An unexpected reactivity of the P460 cofactor in hydroxylamine oxidoreductase

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Hydroxylamine oxidoreductases (HAOs) contain a unique haem cofactor called P460 that consists of a profoundly ruffled c-type haem with two covalent bonds between the haem porphyrin and a conserved tyrosine. This cofactor is exceptional in that it abstracts electrons from a ligand bound to the haem iron, whereas other haems involved in redox chemistry usually inject electrons into their ligands. The effects of the tyrosine cross-links and of the haem ruffling on the chemistry of this cofactor have been investigated theoretically but are not yet clear. A new crystal structure of an HAO from Candidatus Kuenenia stuttgartiensis, a model organism for anaerobic ammonium oxidation, now shows that its P460 cofactor has yet another unexpected reactivity: when ethylene glycol was used as a cryoprotectant, the 1.8 Å resolution electron-density maps showed additional density which could be interpreted as an ethylene glycol molecule covalently bound to the C16 atom of the haem ring, opposite the covalent links to the conserved tyrosine. Possible causes for this unexpected reactivity are discussed.

1. Introduction

Octahaem hydroxylamine oxidoreductases (HAOs) catalyse the oxidation of hydroxylamine (NH2OH) to nitrite (NO2⁻) or nitric oxide (NO). The active sites of these proteins contain a unique, modified haem cofactor called P460 owing to its characteristic peak around 460 nm in the UV–Vis spectra of the dithionite-reduced protein (Erickson & Hooper, 1972). P460 consists of a c-type haem that is covalently linked to a conserved tyrosine residue (Arciero et al., 1993). Among haem redox cofactors, P460 is exceptional in that it abstracts protons from a ligand bound to the iron, whereas other haems involved in redox chemistry usually inject electrons into their iron-bound ligands. Thus, there has been considerable interest in investigating the detailed structure of this cofactor with a view to explaining this deviation from typical haem redox chemistry.

The first structural information on P460 based on mass spectrometry and NMR spectroscopy reported by Arciero and coworkers was consistent with a single covalent bond between a haem meso C atom and a tyrosine-ring C atom in the meta position (Arciero et al., 1993). Later, the 2.8 Å resolution crystal structure of the HAO from Nitrosomonas europaea indeed showed a covalent link between the porphyrin ring and the side chain of a conserved tyrosine. At the time, the structure of the P460 cofactor determined at this medium resolution was modelled with a single covalent bond between the haem C5 and the C' atom, probably influenced by the results obtained by Arciero and coworkers (Igarashi et al., 1997). Later, however, the 2.1 Å resolution crystal structure of...
the Nitrosomonas HAO (Cedervall et al., 2013), as well as the 1.8 Å resolution crystal structure of the HAO from Candidatus Kuenenia stuttgartiensis (Maalcke et al., 2014) published shortly afterwards, both clearly showed a second covalent bond between the C6 atom of the porphyrin macrocycle and the O9 atom of the tyrosine (Fig. 1). As shown in all available crystal structures of HAOs, the covalent attachment to the tyrosine results in a high degree of ruffling of the haem porphyrin macrocycle.

A theoretical study of the HAO mechanism (Fernández et al., 2008) suggested that the unusual electron-abstracting reactivity of P460 is assisted by the presence of the tyrosine residue through the destabilization of a haem–nitric oxide intermediate in which the combined number of iron d and NO \( \pi^* \) electrons is seven; it is therefore denoted the [FeNO]7 complex (Enemark & Feltham, 1974). The destabilization of this intermediate favours its transformation into an [FeNO]8 intermediate by the removal of an electron. However, these calculations were based on the 2.8 Å resolution crystal structure and thus assumed only one covalent bond between the haem porphyrin and the tyrosine. Therefore, it is not yet exactly clear what causes P460 to deviate from typical haem redox chemistry.

Here, we report another unexpected reactivity of P460. In order to obtain the native structure of the HAO from Candidatus K. stuttgartiensis (Maalcke et al., 2014), crystals were cryoprotected using sucrose. We now present the structure of HAO determined using ethylene glycol as the cryoprotectant. In this structure, an ethylene glycol molecule is covalently bound to the P460 cofactor at the C16 atom of the haem moiety. This observation, while probably an artefact, illustrates the pronounced effects of the haem ruffling on the chemistry of the porphyrin ring of P460.

### 2. Materials and methods

#### 2.1. Protein crystallization

Candidatus K. stuttgartiensis HAO was purified as described earlier and crystallized as reported in Maalcke et al. (2014). Briefly, crystals were grown at room temperature in hanging-drop vapour-diffusion setups using 1.3 M ammonium sulfate in 0.05–0.1 M sodium phosphate buffer pH 7.4, 25 mM C-HEGA-10 as the reservoir solution. Dark red rhombic dodecahedra grew to a diameter of 150 μm within 3 d. Crystallization information is summarized in Table 1. One of these crystals was flash-cooled in liquid nitrogen after soaking for 5 min in an artificial reservoir solution containing 25% (v/v) ethylene glycol and stored in liquid nitrogen for subsequent data collection. Data-collection and processing statistics are summarized in Table 2.

#### 2.2. Data collection and structure solution

A 1.8 Å resolution data set was collected on the PXII beamline of the Swiss Light Source at the Paul Scherrer Institute, Villigen, Switzerland. The data were processed in XDS (Kabsch, 2010,a,b) and phased by molecular replacement using Phaser (McCoy et al., 2007; McCoy, 2007) with the known Candidatus K. stuttgartiensis HAO crystal structure (Maalcke et al., 2014) and the structure was refined using REFMACS (Murshudov et al., 1997, 2011). Geometrical parameters for the P460 cofactor and for its covalent bond to an ethylene glycol molecule were obtained from semi-empirical quantum-chemical calculations at the PM-3 level (Steward, 1989) using HyperChem 8.0. Coordinates and structure-factor amplitudes have been deposited in the

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**Table 1**

Crystallization.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Method</td>
<td>Hanging-drop vapour diffusion</td>
</tr>
<tr>
<td>Plate type</td>
<td>24-well Linbro plate, glass cover slip</td>
</tr>
<tr>
<td>Temperature (K)</td>
<td>293</td>
</tr>
<tr>
<td>Protein concentration</td>
<td>( A_{20}^{\text{nm}} = 20 ) (( A_{1}^{\text{nm}} = 95 ))</td>
</tr>
<tr>
<td>Buffer composition of protein solution</td>
<td>25 mM HEPES-KOH pH 7.5, 25 mM KCl</td>
</tr>
<tr>
<td>Composition of reservoir solution</td>
<td>1.3 M ammonium sulfate, 0.05–0.1 M sodium phosphate buffer pH 7.4, 35 mM C-HEGA-10</td>
</tr>
<tr>
<td>Volume and ratio of drop</td>
<td>1 μl + 1 μl</td>
</tr>
<tr>
<td>Volume of reservoir (μl)</td>
<td>800</td>
</tr>
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</table>

**Table 2**

Data collection and processing.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
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<tr>
<td>Diffraction source</td>
<td>PXII, SLS</td>
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<tr>
<td>Temperature (K)</td>
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<tr>
<td>Detector</td>
<td>Pilatus 6M</td>
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</tr>
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<tr>
<td>Total rotation range (°)</td>
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</tr>
<tr>
<td>Exposure time per image (s)</td>
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<tr>
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</tr>
<tr>
<td>a, b, c (Å)</td>
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<tr>
<td>a, b, γ (°)</td>
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<tr>
<td>Mosaiety (°)</td>
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</tr>
<tr>
<td>Resolution range (Å)</td>
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<tr>
<td>Total No. of reflections</td>
<td>459071 (68470)</td>
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<tr>
<td>No. of unique reflections</td>
<td>68117 (10121)</td>
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<tr>
<td>Completeness (%)</td>
<td>99.7 (99.9)</td>
</tr>
<tr>
<td>Multiplicity</td>
<td>6.7 (6.8)</td>
</tr>
<tr>
<td>( R_{	ext{Lbf}} ) (%)</td>
<td>19.6 (3.9)</td>
</tr>
<tr>
<td>( R_{	ext{merge}} ) (%)</td>
<td>7.3 (52.0)</td>
</tr>
<tr>
<td>Overall B factor from Wilson plot (Å2)</td>
<td>26.9</td>
</tr>
</tbody>
</table>

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**Figure 1**

Structure of the P460 cofactor. The porphyrin C atoms C6, C10 and C16 are indicated, as well as the tyrosine O9 and C16.
Table 3
Structure solution and refinement.
Values in parentheses are for the outer shell.

<table>
<thead>
<tr>
<th>Resolution range (Å)</th>
<th>Completeness (%)</th>
<th>σ Cutoff</th>
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</thead>
<tbody>
<tr>
<td>46.1–1.8 (1.9–1.8)</td>
<td>99.7 (99.9)</td>
<td>None</td>
</tr>
</tbody>
</table>

No. of reflections, working set: 64753 (4748)
No. of reflections, test set: 3364 (288)
Final Rmerge: 0.147 (0.197)
Final Rsigma: 0.164 (0.250)
Crucikshank DPI: 0.0880

No. of non-H atoms
- Protein: 3945
- Ion: 15 [3 PO₄³⁻]
- Ligand: 344 [8 haem], 12 [1 C-HEGA-10], 20 [5 ethylene glycol]
- Water: 509
- Total: 4845

R.m.s. deviations
- Bonds (Å): 0.007
- Angles (°): 1.091

Average B factors (Å²)
- Overall: 15.3
- Protein: 18.8
- Ion/ligand: 16.4
- Water: 28.2

Ramachandran plot
- Most favoured (%): 96.2
- Allowed (%): 3.6
- Disallowed (%): 0.2

† This is the conserved His263 which is in a strained conformation.

Protein Data Bank as entry 4rwm. Refinement statistics are summarized in Table 3.

2.3. UV–Vis spectroscopy

For the acquisition of qualitative spectra, the protein was diluted to A₂₈₀ ~ 0.2 and A₄₉₀ ~ 0.9 in 100 mM sodium phosphate buffer solution pH 7.4. Spectra of the reduced enzyme were recorded in 1 ml quartz cuvettes with a 1.0 cm bandwidth using a Jasco V-650 spectrophotometer (Jasco GmbH, Gross-Umstadt, Germany) equipped with a thermostatted sample holder at 25 ºC. The data were processed using the Jasco32 software. The protein was reduced by the addition of a few crystals of solid sodium dithionite (Na₂S₂O₄) to the samples (1 ml volume) and spectra were recorded in the presence and absence of 10% (v/v) ethylene glycol.

3. Results and discussion

No large-scale differences were observed between the crystal structures of native Candidatus K. stuttgartiensis HAO using either sucrose or ethylene glycol as the cryoprotectant. In both cases the asymmetric unit contained one monomer and the haem part of the P₄₆₀ cofactor appeared to be highly ruffled to a similar extent owing to the two covalent bonds between the porphyrin macrocycle C₄ and C₅ atoms and the O⁰ and C⁰ atoms, respectively, of the conserved tyrosine residue Tyr451 from an adjacent monomer in the trimer (Maalcke et al., 2014). However, in the electron-density maps of the ethylene glycol structure additional density was present close to the haem C₁₆ atom of the P₄₆₀ cofactor (Figs. 2a and 2b), indicating that a small molecule had bound to the haem group. Moreover, the density showed that the side chain of Asp262 of the conserved Asp/His pair had rotated away to accommodate the new molecule bound to the haem (Figs. 2a, 2b and 2c). Given the size, shape and position of the density, and considering the various components of the mother liquor and cryoprotection solution for the crystal, the most likely explanation of the density appeared to be a single ethylene glycol molecule covalently bound to the haem C₁₆ C atom with one of its O atoms (Fig. 2). The ethylene glycol was only included at a late stage in the refinement and was modelled with full atom occupancies. The B factors for the ethylene glycol refined to an average value of 24.5 Å², which is somewhat higher than the average B factor of the P₄₆₀ cofactor (16.3 Å²), suggesting that the occupancy was less than 1.0 in the crystal. The refined distance between C₁₆ and the ethylene glycol O atom is 1.46 Å, which is in the range of covalent C—O bond lengths, and is close to the expected value of 1.43 Å obtained from semi-empirical calculations at the PM-3 level (Steward, 1989). The C₁₆-atom geometry can be described as a distorted tetrahedron with angles between the ethylene glycol O atom and the substituents around C₁₆ of between 94 and 99°. The refined geometry of the P₄₆₀–ethylene glycol adduct is very similar to the semi-empirically optimized geometry (Fig. 2d).

Importantly, in the 1.8 Å resolution native data set reported in Maalcke et al. (2014), which was collected from a crystal cryoprotected using 30%(v/v) sucrose in the mother liquor, no additional density bound to C₁₆ was observed, which is consistent with this density being caused by an ethylene glycol molecule. Moreover, UV–Vis spectra of reduced Candidatus K. stuttgartiensis HAO in the presence of 10%(v/v) ethylene glycol did not show the characteristic P₄₆₀ absorption peak, which in Candidatus K. stuttgartiensis HAO is at 468 nm, whereas this absorption is clearly present in spectra of the reduced enzyme without ethylene glycol (Fig. 3).

Importantly, the complex structures of Candidatus K. stuttgartiensis HAO soaked in hydroxylamine, hydrazine and phenylhydrazine reported in Maalcke et al. (2014) were also obtained using ethylene glycol as the cryoprotectant in the ligand-soaking solution. However, in these cases no additional density was observed for an ethylene glycol molecule bound to the haem. It is probable that complex formation with hydroxylamine, hydrazine and phenylhydrazine occurs before ethylene glycol adduct formation and prohibits it, either by reducing the haem or by steric hindrance between the iron ligand and the ethylene glycol.

Apart from the difference density for the ethylene glycol moiety and the rotated side chain of Asp262, there is also difference density suggesting that the distal water molecule in the ethylene glycol-soaked structure is closer to the haem iron (Fe—O distance of 2.2 Å) than in the sucrose-soaked structure (Fe—O distance of 2.5 Å). This is probably owing to partial reduction of the sucrose-soaked crystal (Beitlich et al., 2007) caused by radiation damage during data collection.

The mechanism of ethylene glycol addition to the P₄₆₀ cofactor is unclear. In aqueous solution at approximately
neutral pH, ethylene glycol is not a particularly reactive molecule. Indeed, for the addition of polyethylene glycol to proteins (’PEGylation’), the PEG hydroxyl groups need to be activated using, for example, cyanuric chloride or other highly reactive compounds (Roberts et al., 2012). Oxidation of ethylene glycol results in the formation of glycolaldehyde (2-hydroxyethanal), which may be expected to be more reactive, however, and the possibility that glycolaldehyde causes the observed reaction cannot be excluded. Further oxidation would result in the formation of carboxylic acids such as glycolic acid. However, the reaction of such a compound with the P₄₆₀ cofactor would most likely have resulted in an ester-type bond, and despite the high resolution the electron density offers no evidence for such an adduct.

Covalent adducts of haem groups are well known; haem c cofactors are by definition covalently bound to the protein via thioether bonds between the vinyl groups of the haem and cysteine residues, and in haem d a propionate group can form a γ-spirolactone with a C atom of the porphyrin ring (Sotiriou & Chang, 1988; Murshudov et al., 1996).

Figure 2
Stereofigures showing (a) the simulated-annealing composite OMIT (2mFo−DFo, blue, 1.5σ) and (b) the mFo−DFo (green, +3σ) electron-density maps (Read, 1986) for the P₄₆₀ cofactor in an ethylene glycol-soaked Candidatus K. stuttgartiensis HAO crystal. The maps were calculated by PHENIX (Adams et al., 2010) using the native structure (PDB entry 4n4j; Maalcke et al., 2014) after rigid-body refinement, in which ethylene glycol is not included. The maps are superimposed on the final, refined structure. The conserved Asp262/His263 pair involved in proton abstraction from the substrate (Maalcke et al., 2014) is indicated, and the double covalent link between the P₄₆₀ cofactor and Tyr451 from another subunit (blue) can be seen in the background. (c) Superposition of the native structure (brown) onto the final, refined structure of the ethylene glycol adduct structure (grey). The side chain of Asp262 has been displaced by the presence of the ethylene glycol molecule. (d) Superposition of the P₄₆₀ cofactor as observed in the crystal structure of the ethylene glycol adduct (light grey) and the PM-3 optimized cofactor (dark grey). An ammonia molecule was used to model the proximal histidine ligand in the semi-empirical optimization (blue sphere) and the propionates were omitted.
There are also several examples of covalent modifications at haem meso C atoms: Arciero & Hooper (1997) found a covalent link between a lysine residue and a meso C atom of a c-type haem in the N. europaea monohaem cytochrome P460. This was subsequently confirmed by the crystal structure of this protein determined by Pearson et al. (2007), who also found a hydroxyl adduct to a meso C atom when the protein was heavily oxidized. Haem oxygenase selectively oxidizes the α-meso atom as the first step in haem degradation (Schuller et al., 1999; Matsui et al., 2005). The incubation of horseradish peroxidase with H₂O₂ and chloride resulted in the addition of a chlorine to a haem meso atom (Huang et al., 2005), and the reaction of myoglobin with alkyl hydrazines results in alkylation at the γ-meso atom (Choe & Ortiz de Montellano, 1991).

Thus, while there are several examples of haem modifications at either the vinyl groups or meso C atoms, there are few examples of modifications at the pyrrole C atoms flanking the N atoms such as C₁₆, suggesting these atoms to be comparatively nonreactive under most circumstances.

Apparently, in P₄₆₀ the pronounced ruffling caused by the covalent bonds between haem and tyrosine has increased the reactivity of the C₁₆ atom towards ethylene glycol. In a planar, undistorted porphyrin ring, the C₁₆ atom would have mainly sp² character. In P₄₆₀, the ruffling is likely to cause the C₁₆ atom, which is opposite the covalent bonds to the tyrosine, to adopt a more sp³-like geometry, increasing its reactivity by straining the local structure. Moreover, the remaining hydroxyl group of the ethylene glycol moiety is bound to the side-chain carboxylate group of the conserved Asp262 via a hydrogen bond (Fig. 2). In this way, the protein could assist in adduct formation by binding ethylene glycol in a favourable position and orientation.

Whether the observed adduct has any biological relevance in itself appears doubtful; ethylene glycol is chemically very different from the putative substrate hydroxylamine. However, since this unexpected reactivity of the P₄₆₀ cofactor is likely to be caused by the effects of the ruffling induced by the bonds to Tyr451’ on the chemistry of the porphyrin ring, this observation may assist in elucidating the electronic properties of the P₄₆₀ cofactor, which in turn could shed light on the unique reversal of the electron-flow direction in this singular haem cofactor.

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References


