Supporting Information

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SI Text

Novel pathways make heme and heme d_1 from siroheme.

Discrepancy in Observed Masses for Siroheme, Mono-, and Didecarboxysiroheme. The masses that we observe for siroheme, mono-, and didecarboxysiroheme following HPLC-MS analysis do not directly agree with the theoretically calculated values. For authentic siroheme we observe a value of m/z 912 (M+) for the molecular ion, which is four mass units lower than predicted mass of 916. Following incubation of siroheme with either NirD-L or NirDL-G-H we observe values of m/z 868 (–44) and 824 (–88), respectively, which are consistent with two consecutive decarboxylations (Fig. S4). After removal of the metal ion and esterification to the methyl ester derivatives of the compounds, we obtain the expected values of m/z 975 $[M + H]^+$, 917 $[M + H]^+$, and 859 $[M + H]^+$ for siroheme, mono-, and didecarboxysiroheme, respectively (Fig. S4). Furthermore, after conversion of the didecarboxysiroheme intermediate into Fe-coproporphyrin III the incongruity is no longer observed and we record the expected value of m/z 708 (M+). Although we cannot be certain of the reason for the observed mass discrepancy for these three intermediates, it appears to be due to the properties of the metal-coordinated isobacteriochlorin ring system. It is also worth noting that it is not uncommon to obtain lower than expected mass values for other porphyrins and chlorines, which can be present as $[M - 2]^+$ and is presumably due to the loss of two protons, consistent with the readily oxidizable porphyrin structure (1, 2). We have also observed similar results for other cobalt-isobacteriochlorin complexes. Isobacteriochlorins and their metal complexes are known to be easily oxidizable and will readily form π radical cations (3).

Building Multigene Constructs by Link and Lock. Briefly, the first gene to be cloned is amplified with primers containing an *EcoR*I at the 5′ and *Spe*I and *BamH*I sites at the 3′ end. The remaining genes are amplified with primers containing an *Xba*I at the 5′ end and *Spe*I and *BamH*I sites on the 3′ end. After cloning the first gene into the plasmid via the *EcoR*I and *BamH*I sites, the remaining genes, cut with *Xba*I and *BamH*I restriction enzymes, were cloned consecutively into the plasmid after it had been restricted with *Spe*I and *BamH*I. The *Spe*I- and *Xba*I-restricted fragments form compatible cohesive ends, which after ligation do not reform a restriction site. Thus, by fusing the *Spe*I and *Xba*I sites, these sites can be reused in subsequent cloning steps.

Purification of Multienzyme Cocktail for Sirohydrochlorin Production. In brief, lysate of *Escherichia coli* strain overexpressing the genes 5-aminolevulinic acid dehydratase (*Methanothermobacter thermoautotrophicus*, *hemB*), porphobilinogen deaminase (*Bacillus megaterium*, *hemC*) uroporphyrinogen III synthase (*B. megaterium*, *hemD*), uroporphyrinogen III methyltransferase (*Pseudomonas denitrificans*, *cobA*) and precorrin-2 dehydrogenase (*B. megaterium*, *sirC*) was applied to the Ni-Sepharose column equilibrated with the buffer A. The column was washed in a stepwise manner, first with five column volumes of 50 mM imidazole containing buffer A and then with five column volumes of the 100 mM imidazole containing buffer A. After elution of the proteins from the Ni-Sepharose column using 400 mM imidazole, protein-containing fractions were pooled, transferred to an anaerobic chamber, and buffer exchanged against deoxygenated buffer A using a PD10 column.

NMR Structure Determination. All NMR data were obtained at 298 K using a 14.1 T (600 MHz ^{1}H) Bruker Avance III NMR spectrometer equipped with a TCI cryoprobe. A glove box was used to prepare 0.5 mM samples in 100% deuterium oxide that were subsequently sealed in a septum screw-capped 5 mm NMR tube under an inert atmosphere. NMR assignments were obtained using ^{1}H homonuclear DQF-COSY, NOESY (500 ms), rotating-frame Overhauser effect spectroscopy [ROESY (200 ms)], and total correlation spectroscopy [TOCSY (80 ms)] and ^{13}C, ^{1}H heteronuclear sequential quantum correlation (HSQC), computed tomography-HSQC (27 ms), heteronuclear multiple bond correlation, and HSQC-TOCSY (80 ms) experiments. Mixing/constant time periods are shown in parentheses. NMR data processing was achieved using TopSpin 3.0 and NMR data analysis using the didecarboxysiroheme analysis suite, version 2.0.

1. Chang CK (1985) On the structure of heme d_1—an isobacteriochlorin derivative as the prosthetic group of dissimilatory nitrite reductase. *J Biol Chem* 260:9520–9522.
2. Murphy MJ, Siegel LM, Kamin H (1973) Reduced nicotinamide adenine-dinucleotide phosphate-sulfite reductase of enterobacteria. 2. Identification of a new class of heme prosthetic group—iron-tetrahydroporphyrin (isobacteriochlorine type) with 8 carboxylic-acid groups. *J Biol Chem* 248:2801–2814.
3. Hanson LK, Fajer J, Chang CK (1981) Radicals of Fe(II) isobacteriochlorins—models of siroheme and of nitrite and sulfite reductases. *Biophys J* 33:A78–A78.

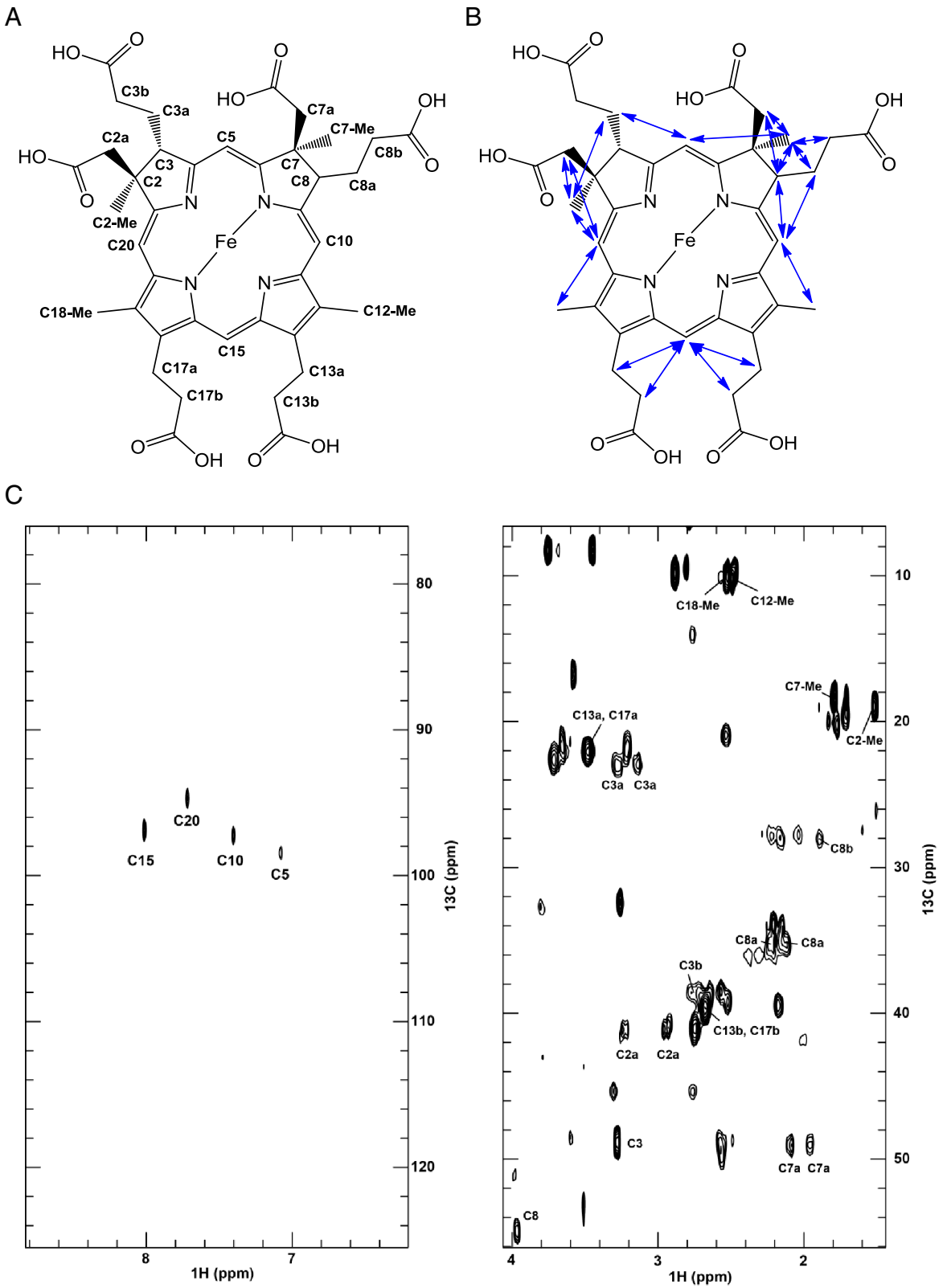

Fig. S1. NMR analysis of 12,18-didecarboxysiroheme. (*A*) The structure of 12,18-didecarboxysiroheme labeled with the atom definitions used for the assignment of the NMR chemical shifts (see Table S2). (*B*) The structure of 12,18-didecarboxysiroheme with the NOE contacts observed in the ROESY spectrum indicated with blue arrows. (*C*) Two regions of the 14.1 T ^{13}C, ^{1}H HSQC of didecarboxysiroheme with major resonances labeled.

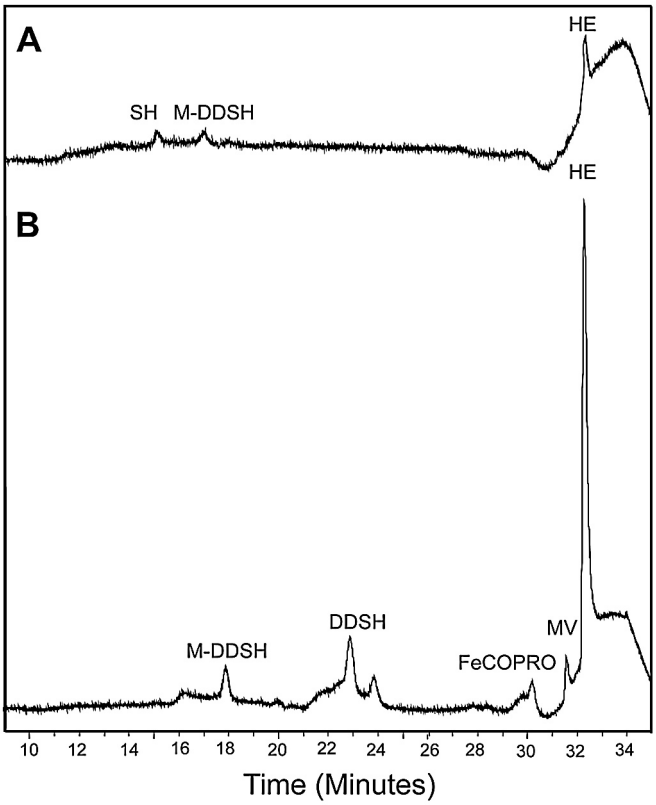

Fig. S2. Conversion of siroheme into heme by cell-free extracts of *Desulfovibrio vulgaris* Hildenborough. HPLC traces of the tetrapyrrole derivatives observed at 390 nm after anaerobic incubation of siroheme with cell-free extracts of *D. vulgaris*, in the presence of NADH and AdoMet. (*A*) Tetrapyrrole derivates observed immediately after the addition of substrates and cofactors to the cell-free extracts: siroheme (SH) and monodecarboxysiroheme (MDSH). (*B*) Tetrapyrrole derivates formed after 18 h of reaction: MDSH, didicarboxysiroheme (DDSH), Fe-coproporphyrin III (Fe-Copro), monovinyl intermediate (MV), and heme (HE). SH, NADH, and AdoMet were used at final concentrations of 10 μM, 500 μM, and 100 μM, respectively.

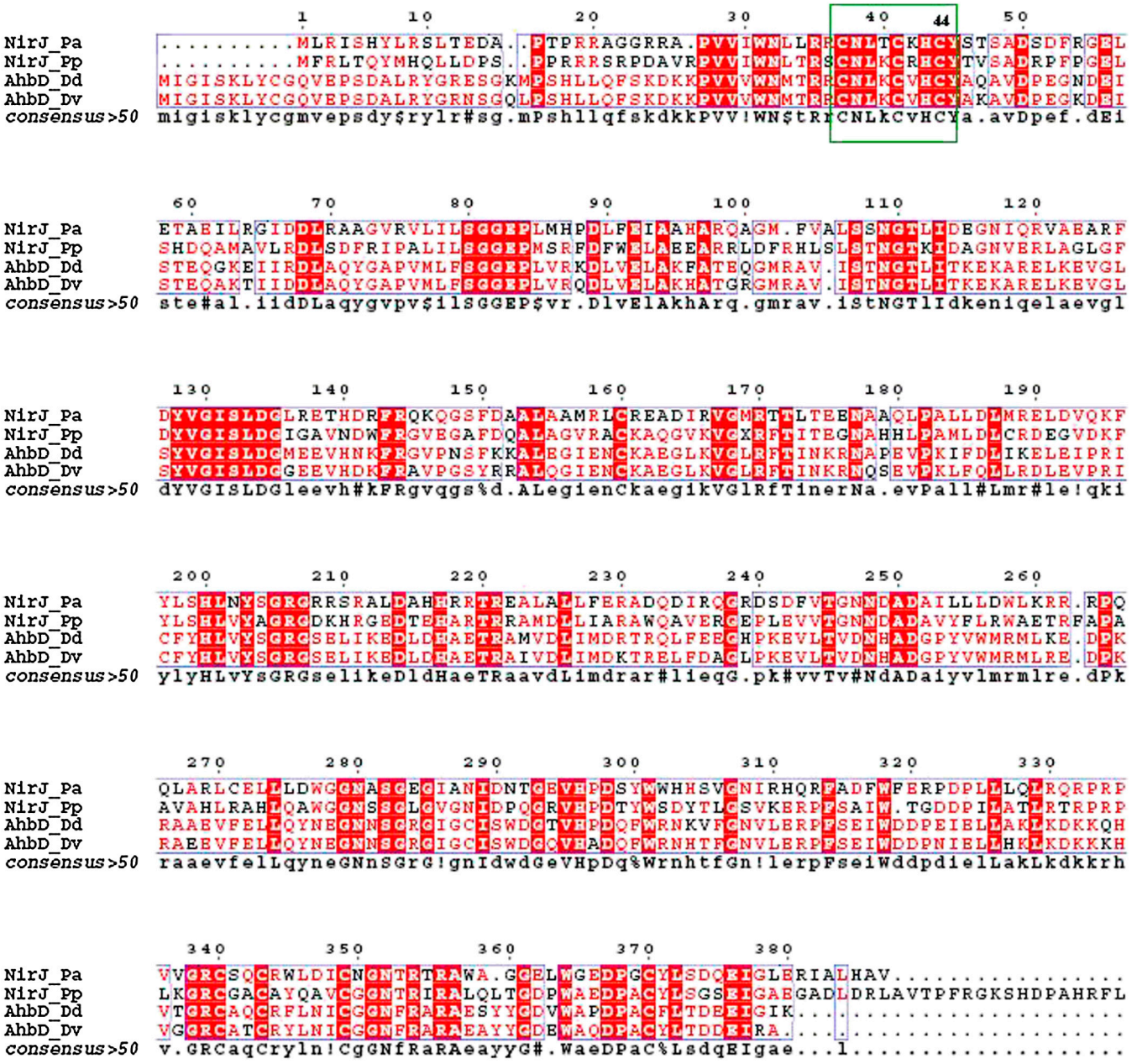

Fig. S3. Alignment of NirJ and AhbD sequences from selected denitrifying and sulfate-reducing bacteria, respectively. The aligned NirJs are from *Pseudomonas aeruginosa* (Pa), *Paracoccus pantotrophus* (Pp), *Desulfovibrio vulgaris* (Dv), and *Desulfovibrio desulfuricans* (Dd). All invariant residues are shown in red. There are two conserved cysteine-rich motifs. The usual iron-sulfur cluster coordinating CX_3CX_2C motif can be seen at the N terminus (in green box), with the last cysteine of motif numbered 44. The second motif consisting of CX_2CX_5C can be seen at the C terminus starting from residue 340.

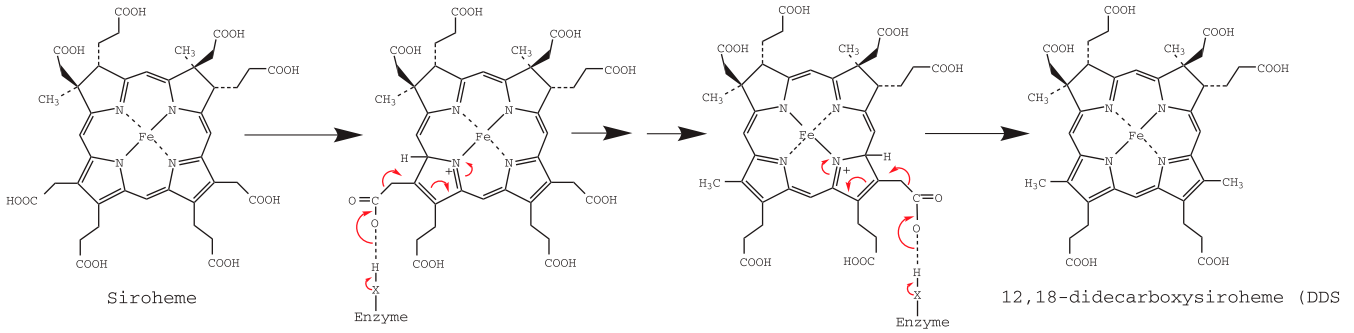

Fig. S4. Proposed reaction mechanism for the decarboxylation of siroheme. Siroheme is acted upon by either a NirD-L, G, H complex (for d_1 heme synthesis) or AhbA, B (for the alternative heme biosynthesis pathway). The reaction involves the formation of an iminium ion that can act as an electron sink. In this case the decarboxylation of the acetate side chain attached to C18 is shown to occur prior to decarboxylation of the acetate side chain on C12, but the actual order is not known.

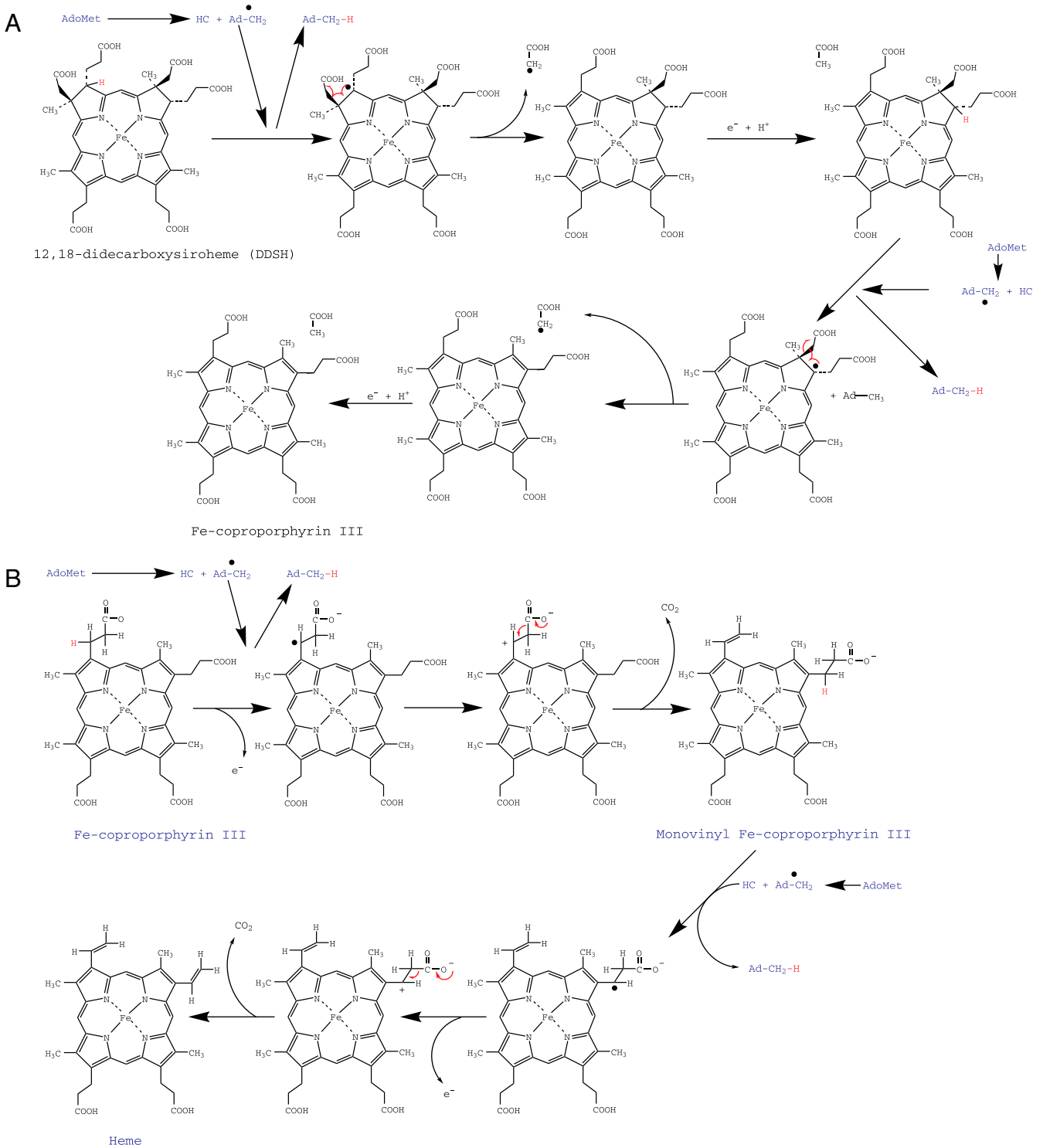

Fig. S5. Proposed reaction mechanisms for the transformation of didecarboxysiroheme into heme. (*A*) Proposed reaction mechanism of AhbC during the synthesis of Fe-coproporphyrin. The initial stage in this reaction requires the formation of an adenosyl radical (Ad-CH_2) and homocysteine (HC) from AdoMet. The adenosyl radical is then able to abstract a hydrogen atom from C3 of the macrocycle. Fragmentation via the formation of a double bond sees the release of the acetate side chain as an acetate radical, which can be converted to acetate by the addition of another electron and a proton. The process is then repeated at the C8 position. The actual order of the release of the acetate side chain is not known. (*B*) Proposed reaction mechanism of AhbD, heme synthase. The initial stage in this reaction requires the formation of an adenosyl radical and homocysteine (HC) from AdoMet. The adenosyl radical is then able to abstract a hydrogen atom from the beta-carbon of the propionate side chain attached to C3. Oxidation via removal of an electron forms a carbon cation, allowing the formation of the vinyl product by loss of CO_2. The procedure is then repeated on the propionate side chain that is attached to C8. Although the order is shown and described in the order of C3 and C8, the actual sequence has not been determined.

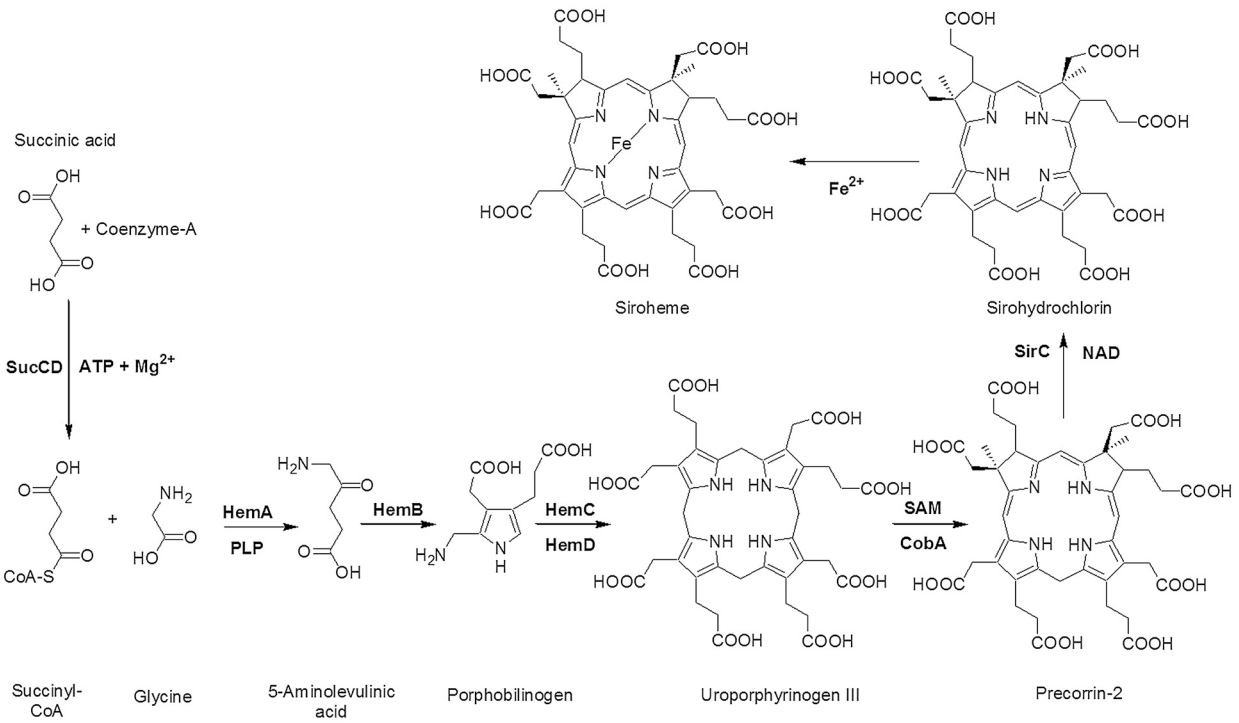

Fig. S6. Enzymatic synthesis of sirohydrochlorin. Biosynthetic reaction steps, catalyzed by the enzymes succinyl-coenzymeA synthetase (SucCD), HemA, HemB, HemC, HemD, CobA, and SirC to synthesize sirohydrochlorin. Uptake of iron by sirohydrochlorin is spontaneous under in vitro reaction conditions.

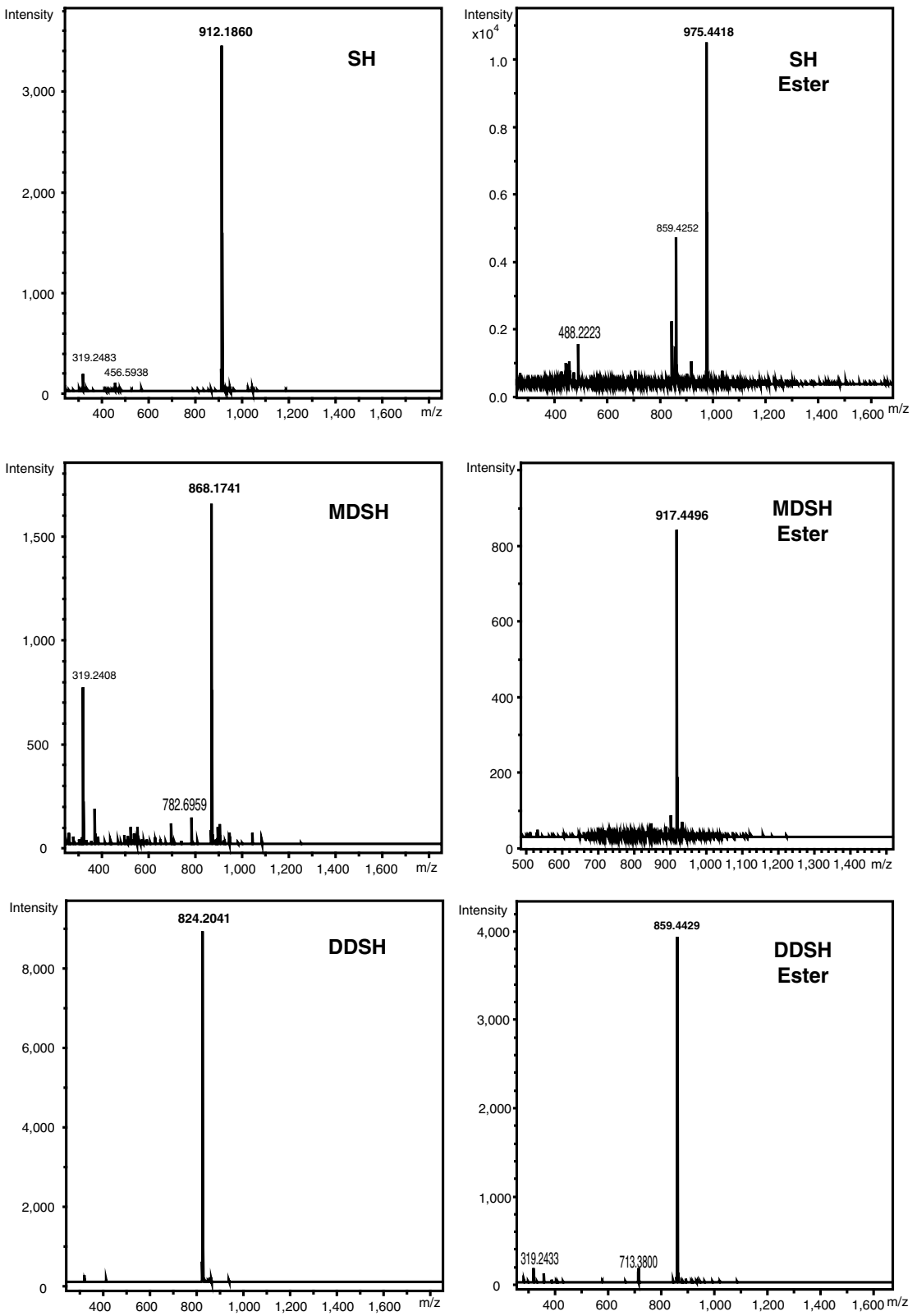

Fig. S7. Mass spectra of siroheme (SH), monodecarboxysiroheme (MDSH), didecarboxysiroheme (DDSH), and their methyl esters. All the compounds were purified by HPLC before their mass analysis. Esterification of SH, MDSH, and DDSH was performed overnight using 95:5 (vol:vol %) of CH_3OH:H_2SO_4 under anaerobic condition.

Table S1. Siroheme decarboxylase activity of Nir and Ahb proteins

Cell-free extract of *Escherichia coli* producing following proteins*	Siroheme (FeSHC)†	Monodecarboxylated siroheme‡	Didecarboxylated siroheme§
Pp-NirD-L	+	40%	ND
Pp-NirD	+	10%	ND
Pp-NirG	+	ND	ND
Pp-NirH	+	8%	ND
Pp-NirD-LGH	+	ND	100%
Pp-NirED-L	+	89%	11%
Pp-NirGH	+	60%	40%
Dv-AhbAB	+	–	100%
Expression vector pET3a	+	ND	ND
Pp-NirD-LGH	–	ND	ND
Pp-NirED-L	–	ND	ND
Pp-NirGH	–	ND	ND

Two-step decarboxylation reaction products separated and identified by liquid chromatography-MS as described in *SI Text*. ND, not detectable; *Paracoccus pantotrophus*, Pp; and *Desulfovibrio vulgaris*, Dy proteins. All the reactions were left overnight to go to completion.

**E. coli* cell-free extract(s) (1.8–2.5 mg of protein) were prepared as described in *SI Text*.

†Siroheme was used at a final concentration of 50 μM in the assays.

‡Monodecarboxylated reaction product has an m/z value of 868.19.

§Didecarboxylated reaction product has an m/z value of 824.23.

Table S2. NMR chemical shift assignments for 12,18-didecarboxysiroheme

Assignment	δ (1H) (ppm)	δ (13C) (ppm)
C2A	2.94	41.1
C2A	3.23	41.1
C2-Me	1.52	18.9
C3	3.28	48.9
C3A	3.14	22.9
C3A	3.28	22.9
C3B	2.72	38.7
C3B	2.78	38.7
C5	7.08	98.5
C7A	1.96	49.0
C7A	2.09	49.0
C7-Me	1.79	18.3
C8	3.97	55.0
C8A	2.16	35.1
C8A	2.23	35.1
C8B	1.89	28.1
C10	7.40	97.3
C12-Me	2.49	10.3
C13A	3.48	22.0
C13B	2.67	39.6
C15	8.01	96.9
C17A	3.48	22.0
C17B	2.67	39.6
C18-Me	2.53	10.1
C20	7.72	94.7