Electronic Supplementary Information (ESI) for *Chem. Commun.*

Unveiling the three-dimensional structure of the green pigment of nitrite-cured meat

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X-ray diffraction data collection and processing

X-ray data were collected at 100 K on a RigakuMSC RU-H3R X-ray generator operated at 48 kV/98 mA to produce Cu/Kα radiation (λ = 1.5418 Å). 1˚ oscillation images of the hh nitriMb nitrite derivative crystal were collected over a range of 200˚ with an exposure time of 5 min per image and a crystal-to-detector distance of 90 mm. Diffraction data were indexed and processed with the d*TREK program (Macintosh v.99D).[1]

Structure solution and refinement

In general, for the R_{free} calculation, 5% of randomly selected reflections were flagged and carried throughout the complete refinement procedure. The *CCP4* program *REFMAC*[2] and the *PHENIX*[3] refinement program were used for the structure refinement. Bulk-solvent modeling and isotropic scaling of the observed and calculated structural amplitudes were employed during the restrained refinements. *COOT*[4]was used for visualization and model building/corrections between refinement cycles.

The phase information was obtained using the molecular replacement method *PHASER* [5] as implemented in *CCP4*. The search model was the 1.45 Å resolution structure of hh MbCO (PDB entry: 1DWR)[6] with all solvent molecules, carbon monoxide and sulfate ions removed. Restrained refinement was carried out for 10 cycles prior to the addition of any solvent, ligand, or sulfate ion, and this resulted in a drop of the *R*-factor to 0.221 from an initial value of 0.289. In the initial F_o-F_c difference electron density map, new electron density was evident at the terminus of the 2-vinyl group of the heme moiety, which indicated that the heme had been chemically modified. A triatomic "O-N-O" fragment was fitted into this new density and the coordinates of the fragment saved into the protein pdb coordinate file. The heme (HEM) group and the added O-N-O fragment were labeled "NTE" (i.e., nitriheme) as a single ligand in the pdb coordinate file.

To generate the NTE ligand library cif file for the needed restrained refinements, we constructed a NTE prosthetic group using *Monomer Library Sketcher* in *CCP4*.[2] To begin, the library file and the coordinate file of the unmodified protoporphyrin IX prosthetic group were loaded from the *CCP4* monomer library as the starting template. The CBB atom was placed into porphyrin plane from its originally near-perpendicular position. An O-N-O fragment was then attached to the CBB atom of the CAB-CBB vinyl group. The N atom was single bonded to the CBB atom and also bonded to two O atoms with delocalized bonds. The "create library descriptions" and "regularize structure" commands were then used to generate the library description and geometry restraints for this NTE molecule. This newly generated NTE cif file was used for further refinements.

One nitrite anion bound to the heme and one sulfate anion were added to the model based on density seen in the initial F_o-F_c electron density map. An additional 10 cycles of restrained refinement in *CCP4* were run to give an R-factor of 0.212%. The structure was checked for sidechains showing low occupancy or multiple conformations. The side chain of residue Ser108 was modeled in two conformations each with 50% occupancy. The C-terminal residues Gln152 and Gly153 were

omitted from the structural model due to the lack of defined electron density. Water molecules were added during additional refinement cycles using *PHENIX*. The crystallographic R and R_{free} for the final model are 0.173 and 0.237, respectively, in the 26.47-1.70 Å range. The hh NMb(ONO) derivative crystallized with one molecule in the asymmetric unit, and the final model of its structure contains 151 amino acid residues, one nitriheme prosthetic group, one nitrite anion, one sulfate anion, and 94 water molecules.

Table 1. X-ray data collection and refinement statistics[a]

Data Collection	
PDB accession code	3VAU
Space group	$P2_1$
Unit cell dimensions (Å)	35.14, 28.42, 62.87, 90, 105.5, 90
Wavelength (Å)	1.5418
Temperature (K)	100
Resolution range (Å)	26.47-1.70
Number of observations	52180
Unique reflections	13475
Average multiplicity	3.87 (3.76)
Completeness (%)	100 (100)
$\langle I/\sigma(I)\rangle$	11.8 (3.4)
R_{merge}[b]	0.053 (0.303)
Refinement	
Number of protein atoms	1188
Number of heteroatoms	158
R-factor[c]	0.173
R_{free}[d]	0.237
Average B-factor (Å^2)[e]	22.3
rms deviations[f]	
bond lengths (Å)	0.03
bond angles (°)	2.3
Ramachandran plot (%)[g]	
favored	98.7
outliers	0
rotamer outliers	0

[a] The data in brackets refer to the highest resolution shell.
[b] $R_{merge} = \Sigma_{hkl}\Sigma_i |I_i(hkl) - \langle I(hkl)\rangle| / \Sigma_{hkl}\Sigma_i I_i(hkl)$, where $I_i(hkl)$ is the ith used observation for unique hkl, and $\langle I(hkl)\rangle$ is the mean intensity for unique hkl.
[c] $R = \Sigma_{hkl}||F_{obs}| - |F_{calc}|| / \Sigma_{hkl}|F_{obs}|$, where F_{obs} and F_{calc} are the observed and calculated structure factors, respectively.
[d] R_{free} was calculated using 5% of the randomly selected diffraction data which were excluded from the refinement.
[e] The average for the polypeptide atoms.
[f] deviations from ideal values[7]
[g] calculated using *MolProbity* as implemented in *PHENIX*[8]

References

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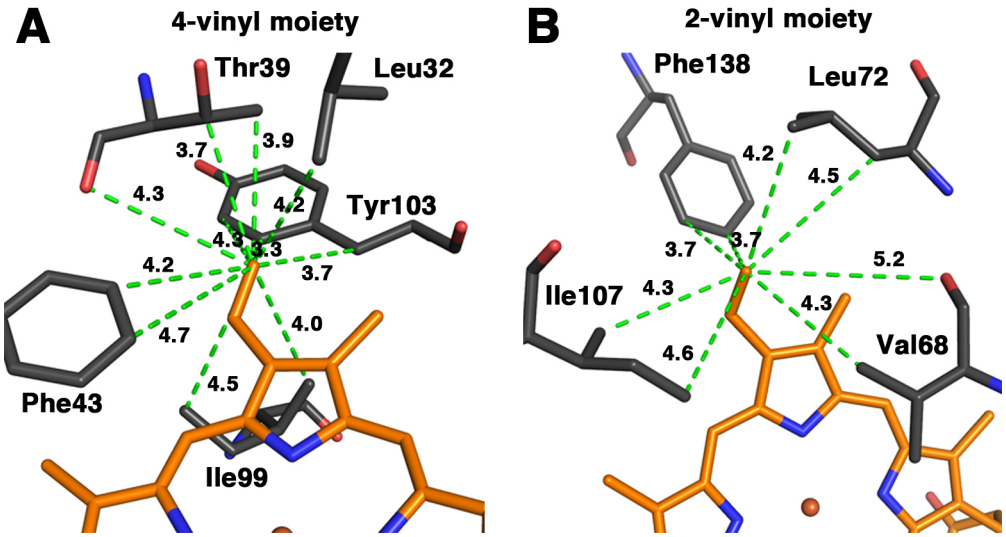

Figure S1. The heme vinyl regions of the ferric hh Mb(ONO) complex (PDB entry: 3LR7, 1.60 Å resolution). The close contacts of the terminal Cβ atom at the 4-vinyl position (**A**) and at the 2-vinyl position (B) with the side chains of nearby residues are shown in green dashed lines.

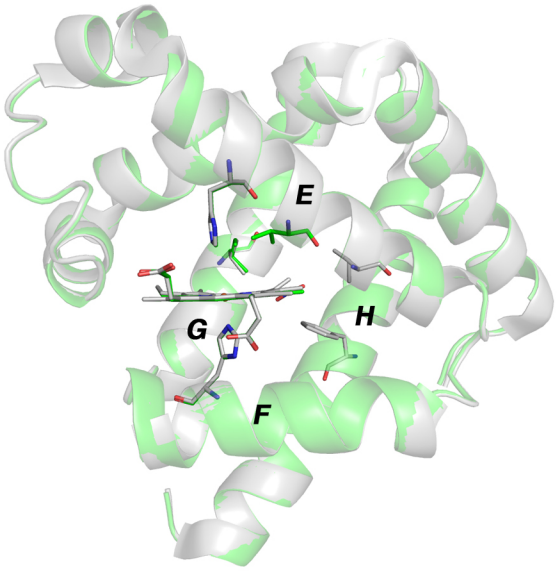

Figure S2. Superimposition of the hh Mb(ONO) complex (light gray, PDB entry: 3LR7) with the hh NMb(ONO) complex (green, this structure). Helices E, F, G and H are labeled.