Supplemental Materials

Insights into the active site of Coproheme decarboxylase from *Listeria monocytogenes.*

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The comparison of coproheme-WT and M149A obtained with and without His-Tag shows that while WT is not affected by the purification technique, the mutant is more sensitive. In particular, qualitatively the protein gives rise to the same mixture of coordination and spin states (5c and 6cLS) but the amount of 6cLS increases in the protein purified using the His-Tag.

Figure S1. UV-Vis absorption and second derivative ($D^2$) spectra (left) and the high frequency region RR spectra (right) of the coproheme complexes with LmChdC WT and its M149A mutant with and without His-Tag. The band wavelengths and frequencies assigned to 5cHS, 5cQS and 6cLS species are indicated in orange, olive green, and magenta, respectively (see text). The spectra have been shifted along the ordinate axis to allow better visualization. Experimental conditions of the RR spectra: 406.7 nm excitation wavelength, laser power at the sample of 5 mW, average of 16 spectra with a 160 min integration time (WT HTag); average of 9 spectra with a 90 min integration time (WT no HTag); average of 3 spectra with a 30 min integration time (M149A no HTag); average of 4 spectra with a 40 min integration time (M149A HTag).
Mutation affects coproheme binding

Figure S2. Coproheme binding to the LmChdC variants. Spectral transitions upon binding of 1 µM coproheme (black line) to 3.5 µM LmChdC M149A (A), Q187A (C), and M149A/Q187A (E). The final, coproheme-bound spectra, are depicted in red (after 5s) and intermediate species (after completion of the first binding phase) are shown in green. Experimental time traces followed at 401 nm and single exponential fits representing biphasic coproheme binding to LmChdC M149A (B), Q187A (D) and time traces and fits at 397 nm for M149A/Q187A (F). The insets show the first fast phases.

S3
Figure S3. Enzymatic activity of the LmChdC M149A, Q187A and M149A/Q187A variants. UV-vis absorption spectra recorded following the stepwise titration of coproheme-LmChdC M149A (A), Q187A (B), and M149A/Q187A (C) with hydrogen peroxide. (D) Normalized absorbance changes after each titration at 565 nm are plotted versus the H$_2$O$_2$/coproheme LmChdC concentration ratio of the variants (M149A, black circles; Q187A, grey squares; M149A/Q187A, white triangles), including sigmoidal fits (M149A, black solid line; Q187A, grey solid line; M149A/Q187A, black dashed line). (E) Kinetics of the H$_2$O$_2$-mediated conversion of coproheme-LmChdC, presented as Hanes plots (M149A, black circles; Q187A, grey squares). All experiments were carried out in 50 mM phosphate buffer, pH 7.0.
Heme \( b \) proteins give rise to spectra typical of a main 6cLS species. In the M149A mutant, two different 6cLS forms are observed.

Figure S4. UV-Vis absorption and second derivative (D\(^2\)) (Panel A) and RR spectra in the high frequency region (Panel B) of the heme \( b \) complexes of the WT and its Q187A, M149A/Q187A, and M19A mutants. The band wavelengths and wavenumbers in magenta indicate the 6cLS species; those in orange and olive green are assigned to minor 5cHS, 5cQS species, respectively. The spectra have been shifted along the ordinate axis to allow better visualization. The 450–700 nm region of the spectra in Panel A is expanded from 6- to 10-fold. Experimental conditions of the RR spectra: 406.7 nm excitation wavelength, laser power at the sample 10 mW, average of 6 spectra with a 30 min integration time (WT), average of 13 spectra with 130 min integration time (Q187A), average of 11 spectra with 110 min integration time (M149A/Q187A) and average of 10 spectra with 100 min integration time (M149A).
The X-band EPR spectrum of the coproheme-WT complex shows the presence of three different species. The most abundant is characterized by g values at 5.90, 5.10, 2.00 (g_{12}= 5.50), which confirms the presence of a 5cQS species.

Figure S5. EPR spectra (experimental in black and simulated in red) of the coproheme-LmChdC WT (300 μM, bottom), coproheme-LmChdC M149A (75 μM, middle), and coproheme-Mb (200 μM, top) complexes at pH 7.0 in 50 mM Hepes buffer. The g values of the bands due to the 5cHS, 5cQS, and 6cLS species are coloured in orange, olive green, and magenta, respectively. The spectra have been shifted along the ordinate axis to allow better visualization. Experimental conditions: temperature 10 K, microwave power 2.1 mW, modulation amplitude 10 G. The asterisk at ca. 330 mT indicates an artifact due to a cavity signal.

Table S1. UV-Vis band assignments of coproheme and the coproheme complexes of WT, the M149A variant and Mb. The second derivative Soret band values are reported in brackets.

| Protein     | 5cQS       | 5cHS       | 6cLS       |
|-------------|------------|------------|------------|
| Mb          |            | 391 (392), 486a, 614 | 406 (406), 525, 555 |
| M149A       | 399 (396), 494, 538, 630 |            | 406 (406), 518, 555 |
| WT          | 393 (396), 494, 538, 630 |            |            |
| Coproheme   | 390 (391), 492, 530, 614 |            |            |

*aThis band is due to the overlapping contributions of the β bands of 5c and 6c HS.
Table S2. RR core size band assignments of coproheme and the coproheme complexes of WT, the M149A variant, and Mb.

| Protein      | \( \nu_3 \) | \( \nu_2 \) | \( \nu_{10} \) |
|--------------|-------------|-------------|--------------|
|              | 5cQS        | 5cHS        | 6cLS         |
| Mb           | 1495        | 1483        | 1508         |
| M149A        | 1503        | 1490        | 1507         |
| WT           | 1503        | 1490        | 1579         |
| Coproheme    | 1493        | 1585        | -            |

The EPR data are in very good agreement with the RR spectra obtained at 80 K. The 80 K RR spectra confirm the presence of a 5cQS species, a 5cHS species, and a 6cLS form for the WT and mainly a 6cLS with only a small amount of 5cHS for the M149 mutant.

Figure S6. RR spectra in the high frequency region at 80 K of the coproheme complexes with WT, M149A, and Mb obtained with \( \lambda_{\text{exc}} \) at 356.4 and 406.7 nm. The frequencies of the bands due to the 5cHS, 5cQS, 6cHS and 6cLS species are coloured in orange, olive green, blue, and magenta, respectively. Experimental conditions: (356.4 nm), laser power at the sample 5 mW and average of 7 spectra with 140 min integration time (WT); laser power at the sample 10 mW, average of 12 spectra with 120 min integration time (M149A); (406.7 nm): laser power at the sample 6 mW (WT and M149A), average of 14 spectra with 280 min integration time (WT), 9 spectra with a 135 min integration time (M149A); laser power at the sample 7 mW, average of 7 spectra with 140 min integration time (Mb). The spectra have been shifted along the ordinate axis to allow better visualization.
The CO conformers of the coproheme-complexes have been identified on the basis of the isotope shift of the ν(FeC) and ν(CO) stretching modes in $^{13}$CO versus $^{12}$CO. 

Figure S7. RR spectra in the low (left) and high (right) frequency regions of the $^{12}$CO (bottom) and $^{13}$CO (top) adducts of coproheme (panel A) and the coproheme complexes with Mb (panel B), WT (panel C), and the M149A (panel D), Q187A (panel E), M149A/Q187A (panel F) mutants. The frequencies of the ν(FeC), $\delta$(FeCO) and ν(CO) modes, are indicated in red. The spectra have been shifted along the ordinate axis to allow better visualization.
Experimental conditions: coproheme and coproheme-Mb: $\lambda_{\text{exc}}$ 406.7 nm, laser power at the sample 5 mW, average of 6 spectra with 60 min integration time and 12 spectra with 120 min integration time in the low and high frequency regions, respectively ($^{12}\text{CO}$-coproheme), average of 9 spectra with 90 min integration time and 11 spectra with 110 min integration time, in the low and high frequency regions, respectively ($^{13}\text{CO}$-coproheme), average of 4 spectra with 40 min integration time and 10 spectra with 100 min integration time, in the low and high frequency regions, respectively ($^{12}\text{CO}$-Mb), average of 4 spectra with 40 min integration time and 7 spectra with 70 min integration time, in the low and high frequency regions, respectively ($^{13}\text{CO}$-Mb); coproheme-WT and its selected mutants, $\lambda_{\text{exc}}$ 413.1 nm, laser power at the sample 1-3 mW, average of 28 spectra with 280 min integration time and 22 spectra with 220 min integration time, in the low and high frequency regions, respectively ($^{12}\text{CO}$-WT), average of 8 spectra with 80 min integration time and 15 spectra with 150 min integration time, in the low and high frequency regions, respectively ($^{13}\text{CO}$-WT), average of 6 spectra with 60 min integration time and 18 spectra with 180 min integration time, in the low and high frequency regions, respectively ($^{12}\text{CO}$-M149A), average of 12 spectra with 120 min integration time and 18 spectra with 180 min integration time, in the low and high frequency regions, respectively ($^{13}\text{CO}$-M149A), average of 9 spectra with 90 min integration time and 15 spectra with 150 min integration time, in the low and high frequency regions, respectively ($^{12}\text{CO}$-Q187A), average of 7 spectra with 70 min integration time and 13 spectra with 130 min integration time, in the low and high frequency regions, respectively ($^{13}\text{CO}$-Q187A), average of 6 spectra with 60 min integration time and 15 spectra with 150 min integration time in the low and high frequency regions, respectively ($^{12}\text{CO}$-M149A/Q187A), average of 7 spectra with 70 min integration time and 12 spectra with 120 min integration time, in the low and high frequency regions, respectively ($^{13}\text{CO}$-M149A/Q187A).
The UV-Vis and RR spectra are typical of the heme b-CO complexes. In the RR spectra the \(\nu(\text{FeC})\) and \(\nu(\text{CO})\) stretching modes are indicated in red and have been identified on the basis of the isotope shift in \(^{12}\text{CO}\) versus \(^{13}\text{CO}\) (Figure S9). Due to the high fluorescent background the \(\nu(\text{CO})\) stretching mode for all the mutants is not clearly defined.

Figure S8. UV-Vis (panel A) and RR (panel B) spectra in the low (left) and high (right) frequency regions of the \(^{12}\text{CO}\) adducts of Mb and the heme b complexes of LmChdC WT and the M149A, M149A/Q187A, Q187A mutants. The frequencies of the \(\nu(\text{FeC})\), \(\delta(\text{FeCO})\) and \(\nu(\text{CO})\) modes, are indicated in red. The spectra have been shifted along the ordinate axis to allow better visualization. Panel A: the 480–700 nm region is expanded 10-fold. Panel B: RR experimental conditions: \(\lambda_{\text{exc}}\) 413.1 nm, laser power at the sample 1 to 2 mW, average of 12 spectra with 120 min integration time in both the low and high frequency regions (Mb), average of 16 spectra with 160 min integration time and 12 spectra with 120 min integration time in the low and high frequency regions, respectively (WT); average of 11 spectra with 110 min integration time (M149A), average of 9 spectra with 90 min integration time (M149A/Q187A), average of 12 spectra with 120 min integration time (Q187A).
The CO conformers of the heme $b$-CO complexes have been identified on the basis of the isotope shift in $^{13}$CO versus $^{12}$CO of the $\nu$(FeC) and $\nu$(CO) stretching modes.

**Figure S9.** RR spectra in the low (left) and high (right) frequency regions of the $^{12}$CO (bottom) and $^{13}$CO (top) adducts of the heme $b$ complexes of LmChdC WT (panel A) and the M149A (panel B), Q187A (panel C), M149A/Q187A (panel D) mutants. The frequencies of the $\nu$(FeC), $\delta$(FeCO) and $\nu$(CO) modes, are indicated in red. The spectra have been shifted along the ordinate axis to allow better visualization. Experimental conditions: $\lambda_{ex}$ 413.1 nm, laser power at the sample 1 to 3 mW, average of 16 spectra with 160 min integration time and 12 spectra with 120 min integration time in the low and high frequency regions, respectively ($^{12}$CO-WT complex), average of 8 spectra with 80 min integration time and 12 spectra with 120 min integration time in the low and high frequency regions, respectively ($^{13}$CO-WT complex), average of 11 spectra with 110 min integration time and 12 spectra with 84 min integration time in the low and high frequency regions, respectively ($^{12}$CO-M149A complex), average of 6 spectra with 60 min integration time and 7 spectra with 70 min integration time in the low and high frequency regions, respectively ($^{13}$CO-M149A complex).
M149A complex), average of 12 spectra with 120 min integration time and 20 spectra with 60 min integration time in the low and high frequency regions, respectively (\(^{12}\)CO-Q187A complex), and average of 12 spectra with 120 min integration time and 28 spectra with 84 min integration time in the low and high frequency regions, respectively (\(^{13}\)CO-Q187A complex), average of 9 spectra with 90 min integration time and 30 spectra with 30 min integration time in the low and high frequency regions, respectively (\(^{12}\)CO-M149A/Q187A complex), average of 11 spectra with 110 min integration time and 30 spectra with 60 min integration time in the low and high frequency regions, respectively (\(^{13}\)CO-M149A/Q187A complex).