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Highly malleable haem-binding site of the haemoprotein HasA permits stable accommodation of bulky tetraphenylporphycenes†

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Iron(III)- and cobalt(III)-9,10,19,20-tetraphenylporphycenes, which possess bulky phenyl groups at the four *meso* positions of porphycene, were successfully incorporated into the haem acquisition protein HasA secreted by *Pseudomonas aeruginosa*. Crystal structure analysis revealed that loops surrounding the haem-binding site are highly flexible, remodelling themselves to accommodate bulky metal complexes with significantly different structures from the native haem cofactor.

Introduction

The substitution of protein cofactors with non-natural analogues is a powerful means to control the function of proteins, and has been intensively investigated, procuring novel biocatalysts,^{1–4} biosensors,^{5–7} and supramolecular structures.^{8,9} Haemoproteins, such as myoglobin (oxygen binding),¹⁰ P450s (metabolism),¹¹ and haem oxygenase (degradation of haem),¹² which contain iron protoporphyrin IX (haem) as their cofactor, are some of the most promising targets for the reconstitution of haem with other metal complexes. The function of haemoproteins has been enhanced by substituting haem for analogues that have been decorated with functional groups and/or exchanged with metals other than iron. Unfortunately, metal complexes with structures differing from haem, such as artificial metal complexes possessing bulky substituents, can generally not be incorporated into wild-type haemoproteins, as their haem-binding cavity has evolved to only accommodate haem. Recently, we reported that the haem acquisition protein HasA secreted by *Pseudomonas aeruginosa* is rather promiscuous

and can accommodate other metal complexes, such as insoluble iron(III)-phthalocyanine, small iron(III)-salophen, and bulky iron(III)-5,15-diphenylporphyrin.^{13,14}

HasA is a haem-binding protein that is secreted by certain pathogens, such as *P. aeruginosa*, when in iron deficient environments. Following acquisition of haem from its host by HasA, haem is transferred to the HasA-specific outer membrane receptor HasR, where it is taken up into the cell and used as an iron source for the pathogen's survival (Fig. 1a).^{15,16} HasA captures haem by using two of its loops like tweezers, where His32 and Tyr75 coordinate each side of the haem iron. Binding of haem to HasA is accompanied by significant structural changes in the His32-loop from the haem-free open-form (apo-HasA) to the haem-bound closed-form (holo-HasA). The crystal structure of holo-HasA reveals a large portion of the haem cofactor protruding out of HasA and exposed to the solvent (Fig. 1a).^{17,18} This unusual solvent-exposed mode of haem binding offers an enticing explanation as to why metal complexes with significantly different structures and properties from haem can be incorporation into HasA. In this study we selected the sterically demanding synthetic cofactor metallo-9,10,19,20-tetraphenylporphycene (Fig. 1b) for incorporation into HasA, as the gram-scale synthesis of 9,10,19,20-tetraphenylporphycenes (Ph₄Pc) has been reported recently.¹⁹ Despite the alluring photophysical and catalytic properties of metallo-9,10,19,20-tetraphenylporphycenes, they have never been considered as potential targets for incorporation into haemoproteins.

Result and discussion

Initially, we attempted to incorporate iron(III)-9,10,19,20-tetraphenylporphycene (Fe-Ph₄Pc) into HasA. A solution of Fe-Ph₄Pc dissolved in DMSO was added to apo-HasA. After dialysis to

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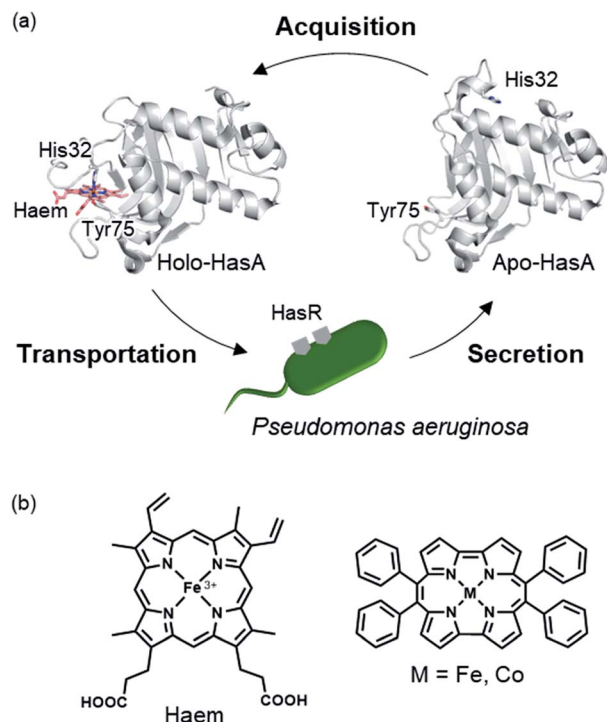


Fig. 1 (a) Schematic of the haem acquisition system of *Pseudomonas aeruginosa*. Under low-iron conditions, apo-HasA (PDB ID: 3MOK) secreted by *P. aeruginosa* captures and transports haem (holo-HasA, PDB ID: 3ELL) to the HasA specific receptor HasR expressed on outer membrane. (b) The structure of haem (left) and metallo-9,10,19,20-tetraphenylporphycene (right).

remove DMSO, HasA containing Fe-Ph₄Pc was purified *via* anion-exchange chromatography. UV/Vis spectroscopic analysis of HasA with Fe-Ph₄Pc exhibited characteristic absorption at 391 and 646 nm, which was assigned to the Soret and Q band of Fe-Ph₄Pc, respectively (Fig. 2a). ESI-TOF mass spectrometry gave a peak at 19 705.0 Da, which corresponded to the mass expected for HasA with Fe-Ph₄Pc (Fig. 2c). Furthermore, we also succeeded in the preparation of HasA capturing cobalt(III)-9,10,19,20-tetraphenylporphycene (Co-Ph₄Pc) using a similar procedure to aforementioned HasA with Fe-Ph₄Pc (Fig. 2b and d).

To comprehend the intricate changes HasA must undergo to enable accommodation of bulky metallo-Ph₄Pcs, X-ray crystal structure analysis of HasA with Fe-Ph₄Pc and Co-Ph₄Pc was attempted, and the structure of HasA capturing Co-Ph₄Pc was successfully determined at 2.5 Å resolution (Fig. 3a). The overall structure of HasA capturing Co-Ph₄Pc resembled that of holo-HasA (root-mean-square deviation (RMSD) over 2–182 amino acid residues for C α atoms against holo-HasA: 0.73). Electron density clearly corresponding to Co-Ph₄Pc was observed in the haem-binding site of HasA (Fig. 3b). Analogous to haem, the cobalt ion of Co-Ph₄Pc was ligated by a nitrogen of His32 (N ϵ) and oxygen of Tyr75 (O η) (Fig. 3b). The two phenyl groups at the 9- and 10-positions of Co-Ph₄Pc were accommodated in a cavity formed by His32(C=O), Pro34, Arg129, His134, and Tyr138, whereas the other two phenyl groups at 19- and 20-positions

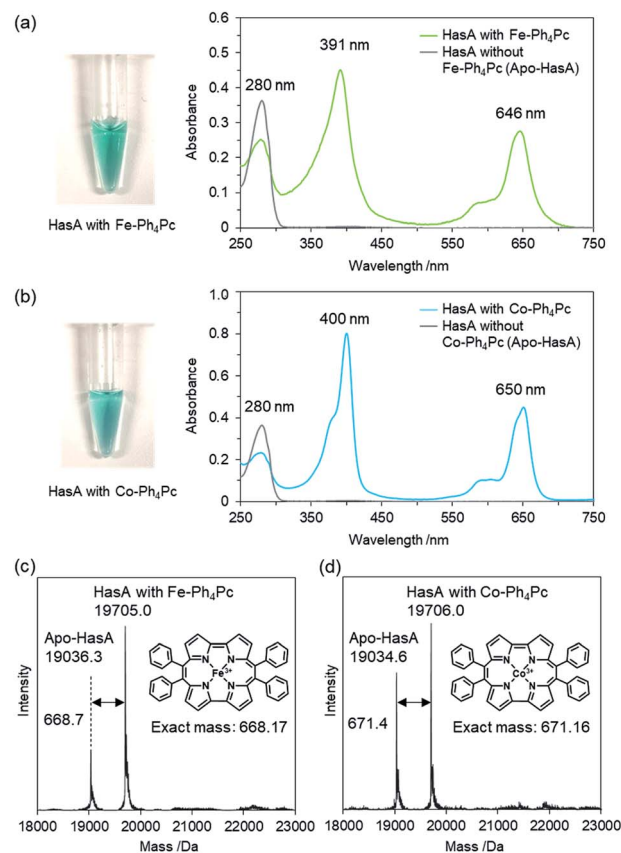


Fig. 2 (a) and (b) UV/Vis spectra of HasA with Fe-Ph₄Pc (green), Co-Ph₄Pc (cyan) and apo-HasA (without metallo-Ph₄Pc, grey). Protein solutions of HasA with metallo-Ph₄Pcs are shown on the left of each corresponding spectrum. (c) and (d) ESI-TOF mass spectra of HasA with Fe-Ph₄Pc (left) and Co-Ph₄Pc (right) in 5 mM ammonium acetate buffer.

were accommodated in another cavity formed by Thr43, Gly44(C=O), Gly45, Phe46, Pro50(C=O), and Phe51 (Fig. 3c). The overall shape of the cavity closely resembled that of HasA with iron(III)-5,15-diphenylporphyrin, which also possesses bulky phenyl groups at a similar position.¹⁴ Superimposition of HasA with Co-Ph₄Pc over haem revealed that the phenyl groups at positions 10 and 20 of Co-Ph₄Pc were positioned where a propionate and vinyl group of haem are usual found, respectively (Fig. 4a). Intriguingly, two different conformations (conformation-A and -B) could be observed in the crystal structure of HasA with Co-Ph₄Pc. Regarding conformation-A, when compared to the structure of holo-HasA, the side chain of Arg129 was flipped to prevent steric repulsion with the 9-phenyl of Co-Ph₄Pc (Fig. 4b). Conversely, Arg129 is not flipped in conformation-B, but steric repulsion is avoided by further extrusion of Co-Ph₄Pc from HasA (Fig. 4c). From these two conformations, we concluded that Arg129 actually interferes with the incorporation of bulky phenyl rings. However, owing to the high flexibility of HasA's loops (Loop-1: Asp29 to Gly44; Loop-2: Gly49 to Arg52 and Loop-3: Thr76 to Leu85), which form the haem-binding pocket, Co-Ph₄Pc can be stably incorporated,

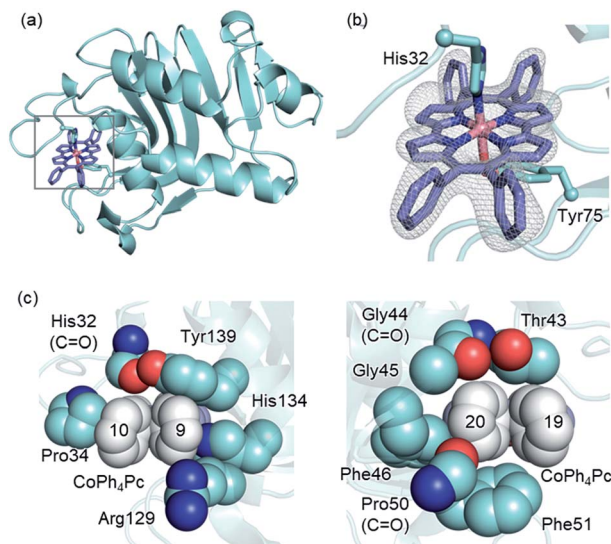


Fig. 3 (a) Overall structure of HasA capturing Co-Ph₄Pc. Co-Ph₄Pc is shown as a purple stick model (PDB ID: 6JLG). (b) Enlarged view of the Co-Ph₄Pc-binding site in HasA. The $F_o - F_c$ electron density map of Co-Ph₄Pc contoured at the 2.5 σ level is shown as a grey mesh. (c) Surrounding amino acids that form the cavity for accommodation of the phenyl groups of Co-Ph₄Pc. Phenyl groups are shown in white.

following adequate changes of the cavity's shape to accommodate bulky phenyl groups (Fig. 4d).

To examine whether HasA with Fe-Ph₄Pc or Co-Ph₄Pc has retained the ability to interact with its receptor HasR, we investigated the inhibition of *P. aeruginosa* growth in the presence of HasA with either Fe-Ph₄Pc or Co-Ph₄Pc. Growth of *P. aeruginosa* was monitored by tracking changes in the optical density at 600 nm (OD_{600}) of bacterial cultures. *P. aeruginosa* was cultured in an M9-based medium containing EDTA as a free iron scavenger and holo-HasA as the sole iron source. Into this, HasA with either Fe-Ph₄Pc or Co-Ph₄Pc was also added. When compared to a control without any added metallo-Ph₄Pcs, both HasA with Fe-Ph₄Pc and Co-Ph₄Pc were observed to significantly inhibit *P. aeruginosa* growth (Fig. 5a). Inhibition of growth is a strong indicator that HasA

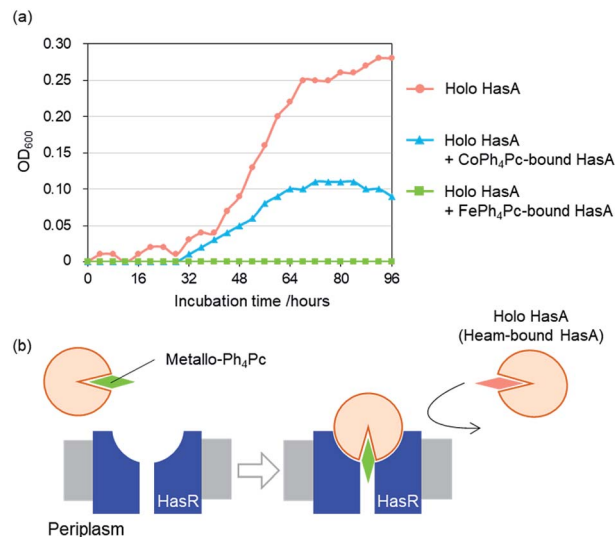


Fig. 5 Effect of HasA with metallo-Ph₄Pc in the presence of haem-bound HasA was monitored via the optical density at 600 nm (OD_{600}). (a) Growth curve of *P. aeruginosa* in iron-limiting medium. (b) Proposed mechanism of growth inhibition by HasA with metallo-Ph₄Pc.

with metallo-Ph₄Pcs retained the ability to interact with HasR. Moreover, these results imply that HasA containing metallo-Ph₄Pcs may stay bound to HasR. As a consequence, the haem-uptake port of HasR is blocked and further acquisition of haem from holo-HasA is inhibited (Fig. 5b). We also confirmed that metallo-Ph₄Pcs showed no cytotoxicity (Fig. S1†). Growth experiments revealed that HasA capturing metallo-Ph₄Pcs works as an inhibitor of *P. aeruginosa* growth by preventing acquisition of haem from holo-HasA via HasR. This indicates that these “fake-HasAs” most probably behave similar to native holo-HasA, as the overall structure of HasA with Co-Ph₄Pc resembles that of native holo-HasA. Interestingly, Ph₄Pc-HasA with iron behaves like a decidedly stronger growth inhibitor than with cobalt, indicating that exchange of metals represents a potent means to regulate the efficiency of growth inhibition (Fig. 5a).

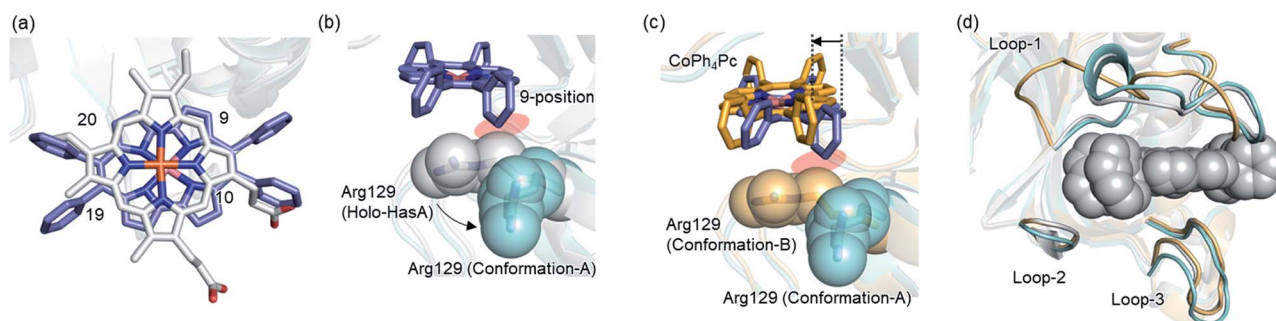


Fig. 4 (a) Superimposed image of HasA-bound haem (white) and HasA with Co-Ph₄Pc (purple). (b)–(d) Superimpositions of the crystal structures of holo-HasA (white) and HasA with Co-Ph₄Pc (conformation-A (cyan) and B (orange)). (b) The side chain of Arg129 was flipped to accommodate the 9-phenyl of CoPh₄Pc. The side chain of Arg129 is shown as spheres. (c) The 9-phenyl was extruded towards the outside of HasA in conformation-B to avoid steric repulsion with Arg129. (d) Conformational change of HasA-loops around haem-binding site. CoPh₄Pcs of both conformations are shown as grey sphere.

Conclusions

We have demonstrated that Fe- and Co-Ph₄Pc can be incorporated into the natural haemoprotein HasA secreted by *P. aeruginosa*. Moreover, we succeeded in the X-ray crystal structure analysis of HasA capturing Co-Ph₄Pc. The crystal structure suggests that HasA possesses an unusually adaptable haem-binding site and reveals a hidden capability of HasA to adjust its haem-binding cavity to accommodate synthetic metal complexes possessing considerably different structures from haem. HasA-loops forming the haem-binding pocket possess sufficient flexibility to accommodate the bulky phenyl groups of Co-Ph₄Pc on the outside of HasA. Our findings also suggest that mutagenesis of amino acids lining the haem-binding cavity may allow for an even richer variety of metal complexes to be incorporated into HasA. Furthermore, we anticipate that HasAs containing synthetic metal complexes may also find use as novel biocatalysts unlocking access to characteristic properties unique to each metal complex. Work along this line is currently underway in our research group and will be reported in future publications.

Materials and methods

Expression and purification of apo-HasA

Expression and purification of truncated HasA derived from *P. aeruginosa* PAO1 was performed according to similar methods reported previously.^{13,14} The details are given in the ESI.† The purity of the protein was checked by SDS-PAGE. Purified apo-HasA solution was flash frozen with liquid nitrogen and stored at –80 °C until use.

Preparation of HasA with iron(III)-9,10,19,20-tetraphenylporphycene (Fe-Ph₄Pc)

The μ -oxodimer of Fe-Ph₄Pc was sufficiently dissolved in DMSO and added to a solution of apo-HasA. The mixture was dialysed into a solution of phosphate-buffered saline (PBS; 140 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, pH 7.3) and filtrated to remove any precipitates derived from excess Fe-Ph₄Pc. To remove Fe-Ph₄Pc-free HasA (apo-HasA), filtered HasA solution was loaded onto an anion exchange column (HiTrap capto DEAE; GE Healthcare) equilibrated in buffer A (100 mM CHES-KOH, pH 9.5) and washed with 1 column volume of buffer A containing 10% (v/v) buffer B (100 mM CHES-KOH, 0.8 M NaCl, pH 9.5). The bound proteins were then eluted over 20 column volumes of a linear gradient from 10% to 80% buffer B, and sample fractions containing the complex of HasA with FePh₄Pc were collected. This partially purified HasA solution was loaded onto the same anion exchange column equilibrated in buffer C (20 mM Tris-HCl, pH 7.5). The bound proteins were eluted over 20 column volumes of a linear gradient from 10% to 80% buffer D (20 mM Tris-HCl, 1 M NaCl, pH 7.5). Eluted fractions containing pure HasA with Fe-Ph₄Pc were concentrated and purified *via* a desalting column (PD-10; GE Healthcare) into a PBS solution. Concentration of HasA with Fe-Ph₄Pc was determined by a bicinchoninic acid (BCA)

method using apo-HasA as a protein standard. The molar extinction coefficient of HasA with Fe-Ph₄Pc was estimated to be 84.1 mM⁻¹ cm⁻¹ at 391 nm.

Preparation of HasA with cobalt(III)-9,10,19,20-tetraphenylporphycene (Co-Ph₄Pc)

HasA capturing Co-Ph₄Pc was prepared using a similar procedure to that described for HasA with Fe-Ph₄Pc. Co-Ph₄Pc-free HasA (apo-HasA) was completely removed in a single-step by anion exchange chromatography using buffer C and D, as used in the 2nd step of the purification of HasA-bound Fe-Ph₄Pc, and a pure solution of HasA with Co-Ph₄Pc could be obtained. After exchanging buffer to PBS solution using a desalting column (PD-10; GE Healthcare), the resulting solution of HasA with Co-Ph₄Pc was flash frozen with liquid nitrogen and stored at –80 °C until use. The molar extinction coefficient of HasA with Co-Ph₄Pc was estimated by BCA assay to be 149.0 mM⁻¹ cm⁻¹ at 400 nm.

Measurement

Ultraviolet-Visible spectra were recorded on a UV-2600 PC spectrophotometer (Shimadzu) and U-3310 spectrophotometer (Hitachi). ESI-TOF mass spectra were recorded on a microTOF II (Bruker Daltonics) using positive mode ESI-TOF method for protein solutions in 5 mM ammonium acetate buffer. FAB-MS measurements were performed on a JEOL JMS-700 instrument.

Crystallisation of HasA with Co-Ph₄Pc

A buffer solution of purified HasA with Co-Ph₄Pc was exchanged with 100 mM KPi (pH 7.0) using desalting column (PD-10; GE Healthcare). After concentration using an Amicon Ultra (Merck Millipore; 3 kDa MWCO), the concentration of HasA capturing Co-Ph₄Pc was determined by UV-Vis spectroscopy. A 1.0 μ L aliquot of concentrated HasA with Co-Ph₄Pc solution (30 mg mL⁻¹ (1.6 mM) in 100 mM KPi, pH 7.0) was mixed with an equal volume of a reservoir solution composed of 1.26 M ammonium sulphate and 100 mM HEPES-HCl (pH 7.5). A drop of the protein-reservoir mixture was equilibrated with 50 μ L of a reservoir solution. HasA with Co-Ph₄Pc was crystallised by sitting-drop vapour-diffusion method at 20 °C for 3 weeks.

Data collection and refinement

Crystals were flash-cooled by liquid nitrogen. X-ray diffraction data sets were collected at SPring-8 (Hyogo, Japan) on the BL26B1 beamline equipped with an EIGER X 4M detector at a wavelength of 1.0 Å at 100 K. The program XDS²⁰ was used for integration of diffraction intensities and scaling. The model structure was solved by molecular replacement using MOL-REP,²¹ with the structure of HasA with haem (3ELL)¹⁸ serving as a search model. Model building and refinement were performed on Coot,²² Phenix,²³ and REFMAC5.²⁴ The model of Co-Ph₄Pc was generated by the PRODRG server²⁵ and SKECHER.²⁶ All protein figures were depicted by using PyMOL.²⁷ The final refinement statistics are summarised in Table S1 (ESI).†

Evaluating the inhibitory effect of HasA with metallo-Ph₄Pcs on *P. aeruginosa* growth

Growth experiments were performed according to a previously reported procedure.¹⁴ The concentration of HasA-bound haem (holo-HasA) was estimated from the maximum absorption of the Soret band and the corresponding extinction coefficient.¹⁷

Evaluating the cytotoxicity of HasA with metallo-Ph₄Pcs

Using M9-based medium without EDTA, the evaluating of cytotoxicity was performed by the same procedure described in the previous section.

Synthesis of metallo-Ph₄Pcs

Synthesis of Co-Ph₄Pc. 9,10,19,20-Tetraphenylporphycene (Ph₄Pc) (61 mg, 0.10 mmol) was mixed with cobalt(II)-acetate tetrahydrate (249 mg, 1.0 mmol) and phenol (10 mL) in a 30 mL flask. The solution was stirred for 3 hours at 170 °C. After cooling to r.t, the reaction mixture was dissolved in dichloromethane (200 mL) and washed 3 times with 50 mL of distilled water, and 6 times with 50 mL of 5% aqueous NaOH. The organic layer was dried over anhydrous Na₂SO₄ and removed *in vacuo*. The resulting solid was washed with methanol to yield Co-Ph₄Pc as a purple solid (45 mg, 67%). HRMS (FAB, *m/z*): calcd (%) for C₄₄H₂₈CoN₄ [M]⁺ 671.1646; found for [M]⁺ 671.1646.

Synthesis of (Fe-Ph₄Pc) μ-oxodimer. 9,10,19,20-Tetraphenylporphycene (Ph₄Pc) (50 mg, 0.081 mmol) and iron(II) acetylacetonate (142 mg, 0.407 mmol) in phenol (15 mL) in a 20 mL pressure-tight microwave tube. The reaction mixture was heated under microwave irradiation (300 W) at 80 °C for 10 min and then at 190 °C for 1 h. After cooling to r.t, the reaction mixture was dissolved in dichloromethane (30 mL) and stirred with 30 mL of a 20% aqueous solution of sodium hydroxide for 30 min. The phases were separated, and the organic layer was washed with distilled water and brine, dried over anhydrous Na₂SO₄, and the solvent was removed *in vacuo* and the crude product was purified using silica-gel column chromatography (100% dichloromethane to remove unreacted Ph₄Pc, then change to dichloromethane/methanol = 95 : 5 to collect target product) to yield (Fe-Ph₄Pc) μ-oxodimer as a black-purple solid (40 mg, 71.4%). HRMS (FAB, *m/z*): calcd (%) for C₉₀H₆₄Fe₂N₈O [M]⁺ 1348.3902; found for [M/2-O]⁺ mono Fe-Ph₄Pc 668.1662. The μ-oxodimer was characterized by single crystal X-ray structure analysis as shown in Fig. S2.†

Data collection and refinement

A crystal of the (Fe-Ph₄Pc) μ-oxodimer was mounted on a loop. Data from X-ray diffraction was collected at 93 K by a Rigaku XtaLAB mini CCD diffractometer equipped with graphite monochromated Mo-Kα radiation (λ = 0.71073 Å). Collected data was integrated, corrected, and scaled using the program CrysAlisPro.²⁸ The structure was refined using SHELXT²⁹ Intrinsic Phasing and SHELXL.³⁰ All non-hydrogen atoms were refined anisotropically. Hydrogen atoms were located at calculated positions and included in the structure factor calculation

but were not refined. The program Olex2 was used as a graphical interface.³¹ Crystallographic data was deposited with the Cambridge Crystallographic Data Centre (CCDC) under deposition No. CCDC-1908492.

Conflicts of interest

There are no conflicts of interest to declare.

Acknowledgements

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