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Supporting Information

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for

Detection of Covalent Intermediates Formed in the Reaction of 4-Amino-4-deoxychorismate Synthase

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Contents

A. Purification of enzymes

i) Growth of cell cultures

Enzymes were expressed in the *E. coli* strain C41:DE3. Cells transformed with the appropriate expression vectors were grown up in 2 × YT media with 50 μg mL^{-1} ampicillin at 37 °C with 250 rpm shaking. Once an optical density (600 nm) of 0.6 – 0.8 was reached protein expression was induced by addition of 1 mM IPTG. Culture growth was continued for 4 – 5 hours. Cells were harvested by centrifugation at 11,000 × *g* for 15 mins, and stored at -80 °C.

ii) Purification of PabB

E. coli PabB was expressed as detailed in i) using the vector pNPB in which the *pabB* gene is under the control of a *tac* promoter. The pNPB vector was generously supplied by Prof. C. T. Walsh (Harvard Medical School). The following steps were carried out at 4 °C. Cells were resuspended in Buffer A (10 mM Tris.HCl pH 8.0, 1 mM EDTA, 1 mM DTT) and lysed by sonication. Cell debris was removed by centrifugation at 39 000 *g* for 30 minutes. A 20% streptomycin sulphate solution was added dropwise with stirring to the cell free supernatant, to a final concentration of 2%. Stirring was continued for 20 minutes, and the precipitated DNA was removed by centrifugation at 39 000 *g* for 30 minutes. The cell-free lysate was applied to a Source Q 16/10 column (Amersham Biosciences). Proteins were eluted with a gradient of 0 to 0.5 M NaCl in Buffer A over eight column volumes. Peak fractions containing PabB were combined and dialysed into Buffer B1 (10 mM potassium phosphate, 1 mM DTT, pH 6.7).

The dialysed preparation was concentrated down to approximately 10 mg mL^{-1} and applied to a ceramic hydroxyapatite column 10/10 (Bio-Rad, Type 1, 20 μm) equilibrated in Buffer B1. Six column volumes of Buffer B1 were run through the column, followed by a rapid gradient to 100% Buffer B2 (1 M potassium phosphate, 1 mM DTT, pH 6.7) over two column volumes PabB that eluted during the initial wash with Buffer B1 was combined and dialysed into Buffer C (10 mM Tris.HCl, 1 mM DTT, 1 mM EDTA, pH 8). The PabB preparation was then concentrated down to approximately 15 mg mL^{-1} and samples applied to a MonoQ 10/10 column. A rapid gradient of 0 – 0.2 M NaCl in Buffer C over two column volumes was applied, followed by a gradient of 0.2 – 0.3 M NaCl in Buffer C over eight column volumes to elute PabB. Fractions containing purified PabB were combined, dialysed into Buffer D (10 mM Tris.HCl, 5 mM DTT, 1 mM EDTA, 5 mM $MgCl_2$, pH 8.0) and stored at -80 °C. The overall yield was 63 mg of PabB per 1 L culture.

The amino acid sequence of the PabB used in this communication differs from that in the SwissProt database with the change Leu12 to Pro12.[1] A mass of 50,953 ± 2 Da was measured

[1] Goncharoff, P.; Nichols, B. P. *J. Bacteriol.* **1984**, *159*, 57-62.

by ESMS and a molar absorption coefficient of 70,800 ± 200 $Lmol^{-1}cm^{-1}$ at 280 nm was determined.

iii) Purification of PabA

PabA from *E. coli* ADCS was expressed as detailed in i) using the vector pNPA in which the *pabA* gene is under the control of a *tac* promoter. The pNPA vector was generously supplied by Prof. C. T. Walsh (Harvard Medical School). The following steps were carried out at 4 °C. Cells were resuspended in Buffer E (10 mM Tris.HCl, 5 mM $MgCl_2$, 1 mM EDTA, 5 mM DTT, 10% glycerol, pH 8.0) and lysed by sonication. Cell debris was removed by centrifugation at 39 000 *g* for 30 minutes. A 20% streptomycin sulphate solution was added dropwise with stirring to the cell free supernatant, to a final concentration of 2%. Stirring continued for 30 minutes and the precipitated DNA was removed by centrifugation at 39 000 *g* for 30 minutes. The cell-free lysate was applied to a Source Q 16/10 column (Amersham Biosciences). PabA eluted immediately from the column while impurities were eluted by a gradient of 0–1 M NaCl in Buffer E. Fractions containing PabA were concentrated and loaded onto a HiLoad Superdex 16/60 column (Amersham Biosciences). The column was run with Buffer F (10 mM Tris.HCl, 5 mM DTT, 10 glycerol, pH 8.0) at 1 mL min^{-1}. PabA eluted after 0.66 column volumes. This preparation was further purified by a applying to a MonoQ 10/10 column (Amersham Biosciences) and eluting with a 0–0.15 M NaCl gradient over twenty column volumes. PabA containing fractions were combined, dialysed into Buffer F, and stored at -80 °C. The yield of purified PabA was 30 mg per 1 L culture.

A mass of 20772 ± 1 Da was measured by ESMS for the PabA preparation, and molar absorption coefficient of 31,400 ± 600 $Lmol^{-1}cm^{-1}$ at 280 nm was determined.

iv) Cloning and purification of K274A PabB

The *E. coli pabB* gene was subcloned into the expression vector mini-pRSETA by Dr Brent K. Nabbs (Department of Chemistry, University of Cambridge). The mini-pRSETA vector was kindly supplied by Mark Proctor (University of Cambridge). This vector was generated by modifying the vector pRSETA (Invitrogen) to encode the N-terminal tag MRGSHHHHHHGLVPRGS, which contains a His_6-tag and a thrombin cleavage site. The polymerase chain reaction (PCR) was used to amplify the *pabB* gene from the vector pNPB and introduce *Bam*HI and *Nco*I restriction enzyme sites to the 5' and 3' ends respectively. The primers were 5'-CGCGGATCCATGAAGACGTTATCTCCCGCTGTG-3' (forward) and 3'-GCATGCCATGGTTACTTCTCCAGTTGCTTCAGGATACG-5' (reverse). The fragment generated was ligated into *Bam*HI and *Nco*I sites of mini-pRSETA to construct the vector *pab*B/mini-pRSETA. The mutation K274A was generated in *pab*B/mini-pRSETA using a QuikChange Site-Directed Mutagenesis Kit (Stratagene). Primers were K274A 5′-CCCGCCCGATTGCAGGC ACGCTACCACGC-3′ (forward) and 5′-GCGTGGTAGCGTGCCTGCAATCGGGCGGG-3′ (reverse).

K274A PabB was expressed from the vector K274A *pabB*/mini-pRSETA as detailed in i). Cells were resuspended in Buffer N1 (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, 1 mM 2-mercaptoethanol, pH 8.0) and lysed by sonication. Cell debris was removed by centrifugation at 39 000 × *g* for 30 min. The cell-free lysate was applied to a 5 mL Ni-NTA column (Qiagen). After washing with 24 column volumes of Buffer N2 (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole, 1 mM 2-mercaptoethanol, pH 8.0), the His_6- tagged K274A PabB was eluted with Buffer N3 (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole, 1 mM 2-mercaptoethanol, pH 8.0). The enzyme was exchanged into 10 mM potassium phosphate, pH 8.0, and stored at -80 °C. The overall yield was 23 mg of K274A PabB from 1 L of culture.

B. Preparation of samples for ESMS

Enzymes were exchanged into MilliQ water using Vivaspin 0.5 mL concentrators (10 000 molecular weight cut-off, Vivascience) spun at 4000 *g* and at 4 °C. Samples were concentrated 25-fold and then diluted into MilliQ water. This process was repeated 8 – 10 times to ensure buffer components were removed from the enzymes, and samples were then made up to a final concentration of 100 μM. For detection of covalent intermediates during the ADCS reactions 50 μM solutions of ADCS were made up by mixing PabA and PabB in a 1:1 ratio. This solution was used as soon as possible to minimize oxidation of the PabA subunit. After the addition of chorismate (100 μM, barium salt), glutamine (10 mM) or DON (10 mM) samples were incubated for 5 min.

Mass spectrometry was performed on a Q-TOF 1 mass spectrometer (Micromass) fitted with a Z-spray ion source. Immediately before injection into an electrospray mass spectrometer, samples were diluted 20-fold in 1% formic acid/10% methanol. Samples were introduced into the mass spectrometer using an injection valve fitted with a 20 μL loop and a flow of 1% formic acid/10% methanol at 20 μL/min, which fed directly into the ESI probe held at 3.3 kV. Spectra were analysed using the software MassLynx (Micromass).

C. Results

i) ESMS of K274A PabB:PabA during the ADCS reaction

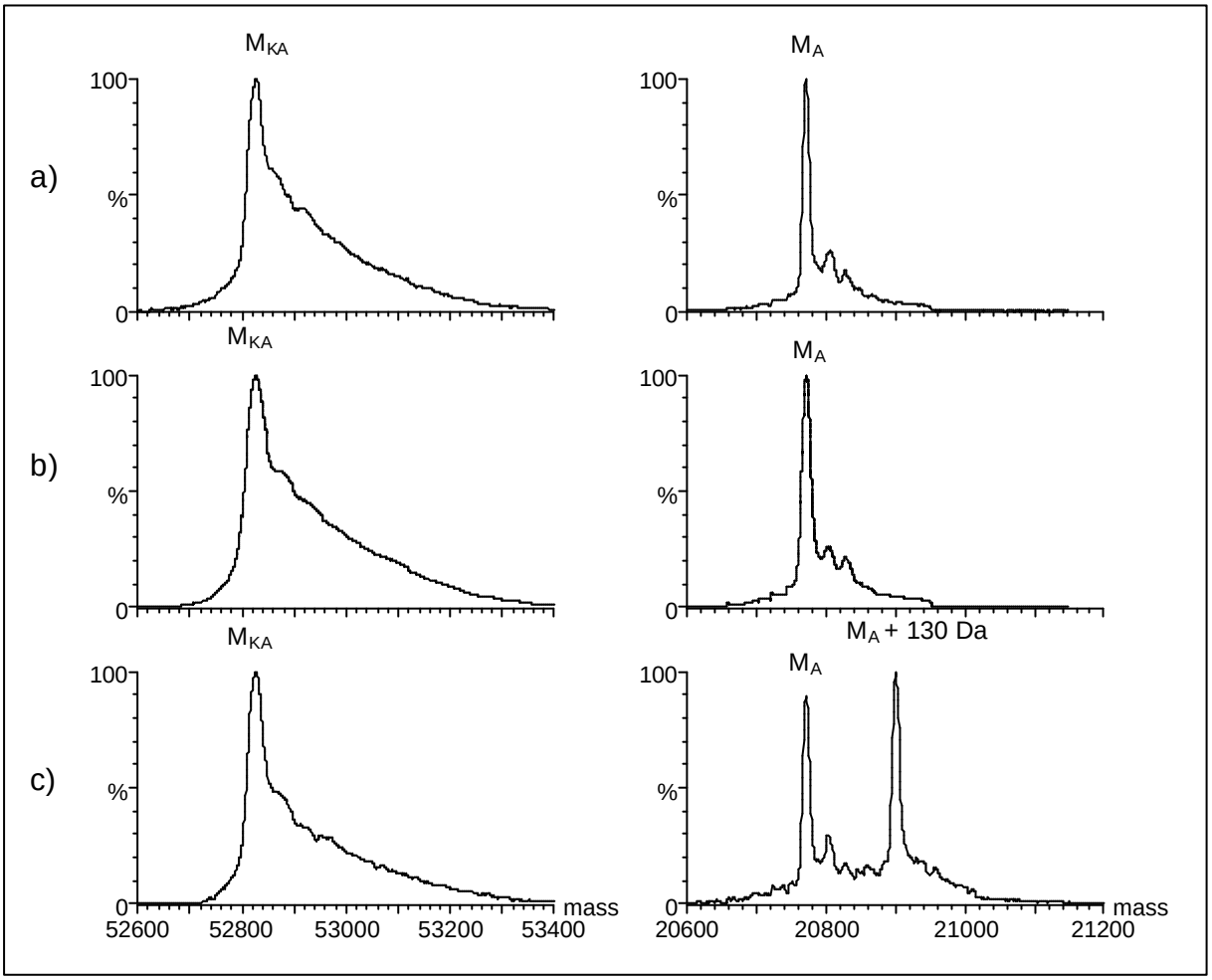

Figure S1: *Charge-deconvoluted electrospray mass spectra of K274 PabB:PabA during the ADCS reaction. K274A PabB (left column) and PabA (right column) shown. a) 50 µM K274A PabB:PabA b) 50 µM K274A PabB:PabA treated with 100 µM chorismate c) 50 µM K274A PabB:PabA treated with 100 µM chorismate followed by 10 mM glutamine. Samples were incubated for 5 min following the addition of substrates, and were prepared for ESMS by a twenty fold dilution in 10% methanol/1% formic acid. The mass of unmodified K274A PabB was 52827 ± 4 Da (M_{KA}) and PabA was 20771 ± 1 Da (M_A).*

ii) Effect of DON on chorismate-modified PabB

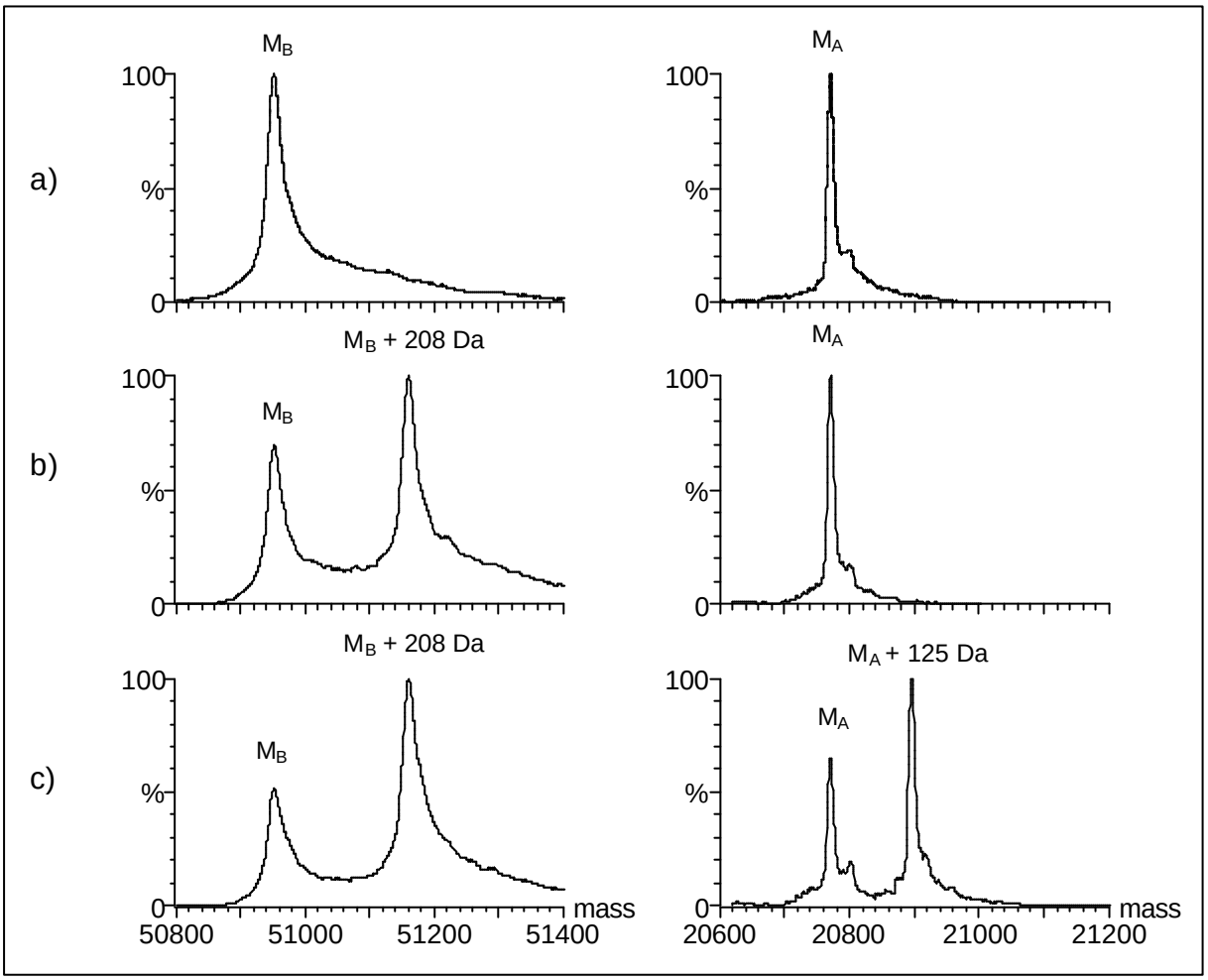

Figure S2: *Charge-deconvoluted electrospray mass spectra showing effect of DON on chorismate-modified PabB. PabB (left hand column) and PabA (right hand column) shown. a) 50 µM ADCS b) 50 µM treated with 100 µM chorismate c) 50 µM ADCS treated with 100 µM chorismate followed by 10 mM DON. Samples were incubated for 5 min following the addition of substrates and were prepared for ESMS by a 20-fold dilution in 10% methanol/1% formic acid. The mass of unmodified PabB was 50953 ± 2 Da (M_B) and PabA was 20771 ± 1 Da (M_A).*

iii) Identification of the site of DON modification of PabA

A solution of PabA (30 μM) was incubated with DON (1 mM) for 1 h at 25 °C. This sample was exchanged into 5 mM NH_4HCO_3, pH 8.0 using a Vivaspin 0.5 mL soncentrator (10 000 molecular weight cut-off, Vivascience) spun at 4000 *g* and at 4 °C. Samples were concentrated tenfold and then diluted into 5 mM NH_4HCO_3. This process was repeated 8–10 times with the sample finally being made up to 30 μM. An aliquot from this sample was diluted 1:1 with 1% formic acid/50% CH_3CN and injected into an electrospray mass spectrometer. Approximately 40% of the PabA was found to have undergone a modification of 125 Da increasing the mass from 20 771 ± 1 Da to 20 896 ± 1 Da.

To identify the site of modification the DON-treated PabA was digested with trypsin. Sequencing grade trypsin (Promega) was added to a final concentration of 1 μg per 100 μg PabA and the sample incubated at 37 °C for 16 h. Analysis of the tryptic digest by matrix-assisted laser desorption/ionization (MALDI) mass spectrometry on a MALDI TOF-TOF instrument (Applied Biosystems 4700 Proteomics Analyser) revealed a novel peptide with a mass of 2136.1 Da, corresponding to peptide 73-92 (2011.1 Da) with a modification of 125 Da. Sequencing of this peptide by tandem mass spectrometry identified Cys79 as the site of modification (Table 1, Figure S3). A possible mechanism for this modification is shown in Figure S4.

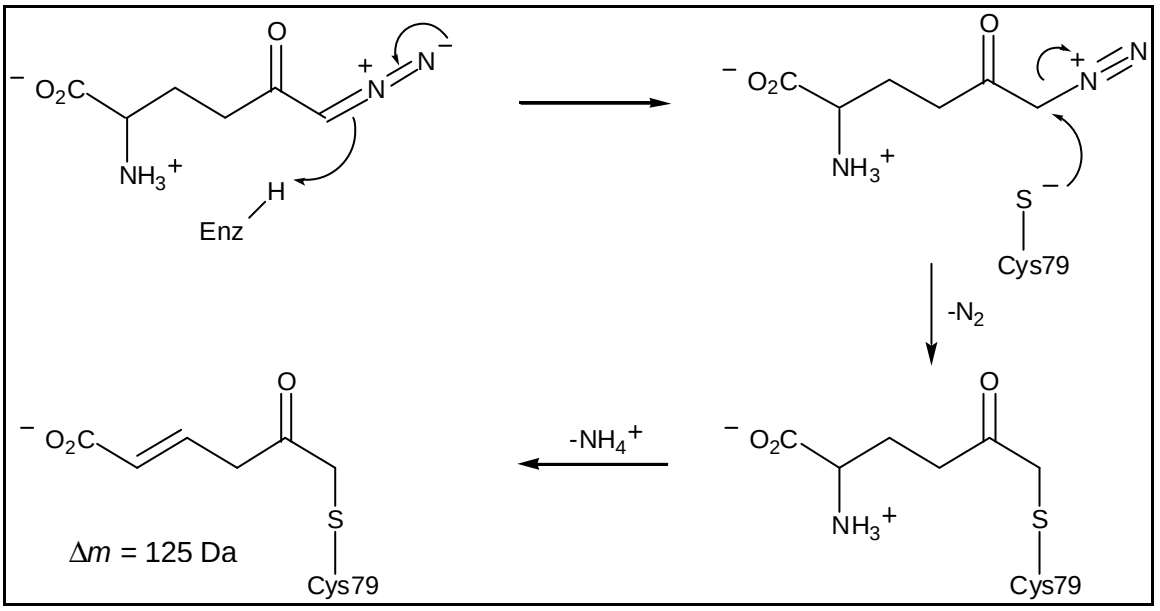

Figure S4*: Possible mechanism for 125.1 Da modification of Cys-79 by DON.*

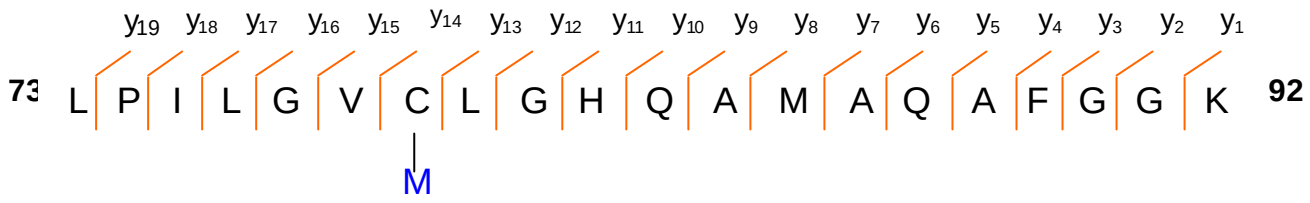

***Figure S3**: Sequencing of peptide 2136.1 Da by tandem mass spectrometry.*

***Table S1**: Tandem mass spectrometry sequencing of peptide 2136.1 Da from tryptic digest of DON-modified PabA.*

y-ion	Expected mass (monisotopic, Da)	Observed mass (monoistopic, Da)	Difference between observed and expected mass (Da)
1	147.1	147.1	-
2	204.1	204.1	-
3	261.2	261.2	-
4	408.2	408.3	-
5	479.3	479.3	-
6	607.3	607.3	-
7	678.4		-
8	809.4	809.4	-
9	880.4	880.4	-
10	1008.5	1008.5	-
11	1145.6	1145.6	-
12	1202.6	1202.6	-
13	1315.7	1315.7	-
14	1418.7	1543.7	125.1
15	1517.7	1642.8	125.1
16	1574.8	1699.8	125.1
17	1687.8	1812.9	125.1
18	1800.9	1926.0	125.1
19	1898.0	2023.1	125.1