Supplemental Information

for

A Dynamic Substrate is Required for MhuD-catalysed Degradation of Haem to Mycobilin

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The degradation of heme to its final product can be given by equation (3):

\[ \text{Heme} \xrightarrow{k_1} \text{Meso} - \text{hydroxyheme} \xrightarrow{k_2} \text{Mycobilin} \] (3)

Simplifying equation (3) to express in terms of A, B and C, we get equation (4):

\[ A \xrightarrow{k_1} B \xrightarrow{k_2} C \] (4)

**Derivation of equation (1).** The rate of heme monoxygenation is given by a psuedo-first order kinetics, because ascorbate is in excess. The differential equation of A can be given by equation (5):

\[ \frac{dA}{dt} = -k_1 [A] \] (5)

where \( k_1 \) is the pseudo-first order rate constant for heme monooxygenation.

Equation (5) can be organized as equation (6):

\[ \frac{dA}{[A]} = -k_1 dt \] (6)

Upon integration and simplification, equation (6) becomes equation (7):

\[ [A] = [A]_0 e^{-k_1 t} \] (7)

where \([A]_0\) is the MhuD–heme concentration at 0 min. Using Beer’s law, equation (7) can be written in terms of Abs as given by equation (8):

\[ A_{\text{Soret}} = A_{\text{Soret},0} e^{-k_1 t} \] (8)

where \( A_{\text{Soret}} \) is the UV/Vis Abs intensity of the Soret band and \( A_{\text{Soret},0} \) is the UV/Vis Abs intensity of the Soret band at \( t = 0 \).
However, since the intermediates and products of MhuD-catalyzed heme degradation have non-zero absorbance at the Soret band wavelength, $A_{\text{Soret}}$ does not reach zero and instead it approaches a nonzero baseline at an infinite time ($\infty$). This situation can be accounted for my modifying equation (8) to yield equation (9):

$$A_{\text{Soret}} = (A_{\text{Soret,0}} - A_{\text{Soret,\infty}})e^{-k_1t} + A_{\text{Soret,\infty}}$$

(9)

where $A_{\text{Soret,\infty}}$ is the UV/Vis Abs intensity of the Soret band at $t = \infty$.

Equation (9) was rewritten in GraphPad Prism 8.0 as equation (10) to fit the data collected from Abs monitored heme degradation assay to extract $k_1$.

$$Y = ((I - F) \ast \exp(-k_1 \ast X)) + F$$

(10)

where I and F are the Soret absorbance at 0 and $\infty$ min. Abs data was used to plot absorbance at 412 nm (Y) as a function of time (X).

**Derivation of equation (2).** The differential equation for the time-course of intermediate B from equation (4) can be written as equation (11):

$$\frac{dB}{dt} = k_1A - k_2B$$

(11)

The concentration of B from equation (4) can then be expressed as shown in equation (12):

$$[B] = \frac{k_1[A]_0}{k_2-k_1}(e^{-k_1t} - e^{-k_2t})$$

(12)

where $k_2$ is the pseudo-first order rate constant for meso-hydroxyheme dioxygenation.

Using the Beer’s law, $[B]$ and $[A]_0$ can be expressed in terms of Abs as be given by equations (13) and (14):
\[
\begin{align*}
[A]_0 &= \frac{A_{620,0}}{\varepsilon_h} \\
[B] &= \frac{A_{620,B}}{\varepsilon_{mh}}
\end{align*}
\]

where \(A_{620,0}\) is the UV/Vis Abs intensity at 620 nm for \(t = 0\), \(A_{620,B}\) is the UV/Vis Abs intensity at 620 nm arising from species B, \(\varepsilon_h\) is the molar extinction coefficient of MhuD–heme at 620 nm, and \(\varepsilon_{mh}\) is the molar extinction coefficient for the meso-hydroxyheme form of MhuD at 620 nm.

Plugging (13) and (14) into equation (12) and rearranging gives equation (15):

\[
A_{620,B} = A_{620,0} \left( \frac{\varepsilon_{mh}}{\varepsilon_h} \right) \left( \frac{k_1}{k_2 - k_1} \right) (e^{-k_1 t} - e^{-k_2 t})
\]

Also, equation (7) can be written in terms of equation (16):

\[
A_{620,A} = A_{620,0} e^{-k_1 t}
\]

where \(A_{620,A}\) is the UV/Vis Abs intensity at 620 nm arising from species A.

As the Abs spectra at 620 nm would have contribution from both species A and B, equation (15) and (16) must be summed to yield equation (17) in terms of an experimental observable:

\[
A_{620} = A_{620,0} \left[ e^{-k_1 t} + \left( \frac{\varepsilon_{mh}}{\varepsilon_h} \right) \left( \frac{k_1}{k_2 - k_1} \right) (e^{-k_1 t} - e^{-k_2 t}) \right]
\]

where \(A_{620}\) is the total UV/Vis Abs intensity at 620 nm.

Equation (17) was rewritten in GraphPad Prism 8.0 as equation (18) to fit the data collected from Abs monitored heme degradation assay to extract \(k_2\) and \(\varepsilon_{mh}\).

\[
Y = A \ast (\exp(-k_1 \ast X)) + \left( \frac{E}{F} \right) \ast (k_1 / (k_2 - k_1)) \ast (\exp(-k_2 \ast X) - \exp(-k_2 \ast X)) + C
\]
where, E and F are the molar extinction coefficients for the meso-hydroxyheme and heme froms of MhuD at 620 nm, respectively. A is the initial Abs intensity at 420 nm and C is a constant to correct for the presence of other species that contribute to the Abs intensity at 620 nm. Abs data was used to plot absorbance at 620 nm (Y) as a function of time (X).
**Figure S1.** FPLC chromatogram for purification of F23W MhuD. The 280 nm absorbance (*black trace*) and percentage of Buffer B (50 mM Tris, 350 mM NaCl, 500 mM Imidazole pH 7.8, *red trace*) are plotted as a function of buffer run through the column. Numbers in boxes on top of the x-axis refer to fractions collected during FPLC.

**Figure S2.** F23W MhuD assessed by SDS-PAGE gel electrophoresis. From left to right, the lanes represent: (A) FPLC fraction 2, (B) FPLC fraction 3, (C) PageRuler Plus prestained protein ladder (Pierce), (D) FPLC fractions 4-6, (E) 1/100 dilution of fractions 4-6, (F) 1/20 dilution of fractions 4-6.
Figure S3. FPLC chromatogram for purification of W66F MhuD. The 280 nm absorbance (black trace) and percentage of Buffer B (50 mM Tris, 350 mM NaCl, 500 mM Imidazole pH 7.8, red trace) are plotted as a function of buffer run through the column. Numbers in boxes on top of the x-axis refer to fractions collected during FPLC.

Figure S4. W66F MhuD assessed by SDS-PAGE gel electrophoresis. From left to right, the lanes represent: (A) Lysate, (B) FPLC fraction 7, (C) FPLC fraction 10-11, (D) PageRuler Plus prestained protein ladder (Pierce), (E) FPLC fractions 14-16, (F) 1/100 dilution of fractions 14-16, (G) 1/20 dilution of fractions 14-16.
**Figure S5.** ESI-MS spectrum of hemin chloride degradation. Control assay of 50 μM hemin chloride, 50 mM potassium phosphate pH 6.0, 150 mM NaCl, 37°C performed in the presence of 5 mM ascorbate, 10 mM EDTA, 840 U/mL of catalase and 167 U/mL of Superoxide Dismutase (SOD).

**Figure S6.** Tandem MS spectra of the m/z 616 ion (Panel A) and authentic hemin chloride (Panel B). Two major daughter fragments of m/z 557 and m/z 498 are observed corresponding to loss of CH₂COOH (red dotted line) and (CH₂COOH)₂ (blue dotted line) respectively.
Figure S7. Tandem MS spectrum of the m/z 611 parent ion. The fragment ions observed during Tandem MS of the m/z 611 parent ion are color matched to their corresponding fragments and can be traced back to mycobilin.

Figure S8. Tandem MS spectrum of the m/z 583 parent ion. The fragment ion of m/z 297 matches up with the loss of the fragment denoted by a red dotted line in the biliverdin structure shown above.
Figure S9. Tandem MS spectra for each chromatographic peak corresponding to m/z 583. Spectra A-C were assigned as α, δ and β-biliverdin respectively, based on their assignments in previous studies. Spectra D has major fragments at m/z 555 and m/z 297, which are assigned to γ-biliverdin. Spectra A-D are present and identified for WT MhuD, A-C for F23W MhuD, and A-B for W66F MhuD.

References