A Journal of the Gesellschaft Deutscher Chemiker

Angewandte International Edition

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Accepted Article

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To be cited as: *Angew. Chem. Int. Ed.* 10.1002/anie.201814708
Angew. Chem. 10.1002/ange.201814708

Link to VoR: http://dx.doi.org/10.1002/anie.201814708
http://dx.doi.org/10.1002/ange.201814708

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10.1002/anie.201814708

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Revisiting the mechanism of anaerobic coproporphyrinogen III oxidases, a radical SAM enzyme involved in heme biosynthesis

Xinjian Ji, Tianlu Mo, Wan-Qiu Liu, Wei Ding, Zixin Deng, and Qi Zhang*

Abstract: HemN is a radical S-adenosyl-L-methionine (SAM) enzyme that catalyzes the oxidative decarboxylation of coproporphyrinogen III to produce protoporphyrinogen IX, an intermediate in heme biosynthesis. HemN binds two SAM molecules in the active site, but how these two SAMs are utilized for the sequential decarboxylation of the two propionate groups of coproporphyrinogen III remains largely elusive. Here we provide evidence supporting that in HemN catalysis, a SAM serves as a hydrogen relay that mediates the radical-based hydrogen transfer from the propionate to the 5′-deoxyadenosyl (dAdo) radical generated from another SAM in the active site. We also observed an unexpected shunt product resulting from trapping of the SAM-based methylene radical by the vinyl moiety of the mono-decarboxylated intermediate, harderoporphyrinogen. These results require a major revision of the HemN mechanism and reveal a new paradigm of the radical-mediated hydrogen transfer in radical SAM enzymology.

Heme is a ubiquitous cofactor in all kingdoms of life and plays essential role in many fundamental biological processes, such as respiration, photosynthesis, and the metabolism and transport of oxygen.[1] The classical heme biosynthetic pathway found in eukaryotes and most bacteria involves oxidative decarboxylation of coproporphyrinogen III (**1**) to produce protoporphyrinogen IX (**3**) (Figure 1).[2] This reaction is catalyzed by coproporphyrinogen III oxidase (CPO), which exists in two evolutionarily and mechanistically distinct families. The oxygen-dependent CPO HemF requires molecular oxygen for activity,[3] whereas the oxygen-independent CPO HemN[4] is an anaerobic enzyme belonging to the radical S-adenosyl-L-methionine (SAM) superfamily.[5]

Radical SAM superfamily is the largest known enzyme family consisting of more than 110,000 members found in all three domains of life.[5-6] These enzymes contain a [4Fe-4S] cluster to bind SAM and reductively cleave its carbon-sulfur bond to produce a highly reactive 5′-deoxyadenosyl (dAdo) radical. This alkyl radical then abstracts a hydrogen from the substrate to produce 5′-deoxyadenosine (dAdoH) and a second radical, thereby leading to highly diverse reactions.[5]

The seminal work by Layer et al. showed that HemN catalyzes the radical SAM-dependent oxidative decarboxylation of **1** in a sequential manner, with the mono-decarboxylated product harderoporphyrinogen (**2**) as an intermediate (Figure 1), although the possibility of an isomer of **2** produced from decarboxylation of the second propionate group of **1** cannot be excluded.[7] Using a series of deuterium-labeled substrates, they identified an allyl-type radical as a result of hydrogen abstraction from the β-position of the propionate side chain of **1**.[7e] HemN binds two SAM molecules, and production of one molecule of **3** consumes two SAMs.[7b, 7c] These observations led to the mechanistic proposal shown in Figure 2. In this mechanism, the dAdo radical produced from SAM cleavage abstracts the pro-*S* hydrogen from the β-carbon of the propionate side chain to produce a β-propionate radical, which undergoes fragmentation to form the vinyl group (Figure 2). Cleavage of the second SAM bound in the active site leads to formation of the second vinyl group in a similar manner to result in **3** formation.

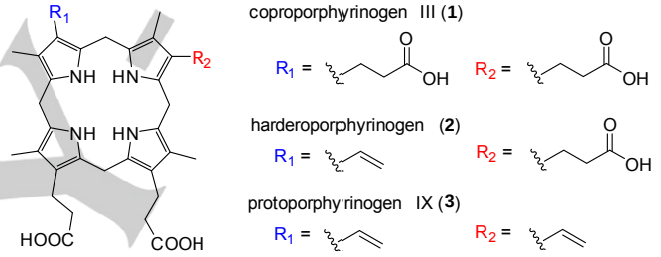

Figure 1. Chemical structures of coprophophyrinogen III (**1**) and its decarboxylated products produced by HemN.

Although the mechanistic proposal shown in Figure 2 has been accepted for a long time,[5, 8] several issues remain unanswered. Because binding of the second SAM (SAM #2) is strictly essential for enzyme activity, as revealed by site-direct mutagenesis,[7c] if both SAMs would be cleaved in the reaction, how could one [4Fe-4S] cluster mediate the cleavage of two SAMs bound at different positions? One hypothesis is that SAM #2 is cleaved by electron transfer from the [4Fe-4S] cluster to SAM #2 via the sulfonium center of the [4Fe-4S]-bound SAM (SAM #1), and later SAM #1 is cleaved by the canonical radical SAM chemistry.[7e] Another hypothesis is that SAM #2 serves as a backup molecule and decarboxylation occurs only at the SAM #1 site. After the first decarboxylation, SAM #2 enters into the SAM #1 site and replaces the methionine and dAdoH products resulting from SAM #1 cleavage.[5b] Although both hypotheses are theoretically feasible, these involve significant movement of SAM and porphyrinogen substrate in the enzyme active site, which is not consistent with the delicate mechanisms observed for many radical SAM enzymes in controlling the high reactivity of radical intermediates during reaction.[5b]

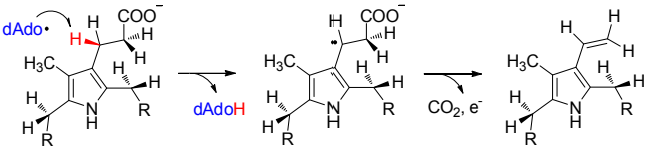

Figure 2. The previously proposed mechanism for the HemN-catalyzed oxidative decarboxylation.

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10.1002/anie.201814708

To investigate the HemN mechanism, we first set out to obtain its substrate **1**. To this end, we knocked out *hemN* in *E. coli* and grew the mutant strain in anaerobic condition, which accumulated a small amount (1～2 mg/L) of coproporphyrin III (**4**), the oxidized form of **1** (Figure 3A). **4** was extracted from the mutant cells and purified by high performance liquid chromatography (HPLC) (Supplementary Methods and Figure S1-S2). Reduction of **4** to **1** was previously achieved by using sodium amalgam,[7a, 9] which, unfortunately, is not commercially available in China, prompting us to test alternative ways for **4** reduction. To our delight, we found that when the reaction was performed with a relatively low concentration of **4** (< 50 μM), quantitative production of **1** was observed by using $NaBH_4$ as a reductant at room temperature (Figure 3A).

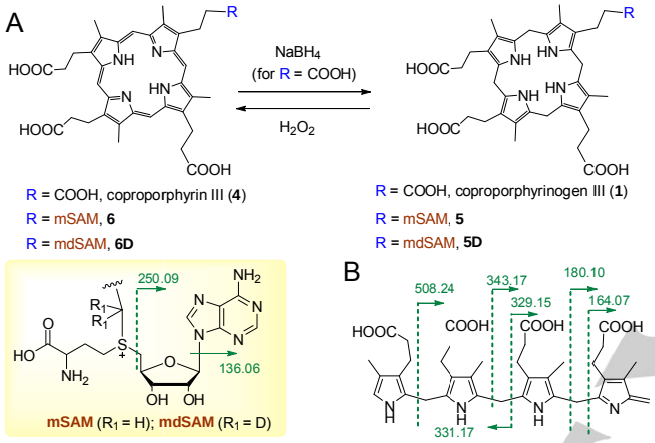

Figure 3. Porphyrin and porphyrinogen derivatives investigated in this study. (A) Interconversion of porphyrin/porphyrinogen derivatives. (B) Common fragment ions observed in the HR-MS/MS spectra of **1**, **5** and **5D**.

We then performed HemN reaction with **1**, SAM, and sodium dithionite, and the reaction mixture was analyzed by liquid chromatography (LC)-high resolution (HR)-mass spectrometry (MS). This analysis showed that both mono-decarboxylated product, harderoporphyrinogen (**2**), and di-decarboxylated product **3** were produced in the reaction mixture (Figure 4A, trace i, and Figure S3). The result is consistent with the early study by Layer and coworkers and confirms that the HemN-catalyzed two-step decarboxylation is distributive.[7f]

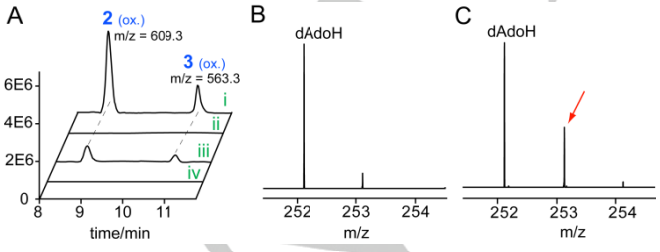

Figure 4. HemN-catalyzed oxidative decarboxylation of **1**. (A) LC-HR-MS analysis of HemN reaction mixtures. The multiple selected ion monitoring (SIM) mode includes $[M + H]^+$ = 609.3 (corresponding to the oxidized form of **2**) and $[M + H]^+$ = 563.3 (corresponding to the oxidized form of **3**) for (i) reaction with SAM, (ii) control reaction with SAM and boiled HemN, (iii) reaction with d_3-SAM, and (iv) control reaction with d_3-SAM and boiled HemN. The MS spectra of **2** and **3** were shown in Figure S3. (B) HR-MS spectra of dAdoH produced from SAM. (C) HR-MS spectra of dAdoH produced from d_3-SAM. The red arrow highlights deuterium incorporation into the resulting dAdoH; approximately 30% of dAdoH is deuterium-labeled. Reactions were performed by incubation of 1.5 μM HemN and 20 μM coproporphyrinogen III with 400 μM SAM or d_3-SAM in the presence of 2 mM sodium dithionate for 6 hrs. The reactions were treated with H_2O_2 (1.5%) before LC-MS analysis.

We next tested the reaction with S-adenosyl-L-[methyl-$^2H_3$]-methionine (d_3-SAM). LC-HR-MS analysis of the reaction mixture showed that both **2** and **3** were produced, but the reaction efficiency is significantly lower than that with non-deuterated SAM (the yields of **2** and **3** were approximately 20% and 15%, respectively) (Figure 4A, trace iii). Importantly, comparative MS analysis revealed apparent deuterium incorporation into the dAdoH produced from the reaction with d_3-SAM (Figure 4B and 4C). These observations strongly argue against the previous mechanism shown in Figure 2 and suggest that dAdo radical produced from SAM #1 abstracts a hydrogen from the methyl group of SAM #2 (or its derivative). Such a dAdo radical-mediated hydrogen abstraction is consistent with the apparent deuterium kinetic isotope effect observed in the reaction (Figure 4A), and is reminiscent of recent studies on the class C radical SAM methyltransferases,[10] which are homologous to HemN.[11]

Intriguingly, careful analysis of HemN reaction revealed a product (**5**) in the reaction with SAM, which exhibited a positive charged molecular ion at m/z = 1013.4531 (calculated m/z = 1013.4550, 2.0 ppm for a molecular formula of $C_{50}H_{65}N_{11}O_{10}S$) (Figure 5A and Figure S4A). HR-MS/MS analysis of **5** showed two fragment ions at m/z = 250.09 and 136.06, which are characteristic of the adenosine-containing compounds (Figure 3A and Figure S5).[12] A series of fragment ions (e.g. m/z = 331.17, 343.17, and 508.24) were also found for **5** (Figure 3B and Figure S5), which are also present in the MS/MS spectrum of **1** (Figure S2). These observations suggest that **5** contains an adenosine moiety and a porphyrinogen ring, and hence are likely a SAM adduct of **2** (Figure 3A). Similarly, a compound (**5D**) exhibiting a molecular ion two mass unit more than **5** (observed m/z = 1015.4642, calculated m/z = 1015.4675, 3.2 ppm for a molecular formula of $C_{50}H_{63}D_2N_{11}O_{10}S$) was observed in the reaction with d_3-SAM (Figure 5A and Figure S4B). **5D** gave a set fragment ions similar to those of **5** (Figure 3B and Figure S6), suggesting **5D** is a deuterium-labeled SAM adduct (Figure 3B). It is noteworthy that in a recent report, a SAM adduct was also observed in the reaction of C10P, a HemN homologous enzyme required for forming the cyclopropane ring in CC-1065.[10k]

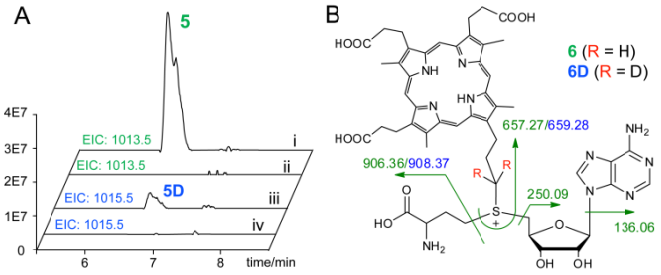

Figure 5. Characterization of the SAM adduct in HemN reaction. (A) LC-HR-MS analysis of HemN reaction mixture, showing the extracted ion chromatograms (EICs) of $[M + H]^+$ = 1013.5 (corresponding to **5**) for (i) reaction with SAM, and (ii) control reaction with SAM and boiled HemN, and the EICs of $[M + H]^+$ = 1015.5 (corresponding to **5D**) for (iii) reaction with d_3-

SAM, and (iv) control reaction with d_3-SAM and boiled HemN. The MS spectra of **5** and **5D** were shown in Figure S4; the HR-MS/MS spectra of **5** and **5D** were shown in Figure S5 and Figure S6. (B) Structure of **6** and **6D** (produced by H_2O_2 oxidation of **5** and **5D**) and the characteristic fragment ions observed in HR-MS/MS analysis (see Figure S7 and Figure S8 for the detailed spectra).

Because the porphyrinogen ring is easily fragmented during MS/MS analysis and hence complicates spectrum interpretation, we treated the reaction mixture with H_2O_2 to convert porphyrinogen to the oxidized form (i.e. porphyrin) (Figure 3A), which is more resistant to fragmentation. HR-MS/MS analysis of **6** and **6D** (the oxidized form of **5** and **5D**) gave a series of fragment ions that clearly indicate that **6** and **6D** are SAM adducts of **4** (Figure 5B), supporting the proposed structure of **5** and **5D** (Figure 3A).

Observation of the SAM adduct **5** provides valuable mechanistic insights into HemN catalysis and suggests the previously proposed mechanism should be significantly revised. In contrast to the direct hydrogen abstraction from the propionate side chain, the dAdo radical generated from SAM #1 cleavage abstracts a hydrogen from the methyl group of SAM #2 to produce a methylene radical **7** (Figure 6A). **7** then abstracts the pro-S hydrogen from the propionate β-carbon to result in decarboxylation. Because of multiple reaction turnovers in the absence of specific electron acceptors, we propose that upon decarboxylation the electron could go back to the [4Fe-4S] cluster to reduce it to the active +1 state, similar to that proposed for DesII catalysis.[13]

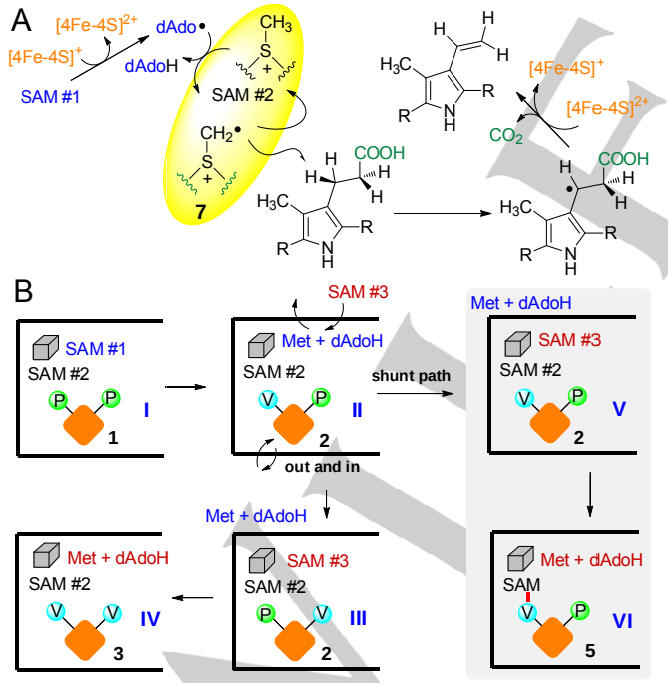

Figure 6. The revised mechanism of HemN catalysis. (A) HemN reaction utilizes SAM #2 (highlighted in a yellow eclipse) as a hydrogen rely for the radical-mediated hydrogen transfer for oxidative decarboxylation of **1**. (B) A working hypothesis for the HemN catalysis. [4Fe-4S] cluster is represented as a cube, and the porphyrinogen ring is represented as an orange diamond. P and V represent propionate and vinyl moieties, respectively.

Apparently, **5** is a shunt product resulting from trapping of the radical intermediate **7** by the vinyl group of **2**. Production of this shunt product suggests that decarboxylation of both propionate side chains occurs at the same site close to SAM #2, and after the first decarboxylation, major conformational change of **2** is required for the second decarboxylation. A working hypothesis for HemN catalysis is shown in Figure 6B. The dAdo radical generated from the cleavage of SAM #1 leads to a cascade of hydrogen transfer to result in the formation of first vinyl side chain on **2** (Figure 6B, II). **2** is likely released from the enzyme and re-enters into the active site with a different, likely a flipped, conformation, in which the second propionate group is placed close to SAM #2 (Figure 6B, III). Such an out-and-in process of **2** is also consistent with the distributive action of HemN observed previously[7f] and in this study. A new SAM (SAM #3) enters into the active site and replaces Met and dAdoH by binding to the [4Fe-4S] cluster, leading to the second decarboxylation to produce **3** (Figure 6B, IV). As the out-and-in process of **2** could be slow, cleavage of SAM #3 can happen without a conformational change of **2** (Figure 6B, V), and in this case, the SAM-based radical **7** can be trapped by the vinyl side chain of **2** to generate the SAM adduct **5**.

Radical SAM enzymes have evolved delicate systems to control the generation and reactivity of radical species to achieve specific catalytic outcomes.[5] However, because of the extremely high reactivity of many radical species, the undesired side reactions could still occur in some cases, as has been extensively studied in reactions catalyzed by the Trp lyase NosL.[14] Characterization of HemN mechanism and finding of the unexpected SAM adduct further demonstrates the intriguing mechanism and remarkable catalytic promiscuity of the radical SAM superfamily enzymes, highlighting the great potential to manipulate these enzymes for novel activities.

Acknowledgements

This work is supported in part by grants from the National Key Research and Development Program (2016 Y F A0501302), and from National Natural Science Foundation of China (31600398 to W.D., and 31670060 to Q.Z.), and from the Open Fund of Key Laboratory of Glycoconjugate Research, Ministry of Public Health.

Keywords: Heme • S-adenosylmethionine • biosynthesis • decarboxylation • radical addition

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10.1002/anie.201814708

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