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Complexes of ferriheme nitrophorin 4 with low-molecular weight thiol(ate)s occurring in blood plasma

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ABSTRACT

Nitrophorins are proteins occurring in the saliva of the blood-sucking insect *Rhodnius prolixus* to carry NO as a vasodilator and blood-coagulation inhibitor into the victim's tissue. It was suggested that the rate of NO release can be enhanced by the blood-plasma component L-cysteine [J.M.C.Ribeiro, Insect Biochem. Mol. Biol. 26 (1996) 899–905]. However, the mechanism of the reaction is not clear. In the attempt to exploit the reaction in detail, complexes of nitrophorin 4 (NP4) with the thiols 2-mercaptoethanol, L-cysteine, and L-homocysteine and with HS^- were formed and characterized under anaerobic conditions using absorption spectroscopy, X-ray crystallography, and EPR spectroscopy. In contrast to met-myoglobin, which is reduced by L-cysteine, all four compounds form low-spin Fe^{III} complexes with NP4. The weak equilibration constants (167–5200 M^{-1}) neither support significant complexation nor the simple displacement of NO *in vivo*. Both amino acid based thiols form additional H-bonds with side chains of the heme pocket entry. Glutathione and L-methionine did not form a complex, indicating the specificity of the complexes with L-cysteine and L-homocysteine. Continuous wave EPR spectroscopy reveals the simultaneous existence of three low-spin systems in each case that are attributed to various protonation and/or conformational stages in the heme pocket. Electron nuclear double resonance (ENDOR) spectroscopy demonstrates that the thiol sulfurs are, at least in part, protonated. Overall, the results not only demonstrate the good accessibility of the NP4 heme center by biologically relevant thiols, but also represent the first structural characterization of a ferriheme protein in complex with L-cysteine L-homocysteine.

1. Introduction

Nitrophorins (NPs) comprise a unique class of ferriheme proteins originating from the saliva of the blood feeding insect *Rhodnius prolixus* [1]. Four nitrophorins, designated NP1–4, were isolated from the insect saliva [2,3] and later recombinantly expressed [4,5]. Another nitrophorin, NP7, was recently established from a cDNA library and then recombinantly expressed [6–8]. The major biological function of these proteins is the transport and delivery of NO from the insect saliva to the blood vessels of a host species where it acts as a vasodilator and blood-coagulation inhibitor [9]. The NO transport is accomplished through the binding of NO to the heme iron. The protein undergoes a significant conformational change when moved from the acidic saliva (pH 5 to 6) [10] to those of the blood plasma (pH ~7.4) which decreases the affinity for NO, for example for NP7 from $>10^9$ M^{-1} (pH 5.5) to 4×10^6 M^{-1} (pH 7.5) [11]. Recently, it was demonstrated that ferriheme NPs, at least *in vitro*, are able to produce NO from NO_2^-, which is an unprecedented feature among the ferriheme proteins [12,13].

In NPs, the heme cofactor is located inside an 8-stranded β-barrel which is an unusual case for a heme protein [14]. The heme iron is coordinated by a His residue where the 6th coordination site is open for the binding of various ligands including the native ligands NO and histamine (Hm) [15]. The protein fold has been classified as a lipocalin type of fold [14], which is a very common fold among the proteome, typically found in proteins that bind lipophilic molecules [16]. A special feature of the lipocalins is the presence of at least one Cys–Cys disulfide; in case of the nitrophorins two Cys–Cys disulfides are present which are formed in a crossed fashion, *i.e.*, Cys_A–Cys_C and Cys_B–Cys_D in a given order …Cys_A…Cys_B…Cys_C…Cys_D… along the peptide sequence.

Where other ferriheme proteins, *e.g.*, met-myoglobin (metMb), are easily reduced by excess NO [17], NPs stabilize the Fe^{III} state through a number of carboxylate residues near the heme pocket [18] together with a ruffled heme geometry, which is induced by several side chains that point toward the distal side of the macrocycle [19]. This way, the reduction potential is established at, for example, –303 mV *vs.* SHE at pH 7.5 for NP1 compared to +63 mV *vs.* SHE at pH 7.0 for horse Mb [20]. The resulting stabilization of the $\{FeNO\}^6$ complex, according to

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the notation of Enemark and Feltham [21], is important for NP function because ferroheme–NO, *i.e.*, $\{FeNO\}^7$, association constants are too large ($K_{eq} = 10^{13} - 10^{14}$ M^{-1}) [11,18] to allow effective NO release *in vivo* [22].

Besides for NO, remarkably high affinities of NP1–4 for Hm were reported ($K_{eq} = 0.4\times10^8 - 1.6\times10^8$ M^{-1} [23,24]) where affinities for imidazole (ImH) are typically ~2 orders of magnitude smaller [11,23]. The comparison of the X-ray crystal structures of these complexes revealed that the high specificity for Hm results from the coordination with the ferriheme center plus the formation of a salt bridge of the Hm:NH_3^+ with Asp30:COO^- in NP4 [14]. Thus, Hm, which is released from mast cells at the site of the bite as an immune stimulus [25], may be trapped by NPs upon the release of NO and, therefore, contribute to the immune response suppression [26] during the time of feeding (10–30 min) [1,27]. Blood concentrations of Hm appear in the range of 1–10 nM [28]; therefore, based on the K_{eq}(Hm) NP1–4 may be able to scavenge Hm during a blood meal. In contrast, the K_{eq}(Hm) of NP7 (10^5 M^{-1} [11]) is too high to allow effective Hm scavenging *in vivo*. The conclusion that the sole physiological purpose of the NPs would be to transport a single NO molecule is, however, puzzling considering the energetic expense paid for translation (184 amino acids) and maturation of the protein; thus, the question should be addressed if NPs may bear other functionalities/activities [29].

This question is not new. Already in 1996, a reaction was described between NP[$\{FeNO\}^6$] in the salivary gland homogenate from *R. prolixus* and the low-molecular weight (LMW) thiols L-cysteine (CysSH), 2-mercaptoethanol (βMESH), *N*-acetyl-L-cysteine, and glutathione (GSH) [30]. Although in the blood plasma, contrary to the cytosol, the major fraction of thiols occurs in the form of disulfides, significant amounts of reduced LMW thiols are also present [31]. As a result of the reaction of NP–NO with RSH, loss of NO and formation of disulfides RS–SR were reported. A proposed mechanism of the reaction involves ferroheme as an intermediate. In a follow-up study, bleaching of the heme of NP2 was described upon administration of 0.5 mM CysSH. The reaction was inhibited by the addition of catalase, *i.e.*, suggesting that H_2O_2 may be transiently formed [32]. Similar, incubation of NP4 and NP7 with $Na_2S_2O_4$ under aerobic conditions resulted in heme bleaching whereas the same experiment under anaerobic conditions left the macrocycle unaffected except for iron reduction [33]. Thus, the "bleaching" is likely a consequence of fast re-oxidation of $Fe^{II} \rightarrow Fe^{III}$ in the presence of O_2 yielding O_2^- that is then able to "bleach" the macrocycle. Rapid mixing kinetic investigations indeed reveal a very fast oxidation of ferroheme NPs with O_2 [29].

For the detailed understanding of the reaction of LMW thiols with NPs, a systematic study of the interaction of NP4 with GSH, CysSH, L-homocysteine (HcySH), βMESH, and Na_2S was conducted under anaerobic conditions. Except for βMESH all of these compounds occur in blood plasma. The resulting complexes were investigated using UV–vis absorption spectroscopy, cw-EPR spectroscopy, and ^{1}H ENDOR spectroscopy. In addition, X-ray crystal structures of all species were solved at high resolutions.

2. Materials and methods

2.1. Materials

HcySH was always freshly prepared from L-homocysteine thiolactone (Sigma-Aldrich) under basic conditions [34,35]. βMESH, L-cystine $(CysS)_2$, and L-homocystine $(HcyS)_2$ were from Sigma-Aldrich. GSH, oxidized glutathione (GSSG), and CysSH were from Serva Electrophoresis GmbH. Bis(2-hydroxyethyl) disulfide ($(\beta MES)_2$) was bought from TCI Europe Fine Chemicals and Na_2S was a product from Alfa Aeser Europe GmbH. Iron 2,4-dimethyldeuteroporphyrin ("symmetric heme") was a gift from Seburo Neya, Department of Physical Chemistry, Chiba University, Japan [36].

NP4 was recombinantly expressed in *Escherichia coli* strain BL21(DE3) (Novagen) and purified and reconstituted as was previously described [7,11,24,37]. Protein preparations were routinely analyzed by SDS-PAGE to be >90% pure. The protein preparations were subjected to MALDI-TOF MS to confirm the correct molecular masses accounting for two Cys–Cys disulfides (calculated for $[NP4+H]^+$: 20,264 Da, observed: 20,279 ± 20 Da). Concentrations of NP4 solutions were determined photometrically using $\epsilon_{404\ nm} = 141{,}000$ M^{-1} cm^{-1} [38].

Experiments under strictly anaerobic conditions were carried out inside an anaerobic chamber (Coy, Inc.) with an atmosphere comprised of 98% N_2/2% H_2 in the presence of Pd catalysts. All solutions were rendered essentially O_2 free by three freeze-pump-thaw cycles performed on a vacuum line.

2.2. Absorption spectroscopy

Absorption spectra were recorded using a Cary-50 absorption spectrophotometer equipped with a fiber optic coupler (Varian, Inc.). Flexible fiber optics (Ocean Optics, Inc.) were used to connect the spectrophotometer with a cuvette holder (Ocean Optics, Inc.) located inside the anaerobic chamber which allows to record absorption spectra under strictly anaerobic conditions. The solvent of the protein was exchanged with O_2 depleted 50 mM 3-morpholinopropane-1-sulfonic acid (MOPS)/NaOH (pH 7.0) and the concentration adjusted thereafter using Biomax-10 ultrafiltration concentrators (Millipore) in a cooled (4 °C) microcentrifuge located inside the anaerobic chamber. In a 1-cm quartz cuvette, the sample was first incubated with the oxidized form of a thiol. In the case of GSSG and $(\beta MES)_2$ 50 mM was added. For $(CysS)_2$ and $(HcyS)_2$ the solvent was saturated because both compounds are poorly soluble (approx. 1 mM). The titration was then performed with the respective thiol, *i.e.*, GSH, βMESH, CysSH, or HcySH. In the case of Na_2S the protein was kept in pure buffer.

2.3. Protein crystallization and X-ray structure determination

Crystals of NP4 were obtained by the vapor-diffusion method using 3.2 M ammonium phosphate (pH 7.4) as precipitant [13,39,40]. For the preparation of the thiolate complexes, crystals were soaked for 5 min at room temperature in 3.2 M potassium phosphate (pH 7.4) with 15 % glycerol where the buffer contained either 2 mM βMESH and 20 mM $(\beta MES)_2$, 10 mM CysSH and saturated with $(CysS)_2$, 10 mM HcySH and saturated with $(HcyS)_2$, or 100 mM Na_2S. Afterwards, the crystals were immediately frozen in liquid N_2 and kept frozen until the measurement. Diffraction data sets were collected at 100 K using the beamline BL14.2 at BESSY II (Berlin, Germany). The data sets were processed with XDS [41] and CCP4 [42]. The molecular-replacement method was applied to determine the phase using an initial model from NP4, PDB: 3MVF [13]. The model building was performed with COOT [43] and the refinement was carried out using PHENIX [44]. The stereochemical properties were checked by RAMPAGE [45]. The bond/angle parameters between the iron and the ligands were refined without restrain.

2.4. EPR spectroscopy

The solvent of the protein was exchanged to O_2 depleted 50 mM MOPS/NaOH (pH 7.5) (or 100 mM MOPS/NaOH (pH 7.5) in the case of Na_2S) containing 20% (v/v) glycerol and the concentration was adjusted to ~100 μM as described above. Upon mixing with either 20 mM βMESH and 50 mM $(\beta MES)_2$, 10 mM CysSH and saturated with $(CysS)_2$, 10 mM HcySH and saturated with $(HcyS)_2$, or 100 mM Na_2S, the sample was incubated for 30 minutes on ice. The samples were quickly transferred into a 3 mm quartz tube and then rapidly frozen in liquid N_2 where they were kept until measurement. cw-EPR spectra were recorded on a Bruker Elexsys E500 spectrometer at X-band equipped with a gas-flow cryogenic system with an ESR900 liquid He cryostat from Oxford Instruments. Spectra were recorded at 15 K with