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Nitric oxide heme interactions in nitrophorin 7 investigated by nuclear inelastic scattering

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Abstract Nitrophorins (NPs) occur in the blood-sucking insect *Rhodnius prolixus*. These proteins use ferric heme to store nitric oxide (NO) in the salivary glands of the insects and transport it to the victim's tissues, resulting in vasodilation and reduced blood coagulation. In this work we present a nuclear inelastic scattering (NIS) study in order to characterize the iron-NO interaction in the isoform nitrophorin 7 (NP7). The NIS data obtained for NP7 complexed with NO show a strong band at $\sim$589 cm^{-1} which is due to modes with significant Fe-NO stretching and bending character. Another conspicuous feature is a significant peak at $\sim$280 cm^{-1} in the region where the heme modes occur. Based on a hybrid calculation method, which uses density functional theory and molecular mechanics, the band at $\sim$280 cm^{-1}is assigned to heme modes with substantial doming character.

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1 Introduction

Nitrophorin 7 (NP7) is one of seven NO-carrying heme proteins in the salivary glands of the bug *Rhodnius prolixus* [1]. The NPs are the first examples of proteins which form stable Fe(III)-NO complexes, where the NO can be stored for a long period of time. When the bug feeds on his victim, the saliva is pumped into the victim's tissue and based on the change of the pH (from approx. 5.5 in the salivary glands to approx. 7.4 in the blood plasma) NO is released [2]. The unbound NO causes vasodilation via a signaling cascade and facilitates the blood meal of the insect. In contrast to the other NPs, NP7 is the only example of a membrane binding NP. Furthermore, the heme binding pocket of NP7 exhibits significant structural differences compared to the other NPs resulting in a pronounced influence on the heme orientation and on the proximal Fe-His bond strength [3, 4]. Therefore, the question arises to which extent the porphyrin, and thus the Fe-NO interaction, might be affected by the NP7 heme pocket. Here we present a nuclear inelastic scattering (NIS) study to address these questions, which are of general importance for the understanding of the structure-function relationships of heme proteins involved in physiological NO signaling.

2 Materials and methods

NP7 was expressed in BL21(DE3) *E. coli* cells (Novagen). The detailed preparation method of the sample can be found in [5]. For the reconstitution with ^{57}Fe-heme, the isotopically enriched cofactor was produced according to the method reported in [6]. The sample was prepared with ∼8 mM ^{57}Fe-enriched NP7 in 100 mM MOPS/NaOH (pH 7.0), 2 % glycerol. In order to study the iron ligand modes of NP7 by means of NIS we used the NO-ligated form of NP7 (NP7-NO). The NIS data were recorded at the Nuclear Resonance Beamline ID 18 of the European Synchrotron Radiation Facility (ESRF) in Grenoble, France, under experiment No. LS-2214. The 6 GeV electron storage ring was operated in hybrid mode. For our experiment the incident beam was monochromatized by a Si(111) double-crystal premonochromator, a refractive beryllium collimator and a high-resolution monochromator to a bandwidth down to 0.8 meV (∼6.5 cm^{-1}). Simulations of the NIS data were performed with GAUSSIAN 09 using the ONIOM method. The heme moiety with protonated carboxylates and its ligands were treated with density functional theory (DFT) using the functional B3LYP with the basis set CEP-31G. The rest of the protein was treated with a molecular mechanics approach using the universal force field UFF [7]. Geometry optimizations and frequency calculations were performed on an unpublished crystal structure of NP7, which was provided from the coauthors M. Knipp and H. Ogata [8]. Normal mode and normal-coordinate structure decomposition (NSD) analysis were performed on the optimized structure [9] using a web engine developed by Sun and Shelnutt [10].

3 Results and discussion

Figure 1a shows the experimentally determined iron partial density of vibrational states (PDOS) of NP7-NO as obtained from NIS measurements performed at 40 K. The PDOS

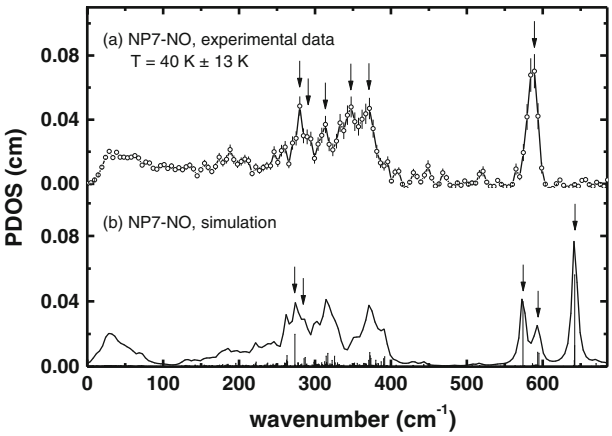

Fig. 1 **a** Experimentally determined PDOS of NP7-NO obtained at 40 K and **b** simulated PDOS of NP7-NO using the ONIOM option of Gaussian 09 (B3LYP with CEP-31G and UFF). The bar graphs display the calculated mode composition factor e^2. The experimental energy resolution of the set-up was ~ 6.5 cm^{-1}

reveals an intense band at 589 cm^{-1}. A similar band with a maximum at 594 cm^{-1} and a shoulder at about 581 cm^{-1} has been also detected for the NO complexed form of NP2 [6]. These bands have been assigned to modes with preferentially FeNO stretching and FeNO bending character. [11]. In addition, Fig. 1a displays five bands in the heme region at 280, 289, 314, 348 and 372 cm^{-1}.

The associated simulation of NP7 with NO bound is shown in Fig. 1b. The calculation of the PDOS using a ferric low-spin heme iron leads to a mode at 642 cm^{-1}, which has significant Fe-NO stretching character. Two bands in the energy region below 600 cm^{-1} display Fe-N-O bending modes at 573 cm^{-1} and 593 cm^{-1}. Both oscillate in phase and the only difference between these modes is the direction of motion. The former mode swings in plane "A" and the latter one in plane "B" which are orthogonal to each other (see Fig. 2a). The reason for the energy difference between these two bending modes might be the geometry of the heme structure. The result of the NSD analysis shown in Fig. 2b allows a closer description of the heme group and reveals that the optimized heme structure shows mainly saddling and ruffling deformation character. Liptak et. al. describe these deformations as follows (see also Fig. 1 in [12]): In case of a saddling deformation the beta carbons of two opposite pyrrole rings of the heme are displaced downward and the other two are displaced upward with respect to the heme plane. A ruffling deformation shows an alternating clockwise and counterclockwise twisting of the pyrrole rings along their Fe-N axes, with two opposite meso carbons displaced downward while the other two are displaced upward with respect to the heme plane) [12]. These two types of out-of-plane deformations are part of the lowest-energy modes which describe the distortion of the porphyrin macrocycle [9]. The degree of saddling of NP7-NO ($\sim$0.49 Å) and NP2-NO ($\sim$0.50 Å [6]) is comparable in their optimized protein structures, whereas the degree of ruffling is about nine times higher in NP2-NO ($\sim$1.6 Å [6]) compared to NP7-NO ($\sim$0.17 Å).

Furthermore, the simulation exhibits two dominant modes at 273 cm^{-1} and 287 cm^{-1} which involve a strong heme doming character and are also present in the experimental data. Heme doming is a concerted out-of-plane bending of all four pyrrole rings [12]. In the energy region between 300 cm^{-1} and 400 cm^{-1} a complex vibrational structure with

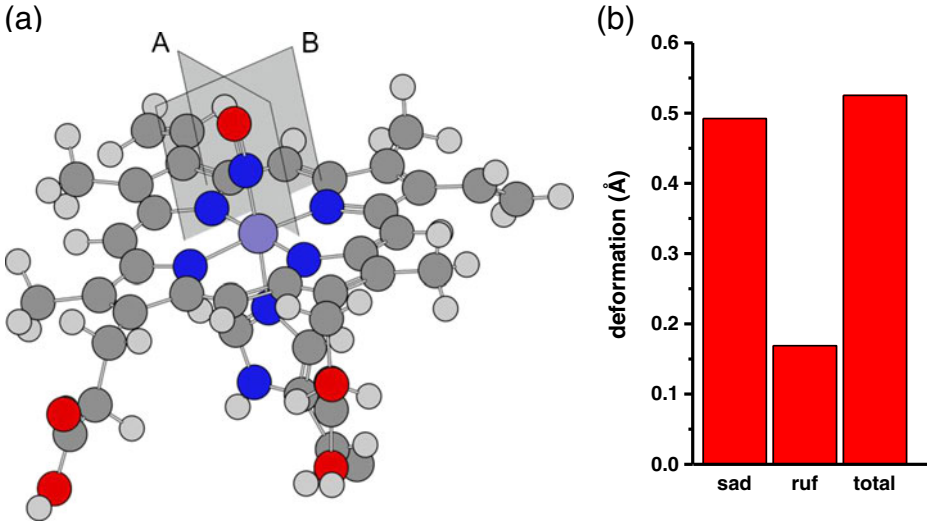

Fig. 2 **a** Heme moiety of NP7 as obtained after energy minimization. For clarity the protein matrix is not shown (iron, purple; nitrogen, blue; oxygen, red; carbon, dark gray; hydrogen, light gray). The iron center of the heme is shown in purple, the nitrogen atoms in blue, the oxygen atoms in red, the carbon atoms in dark grey and the hydrogen atoms in light grey. The two planes "A" and "B" represent the ranges of motion of the protein modes with significant Fe-NO bending character. **b** NSD analysis of the optimized heme structure. *sad* stands for saddling, *ruf* for ruffling and *total* describes the total out-of-plane deformation

many bands caused by in-plane and out-of-plane iron movements coupled with partial heme doming is noticed. These modes are thermally populated at room temperature and especially heme doming may contribute to the process of NO binding that is observed in oxygen storage proteins like myoglobin [12, 13].

The simulations of the PDOS of NP7-NO presented here and in earlier work for NP2-NO [6, 14] reproduce the experimental NIS data in a satisfactory fashion. However, the experimentally observed almost degeneracy of the Fe-NO stretching and bending modes around 590 cm^{-1} is not reproduced by our theoretical QM/MM treatment. Instead, the simulation shows a single stretching mode at 642 cm^{-1}. The reason might be an interaction of protein residues in the heme pocket above the heme plane with the oxygen of the NO ligand possibly communicated via a water hydrogen bond. In order to clarify this issue QM/MM calculations are underway in our laboratory which do not only treat the NO-heme moiety but also selected protein residues near the NO ligand with DFT.

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