

DOI: 10.1002/cbic.200700162

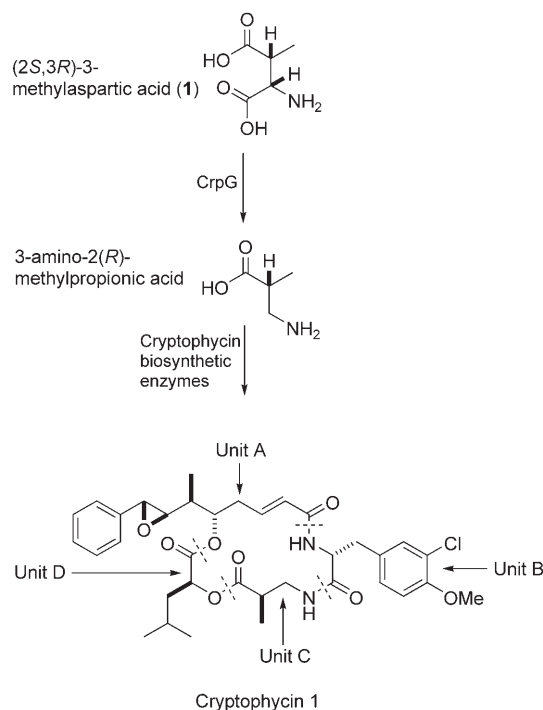
## Characterization of the $\beta$ -Methylaspartate- $\alpha$ -decarboxylase (CrpG) from the Cryptophycin Biosynthetic Pathway

Zachary Q. Beck, Douglas A. Burr, and David H. Sherman<sup>\*[a]</sup>

The cryptophycins are promising therapeutic agents due to their potent selectivity for cancer cells, which include multi-drug-resistant tumor cell lines, and their ability to evade *p*-glycoprotein pumps.<sup>[1,2]</sup> The biosynthetic cluster responsible for production of the cryptophycins has been identified recently in *Nostoc* sp. ATCC 53789 and *Nostoc* sp. GSV224.<sup>[3]</sup> Future efforts to rationally engineer the biosynthesis of novel cryptophycin analogues relies on a detailed understanding of the role for each of the corresponding enzymes involved in construction of these valuable natural products. The majority of the more than 25 naturally occurring cryptophycins are composed of four subunits: unit A, phenyloctanoic acid, unit B, 3-chloro-*O*-methyl-*D*-tyrosine, unit C, 3-amino-2(*R*)-methylpropionic acid, and unit D, *L*-leucic acid (Scheme 1).

Precursor-incorporation studies have indicated that 3-amino-2(*R*)-methylpropionate integrated as unit C into the cryptophycins is generated by decarboxylation of (2*S*,3*R*)-3-methylaspartate (**1**).<sup>[3]</sup> However, there have been no reports of an enzyme with confirmed  $\beta$ -methylaspartate- $\alpha$ -decarboxylase activity in microbial primary or secondary metabolism. In addition, at least five cryptophycin analogues contain  $\beta$ -alanine as unit C. Bioinformatic analysis of the cryptophycin biosynthetic gene cluster<sup>[3]</sup> has revealed that it contains an open-reading frame for a protein product (CrpG) that bears high similarity to pyruvyl-dependent aspartate decarboxylases (Figure 1).

The pyruvyl-dependent aspartate decarboxylases are members of a unique group of mechanistically related enzymes, which include *S*-adenosylmethionine decarboxylase, phosphatidylserine decarboxylase, proline reductase, and bacterial histidine decarboxylase. These enzymes are initially expressed in a proenzyme form ( $\pi$ ), which is proteolytically cleaved at an X–Ser bond by an internal serine residue, to produce a  $\beta$ -subunit that contains a pyruvyl group at its N terminus and an  $\alpha$ -subunit that contains a C-terminal acid (Scheme 2A).<sup>[4]</sup> As a prelude to catalysis, the pyruvyl group forms a Schiff's base with the amine of the amino acid to be decarboxylated (Scheme 2B).



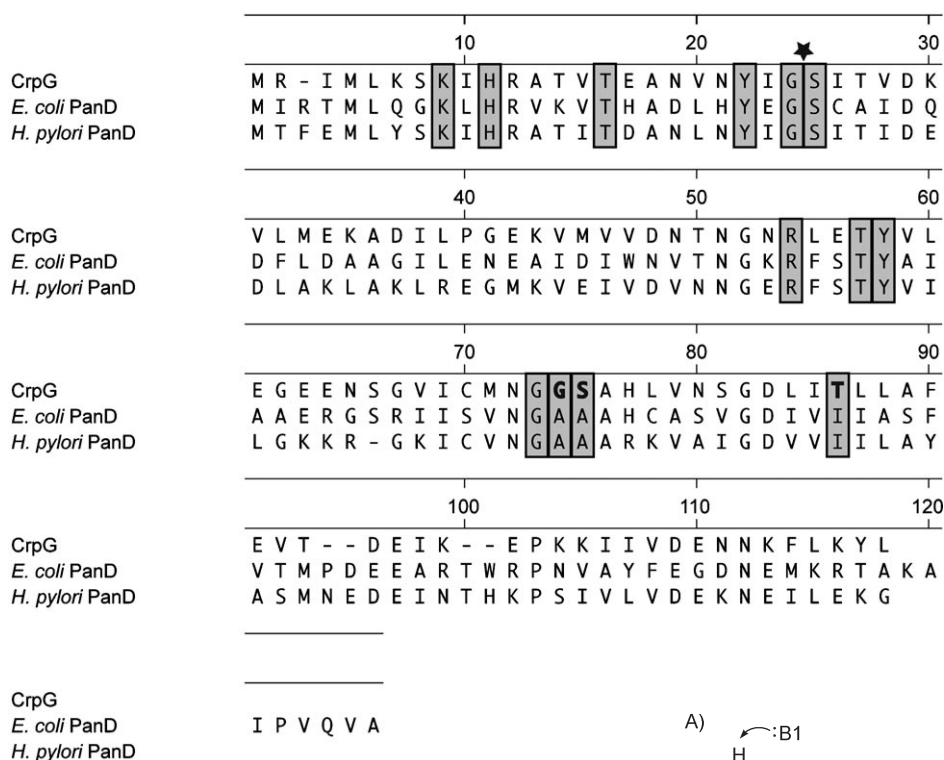
**Scheme 1.** Proposed biosynthesis and incorporation of 3-amino-2(*R*)-methylpropionic acid into unit C of cryptophycin 1.

Previous studies have shown that the aspartate decarboxylase (PanD) from *E. coli* fails to bind all possible stereoisomers of  $\beta$ -methylaspartate.<sup>[5]</sup> Moreover, analysis of the crystal structures of *E. coli* and *Helicobacter pylori* *L*-aspartate decarboxylases revealed highly conserved amino acids in their respective active sites that are in direct contact with the substrate.<sup>[6,7]</sup> Comparison of the identity of these amino acid residues with CrpG indicated that the following three amino acid residues are variant in CrpG: Gly72, Ser73, and Thr84 (Figure 1). This information, in conjunction with the precursor-incorporation study results, led us to hypothesize that the putative *crpG* decarboxylase gene product is responsible for the decarboxylation of **1** to form 3-amino-2(*R*)-methylpropionic acid, which is subsequently incorporated into the cryptophycins (Scheme 1). In this report we describe the cloning, gene over-expression, purification of the *crpG* gene product, synthesis of substrates, and biochemical characterization of CrpG substrate specificity.

The cryptophycin *crpG* expression vector was initially engineered to produce CrpG with a C-terminal His<sub>6</sub>-tag, as this type of translational fusion protein did not interfere with the function of aspartate decarboxylase from *H. pylori*.<sup>[8]</sup> However, the low enzymatic activity of the protein obtained from this construct indicated that the His<sub>6</sub>-tag did significantly compromise CrpG function. Therefore, a new expression vector was designed to produce CrpG without modification to the N or C termini. This protein product was purified by using anion-exchange chromatography to > 95% homogeneity (Figure S1A in the Supporting Information). Mass spectrometry was used to confirm that the proenzyme ( $\pi$ ) was autoprocessed into  $\alpha$  and  $\beta$  subunits with expected masses (Figure S2). The autoprocessing reaction of *E. coli* aspartate decarboxylase has been

[a] Dr. Z. Q. Beck, Dr. D. A. Burr, Dr. D. H. Sherman  
Life Sciences Institute, Departments of Medicinal Chemistry,  
Chemistry, Microbiology, and Immunology, The University of Michigan  
Ann Arbor, MI 48109 (USA)  
Fax: (+1) 734-615-3641  
E-mail: davidhs@umich.edu

Supporting information for this article is available on the WWW under  
<http://www.chembiochem.org> or from the author.



**Figure 1.** Primary sequence alignment of CrpG, *E. coli* PanD, and *H. pylori* PanD. Boxed and shaded amino-acid residues are highly conserved in the active sites of L-aspartate decarboxylases. Amino acids in the active site of CrpG that differ from the highly conserved residues are displayed in bold. The star indicates the site of self-processing.

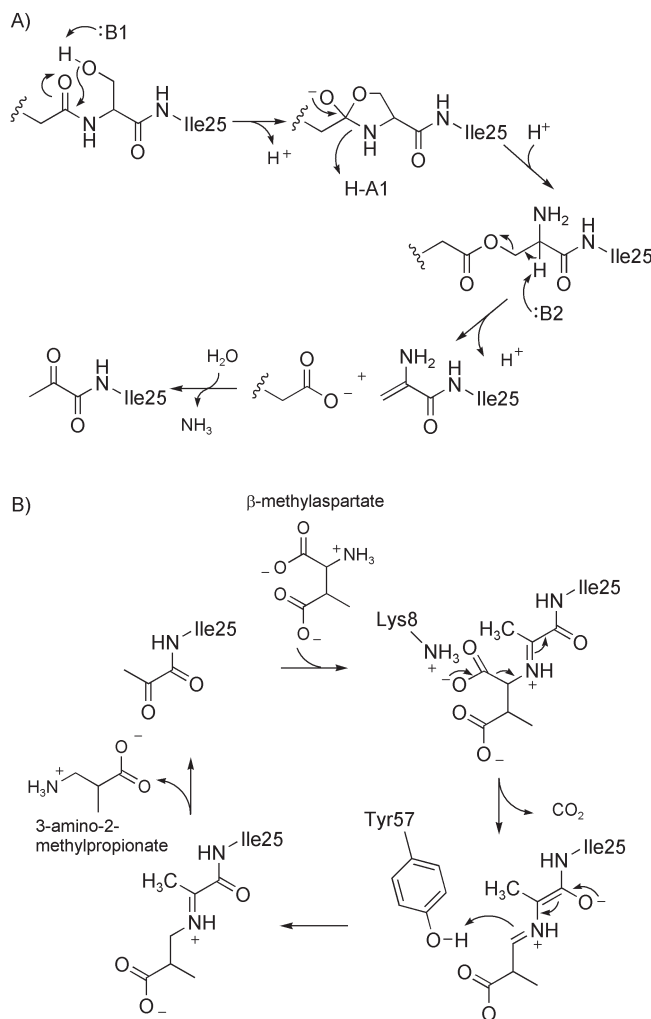
reported to have a half-life of approximately 16 h at 50 °C.<sup>[9]</sup> We determined that the half-life for autoprocessing of CrpG is approximately 8 h at 37 °C and 2 h at 50 °C (Figure S1 B).

CrpG was probed for its ability to decarboxylate L-aspartate, D-aspartate, (2*S*,3*R*)-3-methylaspartate (**1**), (2*S*,3*S*)-3-methylaspartate (**2**), (2*R*,3*R*)-3-methylaspartate (**3**), and (2*R*,3*S*)-3-methylaspartate (**4**). The absolute stereochemistry of **1–4** was confirmed from crystallographic data of the fully protected (2*S*,3*R*)-3-methylaspartate. The synthesis of these compounds is detailed in the Supporting Information. The results of the decarboxylation assay are summarized in Table 1.

Substrate	$k_{cat}$ [s <sup>-1</sup> ]	$K_M$ [μM]	$k_{cat}/K_M$ [s <sup>-1</sup> μM <sup>-1</sup> ]
L-aspartate	0.008 ± 0.001	8100 ± 900	9.8 × 10 <sup>-7</sup> [a] 6.7 × 10 <sup>-7</sup> ± 0.8 × 10 <sup>-7</sup> [b]
D-aspartate	–	–	–
<b>1</b>	0.34 ± 0.04	600 ± 40	5.6 × 10 <sup>-4</sup> [a] 4.5 × 10 <sup>-4</sup> ± 0.8 × 10 <sup>-4</sup> [b]
<b>2</b>	–	–	9.0 × 10 <sup>-8</sup> ± 1 × 10 <sup>-8</sup> [b]
<b>3</b>	–	–	< 1.6 × 10 <sup>-8</sup>
<b>4</b>	–	–	< 1.6 × 10 <sup>-8</sup>

[a] Calculated from  $k_{cat}$  and  $K_M$  values. [b] Calculated by using the reduced form of the Henri–Michaelis–Menten equation: rate =  $k_{cat}/K_M[S]$ , and by assuming that the substrate concentration is considerably below the  $K_M$ .

CrpG catalyzed the decarboxylation of **1** approximately 2–3 orders of magnitude more efficiently than L-aspartate, and 3–4 orders of magnitude more efficiently than **2** as determined by comparing their  $k_{cat}/K_M$  values. The  $k_{cat}/K_M$  value of compound **2** was determined by using a reduced form of the Henri–Michaelis–Menten equation because the solubility was insufficient for determination of individual  $k_{cat}$  and  $K_M$  values. The  $k_{cat}/K_M$  values of L-aspartate and **1** were also determined by using the reduced form of the Henri–Michaelis–Menten equation to allow for direct compari-



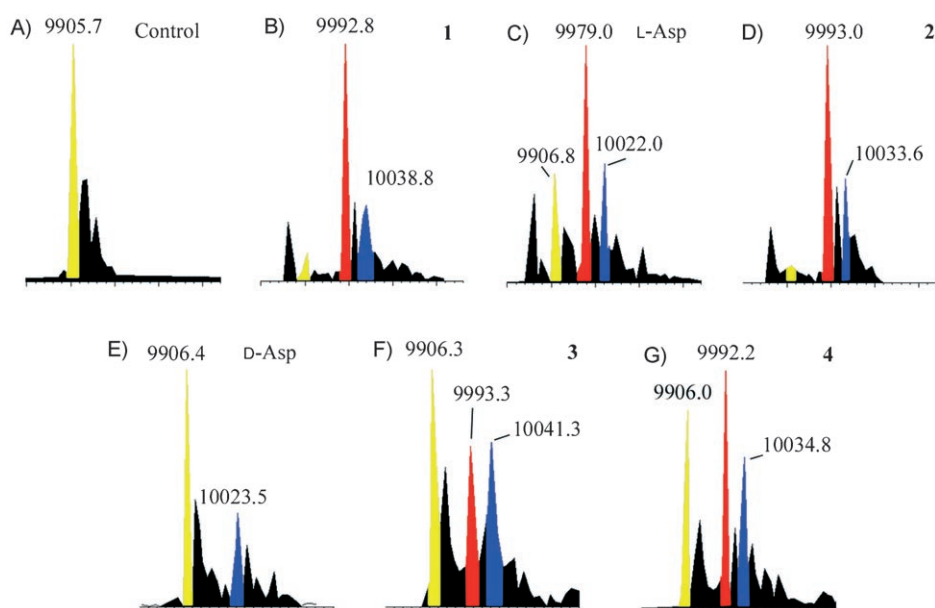
**Scheme 2.** Proposed mechanism for A) formation of the pyruvoyl group between amino acids Gly23 and Ser24 of CrpG, and B) the decarboxylation of β-methylaspartate by CrpG.

son. Decarboxylation of **3** and **4** were detected; however, the rates of the reactions could not be determined accurately due to their extremely low turnover rates. An upper limit for their  $k_{\text{cat}}/K_M$  values was approximated based on the small amount of product that was detected. No product was detected by HPLC when CrpG was incubated with D-aspartate.

The interaction of the decarboxylase with all substrates was probed further by trapping the imine intermediates formed during catalysis to the enzyme by using sodium cyanoborohydride (Figure 2A–G). These data indicated that all substrates tested formed covalent intermediates with the CrpG polypeptide. Interestingly, the decarboxylated form of each substrate bound to CrpG was observed except for D-aspartate; this indicates that D-aspartate can bind the enzyme, but decarboxylation does not occur (Figure 2E).

In conclusion, CrpG preferentially catalyzes the decarboxylation of **1** to form 3-amino-2(R)-methylpropionic acid that serves as the most prevalent unit C precursor in the biosynthesis of the cryptophycins. The ability of CrpG to decarboxylate L-aspartate suggests the possibility that the  $\beta$ -alanine incorporated into some analogues of cryptophycin is generated by CrpG. The results denote the first biochemically validated example of a  $\beta$ -methylaspartate- $\alpha$ -decarboxylase. Amino-acid residues Gly72, Ser73, and Thr84 could play a role in the substrate specificity of CrpG and are attractive targets for future mutagenesis studies directed towards altering the precursor pool for production of cryptophycins.

CCDC-645609 contains the supplementary crystallographic data for this paper. These data can be obtained free of charge from The Cambridge Crystallographic Data Centre via [http://www.ccdc.cam.ac.uk/data\\_request/cif](http://www.ccdc.cam.ac.uk/data_request/cif)



**Figure 2.** Trapping of the imine intermediates formed with the N-terminal pyruvoyl group of CrpG with sodium cyanoborohydride. A) Without substrate, B) with **1**, C) with L-Asp, D) with **2**, E) with D-Asp, F) with **3**, G) with **4**. Yellow shading indicates the processed  $\alpha$ -subunit; red shading indicates decarboxylated substrate bound to the  $\alpha$ -subunit; blue shading indicates substrate bound to the  $\alpha$ -subunit.

## Acknowledgements

We are grateful to Sabine Grünschow and Brian F. Pflieger for assistance with LC-MS. This research was supported financially by NIH grant CA108874 and the Searle Professorship to D.H.S.

**Keywords:** biosynthesis · cryptophycin · decarboxylases · metabolism · natural products

- [1] G. Trimurtulu, I. Ohtani, G. M. L. Patterson, R. E. Moore, T. H. Corbett, F. A. Valeriote, L. Demchik, *J. Am. Chem. Soc.* **1994**, *116*, 4729.
- [2] C. D. Smith, X. Zhang, S. L. Mooberry, G. M. Patterson, R. E. Moore, *Cancer Res.* **1994**, *54*, 3779.
- [3] N. A. Magarvey, Z. Q. Beck, T. Golakoti, Y. Ding, U. Huber, T. K. Hemscheidt, D. Abelson, R. E. Moore, D. H. Sherman, *ACS Chem. Biol.* **2007**, *1*, 766.
- [4] J. M. Williamson, G. M. Brown, *J. Biol. Chem.* **1979**, *254*, 8074.
- [5] M. E. Webb, E. Stephens, A. G. Smith, C. Abell, *Chem. Commun.* **2003**, 2416.
- [6] A. Albert, V. Dhanaraj, U. Genschel, G. Khan, M. K. Ramjee, R. Pulido, B. L. Sibanda, F. von Delft, M. Witty, T. L. Blundell, A. G. Smith, C. Abell, *Nat. Struct. Biol.* **1998**, *5*, 289.
- [7] B. I. Lee, S. W. Suh, *J. Mol. Biol.* **2004**, *340*, 1.
- [8] S. Chopra, H. Pai, A. Ranganathan, *Protein Expression Purif.* **2002**, *25*, 533.
- [9] M. K. Ramjee, U. Genschel, C. Abell, A. G. Smith, *Biochem. J.* **1997**, *323*, 661.

Received: March 30, 2007

Published online on June 28, 2007