Expressed protein ligation to probe regiospecificity of heterocyclization in the peptide antibiotic microcin B17

Ranabir Sinha Roy*†, Owen Allen† and Christopher T Walsh

Background: The *Escherichia coli* peptide antibiotic microcin B17 (MccB17) contains thiazole and oxazole heterocycles derived from a distributive yet directional cyclization of cysteines and serines in the McbA precursor catalyzed by MccB17 synthetase. Whether the formation of upstream rings potentiates downstream heterocyclization has not been previously determined.

Results: McbA fragments (46–61 residues) containing glycine substitutions or homocysteine at select upstream cysteine or serine sites were assembled using expressed protein ligation (EPL). Most of these substrates were only partially cyclized by MccB17 synthetase, in contrast to the efficient processing of wild-type $McbA_{1-61}$. Homocysteine was not processed to the six-membered heterocycle.

Conclusions: The formation of upstream rings in McbA potentiates the cyclization of carboxy-terminal cysteines and serines, probably by selecting against unfavorable substrate conformations. EPL allows structure–function analysis including unnatural amino acid placements to probe the regiospecificity and chemoselectivity of post-translational heterocyclization during antibiotic maturation.

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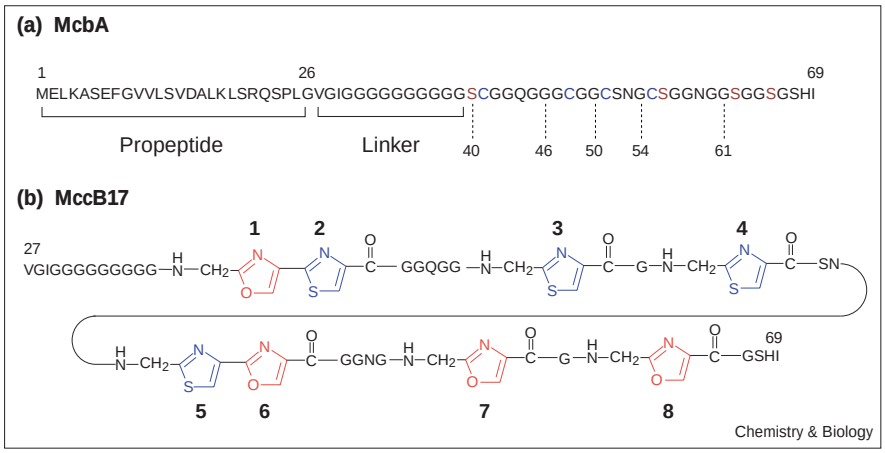
Introduction

Several natural products contain oxazole and thiazole heterocycles derived from precursor peptides containing Xaa–Ser/Cys sequences (where Xaa represents any amino acid) [1]. These molecules often exhibit useful therapeutic properties, ranging from the anticancer activity of the bithiazole-containing drug bleomycin A [2], to the gram-positive antibiotic activity of thiostrepton [3], which is assembled around a pyridine-trithiazole scaffold. Microcin B17 (MccB17), a DNA gyrase inhibitor secreted by certain strains of *Escherichia coli* [4,5], belongs to this family and contains two oxazoles, two thiazoles, a 4,2-linked oxazole–thiazole, and the analogous 4,2-thiazole–oxazole, all within a stretch of 43 residues. The cloning of the genes for MccB17 production (*mcbABCD*) [6], export (*mcbEF*) and immunity (*mcbG*) [7], and subsequent purification of the heterocyclization catalyst (the multimeric MccB17 synthetase enzyme complex composed of the McbB,C,D gene products) [8], have made the biosynthesis of this peptide antibiotic a convenient system for investigating the mechanism and specificity of post-translational heterocyclization of serine and cysteine residues to oxazoles and thiazoles.

Certain structural elements of the MccB17 precursor polypeptide (the 69 amino acid McbA gene product; Figure 1) are important for substrate recognition and heterocyclization by the synthetase. In particular, a *cis*-acting amino-terminal 26-residue helical propeptide sequence is critical for synthetase recognition [8,9], and an adjacent 13-residue polyglycine linker sets the register for downstream Gly–Ser, Gly–Cys, Gly–Ser–Cys and Gly–Cys–Ser sequences that are cyclized to the eight heterocycles in mature MccB17 (numbered consecutively from the amino terminus in Figure 1) [10]. The detection of partially processed McbA intermediates containing 1–7 rings using mass spectrometry (N.L. Kelleher, C. Hendrickson and C.T.W., unpublished observations), together with the purification of partially cyclized MccB17 isoforms from extracts of microcin-producing *E. coli* cells [11], reveals that oxazole and thiazole formation occurs distributively rather than processively, with partially cyclized intermediates being released from the enzyme complex. Tandem mass spectrometry (MS/MS) analysis of these intermediates (N.L. Kelleher, C. Hendrickson and C.T.W., unpublished observations) has shown that the overall heterocyclization of McbA is remarkably directional (amino → carboxyl terminus), given that the modified cysteines and serines are remote in primary sequence from the avidly recognized propeptide helix and are located within a glycine-rich domain ($McbA_{39-65}$) that does not contribute significantly to the binding affinity of the substrate [9]. These observations indicate that the processing

Figure 1



Sequence of (a) the McbA precursor and (b) the mature MccB17 antibiotic. The McbA fragments described in this study are delineated by residue number (dotted lines). The propeptide and linker regions in McbA are also labeled. The four oxazoles (red) and four thiazoles (blue) in mature MccB17 are numbered consecutively from the amino terminus.

of McbA may be influenced by subtle effects that extend beyond the propeptide–synthetase interaction.

To determine whether heterocycle formation influences the directional processing of McbA, we have replaced select upstream Ser/Cys cyclization sites with glycine residues. Whether or not these 'knockout' mutations (corresponding to rings 1–4) affect the heterocyclization of downstream cysteines and serines has been investigated in a set of McbA fragments encompassing one or more heterocyclization sites (rings 2–6). To date, such studies have been precluded by difficulties in the assembly of the requisite mutant McbA sequences. Site-directed mutagenesis efforts have been hampered by codon degeneracy and mispriming in the polyglycine coding regions of *mcbA*, and the solid-phase synthesis of such McbA analogs suffers from low yields because of the high glycine content (up to 48%), which results in poor coupling efficiencies.

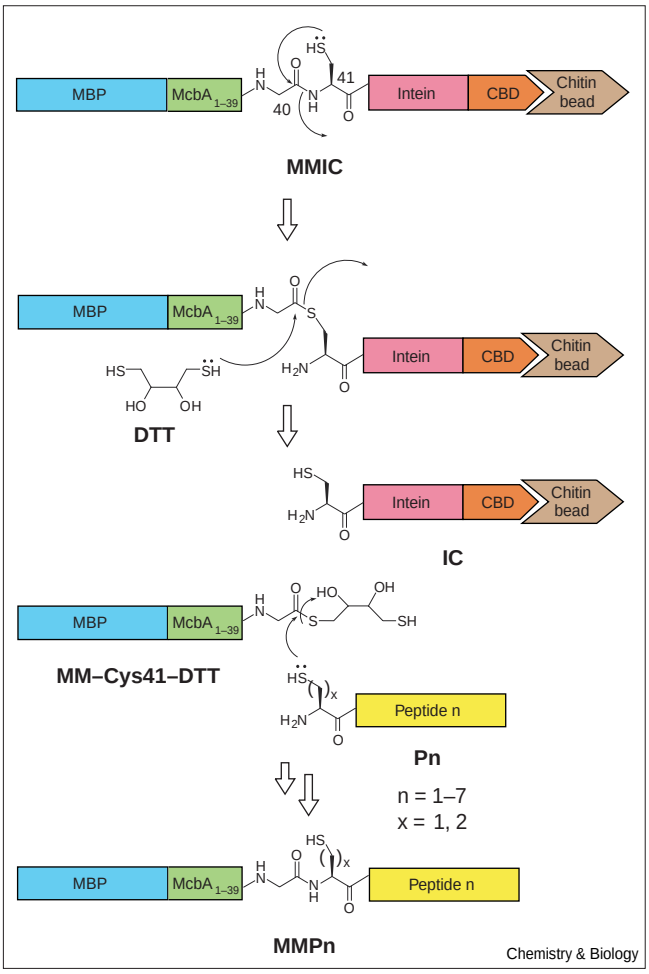
Recently, the method of expressed protein ligation (EPL) has been described as a strategy that exploits the machinery of protein splicing [12] to ligate synthetic peptides to the carboxyl termini of recombinant proteins [13]. We have now utilized this technique to generate the McbA knockout mutants as contiguous sequences derived from synthetic peptides (6–21 residues) that are ligated to the first 40 residues of a mutant McbA protein (containing a Ser40→Gly mutation), which is tagged by maltose-binding protein (MBP). The MBP-tagged $McbA_{1–40(S40G)}$ serves as the substrate-recognition determinant for intein catalysis (Figure 2). The heterocyclization of these mutant McbA domains (46–61 residues) within the ligated MMP (MBP–$McbA_{1–40(S40G)}$–peptide) chimeras, when compared with that of His_6-tagged $McbA_{1–61}$, reveals the importance of upstream rings for the efficient processing of McbA and the biosynthesis of the eight-ring MccB17 antibiotic.

Results

Development of an EPL strategy for the McbA knockout mutants

A tetramodular MBP–$McbA_{1–40(S40G)}$–intein–CBD (MMIC; CBD is a chitin-binding domain) fusion protein containing the MccB17 substrate-recognition domain, an intein domain for protein splicing, and suitable affinity tags for rapid purification was designed to enable assembly of knockout mutants using EPL. The expression vector for recombinant MMIC protein was constructed by fusing sequentially and in frame the genes encoding MBP, a linker containing a thrombin restriction site (LVPRGS, using single-letter amino acid code), and the $McbA_{1–40(S40G)}$ mutant fragment, to the coding sequence of the *Saccharomyces cerevisiae* VMA1 intein [14] fused to a CBD in the commercially available pTYB1 vector (New England Biolabs, MA). The MBP tag provides a useful epitope at the amino terminus of the $McbA_{1–40(S40G)}$ substrate-recognition determinant for immunoblot analysis of the ligation products, does not interfere with heterocyclization by MccB17 synthetase [10], and can be removed by a thrombin digest prior to mass spectrometric analysis of the heterocyclization products. The Ser40→Gly mutation in $McbA_{1–40}$ introduces a Gly40–Cys41 splice junction between the $McbA_{1–40}$ and intein domains (using the numbering scheme of McbA), which favors the formation of a Cys41 α-thioester during intein-mediated splicing. This mutation also corresponds to deletion of the amino-terminal oxazole in processed McbA (ring 1), which allowed the importance of this heterocycle in the cyclization of downstream sites to be determined. The dual-tagged MMIC protein was expressed under the control of the T7 promoter in *E. coli* BL21(DE3) cells, and immobilized on an affinity column prior to the ligation reactions. Although MMIC can be purified using either MBP-directed (amylose) or CBD-directed (chitin) column strategies, the latter gave consistently higher yields of ligated product, and was adopted as the default method for protein purifica-

Figure 2



Construction of McbA knockout mutants using expressed protein ligation (EPL). The tetramodular MMIC (MBP–$McbA_{1–40(S40G)}$–intein–chitin-binding domain) protein is initially immobilized on a chitin column and incubated with dithiothreitol (DTT) and synthetic peptides P1–P7. The intein-catalyzed splicing reaction involves the intermediacy of an α-thioester (MM–Cys41–DTT, using the numbering scheme of McbA), which is captured via transthioesterification by an amino-terminal cysteine (or homocysteine) residue in the target peptide. The ligated sequences in chimeras MMP1–MMP7 encompass 46, 50, 54 or 61 residues of McbA, and selectively elute from the column. The intein–CBD (IC) fragment and unreacted MMIC substrate are retained on the chitin beads.

tion and immobilization. The crude protein extract was loaded on a chitin column, which was washed extensively with column buffer. The chitin beads were then incubated in 30 mM dithiothreitol (DTT) to promote formation of the Cys41-DTT α-thioester (MM–Cys41–DTT; Figure 2), immediately prior to the actual transthioesterification/ligation reaction with target peptides P1–P7.

MM–Cys41-DTT is a viable substrate for EPL

The first target peptide to be tested for EPL-mediated splicing with the MMIC protein and subsequent heterocyclization by MccB17 synthetase was the hexapeptide P1

Table 1

Primary sequence of synthetic peptides used for EPL in this study.

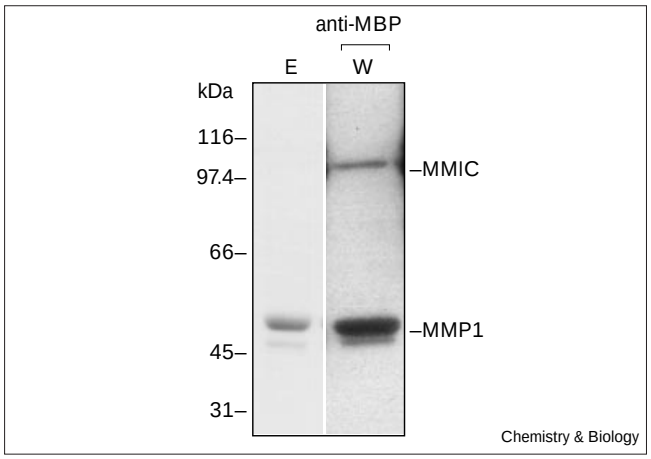
Peptide	Sequence
	41 46 50 54 61 69
$McbA_{41–69}$	C G G Q G G G C G G C S N G C S G G N G G S G G S G S H I
P1	C G G Q G G
P2	C G G Q G G G C G G
P3	C G G Q G G G G G G C S G G
P4	C G G Q G G G G G G G G N G C S G G N G G
P5	C G G Q G G G G G G G G N G C C G G N G G
P6	X G G Q G G
P7	X G G Q G G G C G G

The sequence of $McbA_{41–69}$ is shown for comparison. Cysteine and serine residues that are cyclized to thiazoles and oxazoles in native MccB17 are highlighted in orange, as are potentially cyclizable residues in peptides P1–P7. Two serine residues (underlined) remain unprocessed in the mature antibiotic. Residue substitutions compared with the wild-type sequence are highlighted in red. X, homocysteine.

(NH_2-CGGQGG-$CONH_2$, Table 1), which corresponds to the $McbA_{41–46}$ fragment. The amino-terminal cysteine residue of this target sequence, essential for the ligation methodology (Figure 2), is also the sole residue that can be subsequently cyclized (ring 2 of MccB17). Incubation of the 103.9 kDa MMIC protein (~40 nmoles, immobilized on chitin beads and activated with DTT), with 2 ml of a 2 mM solution of peptide P1 (100-fold molar excess) for 48 hours led to the elution of a lower molecular weight species (lane E, Figure 3), which migrated in sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) with a mass corresponding to that of the anticipated MBP–$McbA_{1–40}$–P1 chimera (MMP1; 46.9 kDa). To confirm its identity, this ligated product was subjected to immunoblot analysis, using either anti-MBP or anti-intein antibodies as probes. The MMP1 chimera was recognized only by anti-MBP antibodies, which target the amino-terminal MBP domain (lane W, Figure 3). The absence of a corresponding immunoblot signal with anti-intein antibodies (data not shown) confirmed the intein→P1 fragment swap that released the MMP1 chimera from the 57.5 kDa intein–CBD portion of MMIC ('IC'; Figure 2), and permitted its selective elution from the chitin beads. Residual full-length MMIC protein and the IC fragment (which was significantly degraded by proteolysis) were subsequently stripped from the chitin beads by an SDS wash and their identities confirmed by similar immunoblot analysis (data not shown). Typically, 5–15 nmoles of purified EPL chimera were obtained after buffer exchange and concentration of the eluate.

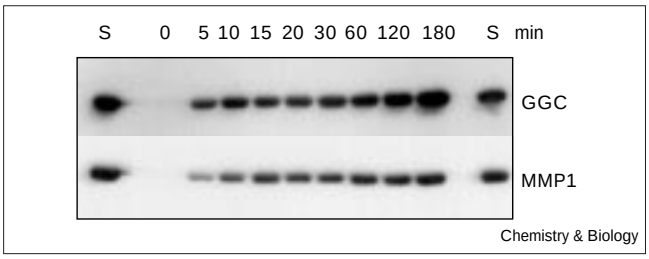
The MMP1 chimera represents an amino-terminal MBP fusion of the contiguous $McbA_{1–46(S40G)}$ fragment, which contains a single Gly39–Gly40–Cys41 sequence that is the precursor to a monothiazole. An authentic sample of this

Figure 3



SDS–PAGE and immunoblot analysis of EPL with the MMIC substrate and peptide P1. The ligated MBP–$McbA_{1–40(S40G)}$–P1 (MMP1) chimera elutes from the chitin column with the anticipated mass (46.9 kDa, lane E). The amino-terminal MBP tag in this protein is recognized by anti-MBP antibodies in the western analysis of the eluate (lane W), which also reveals traces of full-length MMIC protein (103.9 kDa). The faint (< 10%) band at ~46 kDa in each lane is consistent with unligated MBP–$McbA_{1–40(S40G)}$ protein (46.4 kDa) resulting from background hydrolysis of the MM–Cys41-DTT thioester intermediate.

Figure 4



Immunoblot assay for heterocyclization of MBP–$McbA_{1–46(S40G)}$. Samples of this McbA analog prepared by site-directed mutagenesis (MBP–$McbA_{1–46(S40G)}$, 'GGC') or EPL (MMP1) were incubated with MccB17 synthetase and analyzed for heterocycle formation using a western blot assay and anti-MccB17 antibodies as described in the text. A time-dependent immunoblot signal corresponding to formation of the monothiazole species was observed in both cases. Authentic MBP–$McbA_{1–46(S40G)}$ monothiazole was used as a standard (S).

fusion protein (MBP–$McbA_{1–46(S40G)}$, 'GGC') constructed using conventional cloning techniques has previously been reported [15]. Formation of the monothiazole in both samples by affinity-purified MccB17 synthetase [10] was monitored using an immunoblot assay with heterocycle-recognizing (anti-MccB17) antibodies [16]. As anticipated, time-dependent heterocycle formation was observed with the MMP1 chimera (Figure 4), confirming the ability to generate competent heterocyclization substrates using EPL.

Heterocyclization efficiency of chimeras MMP2–MMP5 lacking select upstream Cys/Ser sites

For the next set of substrates, McbA fragments up to 21 residues in length were synthesized (Table 1, peptides P2–P5). These sequences incorporate cyclizable cysteine and serine residues at various loci (precursors to rings 2–6), with glycine substitutions at specific sites (rings 3 and/or 4). Each peptide was ligated to the $McbA_{1–40(S40G)}$ domain in MMIC to generate McbA sequences corresponding to 50, 54 or 61 residues, with a common cyclization site (Gly40–Cys41) being retained at the splice junction. Chimera MMP2 introduces a second cysteine (Cys48, ring 3), whereas MMP3 has a Gly48 knockout at this position with a Gly50–Cys51–Ser52–Gly53 site further downstream (a variant of the Gly50–Cys51–Ser52–Asn53 precursor sequence for ring 4). The P4 and P5 pair of 21-mers each create a ligated $McbA_{1–61}$ with glycine residues in place of Ser40 (ring 1), Cys48 (ring 3), Cys51 (ring 4) and Ser52 (which remains uncyclized in mature MccB17). Chimera MMP4 contains the Gly54–Cys55–Ser56 sequence that forms a 4,2-linked oxazole–thiazole in MccB17 (rings 5 and 6), whereas MMP5 contains a non-native 4,2-bithiazole precursor sequence (Gly54–Cys55–Cys56) at this site.

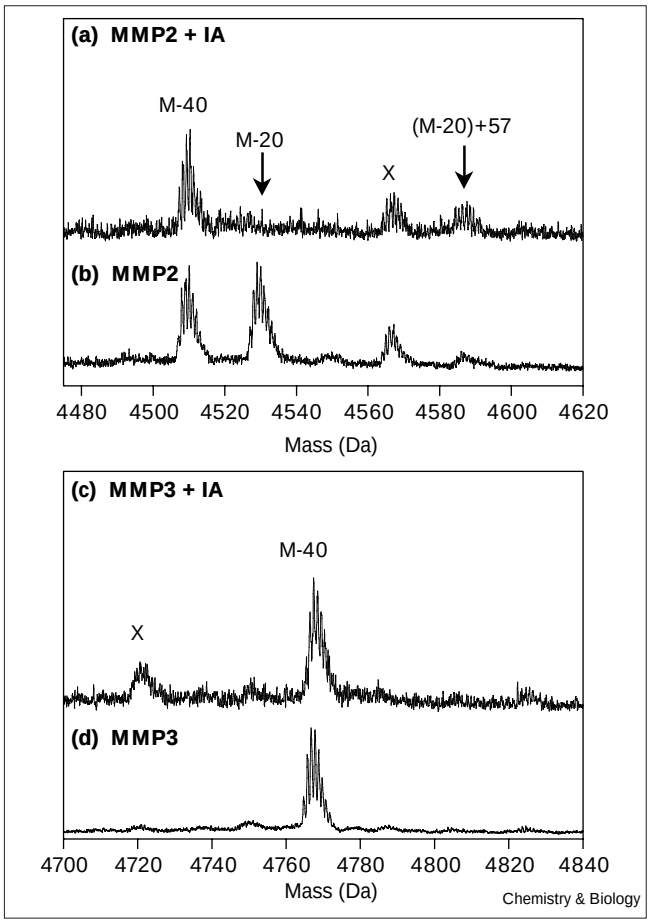
As with the MMP1 substrate, a time-dependent increase in immunoblot signal with anti-MccB17 antibodies corresponding to the formation of heterocyclic products was observed for each chimera MMP1–MMP5 (data not shown), consistent with the cyclization of at least one cysteine and/or serine residue within the corresponding target sequences. To determine whether Cys/Ser residues downstream of the Cys41 (monothiazole) junction were being converted to heterocycles, the products were further analyzed by matrix-assisted laser desorption ionization mass spectrometry (MALDI–TOF MS) after cleavage of the MBP tag with thrombin, which leaves a Gly–Ser dipeptide moiety appended to the amino terminus. This MS analysis allowed the heterocycle content of each cyclized McbA fragment to be determined explicitly (20 Da decrease in mass per heterocycle formed, corresponding to the loss of water and two hydrogen atoms). The MALDI–TOF mass spectrum of the Gly–Ser–$McbA_{1–50}$ mutant sequence from thrombin-digested MMP2 (two potential thiazoles from Cys41 and Cys48), after incubation with synthetase for 24 hours, is illustrated in Figure 5b. Molecular ions corresponding to a 1:1 mixture of monothiazole (M-20) and bithiazole (M-40) species were observed. A similar analysis of processed MMP1 confirmed that Cys41 was quantitatively cyclized to the monothiazole (data not shown). In a separate study, tandem MS of partially processed McbA substrates has revealed that Cys41 is cyclized to the thiazole prior to Cys48 (N.L. Kelleher, C. Hendrickson and C.T.W., unpublished observations). Taken together, these data suggest that it is the downstream Cys48 residue that fails to get completely modified in the MMP2 chimera. The

yield of bithiazole product did improve slightly (59%) upon doubling the incubation time with enzyme, so subsequent substrates were incubated with the synthetase for 48 hours prior to MS analysis.

The $McbA_{1–54}$ sequence in chimera MMP3 contains three potentially cyclizable residues (Cys41, Cys51–Ser52), which should afford one thiazole and a 4,2-linked thiazole–oxazole. Ser52 remains uncyclized during the biosynthesis of MccB17 from native McbA, however [4]. This regiospecificity may be controlled in part by Asn53, because polar residues (e.g. asparagine or histidine) disrupt heterocyclization when inserted immediately downstream of the Gly39–Ser40–Cys41 bisheterocyclization site in $McbA_{1–46}$ [10]. In an attempt to cyclize Ser52 in the MMP3 chimera, Asn53 was substituted by glycine because this residue flanks each site that is completely processed in McbA. MS analysis of MMP3 after a 48 hour incubation with MccB17 synthetase revealed conversion only to a double-ring (M-40) species (Figure 5d), however. This partially processed product was treated with iodoacetamide in a thiol-directed alkylation strategy to determine whether any of the cysteine residues remained uncyclized. As a positive control, the mixture of heterocyclic MMP2 products previously analyzed by MS was also treated with iodoacetamide to confirm alkylation of the unprocessed cysteine residue in the corresponding monothiazole species. As illustrated in Figure 5a, the resulting mass spectrum revealed a shift in the M-20 molecular ion (by +57 Da), corresponding to acetamidylation of the free cysteine in the monothiazole MMP2 intermediate. The fully cyclized bithiazole M-40 product remained unchanged in mass, consistent with the absence of free thiols in this polypeptide. Similarly, the M-40 molecular ion in the mass spectrum of processed MMP3 did not show an increase in mass upon treatment with the alkylating reagent (Figure 5c). These data strongly suggest that it is the serine residue in the Gly50–Cys51–Ser52 site of MMP3 that fails to get modified to the oxazole.

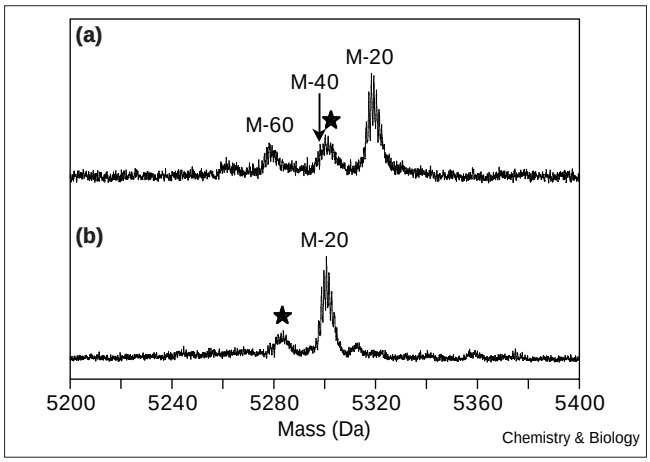
Chimeras MMP4 and MMP5 also contain three cyclizable residues each, including a bisheterocyclic site that is further downstream in primary sequence compared with that in substrate MMP3. The mass spectrum of processed MMP4 (two potential thiazoles from Cys41 and Cys55 and one potential oxazole from Ser56) showed complete conversion only to a monoheterocyclic (M-20) species (Figure 6b). A similar result was obtained with MMP5 (three potential thiazoles from Cys41, Cys55 and Cys56), although traces of the bithiazole (M-40) and trithiazole (M-60) species were also detected (~15% each; Figure 6a). Iodoacetamide alkylation of processed MMP4 resulted in a shift of the (M-20) molecular ion by +57 Da (data not shown), suggesting that one of the two cysteines and the serine residue in this species were not cyclized by the synthetase. Because Cys41 in the splice junction of chimeras MMP1–MMP3 is efficiently processed to the thiazole, and tandem MS has revealed that this residue is precursor to the first heterocycle formed in McbA (N.L. Kelleher, C. Hendrickson and C.T.W., unpublished observations), these data indicate that the downstream Gly54–Cys55–Ser56 site in MMP4 is not detectably modified by the synthetase, and that the corresponding Gly54–Cys55–Cys56 sequence in MMP5 is only inefficiently processed to the monoheterocyclic and bisheterocyclic moieties.

Figure 5



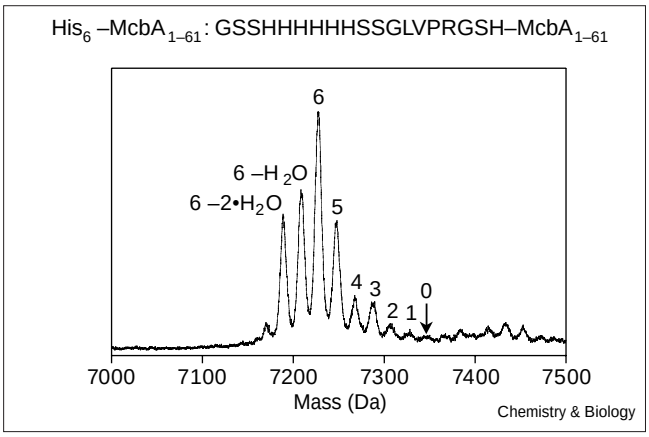
MALDI–TOF MS analysis of heterocyclization in chimeras (a,b) MMP2 and (c,d) MMP3. Mass spectrum of processed MMP2 (b) reveals a 1:1 mixture of monothiazole (M-20; M_{calc} = 4526.2 Da, M_{obs} = 4526.2 Da) and bithiazole (M-40; M_{calc} = 4506.2 Da, M_{obs} = 4507.1 Da) species. Treatment with iodoacetamide (a) causes a shift of the monothiazole molecular ion by + 57 Da (M_{calc} = 4583.2 Da, M_{obs} = 4583.3 Da), corresponding to alkylation of a single cysteine. Mass spectrum of processed MMP3 (d) reveals complete conversion to a bisheterocyclic (M-40; M_{calc} = 4764.3 Da, M_{obs} = 4764.8 Da) species, which is unchanged upon treatment with iodoacetamide (c). Unassigned peaks corresponding to fragments or trace impurities are indicated with an 'X'. These data indicate that although both cysteines in MMP3 are cyclized to thiazoles, the single serine (Ser52) in this substrate remains unprocessed. IA, iodoacetamide.

Figure 6



MALDI–TOF MS analysis of heterocyclization in chimeras (a) MMP5 and (b) MMP4. Incubation of MMP4 with MccB17 synthetase for 48 h resulted in incomplete cyclization to a monoheterocyclic (M-20) species (M_{calc} = 5297.5 Da, M_{obs} = 5299.8 Da). For MMP5, traces (~15%) of bithiazole (M-40) species (M_{calc} = 5296.8 Da, M_{obs} = 5300.0 Da) and trithiazole (M-60) product (M_{calc} = 5276.8 Da, M_{obs} = 5278.2 Da) were observed in addition to the monothiazole (M-20) species (M_{calc} = 5316.8 Da, M_{obs} = 5316.4 Da). Molecular ions marked with a star correspond to neutral loss peaks (loss of ammonia).

Figure 7



MALDI–TOF mass spectrum of processed His_6–$McbA_{1–61}$. Although the amino-terminal His_6 tag incorporates a thrombin restriction site (LVPRGSH), it was retained for the MS analysis. Molecular ions corresponding to species containing 0–6 rings were detected as neutral loss peaks (loss of ammonia). The ring content is listed above each molecular ion. Additional neutral loss peaks (loss of one or two water molecules from the six-ringed species) are also indicated.

Synthetase-mediated processing of His_6–$McbA_{1–61}$ containing intact upstream heterocyclization sites

The His_6-tagged $McbA_{1–61}$ fragment encompasses all four cysteines and two of the four cyclizable serines in full-length McbA, which are located at four distinct sites and are precursors to rings 1–6 of the mature antibiotic. This substrate contains intact upstream sites (corresponding to rings 1–3), some of which were substituted with glycine residues in substrates MMP1–MMP5. In contrast to the incomplete cyclization of most of the chimeric substrates, incubation of His_6–$McbA_{1–61}$ with MccB17 synthetase for 48 hours afforded completely processed (six-ring) polypeptide as the major product (Figure 7). Partially cyclized intermediates (1–5 rings) were also observed, as anticipated for a distributive process. Given that the overall heterocyclization of substrate is directional (amino → carboxyl terminus), these data underline the importance of rings 1–3 for the efficient cyclization of downstream Cys/Ser residues.

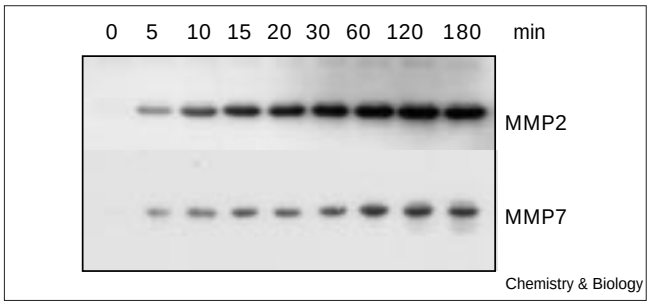
Homocysteine as a substitute for cysteine in ligating peptides

The EPL strategy used with amino-terminal cysteine peptides P1–P5 introduces a Gly40–Cys41 splice junction linking $McbA_{1–40(S40G)}$ to each target peptide in chimeras MMP1–MMP5. We have investigated whether an alternate thiol residue can replace this cysteine to produce a 'silent' splice junction that is not subsequently cyclizable by MccB17 synthetase. The nonstandard residue homocysteine (HCys) was incorporated at the amino termini of target peptides P6 and P7, which are otherwise identical to peptides P1 and P2, respectively (Table 1). The corresponding substrate chimeras MMP6 and MMP7 were readily generated by EPL, although incubation of the peptides with MMIC and DTT was extended to 72 hours, because capture of the MMIC–Cys41-DTT α-thioester by homocysteine is a slower process involving the formation of a six-membered cyclic intermediate. MALDI–TOF MS analysis following thrombin digest of the MBP tags and high performance liquid chromatography (HPLC) purification indicated the correct molecular weight for each McbA analog (data not shown). Because such data cannot confirm rearrangement of the thioester linkages to the amide forms at each homocysteine splice junction [17], each McbA analog was also treated with iodoacetamide to determine the free thiol content. MALDI–TOF analysis revealed quantitative acetamidylation of HCys41 in MMP6 (M_{calc} = 4343.1 Da; M_{obs} = 4343.0 Da), and of both HCys41 and Cys48 in MMP7 (M_{calc} = 4674.2 Da; M_{obs} = 4674.9 Da), confirming that the desired S→N acyl shift had occurred after thiol capture by homocysteine in peptides P6 and P7.

Chimera MMP6 incorporates a Gly40–HCys41 splice junction with no cyclizable cysteines or serines in the ligated sequence. The absence of an immunoblot signal upon incubation of MMP6 with MccB17 synthetase (data not shown) suggested that either the HCys residue was not cyclized to a six-membered heterocycle or the anti-MccB17 antibodies were unable to recognize this modified epitope. MS analysis subsequently revealed that the

MMP6 chimera was indeed not modified by the synthetase, because a single molecular ion corresponding to uncyclized material was observed (M_{calc} = 4286.12 Da, M_{obs} = 4286.21 Da). Chimera MMP7, in contrast, contains an additional Cys48 site downstream of the splice junction, and monothiazole formation in this chimera was confirmed by immunoblot assay (Figure 8). The formation of a single thiazole in MMP7 (from Cys48) compared with two thiazoles in the MMP2 chimera (from Cys41 and Cys48) is predicted to result in a 50% decrease in immunoblot signal intensity with the former, provided the cyclization efficiency remains unchanged. The processing of Cys48 (~59% complete in MMP2) was further reduced in MMP7, however, as revealed by an ~70% decrease in normalized immunoblot signal for the latter (Figure 8). With the proviso that HCys41 does not significantly interfere with the cyclization of downstream Cys/Ser residues, these results identify the thiazole formed from Cys41 in McbA (ring 2) as also being important for increasing the efficiency of downstream heterocyclization.

Figure 8



Immunoblot analysis of processed chimeras MMP2 and MMP7. The Gly40–Cys41 splice junction in MMP2 is replaced by a 'silent' Gly40–HCys41 sequence in MMP7, which does not interfere with downstream heterocyclization. The incomplete formation of thiazole 3 from Cys48 is caused by the absence of oxazole 1 in chimera MMP2 (~59% yield by MS) is more pronounced in MMP7, which lacks both oxazole 1 and thiazole 2 (~48% yield after normalization for heterocycle content).

Discussion

The *E. coli* peptide antibiotic MccB17 undergoes two distinct post-translational modification events to attain antibacterial activity from its 69 amino acid precursor, McbA. The initial cyclodehydration of 14 of the 69 residues at six distinct sites is followed by proteolysis of the first 26 amino acids to produce the 43-residue mature antibiotic containing four oxazoles and four thiazoles [5,16]. Recently, we have detected a ninth heterocycle involving a 4,2-bisheterocycle moiety from Gly50–Cys51–Ser52 in an overproducing strain [11]. It is likely that these tandem heterocycles are DNA intercalating moieties, and initial structure–activity relationship (SAR) studies show that alterations at the Cys55–Ser56 site are more deleterious than those at Ser40–Cys41 for loss of antibiotic activity against sensitive strains of *E. coli* [11].

We have proposed that the heterocyclization of McbA commences when the McbD subunit of the McbB,C,D synthetase complex binds to the amino-terminal propeptide helix of the McbA substrate ($McbA_{1–26}$) [18]. Most of the substrate-binding affinity appears to be derived from this interaction, because the 26 amino acid propeptide fragment alone is a potent competitive inhibitor of the synthetase, with a K_i (2 μM) comparable to the K_M of substrate (2.3 μM) [8]. The McbB subunit is proposed to function as a zinc-dependent cyclodehydratase that converts cysteine and serine residues in McbA (downstream of the $McbA_{27–39}$ polyglycine spacer) to the corresponding oxazole and thiazole heterocycles. The desaturation of these 4,5-dihydro intermediates to oxazoles and thiazoles is presumably catalyzed by McbC, a putative dehydrogenase that contains flavin. Substrate-dependent ATP hydrolysis (most probably by McbD [19]) is critical for the heterocyclization process, and is proposed to provide a conformational switch that either activates the synthetase complex, or serves a motor function in the distributive heterocyclization of all eight Cys/Ser residues.

Although the model for substrate processing by MccB17 synthetase reconciles the proposed chemistry of heterocyclization with the issue of subunit function in the multimeric enzyme complex [18], it does not provide insight into the directional, yet distributive mode of heterocyclization that is observed both *in vitro* [10,11] and *in vivo* [11]. Specifically, it does not explain how the synthetase manages to release partially cyclized intermediates without disrupting the order (amino → carboxyl terminus) in which the six heterocyclization sites (dispersed over 30 residues in McbA) are processed. Because the glycine-rich domain in which these residues are located ($McbA_{39–65}$) presumably adopts a random-coil conformation, and competition assays have revealed that the unprocessed $McbA_{27–69}$ fragment alone is not detectably bound by the synthetase [8,9], a possible explanation for the observed directionality is that the formation of rings proximal to the tightly bound amino-terminal propeptide helix results in conformational or structural perturbations that potentiate the cyclization of remaining sites further downstream in the substrate. To determine the validity of this hypothesis, we have altered the carboxy-terminal third of McbA, focusing on the $McbA_{40–61}$ sequence, which contains six of the eight cyclizable residues (lacking oxazoles 7 and 8).

Two features of the McbA substrate and its recognition by MccB17 synthetase make extensive SAR studies of carboxy-terminal segments problematic. First, the amino-terminal 39 residues must be connected in *cis* [8,9] and second, the run of glycines between residues 30 and 39 renders mutagenesis or chemical synthesis strategies

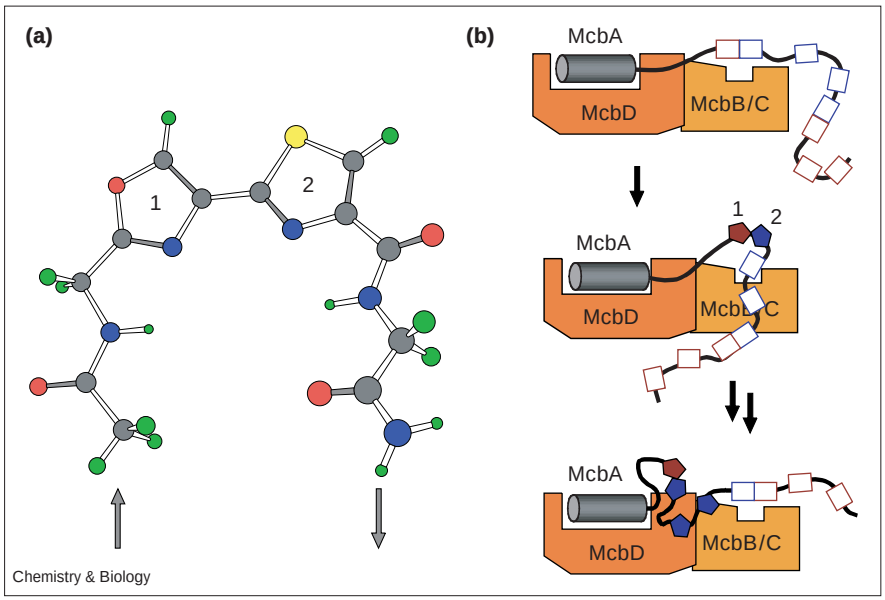
error-prone when exploring variations in Cys/Ser sites between positions 40 and 65. We turned, therefore, to the recently described EPL methodology of Muir *et al.* [13], using the tetramodular MMIC protein as substrate and catalyst. Muir *et al.* [13] utilized thiophenol to facilitate EPL catalysis; we have used DTT instead, such that the substrate for thiol capture by nucleophilic peptides P1–P7 is the nascent MM–Cys41-DTT α-thioester intermediate generated by intein catalysis. An S→N acyl shift subsequently completes the peptide ligation. The EPL strategy is robust, and in its present form can be generalized for constructing McbA analogs with any sequence variation in residues 42–69 to probe determinants of substrate heterocyclization or antibiotic activity. Purification of the ligated MMP products is straightforward and the EPL protocols have been optimized to minimize any competing hydrolysis of MM–Cys41-DTT before capture by the synthetic peptides, which would yield contaminating MBP–$McbA_{1–40(S40G)}$ protein (a competitive inhibitor of MccB17 synthetase).

The processing of EPL chimeras MMP1–MMP7 by affinity-purified MccB17 synthetase was compared with that of His_6-tagged $McbA_{1–61}$, which contains intact sites for rings 1–6. The heterocyclization of each substrate was measured qualitatively using immunoblot assays (with thiazole- and oxazole-recognizing anti-MccB17 antibodies) and quantitatively using MALDI–TOF MS (20 Da decrease in mass per heterocycle formed). A typical distributive pattern of heterocyclization was observed for His_6–$McbA_{1–61}$, with detection of the entire spectrum of species containing 1–6 rings. In contrast, most of the chimeric substrates were only partially processed by the synthetase. Treatment of these species with iodoacetamide to alkylate any unmodified cysteines and MS analysis of the products (57 Da mass increase per uncyclized cysteine) allowed the number of thiazoles (and hence the net heterocycle content) to be unambiguously determined.

The Ser40→Gly mutation in MMIC deletes the amino-terminal oxazole (ring 1 of MccB17) in each EPL chimera. The absence of this heterocycle did not affect the cyclization of Cys41 in MMP1, although the Cys48 site further downstream in chimera MMP2 remained only partially modified, even after a 48 hour incubation with synthetase. Such incomplete cyclization of downstream residues was context dependent, because Cys51 (ring 4) was efficiently processed in MMP3, even though two upstream heterocycles (rings 1 and 3) were absent. This differential processing of downstream sites suggests that the cyclization of specific sites in McbA may be potentiated by conformational changes induced by the upstream heterocycles, which increase the delivery of these residues to the synthetase active site. An alternate hypothesis involving specific recognition of upstream heterocycles by MccB17 synthetase to progressively increase the binding affinity of intermediates appears unlikely, because neither the eight-ring MccB17 antibiotic (50 μM) nor the unprocessed $McbA_{27–69}$ fragment (250 μM) inhibited the processing of MBP-tagged McbA (2 μM, data not shown).

As illustrated in Figure 9, formation of the 4,2-linked oxazole–thiazole (rings 1 and 2) in native McbA can induce a chain reversal in the McbA polypeptide of up to 180° (resembling a β turn), which may facilitate the delivery of Cys48 to the synthetase. Deletion of ring 1 (Ser40)

Figure 9



Induction of secondary structure by the formation of upstream rings may potentiate the heterocyclization of all eight Cys/Ser sites in McbA. **(a)** Dreiding model illustrating the nucleation of a reverse turn (arrows) by the 4,2-linked oxazole–thiazole in McbA (rings are labeled 1 and 2). The peptide termini are *N*-acetylated and *C*-amidated in this model, and double bonds are shaded. C, H, O, N and S atoms are gray, green, red, blue and yellow, respectively. **(b)** The amino-terminal propeptide helix of McbA (gray cylinder) is recognized by the McbD subunit of MccB17 synthetase during the biosynthesis of MccB17. Transient conformational perturbations similar to that shown in (a) for rings 1 and 2 may accompany the formation of each oxazole (red pentagon) and thiazole (blue pentagon), and potentiate the heterocyclization of downstream serine residues (red rectangles) and cysteine residues (blue rectangles) by increasing the frequency of productive collisions between these residues and the enzyme active site (McbB/C).

presumably alters the conformational propensity of this sequence in MMP2, reducing the frequency of productive encounters between Cys48 and the enzyme active site. The cyclization of Cys51 further downstream is less affected by such perturbations, because a change in backbone conformation induced by the cyclization of Cys41 alone (ring 2) is sufficient for the efficient modification of this site in chimera MMP3. Significantly, the Asn53→Gly substitution in this substrate failed to induce the cyclization of Ser52 (one of two unmodified serines in MccB17). Hence, removal of a flanking polar residue known to disrupt bisheterocyclization in $McbA_{1–46}$ analogs [10] does not rescue oxazole formation, indicating that even a slight decrease in conformational rigidity due to the absence of rings 1 and 3 may effectively suppress the modification of this residue in MMP3, especially because the synthetase processes serines significantly slower than cysteines [15]. Such an effect is heightened for the processing of bisheterocyclic sites further downstream in chimeras MMP4 (Gly54–Cys55–Ser56) and MMP5 (Gly54–Cys55–Cys56), which incorporate knockouts at multiple upstream sites (rings 1, 3 and 4). The 4,2-linked bithiazole forms in MMP5 to a small extent, although the native 4,2-thiazole–oxazole moiety in MMP4 does not, again reflecting the general inability to generate downstream oxazoles due to intrinsic chemoselectivity barriers for serine cyclization [15,20].

Muir *et al.* [13] have noted that EPL allows nonstandard amino acids to be incorporated into proteins. We have now demonstrated that homocysteine replaces the amino-terminal cysteine in peptides P6 and P7 for the EPL assembly of chimeras MMP6 and MMP7. As with cysteine, capture of the MM–Cys41-DTT α-thioester by homocysteine is followed by rearrangement of the thioester linkage at the splice junction to afford a native-like amide connectivity in these chimeras. The formation of a six-membered ring is predicted to be slower than that of a five-membered ring, however, which might be one of the reasons why the synthetase is unable to generate a six-ring dihydro or heteroaromatic ring from the Gly40–HCys41 sequence in these chimeras. Because the synthetase does not stall at this unnatural residue, the 'silent' Gly40–HCys41 splice junction allowed thiazole 2 to be deleted for the first time in the chimeric substrates. A progressive decrease in cyclization efficiency of Cys48 upon deletion of ring 1 (MMP2) followed by rings 1 and 2 (MMP7) implicated both heterocycles in facilitating the processing of this residue, and reinforced the importance of upstream heterocyclization for the efficient processing of McbA.

This study demonstrates the potentiation of heterocyclization by upstream rings in a family of McbA sequences assembled by EPL. It is proposed that MccB17 synthetase achieves the directional processing of multiple sites without tight binding of intermediates by exploiting conformational changes that increase the frequency of productive collisions for subsequent heterocyclizations. Such 'conformational filtering' may explain the synergism that controls the heterocyclization process. The EPL methodology lays a foundation for determining the optimal number and placement of heterocyclization sites in McbA chimeras, and can be used with peptidomimetics in thiol peptide libraries to permit selection strategies for heterocyclic peptide antibiotics with increased protease resistance and potency.

Significance

The *Escherichia coli* peptide antibiotic microcin B17 (MccB17) contains thiazole and oxazole heterocycles, derived from the post-translational cyclodehydration and dehydrogenation of cysteine and serine residues between positions 40 and 65 of the 69-residue McbA precursor. These rings are presumably the active moieties that target DNA gyrase in sensitive bacteria. Inhibition of the gyrase catalytic cycle leads to double-strand DNA breaks, reminiscent of the mode of action of quinolone antibacterial drugs. To probe the structure–activity requirements for heterocycle content, placement and identity in MccB17, and to determine which features are necessary and sufficient for antibiotic activity, we have adopted a combination of molecular biology and protein chemistry approaches. The amino-terminal 26 residues of McbA provide high-affinity recognition for the post-translational ring-forming McbB,C,D enzymes, and are essential to any substrate. Hence, the first 40 residues were supplied as an maltose binding protein (MBP)–$McbA_{1–40(S40G)}$ fusion protein. The downstream sequence containing heterocyclizable serines and cysteines was delivered as a series of synthetic peptides of varying length and composition. These peptides contained an amino-terminal cysteine or homocysteine residue to enable intein-mediated expressed protein ligation for the assembly of MBP-tagged McbA analogs (46–61 residues) that were purified and used as substrates for heterocyclization by the McbB,C,D complex. Structure–function studies of these chimeras reveal considerable synergism in the progressive enzymatic cyclization of particular cysteines and serines in MccB17, and should be amenable to analysis of the determinants of antibiotic activity in this class of heterocyclic peptide antibiotics.

Materials and methods

Plasmids, peptides and antibodies

The MBP–$McbA_{1–46(GGC)}$ substrate was purified from plasmid pMSS37 [15]. Plasmid pLARA15b, a pET15b derivative that encodes His_6-tagged McbA, has been reported previously [8]. Plasmid pTYB1 used for construction of the McbA–intein chimera was obtained from New England Biolabs (Beverly, MA), as part of the Impact™ T7 protein purification system. Peptides P1–P7 were synthesized using solid-phase Fmoc chemistry on an automated peptide synthesizer [21], purified using reversed phase C18 HPLC, and characterized by MALDI–TOF MS. PAL-PEG-PS™ resin (PerSeptive Biosystems, MA) was used to obtain carboxy-terminal primary amides. Heterocyclization of these target peptide sequences upon incubation of the corresponding EPL

chimeras with affinity-purified MccB17 synthetase was monitored by western immunoblot assays using rabbit anti-MccB17 antibodies [16] as previously described [10]. Rabbit anti-intein and anti-MBP antibodies were purchased from New England Biolabs.

Construction of plasmids pMMIC and pET15b-McbA$_{1–61}$

Plasmid pMMIC encoding the MBP–McbA$_{1–40(S40G)}$–intein–CBD protein was constructed by polymerase chain reaction (PCR) amplification of the MBP–McbA$_{1–40(S40G)}$ gene as an *Nde*I–*Kpn*I fragment from plasmid pMSS37. Following digestion with *Nde*I and *Kpn*I, the PCR product was inserted into plasmid pTYB1 restricted with the same enzymes, which placed the MBP–McbA$_{1–40(S40G)}$ coding sequence upstream of, and in frame with, the gene encoding the intein–CBD fusion. The resulting plasmid (pMMIC) was transformed into *E. coli* strain DH5α. Plasmid pET15b-McbA$_{1–61}$, which encodes the His$_6$–McbA$_{1–61}$ substrate, was constructed by PCR amplification of the McbA$_{1–61}$ gene as an *Sph*I–*Bam*HI fragment from plasmid pLARA15b. Two sequential stop codons were introduced in *mcbA* after the codon for Gly61. Following digestion with *Sph*I and *Bam*HI, the PCR product was ligated with the 5340 bp fragment obtained from plasmid pET15b-McbA treated with the same restriction enzymes, and the resulting plasmid (pET15b-McbA$_{1–61}$) was transformed into *E. coli* strain BL21(DE3) for protein expression. His$_6$–McbA$_{1–61}$ was expressed using protocols reported for other His$_6$-tagged proteins [18], and purified by immobilized metal-affinity chromatography using Ni-NTA resin (Qiagen, CA) as per the manufacturer's directions.

Overexpression and immobilization of MMIC protein

E. coli strain BL21(DE3) was used as the expression host for recombinant MMIC (MBP–McbA$_{1–40(GGC)}$–intein–CBD) protein. An overnight culture in 10 ml of LB ampicillin (150 μg/ml) was grown at 37°C and used to inoculate 1 l of LB ampicillin. Cultures were grown at 37°C and induced with 1 mM IPTG at an OD_{600} of 0.5–0.7. After 3 h of induction, the cells were harvested and resuspended in 20 ml of buffer A (20 mM Na-Hepes pH 8.0, 500 mM NaCl, 1 mM EDTA) containing protease inhibitors (chymostatin, leupeptin, aprotonin and Pefabloc (Boehringer Mannheim)). The cells were disrupted twice in a French pressure cell (18,000 psi) and cellular debris was removed by centrifugation (18,000 g, 35 min). The supernatant was applied to (and recycled through) a chitin column (2 ml bed volume) at a flow rate of 0.5 ml/min. The column was washed with 20 column volumes of buffer B (buffer A + 0.1% Triton X-100), and the purified MMIC protein was retained on the chitin beads for subsequent ligation with target peptides P1–P7.

Expressed protein ligation

The ligation reactions were performed using the protocols of Muir *et al.* [13] with some modifications. The chitin column containing immobilized MMIC protein was washed with two column volumes of buffer C (buffer A + 30 mM DTT). The column was then loaded with one column volume of a 2 mM solution of target peptide (P1–P7) in buffer C. The column was subsequently disassembled and the chitin beads were transferred to eppendorf tubes, which were shaken at room temperature for 48–72 h. The chitin column was subsequently repacked, and 6 × 1.5 ml fractions were eluted with buffer A. The fractions containing ligated MMP (MBP–McbA$_{1–40(S40G)}$–peptide) chimera were identified using SDS–PAGE, pooled, exchanged into buffer D (50 mM Tris–HCl pH 7.5), and concentrated using a Centricon 30 concentration unit (Millipore, MA). Protein concentration was determined by UV spectroscopy, and extinction coefficients (ε_{280} = 66,350 M^{-1} cm^{-1} for all ligated proteins) were determined by a modification of the Edelhoch method [22,23].

Mass spectrometry and iodoacetamide labeling

Assay mixtures consisted of 50 μM MBP–McbA$_{1–40(S40G)}$–peptide (MMP) ligated substrate, assay buffer (50 mM Tris pH 7.5, 100 mM NaCl, 20 mM $MgCl_2$, 10 mM DTT, 2 mM ATP), and affinity-purified CBP-tagged MccB17 synthetase (0.17 mg/ml, 50 μl) in a total volume of 100 μl. Reactions were incubated at 37°C for 24–40 h. The MBP affinity tag was removed by digestion with thrombin (0.246 units; 2 h at 25°C), which leaves a Gly–Ser dipeptide appended to the amino terminus of the downstream McbA fragment. The cleaved McbA$_{1–40}$ peptide fragments were purified on an analytical C18 reversed-phase HPLC column using a Beckman System Gold HPLC (A = water + 0.1% TFA; B = acetonitrile + 0.1% TFA; 30–42% B linear gradient over 12 min, t_R = 6–8 min). The heterocyclic peptides were collected, lyophilized and analyzed by MALDI–TOF MS as described previously [11]. The heterocyclization products obtained with target peptides P2–P4 were further investigated by iodoacetamide labeling to detect the presence of uncyclized cysteines. The HPLC-purified heterocyclic McbA$_{1–40}$ peptide fragments were dissolved in 100 μl of labeling buffer (50 mM Tris–HCl, pH 8.5, 1 mM EDTA, 5 mM iodoacetamide) and incubated at 25°C for 4 h. The labeling reaction was quenched with 400 μl wash buffer (1% acetic acid, 1% methanol in water), and samples were desalted by passage through a reversed-phase peptide trap (0.1 mm i.d.; Michrom Bioresources, CA) prior to analysis by MALDI–TOF MS. McbA fragments obtained from thrombin-digested chimeras MMP6 and MMP7 were HPLC-purified, treated with iodoacetamide, and analyzed by MALDI–TOF MS to determine free thiol content using the same protocols.

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