

YcaO domains use ATP to activate amide backbones during peptide cyclodehydrations

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Thiazole/oxazole-modified microcins (TOMMs) encompass a recently defined class of ribosomally synthesized natural products with a diverse set of biological activities. Although TOMM biosynthesis has been investigated for over a decade, the mechanism of heterocycle formation by the synthetase enzymes remains poorly understood. Using substrate analogs and isotopic labeling, we demonstrate that ATP is used to directly phosphorylate the peptide amide backbone during TOMM heterocycle formation. Moreover, we present what is to our knowledge the first experimental evidence that the D-protein component of the heterocycle-forming synthetase (YcaO/domain of unknown function 181 family member), formerly annotated as a docking protein involved in complex formation and regulation, is able to perform the ATP-dependent cyclodehydration reaction in the absence of the other TOMM biosynthetic proteins. Together, these data reveal the role of ATP in the biosynthesis of azole and azoline heterocycles in ribosomal natural products and prompt a reclassification of the enzymes involved in their installation.

TOMMs constitute a recently grouped class of peptidic natural products of ribosomal origin containing cysteine-, serine- and threonine-derived azole or azoline heterocycles. The installation of these heterocycles onto a precursor peptide (A peptide) by an enzymatic complex (B, C and D proteins) endows the molecule with conformational rigidity and, in all cases examined to date, is absolutely required for biological activity¹. Since the enzymatic machinery responsible for TOMM biosynthesis was first characterized², it has become evident that this strategy for natural product biosynthesis has been extensively propagated in both bacteria and archaea^{1,3}. Furthermore, the diverse array of biological activities and the pharmacological potential shown by the characterized members of the TOMM family has led to extensive investigations focused on elucidating the molecular underpinnings of their biosynthesis^{2,4-8}. These studies have provided the foundation for understanding the complex nature of substrate processing, but many of the finer details regarding heterocycle formation have remained elusive.

For example, studies performed on the TOMM enzymatic machinery involved in microcin B17 (McbB, McbC and McbD; **Fig. 1a**) and cyanobactin biosynthesis have demonstrated that azole heterocycles are installed over two steps: a cyclodehydration to generate an azoline heterocycle and a subsequent flavin mononucleotide-dependent dehydrogenation to afford the aromatic azole (**Fig. 1b**)²⁻⁴. Although the enzymatic complex responsible for these transformations has been partially characterized, the dehydrogenase (B protein) is the only enzyme with a definitive function⁶. Dissection of the biochemical functions of the C and D proteins has been stymied by the inability to obtain individual activities for either protein; however, cyclodehydratase activity has been demonstrated for a complex of the two proteins^{4,9}. Clearly, the C and D proteins act cooperatively, and thus it is perhaps not surprising that both proteins are found fused as a single polypeptide in roughly half of all known TOMM clusters^{1,3}.

Despite the challenges in separating the enzymatic activities of the C and D proteins, functional assignments have been postulated. The discovery that the C protein of the microcin B17 synthetase, (McbB, named for its position in the gene cluster, not its homology) purified with a stoichiometric amount of Zn²⁺ led to

the hypothesis that the metal was serving as a Lewis acid to facilitate cyclodehydration⁶. Though the Zn²⁺ was later established to serve only a structural role¹⁰, additional support for continuing the assignment of the cyclodehydratase to the C protein comes from sequence similarity to adenylating enzymes involved in microcin C7, molybdopterin and thiamin biosynthesis⁹. As a result of this similarity, TOMM C proteins are routinely misannotated in public databases as being involved in molybdopterin or thiamin cofactor biosynthesis. In contrast, the D protein is homologous only to members of the uncharacterized YcaO/domain of unknown function 181 (DUF181) protein families. Lacking both a biochemically and bioinformatically identifiable function, the D protein has been proposed to have a regulatory role in TOMM biosynthesis by either facilitating synthetase complex assembly and iterative substrate processing or providing substrate recognition^{3,6,8}. On this basis, the D protein has previously been referred to as a docking and scaffolding protein³. Confusing matters further, the cyclodehydration reaction has been shown to require the hydrolysis of ATP to ADP and phosphate (P_i)^{5,8}, yet no characterized C protein contains a bioinformatically recognizable nucleotide-binding site. Rather, the D protein from the microcin B17 synthetase has been implicated in ATP use because it contains sequences of weak resemblance to nucleotide-binding motifs⁸. These residues are not conserved in other D proteins, casting doubt on their importance in TOMM biosynthesis.

The requirement for ATP hydrolysis with respect to azoline formation remains undescribed^{5,8}. Reports of the superstoichiometric hydrolysis of ATP relative to azole formation and continued ATP hydrolysis after complete substrate modification have been used as evidence for the role of ATP as a dynamic regulator of the cyclodehydratase complex, yet direct evidence for such a mechanism has not been reported^{5,8}. Recently, this role for ATP use has been referred to as the 'molecular machine' hypothesis (**Fig. 1c**)⁵. An alternative mechanism implicates ATP hydrolysis in the activation of the peptide backbone during cyclization^{2,5}. Such an activation event would result in the elimination of P_i instead of water during the cyclization and could potentially help drive the cyclodehydration reaction toward completion. Although isotopic labeling studies could readily differentiate between these mechanisms,

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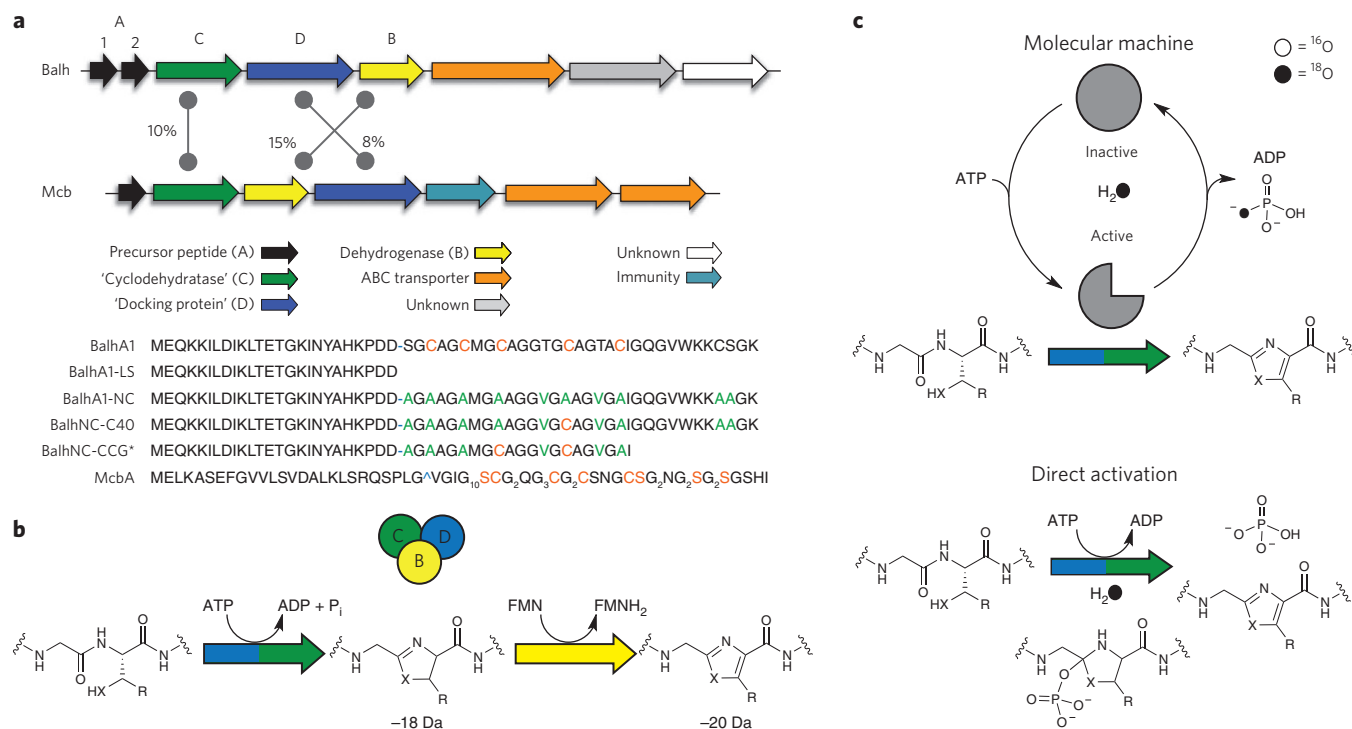


Figure 1 | TOMM biosynthetic gene clusters and possible mechanisms of ATP use during azole formation. (a) The TOMM clusters from *Bacillus* sp. Al Hakam (encoding Balh) and *E. coli* (encoding Mcb) are depicted along with the percentage amino acid identity for each of the three key proteins. Gene assignments are given at the bottom. Note that the dehydrogenase and the 'cyclodehydratase' in the Mcb cluster are assigned as McbC and McbB, respectively. The sequences of the peptide substrates used in this study are shown. Color coding: green, point mutations; orange, residues known to be cyclized *in vitro*¹¹; blue hyphen, putative leader-sequence cleavage site; blue caret, known leader-sequence cleavage site². (b) Heterocycles are installed on a precursor peptide by a heterotrimeric complex composed of a 'cyclodehydratase' (C protein), a 'docking protein' (D protein) and a dehydrogenase (B protein). The cumulative mass change for each step is shown below the modification. (c) The two leading hypotheses for ATP use. ATP hydrolysis could be used to control conformational dynamics (molecular machine) or to directly activate the peptidic substrate as shown. Reactions carried out in [¹⁸O]H₂O will give different products, making it possible to distinguish between these mechanisms. X = S, O; R = H, CH₃.

the relative experimental intractability of previously characterized TOMM synthetases has precluded such a study^{2,3,6}. The discovery of a TOMM biosynthetic cluster from *Bacillus* sp. Al Hakam (*balh*; **Fig. 1a**) yielded robust heterocycle-forming enzymes for *in vitro* characterization¹¹ and allowed us to address two of the foremost issues regarding TOMM biosynthesis: the role of ATP in the mechanism of heterocycle formation and the individual roles of the C and D proteins in the synthetase complex. Our findings demonstrate that the YcaO/DUF181 family member, present in all TOMM clusters, uses ATP to phosphorylate the amide backbone of peptide substrates during the cyclodehydration reaction. As such, the data reported herein provide an improved framework to understand the factors that govern biosynthesis in this class of natural products that has garnered considerable attention^{12–19}.

RESULTS

Minimal requirements for cyclodehydratase activity

To determine the minimal set of proteins required for azoline formation, reactions were initiated with one or more reaction components omitted, and progress was monitored by MALDI-TOF MS (**Fig. 2a**). Samples lacking ATP, BalhC or BalhD did not show a detectable degree of BalhA1 substrate (sequence given in **Fig. 1a**) modification under the conditions used. When the BalhA1 substrate was treated with both BalhC and BalhD, the majority of the peptide was converted to a penta-azoline species as indicated by the loss of 90 Da (each azoline is accompanied by an 18-Da mass loss, as shown in **Fig. 1**). Substrate treated with the full BCD synthetase was converted to a penta-azole species (–100 Da) as previously reported¹¹. In congruence with the microcin B17 and cyanobactin

biosynthetic complexes, the Balh synthetase also hydrolyzed ATP to ADP and P_i during substrate processing (**Supplementary Results, Supplementary Fig. 1**)^{2,5}. Subsequent studies demonstrated that the Balh enzymatic machinery was able to use both ATP and GTP (also analogous to the microcin B17 synthetase)⁸, although ATP was preferred over GTP by approximately a factor of ten owing to differences mainly in *K_m* (**Supplementary Fig. 2**). Substitution of ATP with a nonhydrolyzable analog resulted in no ring formation, providing further evidence of the requirement of nucleotide triphosphate hydrolysis during heterocycle formation (**Supplementary Fig. 3**). The rate at which P_i was generated under varying reaction conditions was monitored by a purine nucleoside phosphorylase (PNP)-coupled assay²⁰. Robust P_i generation was observed only when the C and D proteins were added to samples containing BalhA1 and ATP (**Supplementary Fig. 4**). Addition of the B protein did not significantly (*P* > 0.1) alter the rate of ATP hydrolysis, which is consistent with earlier reports of C-D fusion enzymes forming azoline heterocycles in the absence of the dehydrogenase⁴. In contrast to Balh and the C-D fusion enzymes, the microcin B17 complex was not functional in the absence of the dehydrogenase⁶. The reason for this discrepancy is currently unknown, but the available data suggest that the Mcb dehydrogenase may have a pivotal role in the assembly of an active synthetase complex.

A cyclizable residue stimulates rapid ATP hydrolysis

The molecular machine hypothesis asserts that ATP hydrolysis regulates the conformational dynamics and enzymatic activity of the synthetase complex⁵. Although the mechanism for how this may work has not yet been explicitly stated, one possible route could be

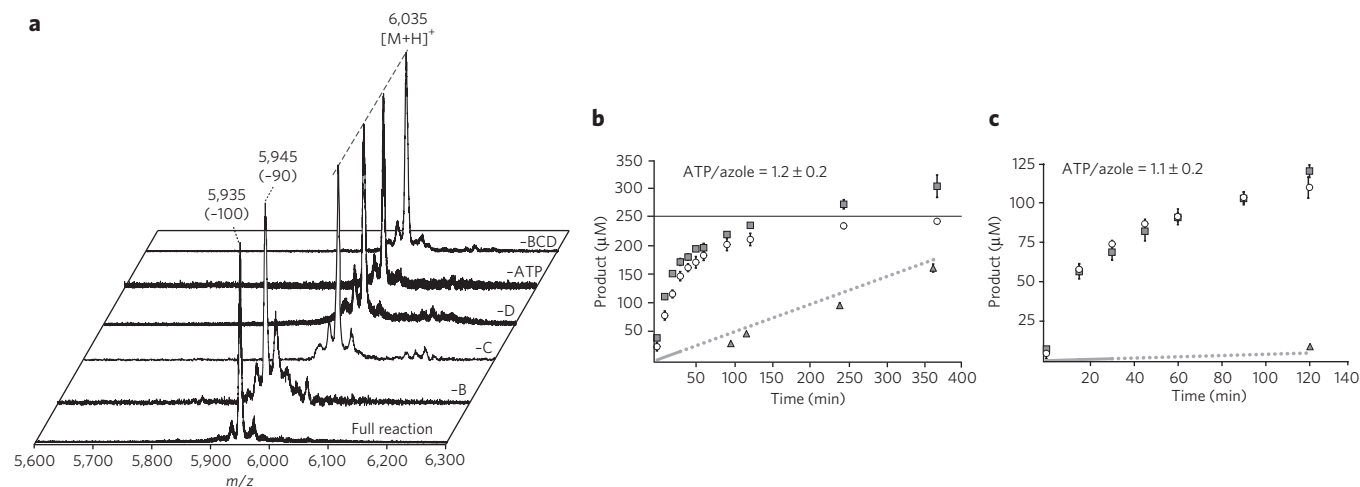


Figure 2 | Minimal requirement for cyclodehydratase activity and ATP-azole stoichiometry for substrate processing. (a) MALDI-TOF spectra monitoring heterocycle formation. Labels represent the component omitted from the reaction (for example, -C lacked BalhC). The mass shift for the major species relative to the unmodified substrate is shown above the appropriate peak. The +1 charge state is shown. (b,c) The stoichiometry of ATP hydrolysis to azole heterocycle formation in the Balh TOMM (b) and the Mcb TOMM (c) is shown. Phosphate (squares), heterocycles (circles), BCD background ATPase activity (solid line, PNP assay; dotted line, extrapolated PNP assay; triangles, malachite green assay), maximum product produced based on the concentration of substrate (solid horizontal line). Error bars represent the s.d. from the mean ($n = 3$). The ATP/azole stoichiometric ratio is shown and is the average of all time points.

that upon peptide-substrate binding, the synthetase may undergo a conformational change that leads to an active complex (Fig. 1c). Previous studies on microcin B17 biosynthesis have demonstrated that the leader sequence of the precursor peptide, as well as a severely truncated (nonprocessed) substrate analog, were incapable of stimulating ATP hydrolysis^{8,21}. These results suggested that the core sequence of TOMM precursor peptides must be important for the ATPase activity of the synthetase. Studies conducted in the absence of BalhA1 demonstrated that a complex of BalhC and BalhD had a measurable degree of ATP hydrolysis that was roughly two orders of magnitude lower than that found in studies conducted at saturating BalhA1 concentrations (Supplementary Fig. 5a). To test whether a noncyclizable (NC), full-length substrate analog could potentiate this basal ATPase activity of the TOMM enzymatic complex, we prepared BalhA1-NC, in which every heterocyclizable residue in the core region was mutated to a hydrophobic amino acid (Fig. 1a). Remarkably, the presence of BalhA1-NC decreased the rate of ATP hydrolysis by a factor of four compared to samples containing only BalhC and BalhD (Supplementary Fig. 5a). Addition of the predicted BalhA1 leader sequence (BalhA1-LS; Fig. 1a) to similar reactions showed a slight, but statistically significant ($P = 0.01$), reduction in the basal ATPase activity. The marked difference between the degrees of inhibition provided evidence that the core sequence was primarily responsible for suppressing the ATPase activity and indicated that there may be a direct interaction between residues of the core sequence and the ATP-binding site. In support of this finding, our previous study demonstrated that the reinstatement of a naturally occurring cysteine (BalhNC-C40; Fig. 1a) yielded a substrate that not only was processed by the synthetase but also increased the rate of ATP hydrolysis to almost that of the wild type¹¹, as shown again here (Supplementary Fig. 5). These data provide substantial evidence that the ATP-binding site and the residues of the core sequence directly communicate. Moreover, the ability of BalhA1-NC to inhibit the basal ATPase activity suggests that the ATP site has greater solvent accessibility in the absence of the precursor peptide. Though not definitive, these results are difficult to reconcile with an ATP-using mechanism consistent with the molecular machine hypothesis, which prompted us to explore alternative mechanistic possibilities.

ATP hydrolysis is tightly coupled to ring formation

The microcin B17 synthetase has been shown to consume approximately 5 mol of ATP per mol of heterocycle formed⁸. Although the superstoichiometric consumption of ATP has not been observed for all TOMM biosynthetic enzymes, this observation cast doubt on the use of ATP to directly activate the substrate during processing and has been used as supportive evidence of the molecular machine hypothesis^{5,8}. To evaluate the stoichiometry of products formed by the Balh synthetase, the rate of P_i and azole production were monitored in parallel time-course assays. An overlay of P_i and azole synthesis illustrated that the formation of these products was tightly coupled during the reaction and that the ATP-to-azole stoichiometry was essentially 1:1 over the entire 6-h time course (Fig. 2b). Furthermore, when BalhA1 was fully heterocyclized, the rate of ATP hydrolysis returned to approximately the basal rate (Fig. 2b). This result was consistent with an earlier report on trunkamide biosynthesis, which uses a C-D fusion protein⁵.

Given the markedly different ATP-azole stoichiometry in the Balh and Mcb systems, we revisited the ATP stoichiometry of microcin B17 processing. We hypothesized that the use of a truncated substrate and a synthetase complex with an abnormal ratio of the B, C and D proteins (due to the purification strategy) could have led to aberrant usage of ATP^{8,12}. Therefore, the ATP-azole stoichiometry of the microcin B17 synthetase was re-established using the full-length substrate (McbA; sequence given in Fig. 1a) and a completely tag-free synthetase complex added in a 1:1:1 molar ratio. As with the Balh synthetase, the concentration of P_i and azole heterocycles mapped almost perfectly over the time course assayed, and the average stoichiometric ratio was calculated to be 1 (Fig. 2c). Taken together, these data demonstrate the tight linkage of ATP hydrolysis to heterocycle formation, which seems to be a general feature of TOMM biosynthesis.

ATP is hydrolyzed by a nonsolvent oxygen nucleophile

Intrigued by our inability to artificially activate ATP hydrolysis and our observation of a 1:1 stoichiometry between ATP consumption and ring formation, we used isotopic labeling to determine the source of the oxygen in the P_i generated during the course of the reaction. If ATP were used to directly activate the substrate,

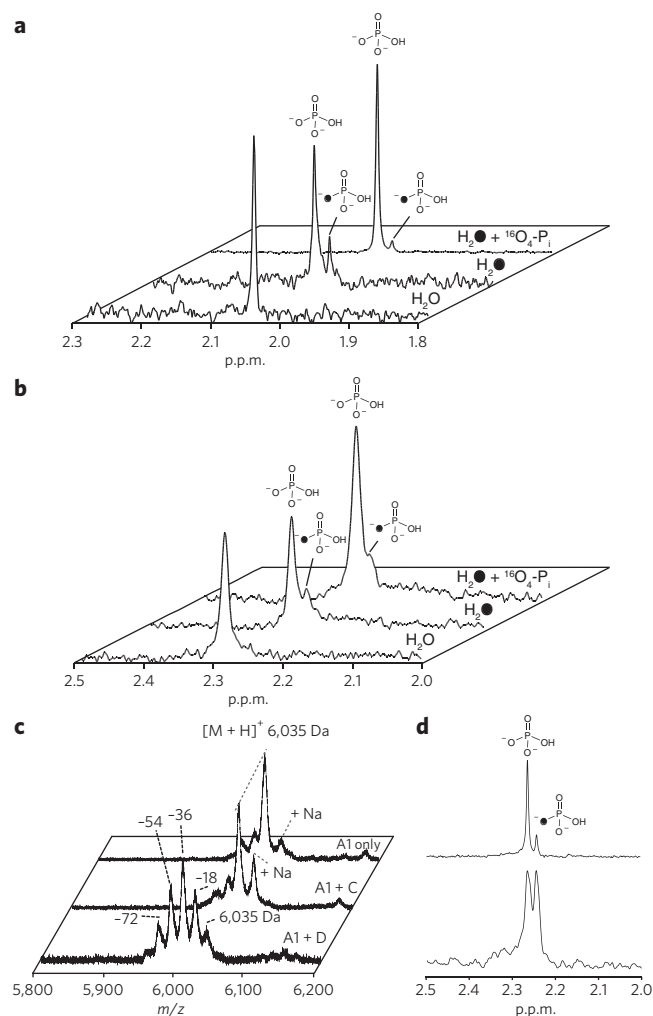


Figure 3 | ATP is used by BalhD to directly activate the amide backbone of the substrate. (a,b) ^{31}P -NMR spectra of reactions with the Balh (a) or the microcin B17 synthetase (b). The identity of the major product, $[\text{O}_4] \text{P}_i$, was confirmed by spiking with authentic $[\text{O}_4] \text{P}_i$. Slight pH differences between the Balh and MccB samples (8.5 and 8.0, respectively) account for the altered chemical shifts. (c) MALDI-TOF MS spectra of BalhA1 treated with a stoichiometric amount of either BalhC or BalhD are shown. The +1 charge state is shown. Mass shifts relative to the BalhA1 control are labeled. (d) A ^{31}P -NMR spectrum of a reaction conducted in ^{18}O - H_2O confirmed the presence of both $[\text{O}_4] \text{P}_i$ and $[\text{O}_3\text{O}^{18}] \text{P}_i$ (bottom). The identity of the $[\text{O}_4] \text{P}_i$ peak was confirmed by spiking the sample with authentic $[\text{O}_4] \text{P}_i$ (top).

the oxygen incorporated into the P_i byproduct would have to originate from the peptide backbone. Thus, running the reaction in ^{18}O H_2O would result in ^{16}O incorporation into phosphate, producing $[\text{O}_4] \text{P}_i$ (Fig. 1c). In contrast, the molecular machine hypothesis demands that the oxygen incorporated into the P_i would originate from bulk solvent (water) in the same manner as with any ATPase (Fig. 1c). The molecular machine mechanism, if acting, would introduce a single ^{18}O label in the P_i produced when the reaction was run in ^{18}O H_2O , which is readily detectable by a roughly 0.02-ppm upfield shift in ^{31}P -NMR compared to $[\text{O}_4] \text{P}_i$ (ref. 22).

Reactions of the Balh TOMM biosynthetic enzymes with the BalhA1 substrate in ^{18}O H_2O resulted in the production of primarily $[\text{O}_4] \text{P}_i$ (Fig. 3a). As would be predicted from the earlier stoichiometry assay (Fig. 2b) and the basal rate of ATP hydrolysis (Supplementary Fig. 5a), these samples also contained a small amount (~15%) of

$[\text{O}_3\text{O}^{18}] \text{P}_i$. The $[\text{O}_4] \text{P}_i$ produced was a consequence of substrate processing and neither was a contaminant of the ATP buffer nor the proteinaceous components of the synthetase reaction as assessed by the malachite green assay. Though the direct activation of the amide carbonyl was the most logical explanation for this data, the possibility existed that $[\text{O}_4] \text{P}_i$ was generated as a consequence of nucleophilic attack at the β -phosphate of ATP or through isotope scrambling with the ADP byproduct. Thus, LC/MS analysis of the ADP formed during the reaction was carried out to determine whether this byproduct contained ^{18}O . The trace clearly demonstrated that ^{18}O was not present in the ADP and provided strong evidence that ATP consumption occurred because of the action of a nonsolvent oxygen nucleophile (Supplementary Fig. 6). This assay was repeated with the microcin B17 synthetase, which yielded the same result (Fig. 3b), though the requirement for a lower-pH buffer gave a slight perturbation in the $[\text{O}_4] \text{P}_i$ chemical shift.

Our data implicate ATP in the direct phosphorylation of the amide carbonyl oxygen preceding the residue undergoing cyclization and also invalidate the molecular machine hypothesis as the major pathway for heterocycle formation. Furthermore, these results demonstrate that this mechanism of substrate modification is shared among distantly related TOMM synthetases (Fig. 1a).

The D protein alone is sufficient for azoline formation

With the elucidation of the role of ATP in heterocycle formation, we sought to more precisely assign functions to the individual BalhC and BalhD proteins, which collectively comprise the cyclodehydratase (Fig. 2a and Supplementary Fig. 4). Given the homology between the C protein, previously annotated as the cyclodehydratase⁵, and MccB, an adenylating protein involved in microcin C7 biosynthesis (Supplementary Fig. 7)²³, we hypothesized that BalhC catalyzes the direct activation of the peptidic substrate. BalhD was suspected to be involved with general substrate handling and enzymatic regulation, thus allowing BalhC to perform multiple turnovers. To test these putative assignments, we performed a pseudo-single-turnover experiment with a stoichiometric amount of the BalhA1 substrate and either BalhC or BalhD under saturating ATP conditions. Unexpectedly, no modifications were detected when BalhC alone was added to BalhA1, but up to four dehydrations (−90 Da) were observed on BalhA1 treated with BalhD alone (Fig. 3c). Subsequent studies showed that these modifications were ATP dependent and were competent intermediates in the production of the penta-azole product (Supplementary Fig. 8). Similar reactions on substrates containing a reduced number of heterocyclizable residues (BalhNC-C40 and BalhNC-CCG*⁺; sequences given in Fig. 1a), subjected to either tandem MS or iodoacetamide labeling, proved that the BalhD-installed modifications were cyclodehydration products (to yield an azoline) and not dehydrations (to yield a dehydroalanine or dehydrobutyrine; Supplementary Figs. 9 and 10). These data confirmed that BalhD, formerly assigned as a docking protein, was necessary and sufficient for azoline formation. Notably, the rate of heterocyclization catalyzed by BalhD only was nearly three orders of magnitude slower than that when BalhC was present (Supplementary Fig. 11). Furthermore, a more detailed analysis of the reaction products catalyzed by BalhD only revealed that the ATP/azoline stoichiometric ratio considerably deviated from 1 (Supplementary Fig. 12). Thus, we recommend that the term cyclodehydratase should be reserved for describing the C–D complex.

The D protein uses ATP for amide backbone activation

The discovery that BalhD could independently install azolines on BalhA1 in an ATP-dependent manner, but that ATP consumption was dysregulated in the absence of BalhC, led us to investigate whether BalhD could independently activate the amide backbone of BalhA1. As before, a reaction was carried out in ^{18}O H_2O , and the $^{16}\text{O}/^{18}\text{O}$ isotopic ratio in the resultant P_i was analyzed by ^{31}P -NMR

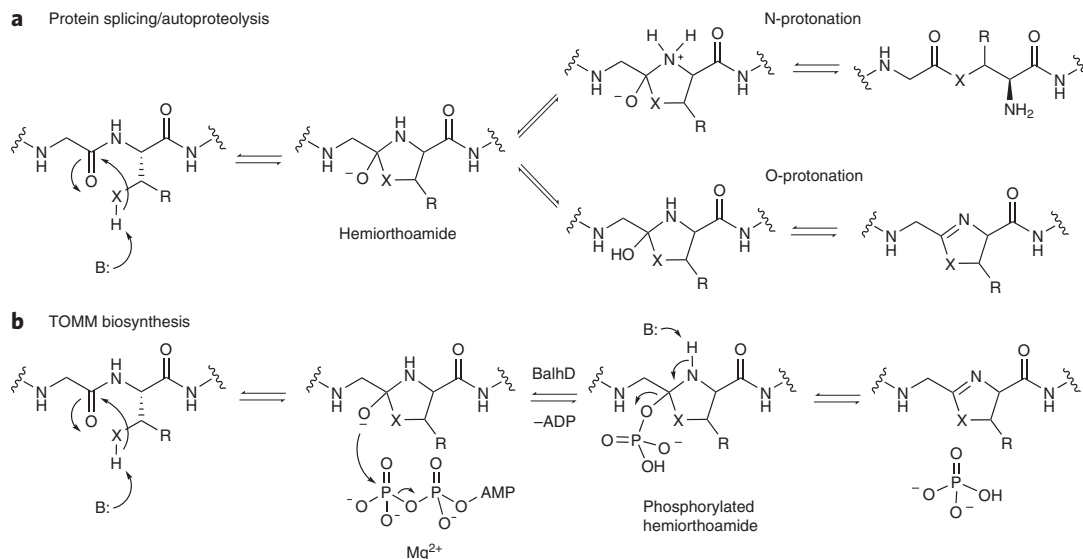


Figure 4 | TOMM azoline installation is reminiscent of intein-mediated protein splicing. (a) The mechanism of protein splicing and autoproteolysis follows the N-protonation pathway to generate an ester or thioester. An azoline heterocycle could also be formed from the hemiorthoamide intermediate if O-protonation occurred. (b) In TOMM biosynthesis, the use of ATP to phosphorylate the substrate could be used to drive the reaction down an O-elimination pathway to generate an azoline. X = S, O; R = H, CH₃.

spectroscopy. Samples containing BalhD, BalhA1 and ATP formed substantial [¹⁶O₄]P_i, as evidenced by the ³¹P-NMR peak (Fig. 3d). However, when any of these essential components were omitted, [¹⁶O₄]P_i was not detectable (Supplementary Fig. 13). Although these data clearly demonstrated that BalhD was responsible for the amide activation event, the nearly identical heights of the [¹⁶O₄]P_i and [¹⁸O₁¹⁶O₃]P_i NMR signals provided further evidence that ATP consumption was dysregulated in the absence of BalhC (Fig. 3d and Supplementary Fig. 12). Unlike the BalhC–BalhD complex, which had a strong enhancement in the rate of ATP hydrolysis upon the addition of BalhA1, BalhD's ATPase activity remained unchanged upon the addition of BalhA1 (Supplementary Fig. 11). These findings implicate the C protein as a potentiator of the D protein, which is the opposite of the original assignments.

DISCUSSION

Prior to this study, the use of ATP hydrolysis for cyclodehydration was poorly understood. Although it had been noted that the initial steps of this transformation could mimic the mechanism of protein splicing by intein domains (an ATP-independent process; Fig. 4), the absolute requirement for nucleotide triphosphate hydrolysis had never definitively been factored into the reaction^{2,5}. Our findings make the TOMM D proteins only the second enzyme class known to use ATP to directly activate an amide carbonyl oxygen, the other being the PurM superfamily. PurM family members conduct similar chemistry on nonpeptide substrates (Supplementary Fig. 14)^{24,25}, and all members whose crystal structures have been solved contain a noncanonical ATP-binding domain^{26–28}. Despite the similarity in ATP use between TOMM D proteins and PurM family members, the two enzyme families share no sequence similarity and represent an example of convergent evolution toward a common mode of ATP usage. Members of the PurM family are proposed to act via the formation of an iminophosphate intermediate²⁶, whereby phosphorylation occurs before nucleophilic attack (Supplementary Fig. 14). Although a similar mechanism can be drawn for azoline formation by the TOMM synthetase (Supplementary Fig. 15), it would require a disfavored 5-*endo*-trig cyclization²⁹ and is inconsistent with the suppression of ATP hydrolysis in the presence of BalhA1-NC. Alternatively, we hypothesize that azoline formation

by the TOMM synthetase occurs through a hemiorthoamide intermediate analogous to that implicated in protein autoproteolytic pathways^{30,31}. During intein splicing and other autoproteolytic events, the hemiorthoamide is resolved by N-protonation and ester or thioester formation (Fig. 4)³². We assert that the phosphorylation of the amide oxygen (in lieu of O-protonation) would direct the hemiorthoamide toward azoline formation and prevent the non-productive breakdown of the intermediate (Fig. 4). Phosphorylation would not only accelerate the elimination reaction (on the basis of the lower pK_a of phosphate relative to water) but also directly couple ATP hydrolysis to cyclodehydration, providing a thermodynamic drive for the reaction. The resolution of the hemiorthoamide via the inclusion of a thermodynamically favorable step is seen in all autoproteolytic pathways³². Further evidence supporting a mechanism in which cyclization precedes phosphorylation (that is, the intein-like mechanism) comes from a recent report of ester formation during microcin B17 biosynthesis³³ and the discovery that engineered intein domains can catalyze azoline formation³⁴. In light of earlier reports, and the data presented here, TOMM cyclodehydration and intein splicing proceed through a common intermediate, which we propose is the hemiorthoamide depicted in Figure 4.

Though previous studies on fused C and D proteins implicated the YcaO/DUF181 domain in the cyclodehydration reaction, the exact role of the D domain in this complex was never elucidated. Besides the TOMM D protein, only one other YcaO/DUF181 family member has had a biological function reported. A recent study linked the function of *Escherichia coli* YcaO to ribosomal thiomethylation but did not reveal any mechanistic details or physiological ramifications of this process³⁵. Thus, our data assign what is to our knowledge the first definitive function to a member of this uncharacterized protein family. As such, the YcaO/DUF181 protein that exists in all TOMM biosynthetic clusters has the potential to provide insight into the activity of an estimated 3,000 conserved or hypothetical genes that remain largely unannotated in GenBank. At the time of writing, approximately 500 of these lie in bioinformatically recognizable TOMM clusters, all of which are likely to perform the reaction described in this report^{1,3}. Although we cannot confidently comment on the function of the remaining 2,500 genes, it is possible that non-TOMM members of the YcaO/DUF181 families

harbor nucleotide-binding sites that either facilitate or otherwise control enzymatic activity.

Our dissection of the cyclodehydratase complex and reclassification of the roles of the C and D proteins raised the question as to the role of the C protein in TOMM biosynthesis. Though we have shown that BalhC acts cooperatively with BalhD to accelerate azoline formation and govern the proper use of ATP, this study does not allow us to conclusively deduce the role of BalhC. Nonetheless, it is conceivable that the C protein could partake in one of three plausible functions: (i) the C protein activates the D protein through an allosteric mechanism, increasing the rate of heterocyclization; (ii) the C protein catalyzes the nucleophilic attack of the preceding side chain by providing the requisite general acid and base residues; or (iii) the C protein forms key contacts with both the substrate and the D protein to facilitate the interaction of the core region of precursor peptide and the D protein active site. Our data do not distinguish between these possibilities, which are not necessarily mutually exclusive.

In summary, the results reported herein not only have elucidated the role of ATP in the heterocyclization of TOMM substrates but also have implicated the hydrolysis of this cosubstrate in the activation of the peptide backbone during substrate processing. Furthermore, we have obtained evidence demonstrating that the D protein is solely responsible for this ATP-dependent cyclodehydration reaction and, as such, have assigned an enzymatic function to the previously enigmatic YcaO/DUF181 protein families. These discoveries pave the way for future work focused on uncovering the complex quaternary interactions involved in regulation of cyclodehydratase activity and the characterization of the catalytic architecture for this poorly understood ATP-using enzyme.

METHODS

Protein overexpression and purification. All proteins were overexpressed as tobacco etch virus protease-cleavable fusions to maltose-binding protein (MBP) and were purified by amylose affinity chromatography by methodology similar to that previously reported³. A detailed protocol can also be found in the **Supplementary Methods**. Unless otherwise stated, all proteins and substrates were used as MBP fusions. The Balh TOMM synthetase proteins are highly active while MBP tagged and are largely insoluble when untagged¹¹.

PNP-based phosphate detection assay. In general, the indicated synthetase proteins were added to a cuvette for a final concentration of 1 μM each. Reactions were initiated via the addition of a 23 $^{\circ}\text{C}$ mixture of peptide substrate, 200 μM 2-amino-6-mercapto-7-methylpurineriboside (Berry and Associates) and 0.2 U of PNP in synthetase buffer (50 mM Tris (pH 7.5), 125 mM NaCl, 20 mM MgCl_2 , 10 mM dithiothreitol and 3 mM ATP). Reaction progress was monitored by the change in absorbance at 360 nm on a Cary 4000 UV-visible spectrophotometer (Agilent). Initial rates of phosphate production were calculated on the basis of linear absorbance change during the first 3 min of the reaction and the extinction coefficient of the resulting guanine analog (11,000 $\text{M}^{-1}\text{cm}^{-1}$). To determine the nucleotide triphosphate kinetic constants, we used a synthetase buffer that lacked ATP, and the reactions were initiated by the addition of the indicated nucleotide. Given the slow rate of ATP hydrolysis without the presence of the precursor peptide, the background ATPase activity was measured using 15 μM MBP-tagged BalhC and BalhD instead of 1 μM . Reactions were carried out as above ($n \geq 3$). Regression analyses to obtain the kinetic parameters were carried out using IGOR Pro version 6.12 (WaveMetrics).

Subtractive activity studies. The effect of each protein on heterocyclization was monitored by MALDI-TOF MS. Reactions were carried out for 16 h at 23 $^{\circ}\text{C}$ with 1 μM of the indicated components, 50 μM MBP-BalhA1 and the synthetase buffer listed above. MBP was proteolytically removed from the substrate by the addition of 2 $\mu\text{g ml}^{-1}$ recombinant tobacco etch virus protease and a 30-min incubation at 30 $^{\circ}\text{C}$. Samples were desalted via C_{18} ZipTip (Millipore) according to the manufacturer's instructions and were analyzed on a Bruker Daltonics UltrafleXtreme MALDI-TOF spectrometer. Spectra were obtained in positive reflector mode using α -cyanohydroxycinnamic acid as the matrix.

Substrate analog assays. Inhibition studies were performed with 15 μM MBP-BalhC and MBP-BalhD and either 50 μM MBP-BalhA1-NC or MBP-BalhA1-LS. Rates were measured with the PNP assay as described above following a 15-min equilibration at 23 $^{\circ}\text{C}$. MBP-BalhNC-C40 reactions were carried out with 1 μM MBP-BcerB (described in **Supplementary Methods**), MBP-BalhC and MBP-BalhD

and 100 μM substrate for 16 h at 23 $^{\circ}\text{C}$. Products were analyzed via MALDI-TOF MS as described above.

Stoichiometry of ATP use. Samples were set up with 2 μM MBP-BalhC and MBP-BalhD, 10 μM MBP-BcerB and 50 μM MBP-BalhA1 in synthetase buffer. Aliquots were removed at the indicated time points (**Fig. 2b,c**) and frozen in liquid nitrogen. The samples were analyzed by LC/MS to detect azole heterocycles and a malachite green assay to detect phosphate (**Supplementary Methods**). The Mcb reactions were carried out in a similar fashion with the following exception: samples containing 1 μM MBP-tagged McbB, McbC and McbD and 20 μM MBP-McbA were digested with thrombin for 4 h before the reaction was initiated with the addition of ATP. The background ATPase activities of both synthetase complexes were analyzed using both the PNP and malachite green phosphate assays.

³¹P-NMR analysis of phosphate isotope incorporation. Two samples containing either the MBP-tagged BCD (4 μM) synthetase components or MBP-BalhA1 substrate (100 μM) with low-salt synthetase buffer (50 mM Tris pH 8.5, 25 mM NaCl, 5 mM MgCl_2 , 10 mM dithiothreitol, 2 mM ATP) were lyophilized separately for 16 h. The resulting solid was reconstituted in 500 μl of 97 atom % ^{18}O H_2O (Cambridge Isotope Laboratories) and incubated at 23 $^{\circ}\text{C}$ with rocking for 8.5 h before quenching the reaction with 5.5 μl of 500 mM EDTA (pH 8.0). Fifty microliters of D_2O were added, and the sample was transferred to a standard 5-mm NMR tube. The ³¹P-NMR spectrum was obtained on a 600-MHz Varian Unity Inova NMR with a 5-mm Varian AutoTuneX probe, 512 transients, 32,768 points and a spectral window of -15 to 5 p.p.m.

The BalhD-only ³¹P-NMR experiment was set up in a similar manner except that the protein concentration was 20 μM and the substrate concentration was 60 μM (to partly circumvent the exceedingly slow reaction and dysregulated ATP usage in the absence of BalhC). After a 3-h incubation at 23 $^{\circ}\text{C}$, the reaction was quenched by the addition of 25% (v/v) acetonitrile to precipitate the protein components and dried via Savant SpeedVac (Thermo Fisher). The sample was reconstituted in 220 μl D_2O with 5 mM EDTA and placed in a D_2O -matched Shigemmi NMR tube. ³¹P-NMR analysis was carried out as before with 1,000 transients.

The MBP-tagged McbB, McbC and McbD proteins were pooled at a 1:1:1 molar ratio and concentrated to 100 μM using a 3-kDa amicon centrifugal filter (Millipore). To this sample, 0.2 μg of thrombin (from bovine plasma) was added, and the sample was proteolytically digested for 4 h at 23 $^{\circ}\text{C}$ to remove the MBP tags. A 600- μl sample of 20 μM MBP-McbA and low-salt synthetase buffer (pH 8.0) was lyophilized for 16 h. The lyophilized protein was reconstituted in 600 μl of ^{18}O H_2O , and the above mixture of McbB, McbC and McbD was added to a final concentration of 5 μM to initiate the reaction. After a 2-h incubation at 23 $^{\circ}\text{C}$, the sample was frozen in liquid nitrogen and lyophilized to dryness. The sample was reconstituted in 220 μl of D_2O with 15 mM EDTA and placed in a D_2O -matched Shigemmi NMR tube. Analysis by ³¹P-NMR was carried out as before with 2,000 transients. Conditions describing the pertinent controls can be found in the **Supplementary Methods**.

Single-turnover activity studies. Individual MBP-tagged synthetase components (45 μM) were set up with MBP-BalhA1 substrate at a 1:1 ratio in synthetase buffer. After an 18-h reaction at 23 $^{\circ}\text{C}$, samples were analyzed by MALDI-TOF MS as described above.

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Author contributions

Experiments were designed by D.A.M., K.L.D. and J.O.M. and were performed by K.L.D. and J.O.M. The manuscript was written by D.A.M. and K.L.D. with critical editorial input from J.O.M. The study was conceived and overseen by D.A.M.

Competing financial interests

The authors declare no competing financial interests.

Additional information

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