Hyperfine Interact (2016) 237:19
DOI 10.1007/s10751-016-1240-6

# Nitric oxide heme interactions in nitrophorin from *Cimex lectularius*

**R. Christmann[1] · H. Auerbach[1] · R. E. Berry[2] · F. A. Walker[2] · V. Schünemann[1]**



**Abstract** The nitrophorin from the bedbug *Cimex lectularius* (cNP) is a nitric oxide (NO) carrying protein. Like the nitrophorins (rNPs) from the kissing bug *Rhodnius prolixus,* cNP forms a stable heme Fe(III)-NO complex, where the NO can be stored reversibly for a long period of time. In both cases, the NPs are found in the salivary glands of blood-sucking bugs. The insects use the nitrophorins to transport the NO to the victim's tissues, resulting in vasodilation and reduced blood coagulation. However, the structure of cNP is significantly different to those of the rNPs from *Rhodnius prolixus*. Furthermore, the cNP can bind a second NO molecule to the proximal heme cysteine when present at higher concentrations. High field Mössbauer spectroscopy on $^{57}$Fe enriched cNP complexed with NO shows reduction of the heme iron and formation of a ferrous nitric oxide (Fe(II)-NO) complex. Density functional theory calculations reproduce the experimental Mössbauer parameters and confirm this observation.

**Keywords** Nitric oxide · Mössbauer spectroscopy · Density functional theory · Nitrophorin · Heme

This article is part of the Topical Collection on *Proceedings of the International Conference on the Applications of the Mössbauer Effect (ICAME 2015), Hamburg, Germany, 13–18 September 2015*

✉ H. Auerbach
auerbach@physik.uni-kl.de

[1] Department of Physics, University of Kaiserslautern, Erwin-Schrödinger-Str. 46, 67663 Kaiserslautern, Germany

[2] Department of Chemistry and Biochemistry, The University of Arizona, Tucson, AZ 85721-0041, USA

Published online: 18 February 2016

## 1 Introduction

The bedbug *Cimex lectularius* has a NO carrying heme protein called nitrophorin (cNP) in the salivary glands which is used to transport the highly reactive signaling NO molecule to the victim's tissues, which facilitates the blood meal of the insect. Like the nitrophorins from the kissing bug *Rhodnius prolixus* (rNP), cNP shows the same pH-dependent reversible NO binding behavior [1]. cNP also forms a stable heme Fe(III)-NO complex, where the NO can be stored for a long period of time but in terms of size, three-dimensional structure, amino acid sequence and as well as the sixth ligand of heme iron it is very different from rNP [1]. The structure of cNP is like a $\beta$-sandwich. The heme is located on the outside of the sandwich and is bound to a cysteine thiolate ligand of the protein [2]. At high concentrations of NO two NO molecules bind, one to the heme iron and the other to the proximal cysteine thiolate, to form a S-nitrosyl conjugate which results in reduction of the heme iron [2]. Nitrosyl heme complexes have been studied by Mössbauer spectroscopy in detail, e.g. for the NO hemoglobin adduct [3, 4] as well as for NO aducts of a variety of heme models [5], Mössbauer studies of NO aducts of heme thiolate proteins have been at least to our knowledge not yet been reported. Therefore, we present a field-dependent Mössbauer spectroscopic study in order to obtain a better understanding of this reversible and concentration-dependent reductive NO heme binding mechanism of cNP.

## 2 Materials and methods

cNP was expressed and purified as reported previously [2]. $^{57}$Fe (95.45% enriched) labeled hemin, prepared as reported previously [6], was inserted and the holoprotein purified as reported previously [2]. Two samples of this $^{57}$Fe-labeled cNP bound to NO were prepared, one at pH 5.5 (50 mM acetate buffer) and the other at pH 7.5 (50 mM phosphate buffer). The protein concentration in both samples was 2 mM. The sample at pH 5.5 was treated with a molar ratio of 2 NO and the sample of pH 7.5 was treated with a molar ratio of 4 NO.

The Mössbauer data on the $^{57}$Fe enriched samples at different magnetic fields have been collected in transmission geometry and constant acceleration mode with a closed cycle cryostat equipped with a superconducting magnet, as described earlier [7]. This set-up exhibits an experimental line width of 0.32 mms$^{-1}$ [7]. The analysis of doublets occurring in the Mössbauer spectra of this study shows a relatively large line-width of 0.40 mms$^{-1}$ (see Table 1). Such a line width is not uncommon for NO heme adducts and was also observed in Hb-NO [4]. Typical measuring time per spectrum was 4 days using a 50 mC $^{57}$Co/Rh source. The spectra were simulated on the basis of Lorentzian line shape or in case of magnetically-split spectra, the spin Hamiltonian approximation using Vinda Add On for Excel 2003 [8]. Isomer shifts are given relative to $\alpha$-Fe at room temperature.

The optimization of the protein structure models for cNP-NO/pH 5.5 (**I**) and cNP-NO/pH 7.5 (**II**) was performed with GAUSSIAN 09 [9] using the ONIOM method [10]. The heme moiety with unprotonated and protonated carboxylates and its neighboring ligands like the cysteine unit and the NO were treated with density functional theory (DFT) using the functional TPSSH in combination with the basis set TZVP. The rest of the protein was treated with a molecular mechanics approach using the universal force field UFF. The structures of **I** and **II** were optimized based on the pdb entries 1NTF.pdb and 1Y21.pdb. Since 1NTF.pdb is the crystal structure of the Fe(III) aqua complex the water molecule has been replaced by a NO molecule.

**Table 1** Mössbauer parameters obtained from the spin-Hamilton analysis (*black solid lines*) shown in Fig. 1 in comparison to the DFT-calculated Mössbauer parameters based on the structural models **I** and **II** shown in Fig. 2

| | $S_{Fe-NO}$ | $\delta$ [$mms^{-1}$] | $\Delta E_Q$ [$mms^{-1}$] | $\Gamma$ [$mms^{-1}$] | $\eta$ | $A/g_n\mu_n$ [T] | g-values |
|---|---|---|---|---|---|---|---|
| cNP-NO/pH 5.5 component **1** | 0 | $0.11 \pm 0.03$ | $1.33 \pm 0.03$ | $0.40 \pm 0.02$ | $0.0 \pm 0.1$ | — | — |
| calc. deprot | 0 | 0.07 | 0.94 | — | 0.2 | — | — |
| calc. prot | 0 | 0.08 | 0.80 | — | 0.2 | — | — |
| cNP-NO/pH 5.5 component **2** | $^1/_2$ | $0.34 \pm 0.03$ | $1.42 \pm 0.03$ | $0.40 \pm 0.02$ | $0.0 \pm 0.1$ | $-24.4 \pm 2.5$ | $2.11 \pm 0.01$ |
| | | | | | | $-24.2 \pm 2.5$ | $2.04 \pm 0.01$ |
| | | | | | | $12.0 \pm 1.0$ | $2.04 \pm 0.01$ |
| cNP-NO/pH 7.5 | $^1/_2$ | $0.34 \pm 0.03$ | $1.42 \pm 0.03$ | $0.40 \pm 0.02$ | $0.0 \pm 0.1$ | $-24.4 \pm 1.0$ | $2.11 \pm 0.01$ |
| | | | | | | $-24.2 \pm 1.0$ | $2.04 \pm 0.01$ |
| | | | | | | $12.0 \pm 1.0$ | $2.04 \pm 0.01$ |
| calc. deprot | $^1/_2$ | 0.25 | 1.12 | — | 0.1 | — | — |
| calc. prot | $^1/_2$ | 0.24 | 1.20 | — | 0.2 | — | — |
| Hb-NO [4] | $^1/_2$ | 0.42 | 1.50 | 0.40 | — | −19.6 | 2 |
| | | | | | | −19.6 | |
| | | | | | | 6.8 | |
| Fe(OEP)NO [5] | $^1/_2$ | 0.26 | 1.27 | 0.28 | 0 | −26 | — |
| | | | | | | −26 | |
| | | | | | | 16 | |

The relative contribution of component **1** is 88 % and 12 % of component **2** to the total area of the spectrum of cNP-NO/pH 5.5. The line-width $\Gamma$ =0.40 $mms^{-1}$ has been determined from the linewidth of component **1** and has been fixed to this value in the spin-Hamilton analysis. The $\delta$ and $\Delta E_Q$ values have been calculated for the case of protonated and non-protonated heme carboxyl residues (prot/deprot)

For the theoretical calculation of the Mössbauer parameters from the optimized layer the program package ORCA [11] was used. The terminal carbon of the cysteine fragment was completed with hydrogen atoms. The functional TPSSH was used in combination with the CP(PPP) basis set for Fe and TZVP basis set for all other atoms.

## 3 Results and discussion

Figure 1a shows the Mössbauer spectra of cNP-NO/pH 5.5 taken at T = 12 K with an external magnetic field of B = 20 mT applied perpendicular to the $\gamma$-beam (upper spectrum) and at T = 15 K with B = 5 T (lower spectrum). The spectra have been analyzed by means of two components. Component **1** with a relative intensity of 88 % has $\delta_1 = 0.11 \pm 0.05$ $mms^{-1}$ and $\Delta E_{Q1} = 1.33 \pm 0.05$ $mms^{-1}$. The protein shows a symmetric doublet at low field and at a high field of 5 T a magnetic splitting which is due only to the external magnetic field.

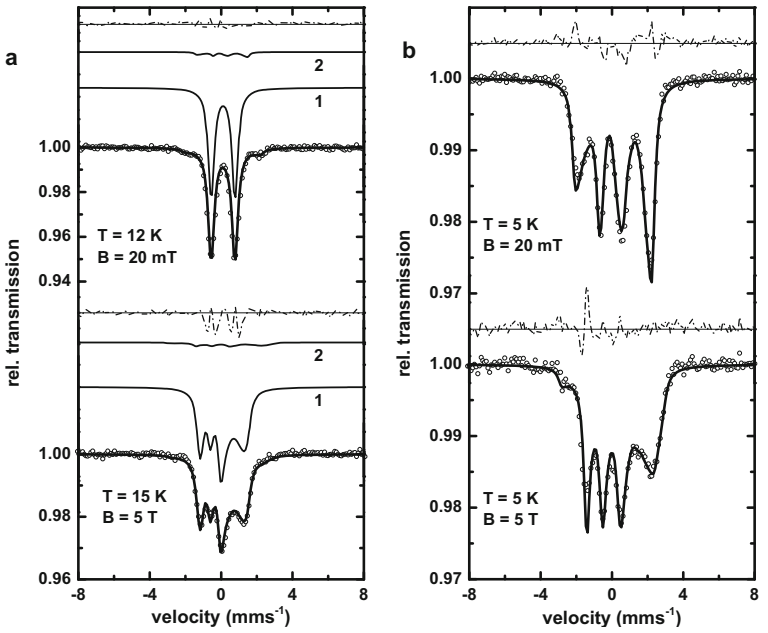


**Fig. 1** Field dependent Mössbauer spectra of cNP-NO/pH 5.5 (**a**) taken at T = 12 K with an external magnetic field of 20 mT applied perpendicular to the $\gamma$-beam (*upper* spectrum) and at T = 15 K with B = 5 T (*lower* spectrum) and of cNP-NO/pH 7.5 taken at T = 5 K (**b**) with an external magnetic field of 20 mT applied perpendicular to the $\gamma$-beam (*upper* spectrum) and with B = 5 T (*lower* spectrum). The *solid lines* are the result of a spin-Hamilton analysis. The *solid line* in (**b**) represents component 2 in (**a**). All resulting parameters are given in Table 1. The residuals are shown as *dashed dotted lines*

This aspect leads to the conclusion that cNP-NO/pH 5.5 has a low-spin ferric heme Fe(III)-NO complex with a diamagnetic ground state (because NO is an $S = {}^1/_2$ radical). The minor component **2** has been analyzed assuming a total spin of ${}^1/_2$ and the following parameters: $\delta_2 = 0.34 \pm 0.05$ mms$^{-1}$, $\Delta E_{Q2} = 1.42 \pm 0.05$ mms$^{-1}$ and a hyperfine coupling tensor $A/g_n\mu_n = (24.4 \pm 2.5, -24.2 \pm 2.5, 12.0 \pm 1.0)$ T. These parameters are characteristic of a low-spin ferrous heme Fe(II)-NO complex which is also formed after the reduction of the heme iron in the presence of higher NO concentrations, as will be shown below.

Mössbauer spectra of cNP-NO/pH 7.5 obtained at T = 5 K in presence of an external magnetic field perpendicular to the $\gamma$-beam at B = 20 mT (upper spectrum) and B = 5 T (lower spectrum) are shown in Fig. 1b. The spectra have been analyzed by a parameter set which is identical to that of component **2** in Fig. 1a. This indicates the full reduction of the heme-NO moiety to a Fe(II)-NO complex. All experimentally-determined parameters are given in Table 1.

Weichsel et al. reported that in the presence of high NO concentrations a second NO molecule forms an S-nitrosyl conjugate with the proximal cysteine [2]. In order to confirm this proposed reaction scheme DFT calculations have been performed based on structural models of **I** and **II**. The Mössbauer parameters calculated for **I** and **II** are in reasonable agreement with the experimentally-determined ones (see Table 1). The protonation state of the carboxyl residues has only a minor effect on the calculated isomer shifts and quadrupole splittings, whereas the characteristic change of the Mössbauer parameters upon reduction

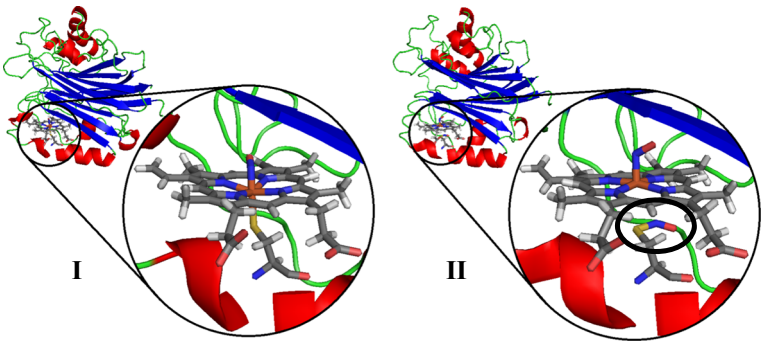


**Fig. 2** Molecular viewgraph of cNP complexed with NO. The zoomed-in regions show the heme with the molecular bonds represented as sticks. The iron (*brown*) and the NO ligand on top of the iron with the nitrogen (*blue*) and the oxygen (red) are visible in the center of the zoomed-in regions. The structures have been obtained via geometry optimization of the models of cNP-NO/pH 5.5 (Fe(III) **I**, *left*) and cNP-NO/pH 7.5 (Fe(II) **II** with Cys-SNO, *right*). In **II** the Cys-SNO is *circled*

from Fe(III) to Fe(II) is fully reproduced by the structural models **I** and **II** which are displayed in Fig. 2.

The field-dependent Mössbauer spectroscopic study presented here confirms the concentration-dependent reductive NO heme binding mechanism of cNP as suggested by Weichsel and coworkers [2]. Accompanying DFT calculations on the heme moieties which reproduce the observed Mössbauer parameters confirm this conclusion.

**Acknowledgments** This work has been supported by the research initiative NANOKAT.

## References

1. Walker, F.A.: J. Inorg. Biochem. **99**(1), 216–236 (2005)
2. Weichsel, A., et al.: PNAS **102**(3), 594–599 (2005)
3. Lang, G., et al.: Proc. Phys. Soc. **87**, 3–34 (1966)
4. Pfannes, H.D., et al.: Hyp. Interact. **91**, 797–802 (1994)
5. Schulz, C.E., et al.: Hyp. Interact. C **5**, 321–324 (2002)
6. Walker, F.A., et al.: J. Am. Chem. Soc. **108**, 5288–5297 (1986)
7. Janoschka, A., et al.: J. Phys. **217**, 1–4 (2010)
8. Gunnlaugsson, H.P., Vinda B.: Excel add-on. http://users-phys.au.dk/hpg/vinda.htm. Accessed 7 August 2015
9. Frisch, M.J., et al.: Gaussian 09, Revision D.01. Gaussian, Inc., Wallingford (2009)
10. Dapprich, S., et al.: J.Mol. Struct., Theochem. **461-462**, 1–21 (1999)
11. Orca- an ab initio density functional and semiempirical program package, Vers. 3.0.3. (2014)