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Nuclear inelastic scattering of heme proteins: from iron ligand vibrations to low energy protein modes

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Abstract The binding of the signal molecule nitric oxide (NO) to the NO transporter protein Nitrophorin 2 (NP2) from the bloodsucking insect *Rhodnius prolixus* has been characterized by Mössbauer spectroscopy as well as nuclear forward scattering (NFS) and nuclear inelastic scattering (NIS). A striking feature of the vibrational spectrum obtained from NP2-NO is a vibration at 594 cm^{-1}. This mode is assigned to a Fe-NO stretching mode via simulation of the NIS data by density functional theory (DFT) coupled with molecular mechanics (MM) methods. At frequencies below 100 cm^{-1} collective motions like heme doming occur which could explain spectroscopic features observed by NIS at these low energies.

Keywords Proteins · Nitric oxide · Nuclear forward scattering · Nuclear inelastic scattering · Heme doming

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1 Introduction

The heme proteins the nitrophorins (NP) are found in the salivary glands of the bloodsucking insect *Rhodnius prolixus*. In order to feed, the saliva containing the NPs is injected into the victim's tissues, and due to the dilution and pH change (from ∼5.5 in the salivary glands to 7.35 in the tissues) the iron ligand nitric oxide (NO) is released. NO then causes dilation of the victim's capillaries and inhibition of platelet aggregation so that more blood flows to the insect [1]. In mammals the signal molecule NO, although highly toxic in high concentrations, is not only involved in vasodilation, but also in inflammatory processes. Therefore we were interested in the question whether the binding of this signal molecule to its transporter protein could be followed by nuclear inelastic scattering (NIS).

2 Materials and methods

The isoform nitrophorin 2 (NP2) was expressed in BL21(DE3) *E. coli* cells (Novagen). Details of the preparation can be found in [2]. Two samples were prepared with ∼8 mM ^{57} Fe-enriched NP2: (i) NO-free (NP2) at pH 7.5 and (ii) NO-bound (NP2-NO) at pH 5.5 with NO added at a molar ratio of 1.5. The samples were transferred into a special sample holder which allows performing Mössbauer spectroscopy as well as NFS and NIS experiments on the same sample. Mössbauer spectra were measured in a He-bath cryostat (Oxford MD 306) and recorded using a conventional spectrometer in the constant-acceleration mode. Isomer shifts are given relative to α-Fe at room temperature. The spectra were analyzed by least-square fits using Lorentzian line shapes. NIS and NFS experiments were performed at ID 18 of the ESRF with an energy resolution of 16 cm^{-1}. Geometry optimizations and frequency calculations were performed with GAUSSIAN 03 [3]. As input for the calculations, the PDB entry 1T68 of NP2-NO was used. QM/MM calculations were performed on the whole protein by applying the method ONIOM [4]. The heme moiety and its ligands were treated with DFT (functional B3LYP with basis set CEP-31G) and the rest of the protein with the universal force field UFF. Normal mode analysis was performed on the optimized structures and NIS spectra were calculated according to [5].

3 Results and discussion

The Mössbauer spectrum of NP2 obtained at T = 77 K shows an asymmetric doublet with $\delta = 0.30$ mms^{-1} and $\Delta E_Q = 2.53$ mms^{-1} (Fig. 1a). These parameters are indicative of a ferric low spin heme iron with two axial ligands [6]. The asymmetry of the spectrum as well as the large line width is caused by paramagnetic relaxation effects [6]. Addition of NO to NP2 leads to a symmetric doublet with $\delta = -0.01$ mms^{-1} and $\Delta E_Q = 1.86$ mms^{-1} (Fig. 1b). These parameters are comparable to those of the NO complex of the isoform nitrophorin 4 ($\delta = 0.00$ mms^{-1} and $\Delta E_Q = 1.84$ mms^{-1} [7]). NFS measurements obtained during the NIS scans of NP2 and NP2-NO (see Fig. 1e,f) are shown in Fig. 1c and d. The spectra exhibit a beating structure which is caused by the presence of quadrupole interaction. The fact that the ΔE_Q-values obtained

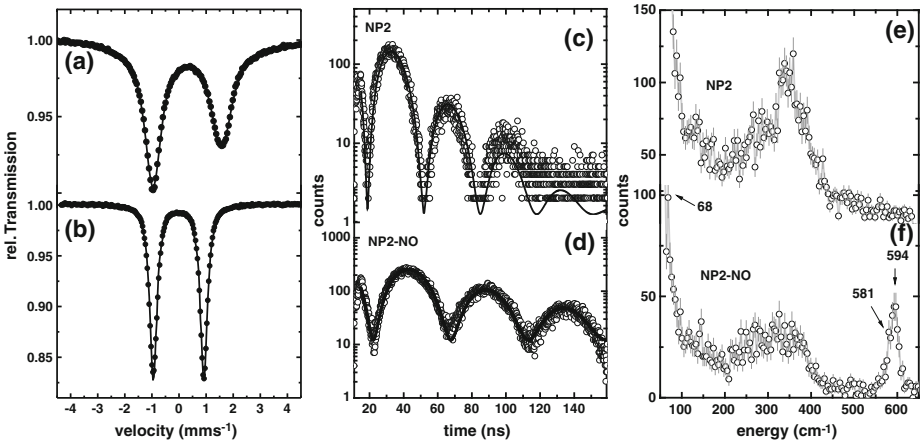

Fig. 1 **a** Mössbauer spectra of NP2 taken at T = 77 K. The *solid line* represents a Lorentzian fit with an asymmetric doublet yielding: $\delta = 0.30 \pm 0.01$ mms^{-1}, $\Delta E_Q = 2.53 \pm 0.01$ mms^{-1}, $\Gamma_1 = 0.86 \pm 0.01$ mms^{-1}, $\Gamma_2 = 1.11 \pm 0.01$ mms^{-1}. **b** Mössbauer spectrum of NP2-NO taken at T = 77 K. The *solid line* is a Lorentzian fit with $\delta = -0.01 \pm 0.01$ mms^{-1}, $\Delta E_Q = 1.86 \pm 0.01$ mms^{-1} and $\Gamma = 0.40 \pm 0.01$ mms^{-1}. **c** NFS spectrum of NP2 taken at T = 100 K. The *solid line* is a simulation using the software Motif [8] yielding $\Delta E_Q = 2.60 \pm 0.05$ mms^{-1} and $t_{eff} = 1.58 \pm 0.05$. **d** NFS spectrum of NP2-NO. The *solid line* has been calculated with $\Delta E_Q = 1.89 \pm 0.05$ mms^{-1} and $t_{eff} = 1.80 \pm 0.05$. Experimental NIS spectra of NP2 (**e**) and of NP2-NO (**f**) obtained at ID 18 of ESRF

from Mössbauer spectroscopy and from NFS (see caption of Fig. 1c,d) are very similar, indicates that no radiation damage had occurred during the NIS experiments. The comparison of the NIS data of NP2 (Fig. 1e) and of NP2-NO (Fig. 1f) shows

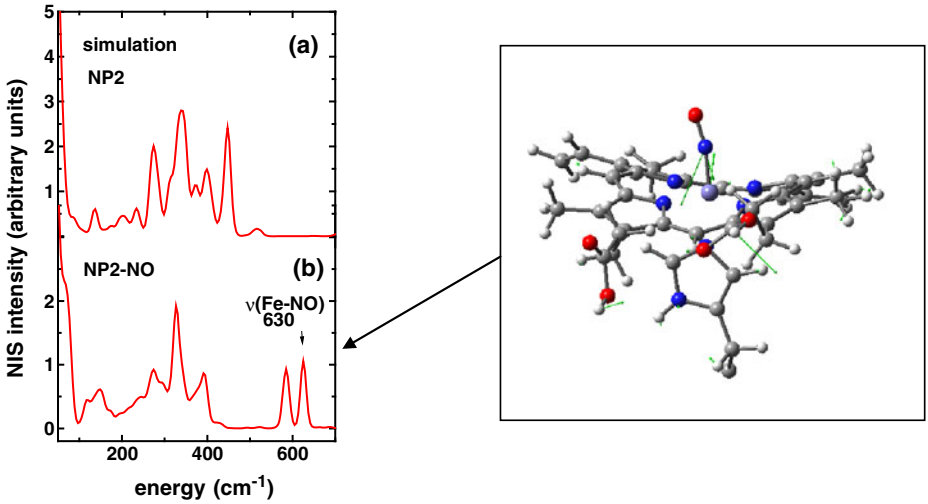

Fig. 2 Simulated NIS-spectra calculated by QM/MM calculations of the whole protein. The optimized heme structure of NP2-NO used for calculating the NIS data presented in Fig. 1e and f is shown in the inset. Only the parts of the molecules treated with DFT are displayed. The residual parts of the protein, which were taken into account by force field calculations, are not shown. The *arrows* describe atom movements of a protein mode with significant Fe-NO stretching character. The calculated energy of this mode is 630 cm^{-1}

that NO binding leads to an intense vibration at 594 cm^{-1}. Simulations of the NIS data are shown in Fig. 2. The simulated NIS spectrum of NP2 (Fig. 2a) has been calculated assuming NH_3 as an axial ligand distal to the heme (pdb entry 2EU7). Albeit the spectrum was calculated with a ferric low-spin heme iron as indicated by Mössbauer spectroscopy and NFS [6] (see Fig. 1a,c), the simulated NIS pattern of NP2 shows a band at 449 cm^{-1} with Fe-NH_3 stretch character which is not present in the experimental data (Fig. 1e). Therefore the sixth ligand is very likely not an NH_3, but another unknown small molecule from the protein expression and purification. In the energy region between 200 and 400 cm^{-1} a complex vibrational structure caused by in-plane as well as out-of-plane iron movements is observed.

Calculation of the NIS spectra of NP2-NO yields a mode at 630 cm^{-1} with strong Fe-NO stretching character (see Movie 1 in supplement) in good agreement with experiment. At energies below 100 cm^{-1} about 300 protein modes have been calculated. Among the most intense are 4 modes at 69, 70, 72 and 74 cm^{-1} all of which involve considerable heme doming (see Movie 2, supplement). It is also striking that the experimental NIS spectrum of NP2-NO has an intense band at 68 cm^{-1}. Based on the simulations presented this band can be assigned to protein modes involving strong heme doming character.

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