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HemQ: an iron-coproporphyrin oxidative decarboxylase for protoheme synthesis in Firmicutes and Actinobacteria

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Abstract

Genes for chlorite dismutase-like proteins are found widely among hemesynthesizing bacteria and some Archaea. It is now known that among the Firmicutes and Actinobacteria these proteins do not possess chlorite dismutase activity but instead are essential for heme synthesis. These proteins, named HemQ, are ironcoporphyrin (coproheme) decarboxylases that catalyze the oxidative decarboxylation of coproheme III into protoheme IX. As purified, HemQs do not contain bound heme, but readily bind exogenously supplied heme with low micromolar affinity. The heme-bound form of HemQ has low peroxidase activity and in the presence of peroxide the bound heme may be destroyed. Thus, it is possible that HemQ may serve a dual role as a decarboxylase in heme biosynthesis and a regulatory protein in heme homeostasis.

Keywords

HemQ; heme synthesis; coproheme decarboxylase; chlorite dismutase-like protein

Introduction

The story of how COG3253, originally annotated as a “chlorite dismutase-like” protein, changed current understanding of bacterial heme biosynthesis as well as models of the pathway’s evolution, is one of gene misannotation and the negative power of established dogma. Heme as an essential cofactor is a near ubiquitous biological compound in aerobic organisms, as well as being found in many anaerobes (1–3). The essential elements of the biosynthetic pathway have been known for over half a century and constitute what has been generally named the “classic” pathway (Fig. 1). The basic tenets of the classic pathway are that eight molecules of an initial committed compound, 5-aminolevulinate (ALA), are converted via six enzymatic steps to the macrocycle protoporphyrin IX, which serves as a

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precursor to both heme and chlorophyll. While two steps have been shown to be catalyzed by distinct oxygen dependent vs oxygen independent enzymes in bacteria, all chemical intermediates in this pathway are conserved. It had been accepted that all heme-containing cells possessed the ability to synthesize their own heme and that the classical pathway was essentially universal. Both of these beliefs were dispelled with the availability of organismal genome sequences. The release of the *Caenorhabditis elegans* genome followed by the elucidation that nematodes cannot synthesize heme, but must acquire it from their diet, clearly demonstrated that not all heme-containing cells synthesize their own heme (1, 4). Additionally the universality of the classic pathway came into question when heme-containing archaea and sulfate-reducing bacteria were found to lack “essential” pathway enzymes, and that many heme-synthesizing bacteria lacked one or more identifiable pathway enzymes (2, 5, 6).

Following the availability of large numbers of microbial genomes it became possible to carry out a comprehensive evaluation of heme synthesis pathways in bacteria. A 2002 review by Panek and O’Brian examined 69 bacterial genomes and found that a significant number of heme-synthesizing bacteria lacked one or more of the then identified heme synthesizing enzymes (7). If that same analysis is extended to the current database of approximately 25,000 bacterial genomes, one finds that a majority of these bacteria lack genes for hemF, hemG, and/or hemY, which had been considered to be essential for heme synthesis. Over the past decade one of our research interests has been to identify and characterize the missing enzymes of heme biosynthesis in bacteria. Towards this end we were able to help fill the gaps for the Proteobacteria (gram negative or diderm bacteria) with the identification of HemY (8) and HemJ (9) as protoporphyrinogen oxidase and biochemical determination that HemG, found mainly in the γ -proteobacteria, is in fact a protoporphyrinogen oxidase (10). Analysis of bacterial genome sequences made it clear that Firmicutes and Actinobacteria (also called gram positive bacteria or monoderm bacteria) lacked identifiable genes for coproporphyrinogen oxidase, the antepenultimate step in heme biosynthesis. While numerous “*hemN*” genes encoding for a putative “oxygen-independent coproporphyrinogen oxidase” are annotated among both diderm and monoderm bacteria, the proteins encoded by many of these genes do not possess the necessary characteristics required for HemN activity (11). In particular, none of the *hemN* genes of gram positive bacteria encode a bona fide coproporphyrinogen oxidase. Since this is a significant void in our knowledge of heme synthesis by gram positive bacteria, we set out to find a valid coproporphyrinogen oxidase in these bacteria.

Bacterial heme biosynthesis

To put the current discussion of the role HemQ plays in heme synthesis in Firmicutes and Actinobacteria into perspective it is necessary to have a basic understanding of bacterial heme synthesis. The overall heme biosynthetic pathway has been reviewed on numerous occasions (1, 2) and is outlined in Figure 1. It is generally accepted that 5-aminolevulinate (ALA) formation is the rate-limiting step and that ALA is the first committed intermediate in the overall tetrapyrrole biosynthetic pathway. This proposal, however, is based largely upon studies of animal ALA synthesis and limited studies in microbial systems. Observations in yeast (12) and some bacteria raise questions as to the universality of the

model, but part of the confusion quite likely arises from the diverse nature of tetrapyrrole end products found in microorganisms (3). It is clear that additional control points exist in some bacteria that produce multiple tetrapyrrole products as part of their normal metabolism (3, 13). Indeed, the overall molecular mechanisms for pathway regulation are not conserved even among the few bacteria examined to date and additional factors regulating bacterial ALA synthesis that were initially proposed over thirty years ago have yet to be resolved at a molecular level (13, 14).

5-Aminolevulinate, the first porphyrin precursor

The formation of ALA is catalyzed by either the “4C” or “5C” path. In the 4C (or Shemin) path, ALA is synthesized from succinyl-CoA and glycine by the pyridoxal phosphate-dependent enzyme ALA synthase (ALAS) (1, 2). Within the bacteria, only α -proteobacteria synthesize ALA via the C-4 pathway and possess ALAS (2, 15). In all bacteria other than α -proteobacteria, as well as in *Archaea* and plants, ALA is synthesized via the 5C path from glutamyl tRNA by glu-tRNA reductase (GltR) and glutamate-1-semialdehyde-2,1-aminomutase (GSAM) (2, 15–17). This pathway is found in over 80% of heme-producing microorganisms currently represented in genomic databases.

The bacterial form of ALAS is highly similar to eukaryotic animal ALAS (49% sequence identity between ALAS of human and *Rhodobacter capsulatus*) (18). Given the high degree of sequence similarity it is perhaps not surprising that the general approach has been to assume similar catalytic and possible regulatory features. However, this may not be totally accurate and clearly the bacterial protein is not subject to the same set of post-translational regulation that is found in higher eukaryotes. Bacterial ALAS is inhibited in vitro by heme at micromolar concentrations (19), but to date there has been no evidence for such inhibition of the animal ALAS. Studies by Neuberger’s group in the 1970s (14) clearly show that ALAS activity in *Rhodobacter sphaeroides* is modulated by an unidentified cellular compound, putatively identified as a “trisulfide.” Under oxidizing conditions, ALAS activity rose in cell extracts and purified “low activity” ALAS was stimulated six- to eight-fold upon addition of the putative activator. However, this redox sensitive regulator has not been identified. ALA synthase as a protein is part of a very large family of pyridoxal phosphate-containing class I and II aminotransferases (20). Given the broad distribution of this family (PFAM 00155.9) and generic catalytic mechanism it is not surprising that during evolution an early ancestor of the α -proteobacteria appropriated and, in time, selected one of these enzymes to carry out the ALA synthesis reaction as is generally proposed by the “patchwork” (or recruitment) model for enzyme evolution (21). Interestingly, Schulze et al. (22) have proposed that ALAS evolved from GSAM, the last enzyme in the C-5 pathway to ALA. In this regard, it would seem clear that the C-5 pathway is the more ancient one and it was the chance acquisition by early animal cells of an α -proteobacterial symbiont as a mitochondrial precursor that resulted in animals possessing ALAS.

ALA synthesized via the C-5 route is regulated by modulation of the reductase, GltR (17, 23). In the majority of γ -proteobacteria as well as some species of α - and β -proteobacteria, *gltR* is not co-localized with *gsam* or the genes encoding the 3-step biosynthesis of uroporphyrinogen III from ALA (below). Instead, it is found in conserved clusters

containing several translation factors, as well as genes involved in isoprenoid and polyisoprenoid biosynthesis. It is tempting to speculate that this pattern is important for coordinating biosynthesis of essential respiratory cofactors (heme and mena- or ubiquinone) with the rate of translation. On the other hand, in the majority of Firmicutes capable of heme biosynthesis, *gltR* and *gsam* are present in a 5-gene cluster with the *pbgd-hmbs-uros* operon. Additionally most Firmicutes possess a second copy of *gsam* that is located outside of the *gltR-pbgd-hmbs-uros* operon cluster. The two copies are highly homologous (~45% identity) and both, from their expression pattern in *Staphylococcus aureus* as determined by global expression profiling over a number of various growth conditions, appear to be synchronized with expression of other heme biosynthetic genes.

Organisms possessing the C-5 pathway generally do not have the C-4 machinery and vice versa – with few exceptions. *Pelagibacter ubique* is an example of a relatively small, but significant number of organisms which have functional heme synthesis yet lack any known genes for either the C-4 or C-5 pathway. This extremely successful group of bacterioplankton accounts for ~25% of the surface pelagic microbial community, yet their genomes are among the smallest known for a free-living microorganism (~1.3 Mb) (24) and appear to be under powerful selection to minimize, known as streamlining (25). This evolutionary trajectory resulted in nearly complete absence of all nonfunctional or redundant DNA, the smallest intergenic spacers known to date, and the elimination of all but the most fundamental metabolic and regulatory functions (26). The source of ALA for *P. ubique* is not clear. While it is possible that a yet undiscovered route of *de novo* ALA synthesis might be present in these organisms, it seems likely that *P. ubique* relies on a high-affinity transporter system (27) for ALA scavenging in its low-nutrient environment.

From ALA to uro'gen III, the common pathway

The next three enzymatic steps responsible for converting eight ALA molecules into one molecule of uroporphyrinogen III represent the most conserved part of the pathway. The three enzymes are almost universally present in tetrapyrrole-producing microbes, and can thus be thought of as the evolutionary core of tetrapyrrole synthesis. The first of these, named ALA dehydratase or porphobilinogen synthase (PBGS or HemB) catalyzes the condensation of two molecules of ALA to form porphobilinogen (PBG) (1, 2, 28, 29). With the exception of a few species predicted to be auxotrophic for porphobilinogen, PBGS is found in all organisms that synthesize tetrapyrroles. The structure of PBGS is usually a homo-octamer composed of four dimers and contains one divalent cation per subunit that is essential for activity (28, 29). Among animals and yeast the cation is zinc, while in plants the metal is magnesium. In most bacteria the metal is magnesium but in some both zinc and magnesium are found. Regardless of the metal specificity, the structures of the zinc and magnesium PBGS enzymes are structurally conserved.

The next enzyme named PBG deaminase or hydroxymethylbilane synthase (HMBS or HemC) catalyzes the head to tail condensation and concomitant deamination of four molecules of PBG to form the linear tetrapyrrole hydroxymethylbilane (1, 2, 29, 30). The enzyme is a homotetramer with the interesting property that it synthesizes its own cofactor during its initial catalytic cycle. It is clear from available genomic data that the protein

sequence is highly conserved. This enzyme is found in all tetrapyrrole-synthesizing organisms and its structure was the first determined for any tetrapyrrole pathway enzyme (30).

The third enzyme of the group, uroporphyrinogen III synthase (UROS or HemD) catalyzes the spiro inversion of the fourth, or D, pyrrole ring as it cyclizes the linear tetrapyrrole into the pathway's first macrocycle, uroporphyrinogen III (1, 2, 29, 31). In the absence of UROS, the product of the previous step, hydroxymethylbilane, will nonenzymatically cyclize under physiological conditions without ring flipping and yield the I isomer of uroporphyrinogen. Uroporphyrinogen I will not serve as a precursor for corrin, siroheme, chlorophyll, or protoheme IX synthesis. The chemical cyclization in humans deficient in UROS activity is the basis for the inherited disease congenital erythropoietic protoporphyria. Thus, the presence of UROS activity is essential for productive tetrapyrrole synthesis and would be expected to be in all tetrapyrrole-synthesizing organisms. However, examination of available prokaryotic genomes reveals that approximately one in twenty do not possess an identifiable *uros* gene.

From uroporphyrinogen III to protoheme IX

The synthesis of uroporphyrinogen III is the last common step for all tetrapyrrole biosynthesis. The first committed step of the classic pathway to protoheme IX synthesis is the decarboxylation of uroporphyrinogen III to coproporphyrinogen III, catalyzed by the enzyme uroporphyrinogen III decarboxylase (UROD or HemE) (1, 2). This enzyme decarboxylates the four acetate side chains of uroporphyrinogen III to methyl groups, releasing four molecules of CO₂ per molecule of uroporphyrinogen III (32). This is in contrast to the siroheme and corrin branch where the first step is the methylation of uroporphyrinogen by the *cobA* or *cysG* gene product (33, 34). Among bacteria *hemE* is present in those organisms that are known to synthesize protoporphyrin IX, often in an operon or cluster with the genes encoding other enzymes of a 3-step protoheme IX biosynthetic module. The crystal structure of HemE has been obtained and shows that the protein is a homodimer with an active site in each monomer (32, 35). The enzyme structure is highly conserved between human and *Bacillus subtilis*, although the bacterial enzyme possesses two additional small loops. Of interest is that this decarboxylase possesses no cofactors, but carries out its catalytic cycle with just amino acid side chains (36).

It is at the next step that accepted dogma and reality part ways and where HemQ makes an impact on heme synthesis. It was long assumed that for protoheme synthesis the next step was the conversion of the tetracarboxylate coproporphyrinogen III to the dicarboxylate protoporphyrinogen IX catalyzed by coproporphyrinogen oxidase. We now know that this pathway, part of the so-called classic heme synthesis pathway, is present in all eukaryotes and many Proteobacteria, but not in Firmicutes, Actinobacteria or Archaea. This step requires the oxidative decarboxylation of two propionate side chains (positions 2 and 4 on the A and B rings, see Fig. 1 for structure labels) to form vinyl groups. There is an oxygen dependent form of coproporphyrinogen oxidase (HemF) in bacteria that is highly similar to the eukaryotic enzyme (1, 2, 37). In bacteria the oxygen-dependent HemF is found in only a limited number of subclasses of proteobacteria, in the Bacteroidetes/Chlorobi group,

Chloroflexi (green nonsulfur bacteria), and the cyanobacteria. It is not found in any Firmicutes or Actinobacteria.

Early studies showed that crude cell-free extracts from some facultative organisms required an odd mixture of cofactors such as ATP and methionine, or S-adenosylmethionine, and NADP to carry out an oxygen independent coproporphyrinogen oxidase reaction (38). Since then the enzyme responsible for this reaction has been identified and named HemN (or CpdH) (2, 39). CpdH is a member of a large class of S-adenosylmethionine (SAM)-dependent “radical SAM” enzymes. The radical SAM class of enzymes is a large and diverse one, and, as with ALAS, recruitment of CpdH from a large protein family represents another example of a patchwork evolutionary enzyme. The crystal structure of the enzyme from *E. coli* has been determined and a catalytic mechanism proposed based upon kinetic and structural data (40). The protein is monomeric, contains both a [4Fe-4S] cluster and SAM and does not utilize molecular oxygen. Unfortunately paralogous radical SAM family proteins, unrelated to heme biosynthesis, have been erroneously annotated as “probable oxygen-independent coproporphyrinogen III oxidases” in genome databases without supporting biochemical or biological data. This nuisance has been noted by researchers in the field (41) and recently clarified with the identification of four distinct protein families within the previous HemN grouping with only one group having CpdH activity (11). Genomic and biochemical studies have demonstrated that CpdH is found in Proteobacteria, cyanobacteria, the Bacteroidetes/Chlorobi group, the Chlamydiae/Verrucomicrobia group, and several other phyla, but does not occur in any sequenced genomes of Firmicutes or Actinobacteria. Thus, Firmicutes and Actinobacteria lack both HemF and HemN and have no apparent means to convert coproporphyrinogen III to protoporphyrinogen IX.

The penultimate step in the classical heme biosynthesis pathway is the oxidation of protoporphyrinogen IX to protoporphyrin IX (1, 2). This is a six electron oxidation and, as with the previous step, there are oxygen-dependent and oxygen-independent forms of the enzyme. The oxidation of protoporphyrinogen to protoporphyrin is now known to be catalyzed by three different enzymes. In the γ and a few β and α proteobacteria HemG is responsible for this step. HemG is a small long chain flavodoxin-like protein that utilizes the cell's respiratory chain as electron acceptor (9, 42). Thus, while a terminal electron acceptor is necessary, this need not be O₂, but can be nitrate, fumarate, etc depending upon the metabolic capacity of the particular cell. Among non- γ proteobacteria, HemJ is the most commonly found bacterial protoporphyrinogen oxidase (ppox) (10, 43). This protein is poorly characterized at present and the nature of the electron acceptor is under debate. HemY also has been identified in multiple bacterial taxa, both monoderm and diderm (8, 42, 44–46). Notably it is hugely underrepresented in Proteobacteria, but appears to be the major ppox type in Firmicutes and Actinobacteria and in the Deinococcus-Thermus group (transitional from monoderm to diderm bacteria). It is a member of the monoamine oxidase superfamily, contains FAD and can utilize molecular oxygen as an electron acceptor (47). All heme-synthesizing eukaryotes possess a HemY-type of ppox.

The terminal step in classical heme biosynthesis is the insertion of ferrous iron into protoporphyrin IX to make protoheme IX (1, 2, 48). This is catalyzed by the enzyme protoporphyrin ferrochelatase. This enzyme is a member of what is named a “class I”

chelatase since it does not require ATP for its action and has been well characterized from both bacteria and eukaryotic sources.

While the classical heme synthesis pathway appears to be present in eukaryotes and most Proteobacteria, in the Archae and denitrifying and sulfate-reducing bacteria a “primitive” or “alternative” pathway exists. This was first proposed in 1998 for *Desulfovibrio vulgaris* (6) and recently characterized for sulfate-reducing and denitrifying bacteria and Archaea (5, 49). These organisms synthesize siroheme, which has uroporphyrinogen III as a precursor, convert siroheme to protoheme via a series of demethylations to form coproheme, and then decarboxylate this to make protoheme (Fig. 2). These enzymes have been named AhbA/B/C/D. All are oxygen independent and some are iron-sulfur-containing radical SAM enzymes.

Identification of HemQ as a protein essential for heme synthesis in Firmicutes and Actinobacteria

With the demonstration that Firmicutes and Actinobacteria lack an identifiable HemF or HemN (11), we sought to find the missing coproporphyrinogen oxidase. In seeking a new enzyme we initially employed complementation of various *Escherichia coli* and *Acinetobacter ADPI* heme auxotrophs since this approach had worked to identify HemJ and HemY (9, 10). However, it did not yield fruit in the search for a Firmicute or Actinobacterial coproporphyrinogen oxidase. We then tried looking for potential heme synthesis operons as well as examining available gene co-expression study data. Identifiable heme synthesis operons in Firmicutes, such as *B. subtilis*, are small, containing few contiguous *hem* genes in any organism. However, among the Actinobacteria, a group of high G+C content monoderm bacteria, we found that *hem* genes were routinely clustered into two uber operons (50). Bioinformatic approaches revealed a gene within these operons that had been annotated as a putative chlorite dismutase as the best candidate for coproporphyrinogen oxidase. This open reading frame encoded a protein that, while related to chlorite dismutases, had distinctive sequence differences and lacked a conserved arginine residue that is essential for enzyme activity. We named the gene *hemQ* (50). This gene is present in all heme-synthesizing Firmicutes and Actinobacteria and not in Proteobacteria nor, more importantly, in any non-heme synthesizing bacteria. However, we and others failed to detect any coproporphyrinogen oxidase activity for the purified, recombinant proteins although other experimental approaches made it clear that HemQ was somehow essential for heme biosynthesis in these organisms (50, 51).

It was noted, although unpublished for many years, that when the genes for the terminal enzymes of heme biosynthesis, HemY and HemH, from *B. subtilis* were expressed in *E. coli* strains that lacked HemG or HemH, the heme auxotrophs were not complemented (50). This was somewhat of a surprise since it was clear that the proteins were being expressed and had activity when assayed in vitro. Furthermore, *hemY* or *hemH* from any tested Proteobacterium, or even the corresponding cDNAs from eukaryotic organisms, complemented the *E. coli* mutants. Since the only discernable difference at that time between HemY and HemH from these other organisms was that they were membrane associated while the HemY and HemH from Firmicutes and Actinobacteria were soluble (8,

46, 48), the explanation offered for the lack of complementation was that the soluble enzymes could not interact properly with the membrane associated enzymes of *E. coli* and/or that the monoderm bacterial enzymes functioned *in vivo* only within a multi-enzyme complex (50). So, it was not entirely a surprise when HemQ did not complement an *Acinetobacter ADP1 hemF* knockout. What was unexpected was that coexpression of *hemQ* + *hemY* + *hemH* in an *E. coli hemH* or *hemG* knockout complemented the heme auxotrophy (50). While various explanations ranging from multi-enzyme complexes to the possibility that HemQ was a regulatory protein were offered, no data were obtained to support these suggestions (50, 51).

The answer to the conundrum came after a reexamination of heme synthesis in Firmicutes and Actinobacteria. While it was always assumed that monoderm bacteria utilized the same classical pathway as is found in diderm bacteria, there had never been any study that demonstrated this and protoporphyrin had never been identified in any monoderm bacterium (52, 53). Indeed, the short answer to the long unsolved mystery is that in monoderm bacteria HemY, initially identified as a protoporphyrinogen oxidase, actually functions to oxidize coproporphyrinogen to coproporphyrin (11). In addition HemH, which in all other organisms had been shown to catalyze the insertion of ferrous iron into protoporphyrin to make protoheme, functions in monoderm bacteria to insert iron into coproporphyrin (11). Additionally these organisms do not possess an enzyme to convert coproporphyrinogen III to protoporphyrinogen IX. The role that HemQ plays is to oxidatively decarboxylate coproheme formed by HemH into protoheme (Fig. 3) (11). This conversion appears to require two consecutive decarboxylations. *In vitro* this occurs with the release of the intermediate mono-vinyl, mono-propionyl deuteroheme IX, but it is not known if this occurs *in vivo*. The order in which the decarboxylations occur has not yet been determined.

An interesting bioinformatics-based discovery that came out of this same study (11) revealed that AhbD, which had previously been identified as an anaerobic radical SAM coproheme decarboxylase employed in the AhbA/B/C/D-based conversion of siroheme into protoheme by sulfate-reducing bacteria (5, 54), is more commonly found without AhbA/B/C. The data strongly suggest that the conversion of coproheme into protoheme in Firmicutes and Actinobacteria may be mediated under aerobic conditions by HemQ and anaerobically by the radical SAM enzyme AhbD. This, then, mirrors the similar decarboxylation of coproporphyrinogen into protoporphyrinogen in the Proteobacteria by HemF aerobically and by the radical SAM enzyme HemN (CpdH) anaerobically (Fig. 1).

Presence of HemQ in Archaea

Analysis of 981 representative microbial genomes reveals that *hemQ* is largely, although not entirely, restricted to Actinobacteria and Firmicutes and there is minimal overlap between organisms that possess *hemN* and/or *hemF* and those that possess *hemQ* and/or *AhbD* (Fig 4) (11). Exceptions to this are bacteria that represent “evolutionary intermediates” or transitional bacteria such as *Deinococcus* and *Thermus*. An apparent gene for *hemQ* is also found in some Archaeal genomes, although only five Archaeal genomes within the 981 representative set have *hemQ*. One organism, *Nitrosopumilus maritimus* SCM1, possesses a gene for HemQ that appears similar to the eubacterial HemQs. Interestingly this organism

possesses *ahbA* and *ahbB* of the alternate heme pathway (AhbA, B, C, D) that exists in heme synthesizing Archaea to convert siroheme to protoheme. However, *ahbC* is lacking so how the essential AhbC reaction is catalyzed remains unknown. The second homolog of *hemQ* genes found in the Archaea possess a *hemQ* gene fused to an additional domain that is annotated as a “putative monooxygenase domain”. Four examples of this in the 981 representative genomes are *Halobacterium* sp NRC-1, *Halomicrobium mukohataei*, *Halorhabdus utahensis*, and *Natronomonas pharaonis*. One published study on the HemQ-monooxygenase fusion is a protein named PitA from *Haloferax volcanii* (55). Unlike the HemH-HemQ fusion of *Propionibacterium acnes*, where HemH is fused to the amino terminus of HemQ, in PitA the fusion has the HemQ homolog at the amino terminus of the monooxygenase. The function that PitA plays is unknown and while the protein has been purified, it has not been characterized. Among the HemQ homologs with putative monooxygenase domains the presence of necessary heme pathway enzymes is variable. For instance *Halobacterium* sp. NRC-1 (and two other genomes) possess only *ahbC*, but not *ahbA* and *ahbB*, as well as *ahbD*. *H. utahensis* stands out since it has *ahbC* and *ahbD* along with genes for homologs of eubacterial HemY, HemG, and HemH. Clearly defining a role for archaeal HemQ homologs as participants in heme biosynthesis will require biochemical characterization of the individual enzymes.

Characteristics of HemQ and some comparisons to chlorite dismutase

While considerable structure/function data now exist for chlorite dismutases, much less is known about HemQs. Examination of primary HemQ sequence alignments show that within the Actinobacteria and Firmicutes there is significant sequence identity, but there is much less homology between the Actinobacterial and Firmicute HemQs (50) (Fig 5). Indeed, if one were to have only sequence and structural information without any enzymatic data, it would be easy to imagine that the two proteins serve different functions. Interestingly the areas of differences within the structures are both internal and surface-located, although the most obvious differences are on the surface surrounding the active site pocket (Fig 6). HemQs of Firmicutes have an additional short loop at the mouth of the putative active site (116–125 *B. subtilis* numbering) that is not found in Actinobacterial HemQ (Fig. 5). The basis for this difference is unknown, but probably has to do in part with potential interacting protein partners. With regard to this there are two items of interest. First is that the preceding pathway enzyme HemH differs significantly with the Actinobacterial proteins possessing a [2Fe-2S] cluster while HemH of the Firmicutes lack this feature and the corresponding carboxyl-terminal end extension that, in part, coordinates this cluster (48, 56). Second is the nature of the electron acceptor. While the reaction proceeds under aerobic conditions, it appears that molecular oxygen is not the terminal electron acceptor. At present available data show that H₂O₂ or FMN, but not NAD, NADP, or FAD, serve as electron acceptors. Yet untested are physiological small proteins such as flavodoxins, ferredoxins, or cytochromes from appropriate bacterial sources or quinones or as yet unidentified redox proteins. Given the considerable physiological diversity among HemQ-containing bacteria, it would not be surprising to see some diversity in the preference/specificity for electron acceptors.

To date there are no Actinobacterial HemQ structures in the PDB, but there exist three structures of HemQs from Firmicutes in the PDB: 1T0T (*Geobacillus stearothermophilus*), 3DTZ (*Thermoplasma acidophilum*), and 1VDH (*Thermus thermophilus* HB8). While all three were initially annotated as chlorite dismutases, it is clear from their sources and sequences that they are HemQ. Structurally all three PDB structures show assumed biological molecules as homopentamers although the 1T0T structure also presents a second biological assembly that is approximately a 250 Å diameter 60-mer nanosphere. There are no experimental data to support a biological role for this crystal structure but it is of interest since preliminary structural studies on an Actinobacterial HemQ also yielded a 60-mer nanosphere crystal structure (unpublished data). Ongoing experiments should clarify if a nanosphere is a biologically significant molecule or if HemQ functions as a pentamer or even monomer. Unfortunately none of the available structures possess bound heme so the position of the substrate coproheme or product protoheme can only be guessed by analogy to known holo-chlorite dismutase structures. Additionally there is no evidence to reveal if catalysis or product release involves any molecular movement as has been shown for human ferrochelatase. There are a collection of highly conserved putative active site residues between HemQ and chlorite dismutases. Utilizing the numbering of *Mycobacterium tuberculosis* HemQ (Fig. 5), these residues include D67, R/K137, W141, W/Y142, R149, H156, and D/E205. The conserved active site arginine of chlorite dismutase that serves as an axial ligand to the heme iron (57–59) is not conserved in HemQs, being replaced by alanine in Actinobacteria and glutamine/serine in Firmicutes. Previous studies identified H156 as an axial ligand to a bound heme iron in *M. tuberculosis* HemQ, leaving the sixth iron coordination site open to water, CN, imidazole, or other weak-binding small ligand (50). Interestingly of the HemQ variants R137L, W141L, H156A, and W181L, only the W141L variant was reported to be nonfunctional in complementation assays (50). This observation is of interest given that a W to V replacement in the corresponding residue of *Candidatus Nitrospira defluvii* chlorite dismutase had a “modest impact on enzyme kinetics” (58).

An excellent kinetic, physical, and crystallographic study of chlorite dismutase by Hofbauer et al. has allowed for a critical examination of how this enzyme works (58). Not unexpectedly, there appears to be minimal structural reorganization during catalysis. However, unlike chlorite dismutase where heme remains protein-bound as a cofactor throughout the catalytic cycle, HemQ utilizes coproheme as a substrate and releases protoheme after decarboxylation. Thus, HemQ functions more like ferrochelatase which must release a metallated heme as a product and in doing so it undergoes considerable structural movement (60–63). In the published HemQ structures there is a flexible loop covering or near to the putative heme-binding pocket of the heme-free protein. Given that a catalytic cycle involves metallated macrocycle binding, decarboxylation and heme release, it will be of interest to see if and how the flexible loop may move, what function that movement serves and what controls the movement.

Two additional items deserve mention about HemQ. First, while there are no published data in support of a stable multiprotein complex, *P. acnes* possesses a fusion of HemH with HemQ which hints that these proteins functionally interact (50). It also has been reported that *M. tuberculosis* HemY activity is stimulated in the presence of HemQ. This is best

explained as a protein-protein interaction since there is an additional catalytic step (HemH) between HemY and HemQ (50). Second, it is well documented that HemQ with protoheme bound will function as a poor peroxidase (50, 51). However, it exhibits a high heme turnover rate when functioning as a peroxidase. Thus, HemQ may function both to form protoheme as well as to catalyze the destruction of the protein-bound heme that is not removed for cellular metabolism from HemQ thereby suggesting that HemQ serves dual functions in heme metabolism.

In conclusion HemQ adds the new catalytic function of an oxidative decarboxylase to the “chlorite dismutase-like” family of enzymes. The HemQ (or coproporphyrin-dependent) pathway is found only in monoderms and the protoporphyrin-dependent pathway is present only in diderms meaning that coproheme and protoporphyrin represent metabolic markers for the presence of monoderms and diderms, respectively, in environmental microbial samples. While relatively little is currently known about HemQ, its essential role in heme synthesis by Actinobacteria and Firmicutes, some of which are troublesome pathogens, should insure a rapid expansion in the study of this protein. Given the uniqueness of HemQ to Firmicutes and Actinobacteria, this enzyme presents an excellent new antibiotic target. Additionally the diverse conserved regions of the Actinobacterial vs Firmicute HemQ hint at potentially significant distinctions between these two HemQs in their in vivo interactions and functioning.

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Highlights

- HemQ is a chlorite dismutase-like protein found in gram positive bacteria.
- HemQ catalyzes the oxidative decarboxylation of coproheme into protoheme.
- HemQ is the terminal enzyme of the coproporphyrin-dependent heme synthesis pathway.
- Given its essential nature HemQ represents a valid antimicrobial target.

Figure 1. Bacterial heme biosynthetic pathways

Abbreviations are: succCoA, succinyl-coenzyme A; Glut-tRNA, glutamyl-tRNA; ALA, 5-aminolevulinic acid; PBG, porphobilinogen; HMB, hydroxymethylbilane; URO'GEN, uroporphyrinogen; COPRO'GEN, coproporphyrinogen; COPRO, coproporphyrinogen III; COPROHEME, iron coproporphyrin III; PROTO'GEN, protoporphyrinogen IX; and PROTO, protoporphyrin IX. In the classic pathway (red box) the conversion of coproporphyrinogen to protoporphyrinogen is catalyzed under aerobic conditions by HemF and anaerobically by HemN. Conversion of protoporphyrinogen to protoporphyrin may be catalyzed aerobically by HemY and anaerobically by respiratory chain linked HemG or HemJ. In the alternate pathway (purple box) uroporphyrinogen is converted to siroheme and that may be converted to protoheme (see figure 2 and text). In the HemQ pathway (orange box) HemY and HemH differ for the similarly annotated enzymes in the classical pathway in that HemY catalyzes the oxidation of coproporphyrinogen to coproporphyrin and HemH inserts iron into coproporphyrin to make coproheme.

Figure 2. Intermediate metabolites in the alternative and classical heme synthesis pathways Tetrapyrrole and heme structures from uroporphyrinogen III (uro'gen) to protoheme IX are shown for the alternative heme biosynthetic pathway (5) on the upper line and those for the classical pathway are shown in the lower line. As described in the text multiple steps are required to convert uroporphyrinogen into siroheme. AhbA,B,C convert siroheme into coproheme and AhbD catalyzes the oxidative decarboxylation of coproheme into protoheme.

Figure 3. Coproheme decarboxylation is catalyzed by HemQ

The oxidative decarboxylation converts the propionyl side chains at positions 2 and 4 on the A and B rings to vinyl groups. Available data suggests that the reaction is step-wise and goes through a mono-vinyl, mono-propionyl deuteroporphyrin IX intermediate (50). Since there are no data available to identify if there is a stereospecific decarboxylation for the first step, the intermediate is not shown.

Figure 4. Venn diagram showing the overlap of HemQ with AhbD and HemN with HemF

Data to construct the diagram are the 981 representative genomes detailed in http://pubseed.theseed.org/SubsysEditor.cgi?page=ShowSubsystem&subsystem=Heme_Biosynthesis%3A_protoporphyrin-_and_coproporphyrin-dependent_pathways, discussed in the text and reference 11. The size of the circles reflect in part the difference in the number of available genomes present in the database (fewer currently available monoderm genomes compared with diderm genomes).

Figure 5. ClustalW alignment of selected Firmicute and Actinobacterial HemQs

Listed are the sequences of HemQ from the Firmicutes *B. subtilis*, *B. anthracis*, *Staphylococcus aureus*, *Listeria monocytogenes*, and *Oceanobacillus iheyensis* and the Actinobacteria *Mycobacterium tuberculosis*, *Nocardia farcinica*, *Corynebacterium diphtheriae*, *Streptomyces coelicolor*, and *Propionibacterium acnes*. Residues that are identical between all sequences are boxed in red. Residues conserved among Firmicute HemQ are boxed in orange and those conserved among Actinobacterial HemQ are boxed in blue.

Figure 6. Surface representation of HemQ (1T0T) showing residues conserved between HemQ of Firmicutes and Actinobacteria

The crystal structure of HemQ of the firmicute *Geobacillus stearothermophilus* (PDF #1T0T) is shown. One subunit is highlighted in green and the remaining four subunits are in light gray. Residues that are identical among all known HemQs are shown in red. Most of these are not visible on the surface diagram since they are within the putative active site. Residues that are conserved among Firmicute HemQs are shown in yellow with dots, and residues conserved in Actinobacterial HemQ are in blue. Residues that are conserved within Firmicutes and within Actinobacteria, but different between the two bacterial groups are shown in black. The red arrow points at the putative active site pocket. The red circle outlines a segment (residues 116–125 in *B. subtilis* numbering) that is present in Firmicute HemQ, but not in Actinobacterial HemQs.