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## The HemQ coprohaem decarboxylase generates reactive oxygen species: implications for the evolution of classical haem biosynthesis

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### ABSTRACT

Bacteria require a haem biosynthetic pathway for the assembly of a variety of protein complexes including cytochromes, peroxidases, globins, and catalase. Haem is synthesised via a series of tetrapyrrole intermediates including non-metallated porphyrins such as protoporphyrin IX, which is well-known to generate reactive oxygen species (ROS) in the presence of light and oxygen. *Staphylococcus aureus* has an ancient haem biosynthetic pathway that proceeds via the formation of coproporphyrin III, a less reactive porphyrin. Herein, we demonstrate for the first time that HemY of *S. aureus* is able to generate both protoporphyrin IX and coproporphyrin III, and that the terminal enzyme of this pathway, HemQ, can stimulate the generation of protoporphyrin IX (but not coproporphyrin III). Assays with hydrogen peroxide, horseradish peroxidase, superoxide dismutase, and catalase confirm that this stimulatory effect is mediated by superoxide. Structural modelling reveals that HemQ enzymes do not possess the structural attributes that are common to peroxidases that form compound I [ $\text{Fe}^{\text{IV}}=\text{O}$ ]<sup>+</sup>, which taken together with the superoxide data leaves Fenton chemistry as a likely route for the superoxide-mediated stimulation of protoporphyrinogen IX oxidase activity of HemY. This generation of toxic free radicals could explain why HemQ enzymes have not been identified in organisms that synthesise haem via the classical protoporphyrin IX pathway. This work has implications for the divergent evolution of haem biosynthesis in ancestral microorganisms and provides new structural and mechanistic insights into a recently discovered oxidative decarboxylase reaction.

**KEYWORDS:** Coproporphyrinogen, protoporphyrinogen, haem biosynthesis

**SUMMARY STATEMENT:** This study characterises an interaction between enzymes of classical and coproporphyrin-dependent haem biosynthesis that is mediated by toxic reactive oxygen species. This finding has important implications for why evolution has not favored microorganisms that synthesise haem via both routes.

### INTRODUCTION

Haem is an important cofactor required for a variety of functions including the transport of oxygen and carbon dioxide in multicellular organisms, cytochrome-mediated electron transfer in respiratory chains, and detoxification of nitric oxide by bacterial globin proteins. In addition, peroxidases, catalases, and cytochrome P450s also utilise haem as a cofactor. Until

recently, it was generally accepted that the terminal stages of haem synthesis were catalysed by four enzymatic steps via a pathway from uroporphyrinogen III ending in the insertion of ferrous iron. In the so called classical pathway, uroporphyrinogen III undergoes decarboxylation to form coproporphyrinogen, whereby acetyl side chains are converted to methyl groups with concomitant release of CO<sub>2</sub>. Coproporphyrinogen III is then converted to protoporphyrinogen IX via an oxidative decarboxylation reaction that removes carboxyl groups from the propionates on rings A and B leaving vinyl substituents. There are two alternative enzymes that can catalyse this process, HemF and HemN. The reaction involving HemF is oxygen-dependent, with molecular oxygen being used as the final electron acceptor. This form of coproporphyrinogen oxidase is mainly found in eukaryotic organisms, whereas the HemN enzyme that is found in many Gram negative bacteria is oxygen independent [1]. The penultimate step in the synthesis of haem via this pathway is the six-electron oxidation of protoporphyrinogen IX to protoporphyrin IX by protoporphyrinogen IX oxidase (also known as PPO). As with the coproporphyrinogen oxidase reaction there are oxygen-dependent and oxygen-independent PPO enzymes. The oxygen dependent reaction, which is catalysed by the HemY enzyme, uses oxygen for the terminal electron acceptor, whereas the oxygen independent HemG and HemJ enzymes shuttle the electrons into the respiratory chain [2-4]. Finally, a ferrous iron is inserted into protoporphyrin IX by the terminal enzyme of the pathway, ferrochelatase.

An alternative pathway for haem biosynthesis was discovered recently that produces haem *via* a sirohaem precursor [5], which has now been observed in sulphate reducing bacteria and methanogenic archaea [6-8]. More recently, a third coproporphyrin-dependent pathway has been identified in Gram-positive bacteria [9, 10] whereby coproporphyrinogen III is converted to protohaem via coproporphyrin III and Fe-coproporphyrin III intermediates. This pathway (Fig. 1, blue route) has been reported to be more widely distributed and older than the classical protoporphyrin IX pathway (Fig. 1, red route), pre-dating oxygenic photosynthesis [9]. It has long been known that certain Gram-positives such as *S. aureus* accumulate coproporphyrin III and not protoporphyrin IX [11]. Interestingly the enzyme HemY from *Bacillus subtilis* has been shown to oxidise both coproporphyrinogen III and protoporphyrinogen IX [12, 13]. Further studies reported that a haem-binding protein HemQ that is required for the terminal steps of haem synthesis in species of Actinobacteria and Firmicutes had peroxidase and catalase activity and could stimulate the oxidation of protoporphyrin IX by HemY [14]. *S. aureus hemQ* mutants were then shown to accumulate coproporphyrin III and the HemQ enzyme was reported to lyse haem in the presence of peroxide/chlorite [15]. Structural analyses then confirmed the HemQ family of proteins to be distinct from the original assignment to the family of chlorite dismutase enzymes [16], these differences defined by variations in conserved proximal/distal pocket residues [17]. However, an overlay of chlorite dismutase coordinates with the crystal structure of apo-HemQ from *Listeria monocytogenes* revealed the chlorite dismutase haem docks neatly in the HemQ active site cleft [18]. The true *in vivo* role for HemQ was revealed in 2015 when HemQ was confirmed to catalyse the decarboxylation of coprohaem for the noncanonical coproporphyrin-dependent pathway [9, 10]. Bona fide coproporphyrinogen oxidases were shown to be absent in Actinobacteria and Firmicutes explaining the lack of protoporphyrin IX in these organisms observed over four decades earlier [11]. The lack of a functional coproporphyrinogen oxidase in *S. aureus* was then validated by report of a lack of activity for the purified HemN protein [10], which appears to have been mis-annotated [9]. Finally, the HemQ reaction was later shown to proceed via an unusual peroxide-dependent reaction via a harderohaem III intermediate [19], and FMN has also been shown to be an effective electron acceptor [9].

The current study seeks to investigate mechanisms that have driven the evolution of the classical protoporphyrin IX-dependent pathway present in proteobacteria and eukaryotes. Since the HemQ component of the coproporphyrin pathway has been identified in evolutionarily early-branching (Acidobacteria and Planctomyces) and transitional (Deinococcus-Thermus group) diderm phyla [9, 20, 21], it is highly likely that this pathway is an evolutionary precursor to the classical protoporphyrin IX pathway. However, the

presence of a non-functional HemN homologue in the vast majority of Firmicutes/Actinobacteria [9], the identification of *hemF* genes in a small proportion of these families [9], and demonstrations that Gram-positive HemY enzymes can oxidise both protoporphyrinogen IX and coproporphyrinogen III [9, 13, 14], all suggest that ancestral species of Firmicutes/Actinobacteria may have once produced both coproporphyrin III and protoporphyrin IX intermediates. The reported lack of protoporphyrin IX in Actinobacteria and Firmicutes [11] indicates that HemQ cannot convert coproporphyrin III to protoporphyrin IX, which is consistent with the requirement for a metallated porphyrin to participate in the peroxide-dependent HemQ mechanism [19].

Hence, one plausible route for the protoporphyrin-dependent pathway to have evolved is: i) the necessary appearance of HemN/HemF enzyme(s) that decarboxylate coproporphyrinogen III to protoporphyrinogen IX, ii) emergence of HemY variants that can oxidise both coproporphyrinogen III *and* protoporphyrinogen IX, iii) emergence of HemH ferrochelatases that can catalyse iron insertion into coproporphyrin III *and* protoporphyrin IX, and finally iv) loss of Fe-coproporphyrin III decarboxylases (HemQ/AhbD). Since there is a requirement for haem (or a significant growth advantage to synthesising it) in most organisms that have a functional haem biosynthetic pathway, it is plausible that the protoporphyrin- and coproporphyrin-dependent pathways co-existed before the protoporphyrin-dependent pathway came to predominate in proteobacteria and eukaryotes. Following this branching event, major clades have some interesting characteristics: HemQ/AhbD enzymes are largely absent from proteobacterial species that oxidise protoporphyrinogen via the classical pathway, and Firmicutes/Actinobacteria possess HemQ/AhbD enzymes yet do not oxidise protoporphyrinogen IX to protoporphyrin IX. This exclusive presence of a classical *or* a coproporphyrin-dependent haem pathway is particularly intriguing in light of demonstrations that HemQ stimulates protoporphyrinogen oxidation by *Propionibacterium acnes* HemY [14]. Since HemQ of *S. aureus* decarboxylates Fe-coproporphyrin III via a peroxide-dependent mechanism [19], it was hypothesised that that radical generation by HemQ has a greater impact upon protoporphyrinogen oxidation compared to coproporphyrinogen oxidation (Fig. 1), resulting in the generation of reactive oxygen species that are toxic to the cell. It was therefore of interest to re-evaluate substrate specificity in *S. aureus* HemY and to examine how the likely co-existence of HemY and HemQ influenced flux through the protoporphyrin IX (classical) and coproporphyrin III pathways. Herein, we investigate this ancestral process in terms of how a combination of metalloporphyrin-bound HemQ and hydrogen peroxide influences the oxidation of porphyrinogen intermediates in both pathways (Fig. 1).

## EXPERIMENTAL PROCEDURES

### Protein purification

Recombinant *S. aureus* HemY and HemQ proteins were overexpressed and purified essentially as reported previously for other HemY proteins [22-25]. *E. coli* BL21 pLysS cells were transformed with pET28a-*S. aureus hemY* [10] and pET14b-*S. aureus hemQ* [10] and starter cultures were grown overnight at 37 °C and 160 rpm. Baffled flasks (2L) containing LB (1L) were inoculated and incubated at 37 °C and 160 rpm until an OD<sub>600</sub> of 0.6 had been reached, and 0.4 mM IPTG and 0.75 µg mL<sup>-1</sup> riboflavin were added at this point. Cultures were then grown for 16 h at 19 °C and 160 rpm. Cells from 1 L cultures were harvested by centrifugation and resuspended in buffer (50 mM Tris/MOPS pH 8.0, 100 mM KCl, 0.5 % Tween-20 (v/v)) containing 1 mg mL<sup>-1</sup> phenylmethylsulfonyl fluoride (PMSF), sonicated on ice for 6 x 30 s, and centrifuged at 100,000 xg (30 min) to remove membranes and insoluble proteins. The supernatant was applied to cobalt-chelate columns (2.5 mL bed volume) that were subsequently washed with 20 column volumes of buffer and a further 20 volumes of buffer containing 15 mM imidazole. The purified proteins were eluted with buffer containing 300 mM imidazole and concentrated to <1 mL. The imidazole was subsequently removed using PD10 desalting columns (GE Healthcare). Haem- and porphyrin-bound derivatives of HemY and HemQ were purified following addition of tetrapyrroles prior to affinity

chromatography, and the bound ligands were verified by absorption spectroscopy. Protein concentrations were determined spectrophotometrically using a Markwell assay [26].

### **Tetrapyrrole preparation**

3 mg of protoporphyrin IX was solubilised with 4 drops of 30 % ammonium hydroxide, and or coproporphyrin III was solubilised with a few drops of DMSO. These porphyrins were then dissolved in 2.5 mL of freshly prepared 10 mM KOH containing 20 % ethanol. This was then diluted with 2.5 mL of 10 mM KOH solution. Sodium amalgam was prepared, as previously described [27], by mixing 3.5 g of sodium metal with 75 g of mercury under a stream of nitrogen in a fume hood. The hot amalgam was cooled to room temperature, broken into 1 cm<sup>3</sup> pieces, and stored under nitrogen for up to one week. Immediately prior to use, 20 g of amalgam was pulverized to with a mortar and pestle and transferred to a flask under nitrogen in the dark. The 5 mL solution of porphyrin was transferred to the flask. The flask was sealed and shaken until the solution became colourless, making sure to vent any evolved gas. The pH was adjusted to pH 8.0 using a solution of 4 M MOPS. The resultant porphyrinogen solution (proto or copro) was filtered (using a 0.2 µm syringe filter), placed in the dark under nitrogen, and used immediately.

Haemin powder (Sigma) was solubilised in DMSO (Fluka) and was subsequently diluted in water. Protohaem IX solutions were quantified using a pyridine haemochrome assay [28] by mixing in a 1:1 ratio with a solution containing 4.2 M pyridine (Sigma) and 0.4 M NaOH. Reduced (sodium dithionite) *minus* oxidised (potassium ferricyanide) difference spectra were recorded and haem concentrations were determined using an extinction coefficient of  $\epsilon_{557-541} = 20.7 \text{ mM}^{-1}\text{cm}^{-1}$  [29].

### **Assay of protoporphyrinogen oxidase and coproporphyrinogen oxidase activities**

HemY activity was assayed using a continuous assay essentially as described previously [25]. PPO activity was monitored using a continuous assay *via* the detection of porphyrin fluorescence. This was performed on a FLUOstar OPTIMA plate reader using excitation and emission wavelengths of 520 nm and 620 nm, respectively.

The instrument was calibrated with known concentrations of protoporphyrin IX and coproporphyrin III. An opaque, clear bottom, 96-well plate was used for the assays. The reaction mixtures were pre-incubated at 37 °C in the dark for 3 min. The reactions were started by the addition of HemY (0.5 µM), which was also preincubated at 37 °C, and the evolution of porphyrin was monitored for 30 min. The nonenzymatic rates were determined concomitantly and were subsequently subtracted. The total volume of the reaction mixture was 100 µL and contained 50 mM NaH<sub>2</sub>PO<sub>4</sub> pH 8.0, 0.2 % Tween-20 (v/v). The concentration of porphyrinogen substrate was determined retrospectively after the complete auto-oxidation of the stock solution to the porphyrin product, which was quantified spectrophotometrically. Extinction coefficients for protoporphyrin IX and coproporphyrin III were  $\epsilon_{408} = 262 \text{ mM}^{-1}\text{cm}^{-1}$  (in 2.7 N HCl) and  $\epsilon_{400} = 489 \text{ mM}^{-1}\text{cm}^{-1}$  (in 0.1 N HCl), respectively [30]. HRP (120-180 units/mg) and SOD ( $\geq 3000$  units/mg) were obtained from Sigma and were included in the assays at concentrations indicated in the figure legends.

### **Structural modelling**

Structural modelling was performed for HemQ enzymes using the RaptorX server at the University of Chicago [31, 32]. Models for HemQ from *S. aureus* (SaHemQ) and *P. acnes* HemHQ residues 450-683 (PaHemQ) were generated using the crystal structure of HemQ from *Thermus thermophilus* (1VDH [33]) as a template ( $\alpha$ -carbon RMSD values for SaHemQ and PaHemQ were 0.34 Å and 0.75 Å, respectively). Structural modelling for HemQ from *M. tuberculosis* (MtHemQ) was performed using the crystal structure of HemQ from *Listeria monocytogenes* (4WWS [18]) as the most suitable template ( $\alpha$ -carbon RMSD = 0.74 Å). To model a haem cofactor in the binding clefts, the  $\alpha$ -carbon atoms of the closely related chlorite dismutase from *Candidatus Nitrospira defluvii* (3NN1, [34]) were superposed (RMSD values for SaHemQ, PaHemQ, and MtHemQ were 1.72 Å, 1.51 Å and 1.68 Å, respectively).



## RESULTS

### ***S. aureus* HemY catalyses the oxidation of both protoporphyrinogen IX and coproporphyrinogen III**

HemY enzymes from *B. subtilis* (a Firmicute), *P. acnes*, and *M. tuberculosis* (both Actinobacteria) and have previously been shown to catalyse the oxidation of both protoporphyrinogen IX and coproporphyrinogen III [9, 13, 14], supporting the hypothesis that the classical haem pathway evolved from the coproporphyrin-dependent route. Hence, it was of interest to investigate if the substrate specificity of HemY from *S. aureus* (a Firmicute) was consistent with these findings. Our kinetic analyses revealed  $k_{\text{cat}}$  values of  $0.44 \pm 0.03$  and  $0.46 \pm 0.02 \text{ min}^{-1}$  for protoporphyrinogen IX and coproporphyrinogen III, respectively (Fig. 2). The  $K_m$  for coproporphyrinogen III was determined to be  $6.7 \pm 0.8 \mu\text{M}$ , whereas the  $K_m$  for protoporphyrinogen IX is likely to be lower than this but could not be accurately determined due to detection constraints at low substrate concentrations.

### **Haem-bound HemQ stimulates HemY-mediated oxidation of protoporphyrinogen but not coproporphyrinogen III**

A major focus of this study was to investigate why the classical pathway and coporphyrin-dependent pathways do not co-exist. One hypothesis is that haem-bound HemQ produces toxic free radicals in the presence of protoporphyrin IX and peroxide, whereas the less reactive coproporphyrin III product will not support this radical generation. There is a precedent for this, as the generation of superoxide by cytochrome *c* has previously been shown to stimulate the oxidation of protoporphyrinogen IX by a eukaryotic HemY enzyme [35]. We therefore aimed to test the hypothesis that any stimulation of HemY-catalysed protoporphyrinogen oxidation by haem-loaded HemQ, as observed previously for *P. acnes* HemY/HemQ [14], could only be observed when protoporphyrin IX is a reaction product (and not coproporphyrin III) and HemQ is specifically binding its haem product. Our data demonstrate that the presence of haem-loaded HemQ increased the  $k_{\text{cat}}$  for protoporphyrinogen oxidation by 2.6-fold (Fig. 3), whereas the same experiment when coproporphyrinogen III was used as a substrate yielded no significant increase in activity in the presence of HemQ. To support the hypothesis that peroxidase type chemistry of HemQ was contributing to this rate stimulation, HemY was assayed in the presence of HemQ that was loaded with bound haem, protoporphyrin IX, and coproporphyrin III (Fig. 4). These data demonstrate that only haem bound HemQ stimulates the HemY reaction, suggesting the involvement of peroxidase-like chemistry in this rate stimulation.

### **Hydrogen peroxide participates in the HemQ-mediated stimulation of protoporphyrinogen IX oxidation via the generation of superoxide**

We hypothesised that hydrogen peroxide produced during HemY catalysis was participating in the HemQ-mediated rate stimulation. To confirm the involvement of hydrogen peroxide, HemY-catalysed protoporphyrinogen IX oxidation was assayed in the presence and absence of haem-loaded HemQ at various concentrations of peroxide (Fig. 5A). In the absence of HemQ, peroxide elicited a gradual decrease in activity, whereas in the presence of HemQ the peroxide caused a significant stimulation of protoporphyrinogen IX oxidation. The same experiment was performed with coproporphyrinogen III as a substrate, and peroxide did not elicit any changes in HemY catalysis in the presence or absence of HemQ (Fig. 5B). To test the hypothesis that peroxidase chemistry was involved in the HemQ-mediated stimulation of HemY, protoporphyrinogen IX oxidation was measured in the presence of horseradish peroxidase (HRP, Fig. 6), which demonstrated the same pattern of rate stimulation as haem-loaded HemQ (i.e. only when protoporphyrinogen IX was used as a substrate). To confirm that superoxide generation was involved in the HemQ-mediated stimulation of HemY, protoporphyrinogen IX oxidation was measured in the presence of superoxide dismutase (Fig. 6), which completely abrogated the stimulatory effect of HRP. Since exogenous peroxide caused some inhibition of HemY in the absence of HemQ (Fig. 5A), it was necessary to verify that the HRP stimulatory effect was not due to *removal* of peroxide. The presence of

catalase in HemY assays did not elicit a stimulatory effect (Fig. 6), confirming that peroxide removal was not responsible for the stimulation of HemY-catalysed protoporphyrinogen IX oxidation.

### **HemQ enzymes possess a haem environment that is distinct from classical peroxidases**

HemQ enzymes have recently been reported to lack a hydrogen-bonding network in the proximal haem-binding pocket that is present in closely-related chlorite dismutase enzymes [17, 18]. Such features are common in peroxidase-like enzymes that induce imidazolate character upon the axial histidine ligand that is important in catalysing the O-O bond cleavage reaction of haem-bound peroxides via the well-known ‘push pull’ model [36] (to stabilise higher oxidation states of the haem iron during the peroxidase catalytic cycle). Since HemQ has recently been reported to proceed via a peroxide-dependent reaction [19] and the current data support the generation of superoxide by HemQ, it was of interest to re-visit the structural components of the haem binding cleft. Furthermore, previous structural analyses on HemQ-type enzymes have overlooked the members of this family that have been kinetically characterized and verified to be *bona fide* HemQ enzymes [17]. Hence, structural models for HemQ enzymes from *S. aureus*, *P. acnes*, and *M. tuberculosis* were generated using the RaptorX server [31, 32] and the haem cofactor from the closely related chlorite dismutase from *Candidatus Nitrospira defluvii* (PDBid = 3NN1 [34], Fig. 7H) was overlaid (Fig. 7A-C). Similarly, apoprotein crystal structures for enzymes that have been assigned to the were overlaid with the haem cofactor from 3NN1. These include 1VDH from *T. thermophilus* [33], 1T0T from *Bacillus stearothermophilus*, 4WWS from *L. monocytogenes* [18], and 3DTZ from *Thermoplasma acidophilum* (Fig. 7D-G).

Initial comparisons of the distal pockets confirm that HemQ enzymes lack the conserved arginine that is common to chlorite dismutases (e.g. R173 in 3NN1): this position is labelled in red in Fig. 7A-H and is most commonly occupied by a glutamine residue (e.g. Q185 in *S. aureus* HemQ), although this sidechain is not necessary for the decarboxylation of Fe-coproporphyrin III as evidenced by alanine residues in at this position for HemQ models for *P. acnes* and *M. tuberculosis* (Fig. 7B-C). A multiple sequence alignment (Fig. 7I) highlights a conserved tyrosine (Y145 in *S. aureus* HemQ) at the periphery of the distal cleft of HemQ enzymes (labelled in blue in Fig. 7A-H), and an adjacent proline that is also fully conserved (P146 in *S. aureus* HemQ). Another notable observation is a conserved tryptophan residue adjacent to the haem propionates in Fig. 7 (labelled in green in Fig. 7A-H), although this residue is a phenylalanine in 3DTZ. There are no fully-conserved residues in close proximity to the ubiquitous axial histidine in the proximal pocket for HemQ family members, although a lysine/arginine residue is present (K149 in *S. aureus* HemQ, labelled in purple in Fig. 7A-H) that could potentially stabilise haem propionates or contribute to a proximal hydrogen bonding network as in 3NN1 (albeit via water molecules). To gain greater insights into sidechain requirements in and around the haem-binding cleft, residues that are fully or functionally conserved in the HemQ family were identified (asterisks on Fig. 7I) and displayed on the model of *S. aureus* HemQ (Fig. 8). This analysis revealed fully conserved tryptophan/tyrosine pair buried deep in the cleft (Fig. 8, W157/Y158), and fully conserved arginine residues at opposite ends of the distal pocket (Fig. 8, R165/R218). In addition, a fully conserved glutamate residue is adjacent to the conserved tryptophan (Fig. 8, E197/W198) in the distal cleft. These observations provide insights into the required machinery for HemQ-mediated decarboxylation of Fe-coproporphyrin III, and also have implications for the ability of HemQ enzymes to generate radicals that participate in the oxidation of porphyrinogen intermediates.

## **DISCUSSION**

This work examines the hypotheses that *S. aureus* HemY can participate in both the coproporphyrin-dependent and classical pathways and that the generation of ROS by the HemQ enzyme has a more profound effect upon macrocycle oxidation in the classical pathway compared to the coproporphyrin-dependent pathway. These investigations will aid

our understanding of how the classical pathway evolved from the more ancient coproporphyrin-dependent pathway [9].

Kinetic analysis of *S. aureus* HemY (Fig. 2) revealed  $k_{\text{cat}}$  and  $K_m$  values of  $0.46 \pm 0.02 \text{ min}^{-1}$  and  $6.7 \pm 0.8 \text{ }\mu\text{M}$  for coproporphyrinogen III oxidation compare to previous measurements of  $1.33 \pm 0.04 \text{ min}^{-1}$  and  $0.31 \pm 0.01 \text{ }\mu\text{M}$  [10], respectively. Previously, the oxidation of protoporphyrinogen IX for *S. aureus* HemY could not be detected [10], so this reaction was re-examined. The data herein clearly show activity for the protoporphyrinogen IX substrate ( $k_{\text{cat}} = 0.44 \pm 0.03 \text{ min}^{-1}$ ), although the background rate and signal:noise was significantly higher for the protoporphyrinogen IX reaction (Supplemental Data, Fig. S1), possibly explaining why enzymatic activity could not be detected in previous studies [10]. This pattern of substrate specificity is consistent with previous observations for HemY enzymes from numerous Actinobacteria/Firmicutes [9, 13, 14], and supports the hypothesis that evolutionary precursors to these HemY enzymes may once have also oxidized protoporphyrinogen IX *in vivo* in ancestral organisms. This is also supported by previous observations that the HemH ferrochelatases from Gram-positives can insert iron into both coproporphyrin III and protoporphyrin IX [9].

If we accept the compelling evidence that the classical pathway evolved from the coproporphyrin-dependent pathway, an important question presents itself: why do the majority of Actinobacteria/Firmicutes now lack a functional coproporphyrinogen oxidase that prevents the formation of protoporphyrinogen IX and protoporphyrin IX? One potential explanation would be that the peroxide-dependent mechanism of HemQ can generate free radicals, which will have a greater cytotoxic effect in combination with protoporphyrin IX compared to the less reactive coproporphyrin III. This is consistent with a previous report that the more reactive protoporphyrin IX causes greater cellular damage [37]. Hence, it was hypothesised that stimulation of HemY-mediated protoporphyrinogen IX oxidation by HemQ [14] was due to the evolution of ROS, as previously reported for cytochrome *c* and eukaryotic HemY [35], and that this stimulation would be exacerbated when protoporphyrin IX is a reaction product (compared to the less reactive coproporphyrin III). HemY assays with both substrates in the presence and absence of HemQ were consistent with this hypothesis (Fig. 3), and yielded the surprising observation that HemQ did not stimulate the oxidation of coproporphyrin III at all. Further kinetic analysis with haem/porphyrin loaded ligands confirm that haem, the product of the HemQ reaction, must be present for the stimulation of HemY activity (Fig. 4). Further assays with varying hydrogen peroxide (Fig. 5) demonstrate that this ROS-generator stimulates the HemY reaction only when HemQ is present, but causes inhibition in the absence of HemQ. Since the addition of high concentrations of peroxide resulted in a negligible stimulation in nonenzymatic oxidation of porphyrinogen substrate (Fig. S1), the most simple explanation for the inhibition of HemY by peroxide in the absence of HemQ is due to classical product inhibition. However, it is not clear why this does not take place when coproporphyrinogen III is used as a substrate (Fig. 5B), although conformational differences in active site structure are likely to exist when additional propionates are present on the porphyrinogen substrate.

The stimulation of HemY activity by peroxide and HRP combined with the abrogation of this rate enhancement by SOD (Fig. 6) provide clear confirmation that it is the peroxidase activity of HemQ that enhances HemY-mediated protoporphyrinogen IX oxidation. However, the previously reported rate constant for the peroxide-dependent evolution of protohaem of  $1.6 \text{ min}^{-1}$  is very slow [16], which is consistent with a peroxidase-like active site for HemQ that lacks key features such as a distal pocket arginine (that would stabilise a developing negative charge on the leaving group) and a proximal pocket hydrogen bonding network (that would give the axial histidine imidazolate character to stabilise higher oxidation states of the iron during the peroxidase catalytic cycle). Previous structural analyses have focused on HemQ family members that lack biochemical confirmation as Fe-coproporphyrin III decarboxylases [17, 18], so it was of interest to investigate HemQ enzymes from *S. aureus*, *P. acnes*, and *M. tuberculosis*, which have all been experimentally validated as HemQ enzymes. The structural modelling studies herein (Fig. 7) suggest that imidazolate character on the axial histidine is not necessary for catalysis, although some



HemQ enzymes do possess hydrogen bonding residues that could potentially polarize this residue. This is the case for *S. aureus* HemQ (Fig. 7A), although previous resonance Raman data report a  $\nu_{\text{Fe-His}}$  stretching mode of  $213\text{ cm}^{-1}$  [16] which is consistent with a neutral axial ligand, although it is not clear whether haem or Fe-coproporphyrin III was bound in this study. Furthermore, while the majority of HemQ enzymes do possess a glutamine residue in place of the conserved distal arginine found in classical peroxidases, which could stabilise a positively charged leaving group, this residue is not necessary for HemQ activity as evidenced by alanine residues in this position for HemQ enzymes from *P. acnes* and *M. tuberculosis* (Fig. 7B-C). Another notable absence in HemQ enzymes is a distal histidine residue, which in classical peroxidases acts as a base toward  $\text{H}_2\text{O}_2$  catalysing the formation of the initial FeIII-OOH (Compound 0) intermediate [38]. In peroxidase mutants lacking an active site base, formation of Compound 0 can be slowed by up to 5 orders of magnitude [39, 40]. Furthermore, HemQ enzymes lack a conserved tryptophan residue that is found adjacent to the proximal histidine in classical peroxidases, which is the site of radical formation in compound I ( $[\text{Fe}(\text{IV})=\text{O Trp}(\bullet+)]$ ). While peroxidases that lack such a proximal aromatic residue are likely to proceed via a porphyrin pi-cation radical mechanism, the fully-conserved distal tryptophan and tyrosine residues in HemQ enzymes (Fig. 7I, Fig. 8 Y145, W198) are candidates for participation in radical-mediated decarboxylation of Fe-coprohaem. While the conserved doublet of W157/Y158 would provide a convenient tool for the decarboxylation of two propionate groups, a significant conformational change would be necessary to bring them into close proximity of the target  $\beta$ -carbon atoms. Together, these analyses are consistent with a HemQ enzyme that has evolved to have slow peroxidase activity ( $1.6\text{ min}^{-1}$  [16]), which fits well with measurements of the preceding ferrochelatase step ( $1.8\text{ min}^{-1}$  for *M. tuberculosis* HemH [9]).

Returning to the model in Fig. 1, the current data are consistent with ROS generation by HemQ stimulating the HemY-catalysed oxidation of protoporphyrinogen IX only (and not the HemY-mediated stimulation of coproporphyrinogen III oxidation). However, one should not overlook the profound effect that HemQ has on the nonenzymatic oxidation of protoporphyrinogen IX (Supplemental Data, Fig. S1). While HemQ does appear to also elevate the nonenzymatic oxidation of coproporphyrinogen III, the overall rates are an order of magnitude lower than those for protoporphyrinogen IX that are comparable to the HemY-catalyzed turnover rates in Fig. 2. This additional nonenzymatic rate enhancement is very likely to involve superoxide (from HemQ and possibly porphyrin-derived), adding weight to the hypothesis that peroxidase activity by HemQ will enhance the production of ROS in combination with tetrapyrrole intermediates of the classical pathway. Indeed, this mechanism is supported by our observations that reduced glutathione, a superoxide scavenger and common component of HemY assays, abolishes the stimulation of HemY-mediated protoporphyrinogen oxidation: this is why reduced glutathione is not present in the assays described herein. From an evolutionary perspective, this generation of toxic ROS would provide a selection pressure to retain either the coproporphyrin-dependent pathway *or* the classical pathway, but not both. This model is also consistent with observations that HemQ enzymes may utilise alternative electron acceptors such as FMN, negating the requirement for HemY-derived peroxide in the coproporphyrin-dependent pathway. This may also explain why in proteobacteria that commonly utilise the classical pathway, HemJ or HemG enzymes that do not require oxygen nor produce hydrogen peroxide are the most common forms of protoporphyrinogen oxidase [4, 9].

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#### DECLARATIONS OF INTEREST

The authors declare no competing financial interests.

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## AUTHOR CONTRIBUTION

CH performed the experiments and MS drove the experimental design. CH and MS analysed the data. CH, HAD, and MS wrote the paper.

## REFERENCES

- 1 Layer, G., Reichelt, J., Jahn, D. and Heinz, D. W. (2010) *Prot. Sci.* **19**, 1137-1161
- 2 Mobius, K., Arias-Cartin, R., Breckau, D., Hannig, A.-L., Riedmann, K., Biedendieck, R., Schroder, S., Becher, D., Magalon, A., Moser, J., Jahn, M. and Jahn, D. (2010) *Proc. Natl. Acad. Sci. U.S.A.* **107**, 10436-10441
- 3 Jacobs, N. J. and Jacobs, J. M. (1976) *Biochim. Biophys. Acta* **449**, 1-9
- 4 Kobayashi, K., Masuda, T., Tajima, N., Wada, H. and Sato, N. (2014) *Genome Biol. Evol.* **6**, 2141-2155
- 5 Buchenau, B., Kahnt, J., Heinemann, I. U., Jahn, D. and Thauer, R. K. (2006) *J. Bacteriol.* **188**, 8666-8668
- 6 Palmer, D. J., Schroeder, S., Lawrence, A. D., Deery, E., Lobo, S. A., Saraiva, L. M., McLean, K. J., Munro, A. W., Ferguson, S. J., Pickersgill, R. W., Brown, D. G. and Warren, M. J. (2014) *Mol. Microbiol.* **93**, 247-261
- 7 Lobo, S. A. L., Lawrence, A. D., Romao, C. V., Warren, M. J., Teixeira, M. and Saraiva, L. M. (2014) *BBA-Proteins Proteomics* **1844**, 1238-1247
- 8 Bali, S., Lawrence, A. D., Lobo, S. A., Saraiva, L. M., Golding, B. T., Palmer, D. J., Howard, M. J., Ferguson, S. J. and Warren, M. J. (2011) *Proc. Natl. Acad. Sci. U.S.A.* **108**, 18260-18265
- 9 Dailey, H. A., Gerdes, S., Dailey, T. A., Burch, J. S. and Phillips, J. D. (2015) *Proc. Natl. Acad. Sci. U.S.A.* **112**, 2210-2215
- 10 Lobo, S. A., Scott, A., Videira, M. A., Winpenny, D., Gardner, M., Palmer, M. J., Schroeder, S., Lawrence, A. D., Parkinson, T., Warren, M. J. and Saraiva, L. M. (2015) *Mol. Microbiol.* **97**, 472-487
- 11 Jacobs, N. J., Jacobs, J. M. and Brent, P. (1971) *J. Bacteriol.* **107**, 203-209
- 12 Dailey, T. A., Meissner, P. and Dailey, H. A. (1994) *J. Biol. Chem.* **269**, 813-815
- 13 Hansson, M. and Hederstedt, L. (1994) *J. Bacteriol.* **176**, 5962-5970
- 14 Dailey, T. A., Boynton, T. O., Albetel, A.-N., Gerdes, S., Johnson, M. K. and Dailey, H. A. (2010) *J. Biol. Chem.* **285**, 25978-25986
- 15 Mayfield, J. A., Hammer, N. D., Kurker, R. C., Chen, T. K., Ojha, S., Skaar, E. P. and DuBois, J. L. (2013) *J. Biol. Chem.* **288**, 23488-23504
- 16 Celis, A. I. and DuBois, J. L. (2015) *Arch. Biochem. Biophys.* **574**, 3-17
- 17 Hofbauer, S., Howes, B. D., Flego, N., Pirker, K. F., Schaffner, I., Mlynek, G., Djinovic-Carugo, K., Furtmuller, P. G., Smulevich, G. and Obinger, C. (2016) *Biosci. Rep.* **36**
- 18 Hofbauer, S., Hagmuller, A., Schaffner, I., Mlynek, G., Krutzler, M., Stadlmayr, G., Pirker, K. F., Obinger, C., Daims, H., Djinovic-Carugo, K. and Furtmuller, P. G. (2015) *Arch. Biochem. Biophys.* **574**, 36-48
- 19 Celis, A. I., Streit, B. R., Moraski, G. C., Kant, R., Lash, T. D., Lukat-Rodgers, G. S., Rodgers, K. R. and DuBois, J. L. (2015) *Biochemistry* **54**, 4022-4032
- 20 Gupta, R. S. (1998) *Microbiol. Mol. Biol. Rev.* **62**, 1435-1491
- 21 Gupta, R. S. (2011) *Antonie Van Leeuwenhoek* **100**, 171-182
- 22 Dailey, T. A. and Dailey, H. A. (1996) *Prot. Sci.* **5**, 98-105
- 23 Dailey, H. A. and Dailey, T. A. (1996) *J. Biol. Chem.* **271**, 8714-8718
- 24 Wang, K. F., Dailey, T. A. and Dailey, H. A. (2001) *FEMS Microbiol. Lett.* **202**, 115-119
- 25 Shepherd, M. and Dailey, H. A. (2005) *Anal. Biochem.* **344**, 115-21

- 26 Markwell, M. A. K., Haas, S. M., Bieber, L. L. and Tolbert, N. E. (1978) *Anal. Biochem.* **87**, 206-210
- 27 Brenner, D. A. and Bloomer, J. R. (1980) *Clinica Chimica Acta* **100**, 259-266
- 28 Berry, E. A. and Trumpower, B. L. (1987) *Anal. Biochem.* **161**, 1-15
- 29 Smith, K. M. (1975) *Porphyrins and metalloporphyrins*, Elsevier, New York
- 30 Falk, J. E. (1964) *Porphyrins and metalloporphyrins: their general, physical and coordination chemistry, and laboratory methods*, Elsevier, Amsterdam
- 31 Kaellberg, M., Wang, H., Wang, S., Peng, J., Wang, Z., Lu, H. and Xu, J. (2012) *Nat. Protoc.* **7**, 1511-1522
- 32 Peng, J. and Xu, J. (2011) *Proteins* **79**, 161-171
- 33 Ebihara, A., Okamoto, A., Kousumi, Y., Yamamoto, H., Masui, R., Ueyama, N., Yokoyama, S. and Kuramitsu, S. (2005) *Journal of Structural and Functional Genomics* **6**, 21-32
- 34 Kostan, J., Sjoebloom, B., Maixner, F., Mlynek, G., Furtmueller, P. G., Obinger, C., Wagner, M., Daims, H. and Djinojic-Carugo, K. (2010) *J. Struct. Biol.* **172**, 331-342
- 35 Shepherd, M. and Dailey, H. A. (2009) *Cell. Mol. Biol.* **55**, 6-14
- 36 Poulos, T. L. (1996) *J. Biol. Inorg. Chem.* **1**, 356-359
- 37 Aravind Menon, I., Persad, S. D. and Haberman, H. B. (1989) *Clin. Biochem.* **22**, 197-200
- 38 Poulos, T. L. and Fenna, R. E. (1994) *Met. Ions Biol. Syst.* **30**
- 39 Hiner, A. N., Raven, E. L., Thorneley, R. N., García-Cainovas, F. and Rodríguez-Loípez, J. N. (2002) *J. Inorg. Biochem.* **91**
- 40 Erman, J. E., Vitello, L. B., Miller, M. A., Shaw, A., Brown, K. A. and Kraut, J. (1993) *Biochemistry* **32**, 9798-9806

## FIGURE LEGENDS

**Figure 1. The classical and coproporphyrin-dependent pathways of haem synthesis.** This figure depicts haem synthesis in a hypothetical ancestral organism where the oxidation of coproporphyrinogen III to coproporphyrin III (blue) and the classical route involving a protoporphyrin IX intermediate (red) both take place. HemQ is hypothesised to generate reactive oxygen species (ROS), which in turn are hypothesised to impact upon protoporphyrinogen IX oxidation to a greater extent than coproporphyrinogen III.

**Figure 2. Kinetic analysis of the HemY protoporphyrinogen oxidase utilising protoporphyrinogen IX and coproporphyrinogen III substrates.** Nonlinear regression analysis reveals  $k_{cat}$  values of  $0.44 \pm 0.03$  and  $0.46 \pm 0.02 \text{ min}^{-1}$  for protoporphyrinogen IX (●) and coproporphyrinogen III (○), respectively. The  $K_m$  for coproporphyrinogen III is  $6.7 \pm 0.8 \mu\text{M}$ .

**Figure 3. Haem-loaded HemQ stimulates HemY-mediated oxidation of protoporphyrinogen but not coproporphyrinogen III.** Steady state kinetics for HemY in the presence (○) and absence (●) of haem-loaded HemQ (1  $\mu\text{M}$ ) using protoporphyrinogen IX (top panel) and coproporphyrinogen III (bottom panel) substrates. Nonlinear regression analysis reveals HemQ-mediated 2.6-fold increase in  $k_{cat}$  when protoporphyrinogen IX is used as a substrate, and a HemQ-mediated 1.1-fold increase in  $k_{cat}$  when coproporphyrinogen III is used as a substrate.

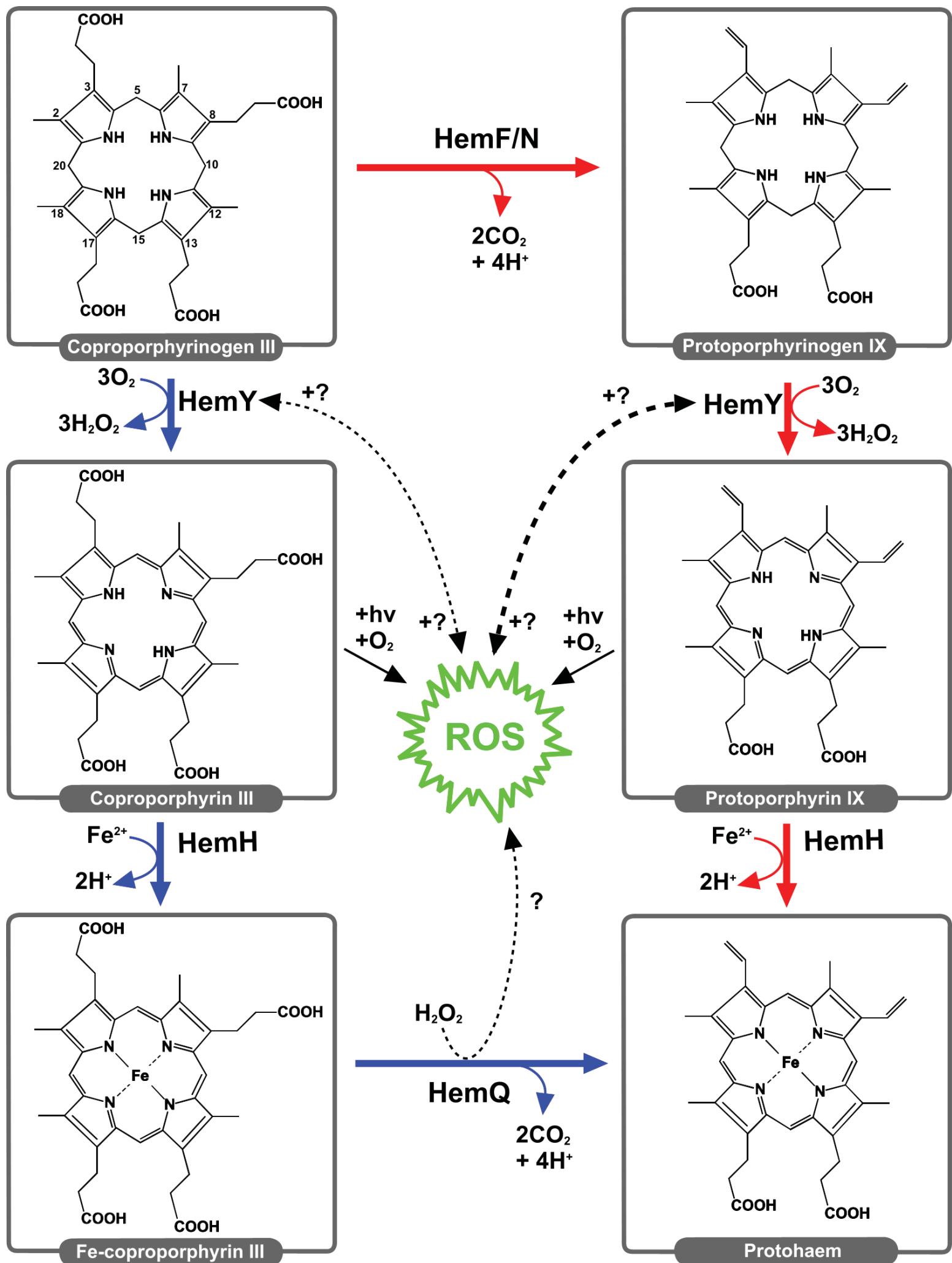
**Figure 4. HemQ must bind haem for stimulation of protoporphyrinogen oxidase activity.** Protoporphyrinogen oxidase activity of HemY was assayed in the presence of HemQ (1  $\mu\text{M}$ ) and protoporphyrinogen IX (10  $\mu\text{M}$ ) with different tetrapyrroles bound. Error bars represent SD values. Asterisk indicates that rates measured are significantly different from those measured using HemY alone (Student's *t*-test,  $P < 0.05$ ).

**Figure 5. Hydrogen peroxide stimulates protoporphyrinogen oxidase activity in the presence of HemQ.** Protoporphyrinogen oxidase activity of HemY was assayed in the presence (1  $\mu$ M) and absence of HemQ at various [hydrogen peroxide] using 9.5  $\mu$ M protoporphyrinogen IX (A) and 5.8  $\mu$ M coproporphyrinogen III (B) as substrates. Error bars represent SD values. Asterisks indicate that rates measured are significantly different from those measured in the absence of peroxide (Student's *t*-test,  $P < 0.05 = *$ ,  $P < 0.01 = **$ ,  $P < 0.001 = ***$ ).

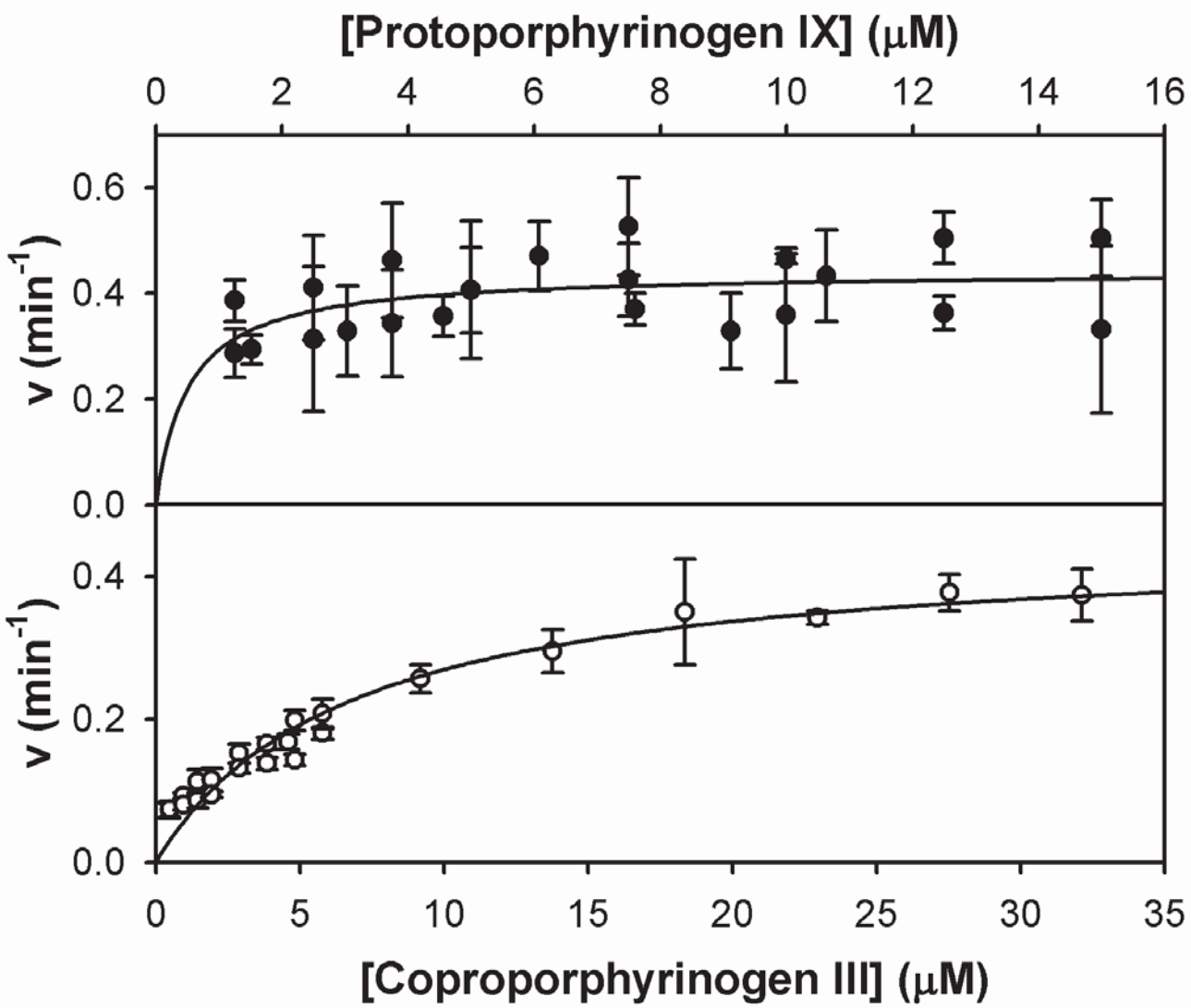
**Figure 6. Peroxidase-derived superoxide stimulates HemY-mediated oxidation of protoporphyrinogen IX.** Kinetic assays showing the effect of HRP (1  $\mu$ M), superoxide dismutase (1  $\mu$ M) and catalase (1  $\mu$ M) upon the activity of HemY (0.5  $\mu$ M) using protoporphyrinogen IX (8.6  $\mu$ M) and coproporphyrinogen III (8.2  $\mu$ M) substrates. Abbreviations: HRP, horseradish peroxidase; SOD, superoxide dismutase; Cat, catalase; P'gen IX, protoporphyrinogen IX; C'gen III, coproporphyrinogen III. Asterisks indicate that rates measured are significantly different from those measured in the absence of peroxide (Student's *t*-test,  $P < 0.001 = ***$ ).

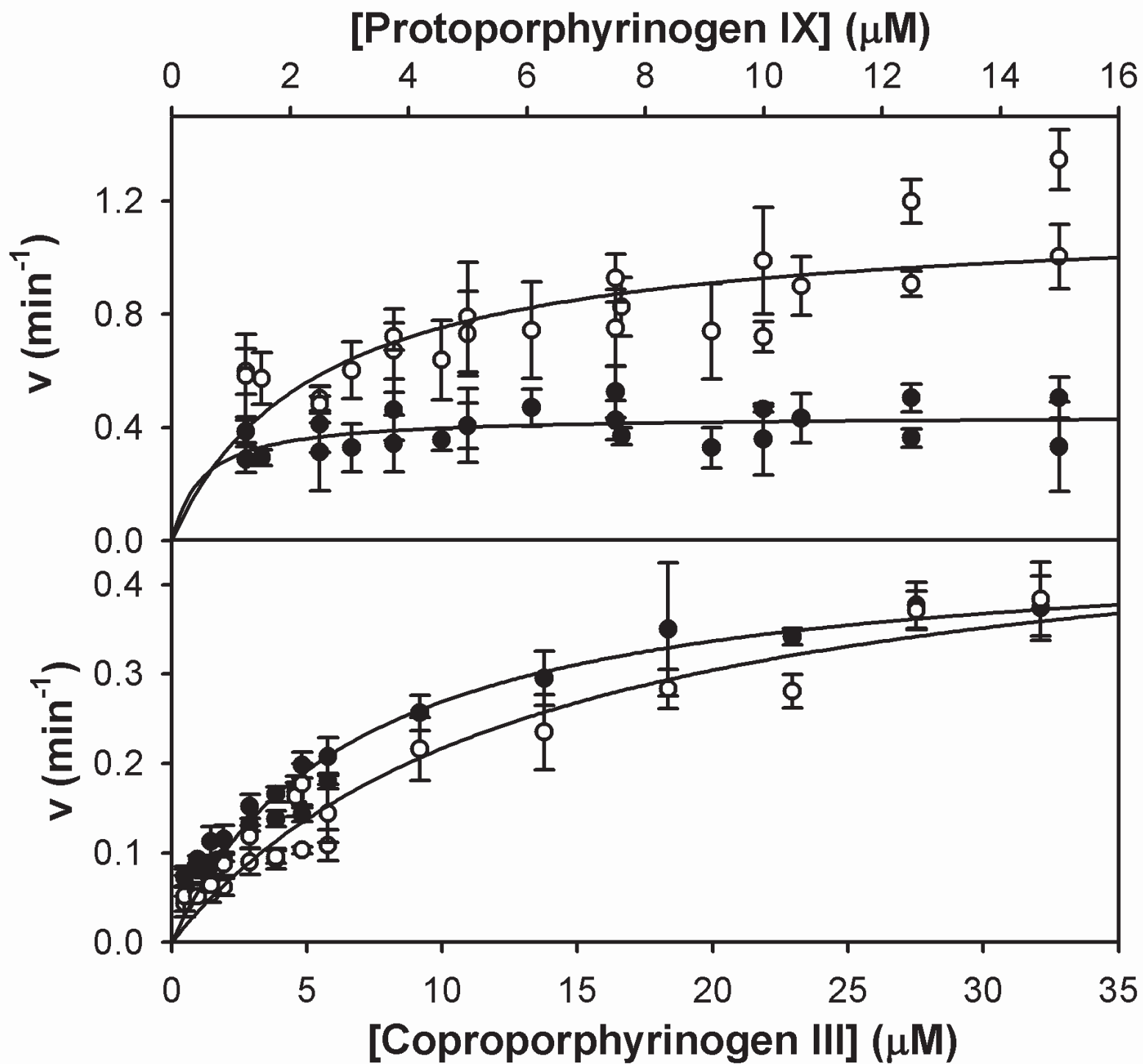
**Figure 7. Structural analysis of the haem-binding cleft of HemQ family members.** Structural models of haem-binding clefts for HemQ enzymes from *S. aureus* (A, SaHemQ), *P. acnes* (B, PaHemQ), and *M. tuberculosis* (C, MtHemQ) were produced using the haem cofactor from the chlorite dismutase from *Candidatus Nitrospira defluvii* (H, 3NN1 [34]). Panels D-G show the apoprotein crystal structures of HemQ enzymes from *T. thermophilus* (1VDH [33]), *B. stearothersophilus* (1T0T), *L. monocytogenes* (4WWS [18]), and *T. acidophilum* (3DTZ), all with the haem cofactor from 3NN1 superimposed. Panel I shows a multiple sequence alignment of sections of the proteins described above with residue numbers for SaHemQ marked at the top and functionally conserved residues marked with an asterisk.

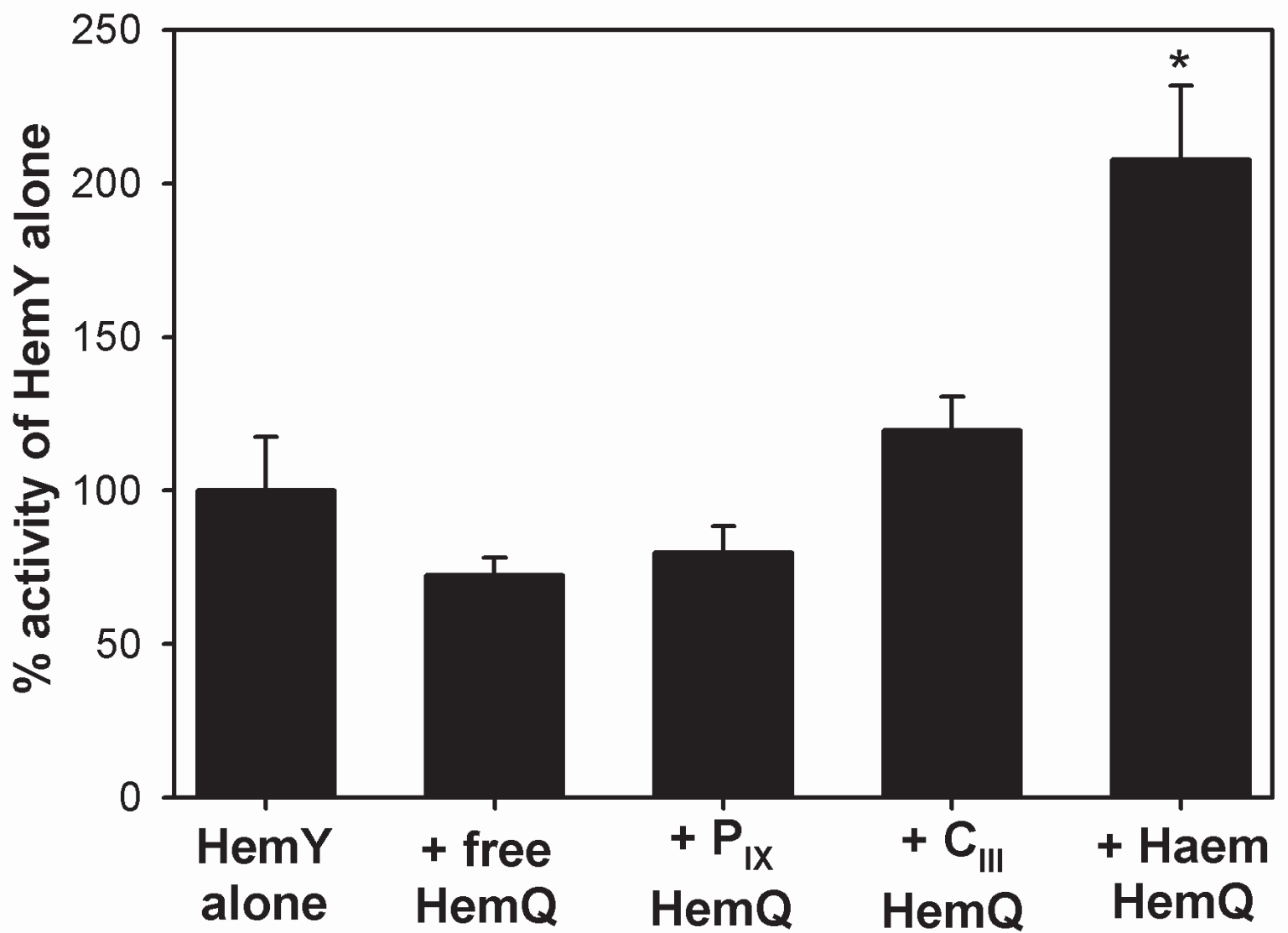
**Figure 8. Conserved residues in the haem binding cleft of HemQ family members.** Fully and functionally conserved residues in HemQ proteins from Fig. 7I are represented on the structural model of *S. aureus* HemQ viewed from the entrance to the haem cleft (A) and from behind rotated 180° around the y-axis (B).





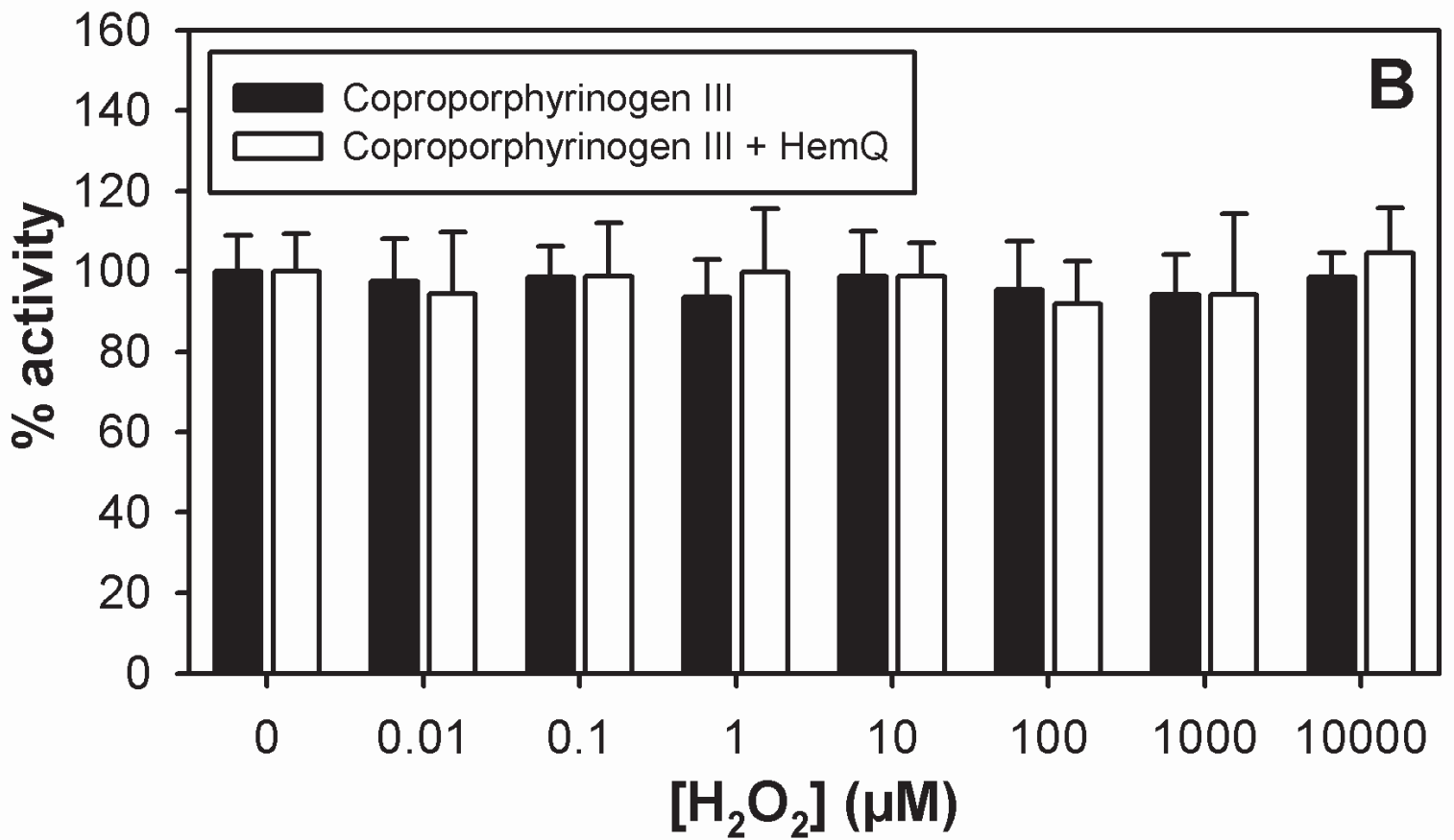
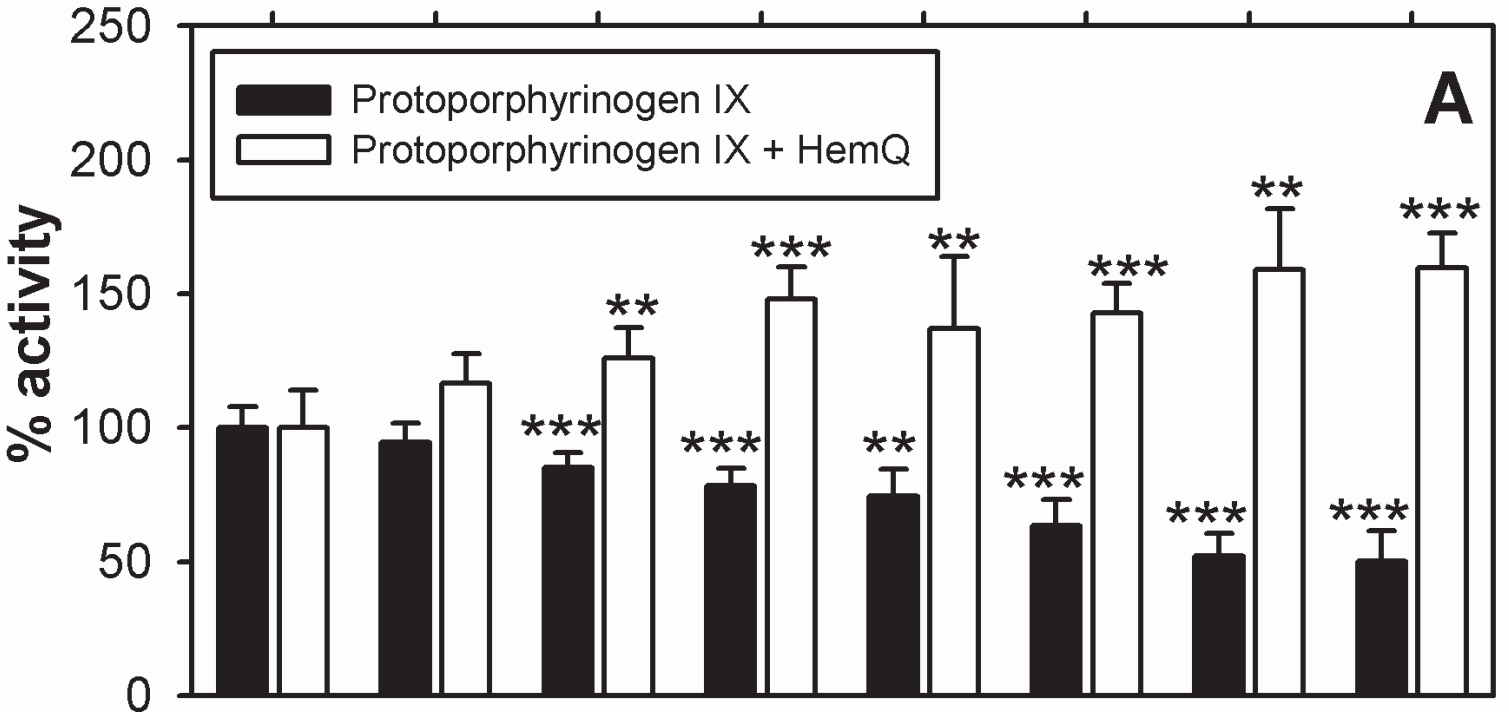


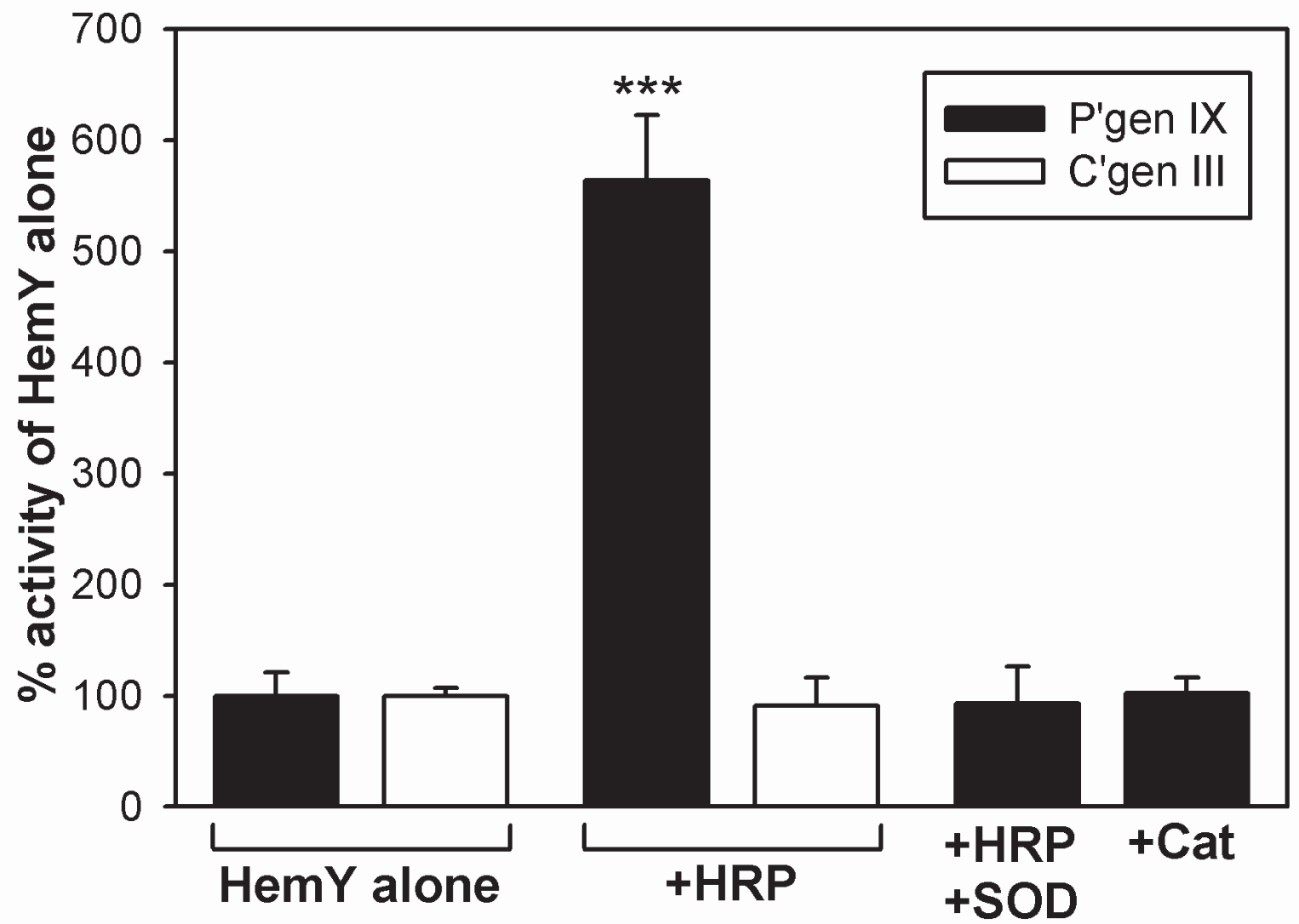




[H<sub>2</sub>O<sub>2</sub>] (μM)

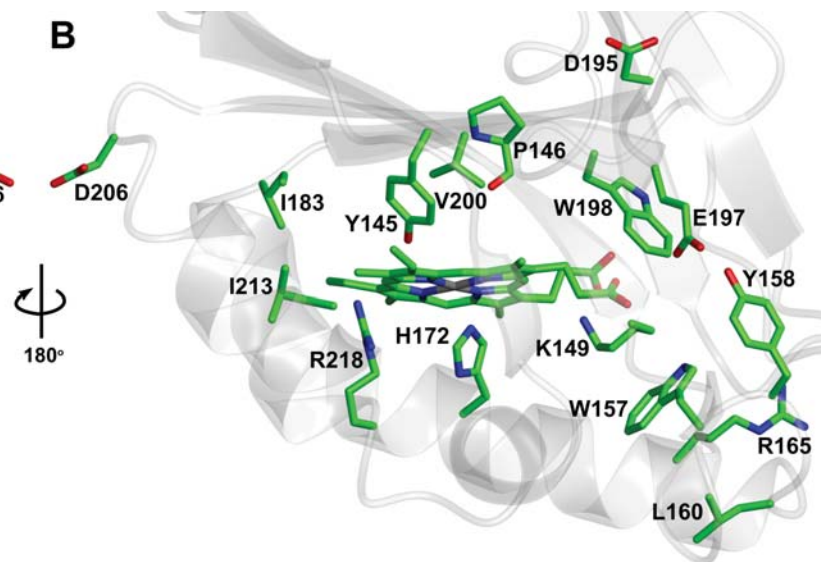
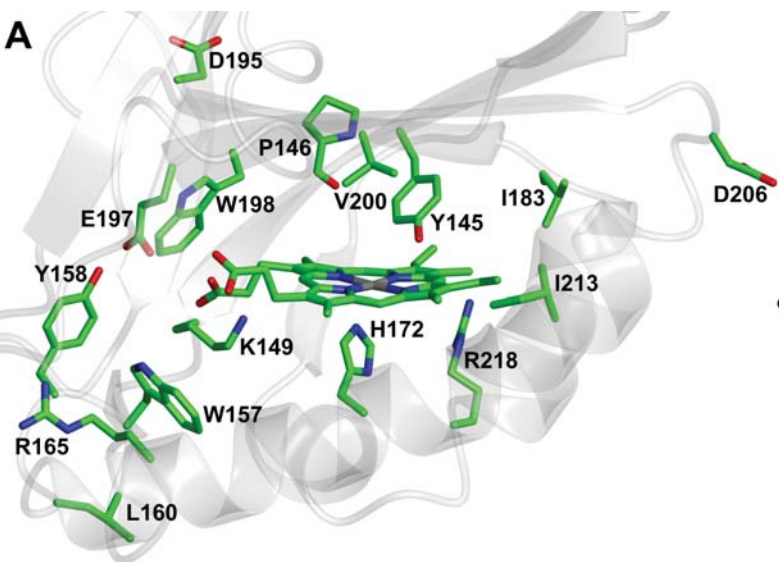
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**The HemQ coprohaem decarboxylase generates reactive oxygen species: implications for the evolution of classical haem biosynthesis**

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**Fig. S1. HemQ stimulates the nonenzymatic oxidation of protoporphyrinogen IX with and without exogenous hydrogen peroxide.** The nonenzymatic oxidation of porphyrinogens was assayed in the presence (1  $\mu$ M) and absence of HemQ at various [hydrogen peroxide] using 9.5  $\mu$ M protoporphyrinogen IX (A) and 5.8  $\mu$ M coproporphyrinogen III (B) as substrates. Control assays (Ctrl) contained no hydrogen peroxide. Error bars represent SD values. Abbreviations: P'gen IX, protoporphyrinogen IX; C'gen III, coproporphyrinogen III.

