum of DNR (plotted curves) is compared with various high-spin (HS) and low-spin (LS) coordination models of the heme iron in heme models and hemeproteins (solid lines). From bottom to top: acid aquomet-myoglobin, His-Fe(III)-H$_2$O; 1,2 methyl-imidazole (penta-coordinate) hemin dissolved in SDS micelles, His-Fe(III); Fe(III)-tetraphenyl-porphyrin-(Imid)$_2$, His-Fe-His: P450, Cys-Fe(III)-H$_2$O; alcaline aquomet-myoglobin, His-Fe(III)-OH; Cytochrome C, His-Fe(III)-Met and neuroglobin (His-Fe(III)-His). The last derivative is fastly photo-reduced under X-ray irradiation.

The XANES spectrum of the chemically reduced (by sodium dithionite excess) ferrous DNR is shown in Figure 3 (lower curve, solid line) and compared with the formally ferric, photoreduced species (lower curve, dotted line). The most important change is observed at 7117 eV (pointed out by an arrow), where a shoulder emerges in the low rising edge of ferrous DNR. The shoulder appears as a further peak in the derivative spectrum. This feature is absent in any of the coordination models of Figure 2, but is typical (and very pronounced) in tetra-coordinated square-planar metallo-porphyrins (Zn, Cu, Fe, Ni) being assigned to a dipole allowed 1s$\rightarrow$4p$_z$ transition[8, 9]. Hemin dissolved in 10% sodium dodecyl sulphate (SDS) and reduced by sodium dithionite was used to represent the XANES spectrum of tetra-coordinate Fe(II)-protoheme in Figure 3, upper curve.

**Figure 3.** Fe K-edge XANES spectrum of ferrous DNR (lower curve, solid line), formally ferric, photoreduced DNR (dotted line), and Fe(II)-porphyrin in SDS micelle, a model for tetra-coordinate hemes (upper curve). In the insets, their respective derivative spectra are depicted. The feature at 7117 eV is put in evidence by an arrow.

DNR was 1.6 mM, in 32 mM phosphate buffer, pH 7.4, 240 mM NaCl, slight excess of sodium dithionite, gly 20%
The appearance of the shoulder at 7117 eV gives a clue for a partial destabilization of the axial ligation in ferrous DNR, resulting in an equilibrium between two species, an hexa- and a tetra-coordinate one. Thus we have tried to fit the derivative spectrum of ferrous DNR as a linear combination of a bis-histidine adduct and a tetra-coordinate Fe-porphyrin. The resulting best fit is shown in Figure 4. According to our equilibrium model, about 35% of the hemes inside the DNR hydrophobic core is tetra-coordinate.

Figure 4. XANES derivative spectrum of ferrous DNR (dotted line). The solid line represents the best fit result of a linear combination (0.65:0.35) of the formally ferric, photoreduced DNR and the tetra-coordinate Fe(II) porphyrin.

4. Discussion

The Fe-heme, inside the hydrophobic core of the ferrous holo-DNR, seems to be in dynamical equilibrium between two species: a bis-histidine hexacoordinate one, and a tetra-coordinate one.

The presence of a percentage of tetra-coordinate heme-iron gives support for its role as the cofactor involved in NO sensing activity of DNR. It is well known [10] that the binding equilibrium constant of NO to a tetra-coordinate heme iron is extraordinarily large with respect to other gaseous ligands like CO and O₂ and, contrary to them, NO binding is anticooperative with respect to a trans-ligand, i.e. NO binds better without a trans-base, and correspondingly, the binding constant for a trans-imidazole is decreased by about 3-fold upon addition of NO.

Thus, a picture of the NO sensing activity in homodimeric DNR “in vivo” can be summarized as follows: in the presence of NO, the dynamical bis-histidine/tetra-coordinate equilibrium can be shifted by a rapid ligation of NO to a tetra-coordinate species, resulting in an activated functional form of the protein, very likely having a penta-coordinate nitrosyl-heme. Activation could include tertiary or quaternary changes following, as key steps, either the breaking of the Fe-histidine bond, like in guanylate cyclase [11], either the distortion of the peripheral heme groups due to formation of the penta-coordinate adduct.

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