

Mechanistic Investigations of PoyD, a Radical S-Adenosyl-L-methionine Enzyme Catalyzing Iterative and Directional Epimerizations in Polytheonamide A Biosynthesis

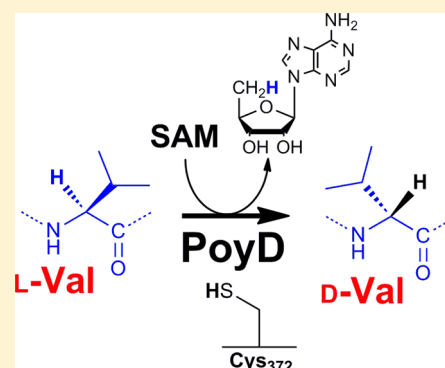
Aubérie Parent,^{†,§} Alhosna Benjdia,^{†,§} Alain Guillot,^{†,§} Xavier Kubiak,^{†,§} Clémence Balty,[†] Benjamin Lefranc,[‡] Jérôme Leprince,[‡] and Olivier Berteau^{*,†,§}

[†]Micalis Institute, ChemSyBio, INRA, AgroParisTech, Université Paris-Saclay, 78350 Jouy-en-Josas, France

[‡]Inserm U1239, PRIMACEN, University of Rouen Normandy, 76000 Rouen, France

Supporting Information

ABSTRACT: Ribosomally synthesized and post-translationally modified peptides (RiPPs) are a growing family of bioactive peptides. Among RiPPs, the bacterial toxin polytheonamide A is characterized by a unique set of post-translational modifications catalyzed by novel radical S-adenosyl-L-methionine (SAM) enzymes. Here we show that the radical SAM enzyme PoyD catalyzes in vitro polytheonamide epimerization in a C-to-N directional manner. By combining mutagenesis experiments with labeling studies and investigating the enzyme substrate promiscuity, we deciphered in detail the mechanism of PoyD. We notably identified a critical cysteine residue as a likely key H atom donor and demonstrated that PoyD belongs to a distinct family of radical SAM peptidyl epimerases. In addition, our study shows that the core peptide directly influences the epimerization pattern allowing for production of peptides with unnatural epimerization patterns.



INTRODUCTION

Ribosomally synthesized and post-translationally modified peptides (RiPPs) form an expanding family of natural products that was recently unified.^{1,2} This large family of natural products contains diverse classes of peptides including lanthipeptides, thiopeptides and microcins, which have relevant biological properties, notably antibiotic and anticancer activities. These biological properties are one of the reasons behind the renewed interest in RiPPs. Indeed, RiPPs appear not only as promising natural products to address the antibiotic resistance crisis, but also as a source of novel molecules to regulate the human microbiota.^{3–5}

RiPPs are produced according to a simple biosynthetic logic, a precursor peptide containing a leader or a follower sequence is synthesized and modified to various extent by tailoring enzymes, before being generally secreted and the leader (or follower) cleaved off.^{6,7} RiPPs have been shown to contain a wealth of post-translational modifications such as thioether^{8,9} and carbon–carbon^{10–12} bonds, unusual C-methylation^{13–16} and epimerization.^{5,17} In a unique manner, the so-called radical S-adenosyl-L-methionine (SAM) enzymes, an emerging super-family of metalloenzymes^{4,18,19} have been shown to catalyze all these various and chemically unrelated modifications.² Indeed, radical SAM enzymes, despite a core mechanism involving the coordination of SAM to an [4Fe-4S]^{2+/1+} cluster in a bidentate fashion^{20,21} and the generation of the 5'-deoxyadenosyl radical (S'-dA•)²² to initiate catalysis, have evolved an unsurpassed but still ill-understood diversity of mechanisms and reactions.

Among RiPPs, polytheonamide A is so far unique by requiring three radical SAM enzymes (PoyB, PoyC and PoyD) to introduce two types of post-translational modifications (i.e., methylation and epimerization).¹⁷ Another fascinating feature of polytheonamide A is the extent of post-translational modifications introduced by these three enzymes. Indeed, the two B₁₂-dependent radical SAM enzymes: PoyC and PoyB, have been recently shown in vitro¹⁵ and in vivo²³ to be responsible for the formation of the 13 C_β methylations and the N-terminal *ter*-butyl group (Figure 1a). By coexpressing PoyD with various truncated forms of the precursor peptide PoyA in *E. coli*,^{17,23} it has been shown that PoyD catalyzes the 18 epimerizations found in polytheonamide A in a likely C-to-N directionality (Figure 1). On the basis of its sequence and these unique properties, PoyD has been predicted to form a distinct class of radical SAM enzymes.^{2,5,24} To understand the mechanism of this enzyme and unravel how it introduces a unique pattern of epimerizations within a peptide backbone, we undertook the biochemical characterization of the radical SAM enzyme PoyD.

RESULTS

PoyD Is a Radical SAM Enzyme Catalyzing in Vitro Peptide Epimerization. PoyD was expressed as a Strep-tag fusion protein in *E. coli* (Figure 2a). The purified protein

Received: August 8, 2017

Published: December 18, 2017

containing the residues +1 to +15, could serve as substrate for PoyC, the B₁₂-dependent radical SAM enzyme catalyzing valine C-methylation.¹⁵ However, because of the high content of hydrophobic residues (i.e., Ile, Val and Ala), we had to insert an N-terminus stretch of Lys residues to obtain a soluble substrate. With this substrate, PoyC catalyzed methylation of Val-14 but not of the five other Val residues located between positions +5 to +10, presumably because of the presence of the Lys-stretch.

Interestingly, the leader sequence of PoyA contains charged amino acid residues (Asp) that could be exploited to improve solubility and make peptides more suitable for LC-MS analysis. We thus synthesized PoyA derivatives containing residues -9 to -1 from the leader sequence and residues +1 to +10 from the core region (Figure 1c). To simplify the detection and analyses by HPLC and LC-MS, we also introduced either one N-terminal Trp residue (peptide 1) or substituted the residue Gln -5 by a Lys moiety (peptide 2) (Figure 1c). Each peptide was assayed with PoyD under anaerobic and reducing conditions in the presence of the S-adenosyl-L-methionine (SAM) cofactor.

In each condition, PoyD catalyzed the reducing cleavage of SAM into 5'-dA (Figure 2d,e; $[M + H]^+ = 252.1$) demonstrating its activity as a radical SAM enzyme. Incubation of PoyD with peptide 1 led to the formation of three peptides (peptides 4, 5 and 6, eluting at 30.1, 30.4, and 30.9 min, respectively) (Figure 2f). Mass spectrometry analysis revealed no mass difference between peptide 1 and the products formed (Supplementary Figure S2). In order to ascertain the nature of the modification and to identify the modified residues, we performed the reaction in deuterated buffer. Indeed, *in vivo*^{23,24} and *in vitro*⁵ investigations of radical SAM epimerases have shown that they introduce solvent derived H atoms into their products. Under these conditions, the peptides produced had their masses shifted from $[M + 2H]^{2+} = 969.6$ to $[M + 2H]^{2+} = 970.6$ for peptides 4 and 5 and to $[M + 2H]^{2+} = 971.1$ for peptide 6 (Figure 3a).

These results indicated that two deuterium atoms were introduced into peptides 4 and 5 while three deuterium atoms were introduced into peptide 6 during the reaction. LC-MS/MS analysis of peptide 4 showed that one deuterium atom was located in Ala-8 and another one in Val-10 (Figure 3b, Supplementary Figure S3 and Tables S1 and S2). Analysis of peptide 5 revealed a different pattern with one deuterium atom introduced into Val-9 and one into Val-7 (Supplementary Figure S4a and Table S3). Finally, peptide 6 proved to contain three deuterium atoms located in Val-5, Val-7 and Val-9 (Supplementary Figure S4b and Table S4). The modified peptides were further purified by HPLC and their amino acid content analyzed by LC-MS/MS (see Supplementary Methods). Comparison with authentic standards showed that, in each peptide produced (i.e., peptides 4, 5 and 6), D-Val and D-Ala residues were present (Figure 3c and Supplementary Figure S5). These results established that peptides 4, 5 and 6 are diastereoisomers of peptide 1 and that PoyD is a peptidyl epimerase which requires only an [4Fe-4S] cluster and the SAM cofactor to convert *in vitro* L-Val and L-Ala into their D-configured counterparts.

PoyD Substrate Specificity. As shown above, incubation of peptide 1 led to the formation of three peptides with two epimerization patterns (i.e., natural pattern: Ala-8 and Val-10 and unnatural pattern: Val-5, Val-7 and Val-9) involving either the C-terminal or the penultimate residue with alternating epimerizations (Figure 3a,b). With peptide 2, only one new

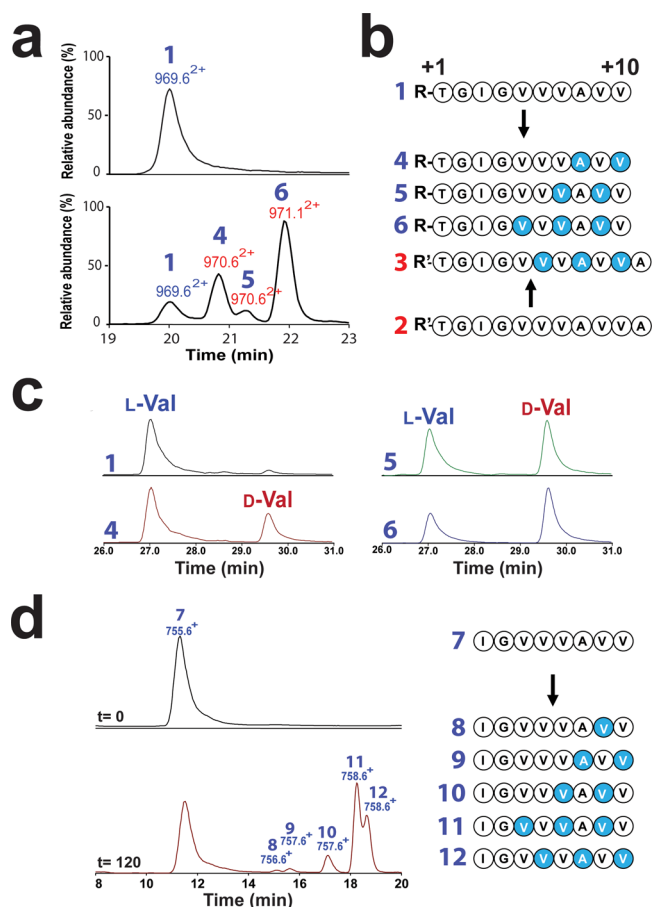


Figure 3. Characterization of the products formed *in vitro* by PoyD. (a) LC-MS/MS analysis of peptide 1 incubated with PoyD. Upper trace peptide 1. Lower trace, peptide 1 incubated with PoyD for 2 h, in deuterated buffer. See Supporting Information for experimental conditions. Numbers refer to the corresponding peptides. (b) Sequence of the different products formed by PoyD *in vitro* after incubation with peptide 1 or peptide 2. See Figure 1 for the amino acid residues corresponding to R and R' and Supplementary Figures S3–4 and S6 and Supplementary Tables S1–S6 for complete peptide assignment. (c) LC-MS/MS analysis of the L-/D-Val content in peptide 1 and the products: peptides 4, 5, and 6 obtained after incubation with PoyD. Amino acids were analyzed after hydrolysis and derivatization by L-FDVA and detected by LC-MS after ion current extraction in MS/MS experiments using the transition 398 > 352 for Val-FDVA derivatives. Numbers indicate peptides analyzed. (d) LC-MS/MS analysis of peptide 7 incubated with PoyD. Analysis was performed at $t = 0$ and after 2 h incubation, upper and lower traces, respectively. Sequences of the peptides produced are indicated. See Supplementary Figures S8–S13 and Supplementary Tables S7–S12 for full peptide assignment. Numbers refer to the corresponding peptides.

peptide was produced, albeit at low level (Supplementary Figure S6). This novel peptide (peptide 3) had a mass increment of +3 Da when the reaction was performed in deuterated buffer, consistent with the incorporation of three deuterium atoms. LC-MS/MS analysis allowed to position deuterium incorporation into Val-6, Ala-8 and Val-10 (i.e., the natural epimerization pattern) (Figure 3b, Supplementary Figure S7 and Tables S5 and S6).

In order to determine the influence of the leader peptide on the activity and specificity of PoyD, we synthesized a peptide containing the residues +3 to +10 (peptide 7, Figure 3d). With

this peptide, devoid of residues from the leader peptide, PoyD catalyzed the formation of five peptides (peptides 8, 9, 10, 11 and 12) (Figure 3d). LC-MS/MS analysis showed that the main products formed were two peptides with three epimerized residues (i.e., Val-5, Val-7 and Val-9 or Val-6, Ala8 and Val-10, peptides 11 and 12, respectively), two peptides with two epimerized residues (i.e., Val-7 and Val-9 or Ala-8 and Val-10, peptides 10 and 9, respectively) and a very low amount of a monoepimerized peptide (peptide 8) (see Supplementary Figures S8–S13 and Supplementary Tables S7–S12).

Thus, this short substrate, despite lacking residues from the leader peptide, recapitulated the different epimerization patterns obtained with peptides 1 and 2.

Collectively, these data showed that subtle variations in the sequences of the substrates led to the formation of peptides with the epimerization pattern found in polytheonamide A (i.e., peptides 3, 4, 9 and 12) but also peptides with an unnatural epimerization pattern (i.e., peptides 5, 6, 8, 10 and 11). However, PoyD always catalyzed epimerization of residues from the core sequence at 1,3-positions but never of residues from the leader sequence. In addition, these experiments support that the activity of PoyD is largely independent of the leader peptide. Of note, PoyD produced several peptides with epimerization located on the last residue (i.e., peptides 4, 9 and 12), in sharp contrast with a recent *in vivo* study which suggested that PoyD cannot modify the last residue of truncated PoyA peptides.²³ Finally, because the post-translational modifications accumulated at the C-terminal end of the various peptides assayed, our results are consistent, as recently suggested by *in vivo* experiments, with PoyD having a directional activity from the C-terminal toward the N-terminal end of the peptide.

To date, several radical SAM enzymes such as Alba^{8,9} and YydG⁵ have been shown to introduce multiple post-translational modifications in their substrate, *in vitro*. However, there is no evidence that one molecule of enzyme is responsible for the insertion of several post-translational modifications on one molecule of substrate. Only for the radical SAM enzyme lipoyl synthase, it has been shown that one molecule of enzyme introduces sequentially two modifications in its substrate (i.e., insertion of two sulfur atoms into its fatty acyl substrate).²⁵ However, the recently solved structure of lipoyl synthase has shown that the incorporation of the two sulfur atoms is part of the same catalytic event.²⁶ The production by PoyD of peptides containing several epimerized residues indicated either the combined action of several enzymes on a same peptide backbone or a processive activity of PoyD.

Mechanistic Investigation of PoyD. Having developed an *in vitro* assay for PoyD, we were able to interrogate its mechanism. MS analysis of the epimerized amino acid residues (i.e., D-Val and D-Ala) produced by PoyD in deuterated buffer showed a mass shift of +1 Da compared to their L-configured counterparts (Figure 4a and Supplementary Figure S5). This result, in line with *in vivo* studies,^{23,24} confirmed that one solvent-derived deuterium atom was incorporated during catalysis. However, MS analysis of the 5'-dA produced ($[M + H]^+$: 252.1) showed no deuterium incorporation consistent with PoyD abstracting a substrate nonexchangeable H atom (Figure 4b). To further validate this conclusion, we incubated PoyD in deuterated buffer but omitted the peptide substrate. Under these conditions, the molecular weight of 5'-dA shifted to $[M + H]^+$: 253.1 (Figure 4b, middle panel) indicating that in

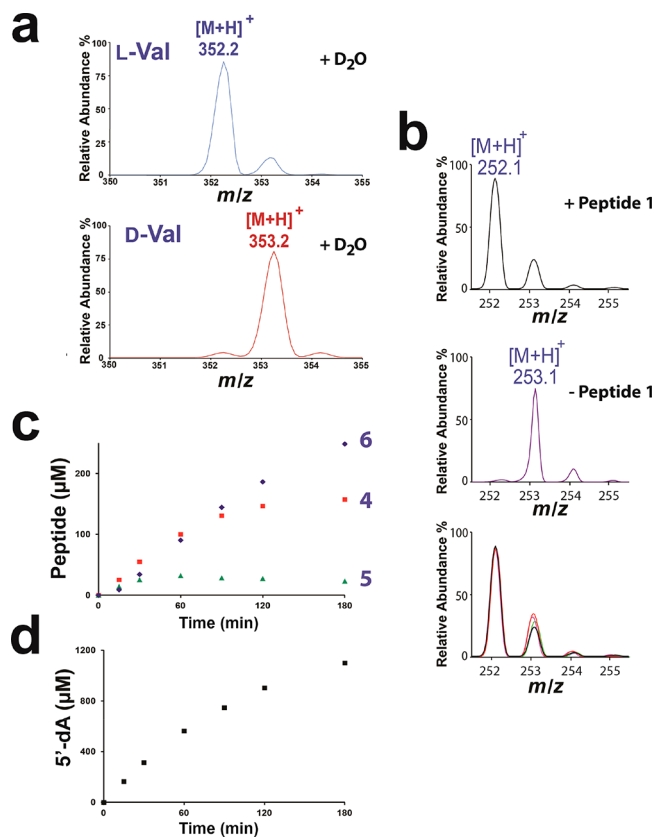


Figure 4. Mechanistic and kinetic analysis of the reaction catalyzed by PoyD. (a) MS/MS spectra of L-Val (upper traces) and D-Val (lower traces) obtained after incubation of PoyD with peptide 1 in deuterated buffer. Peptide products were purified and after acid hydrolysis, amino acids were derivatized with L-FDVA and analyzed by LC-MS/MS. See Supporting Information for experimental conditions. (b) MS spectra of 5'-dA produced by PoyD. PoyD was incubated in deuterated buffer under anaerobic and reducing conditions in the presence (upper trace) or in the absence of peptide 1 (middle trace). The lower trace shows MS spectra of 5'-dA produced over time (from $t = 0$ to $t = 240$ min) in the presence of peptide 1 in deuterated buffer. Production of epimerized peptides (c) and 5'-dA (d) by PoyD. PoyD was incubated in the presence of peptide 1 under anaerobic conditions with sodium dithionite and SAM. Numbers refer to the corresponding peptides formed.

absence of its substrate, PoyD still generated 5'-dA[•] but that this latter reacted with buffer components (Figure 4b).

Kinetic experiments performed with peptide 1 showed that formation of peptide 4 stopped after 90 min (Figure 4c) while peptide 6 was produced over 3 h with an estimated k_{cat} (per epimerization) of 0.02 min^{-1} and 0.03 min^{-1} , respectively (Figure 4c). Thus, peptide 6, with the unnatural epimerization pattern, was the most efficiently produced peptide, *in vitro*. Interestingly, after an initial accumulation, peptide 5 tended to disappear while production of peptide 6 still proceeded. This result suggested that peptide 5 could serve as substrate for PoyD and was further converted into peptide 6. Since peptides 4 and 5 have two modifications and peptide 6 has three modifications, production of 5'-dA ($\sim 1100 \mu M$) and the three epimerized peptides (peptide 4 ($157 \mu M$), peptide 5 ($23 \mu M$) and peptide 6 ($248 \mu M$)) indicated a good correlation between epimerization events and SAM consumption (Figure 4c,d). In addition, LC-MS analysis of 5'-dA produced overtime in deuterated buffer exhibited no deuterium incorporation. Only

when the substrate became limiting (after 1 h), we monitored <10% deuterium incorporation in 5'-dA (Figure 4b, lower trace) while no labeling was measured in the remaining SAM. Altogether, these results were consistent with one molecule of SAM being used by epimerization event.

Identification of a Potential Critical H Atom Donor.

We have recently discovered a peptidyl epimerase in *Bacillus subtilis*. This enzyme called YydG possesses, in addition to the radical SAM cluster, an additional [4Fe-4S] cluster.⁵ This auxiliary cluster has been proposed to assist radical quenching during catalysis.⁵ Interestingly, the only other radical SAM epimerase characterized in vitro, NeON²⁷ which epimerizes the C-5'' of neomycin, also contains an additional [4Fe-4S] cluster²⁷ in a SPASM-like domain.^{12,28,29}

Sequence analysis of PoyD revealed no obvious motif for the coordination of an additional [4Fe-4S] cluster among the 10 cysteine residues present within the protein sequence. To confirm this hypothesis, we replaced the cysteine residues of the radical SAM motif (CxxxCxxC) by alanine residues and probed for the presence of additional [4Fe-4S] clusters. After purification and anaerobic reconstitution, the triple Cys → Ala mutant (A3 mutant) exhibited a distinct UV–visible spectrum from the wild-type enzyme (Figures 5a and b). Determination of the iron content indicated that the A3 mutant contained 0.3 ± 0.2 mol of Fe per polypeptide consistent with its UV–visible spectrum showing the absence of iron–sulfur clusters. To assay its activity, we coexpressed in vivo the A3 mutant with PoyA. As shown (Figure 5c), no epimerized residues could be identified in PoyA supporting the critical role of the radical SAM cluster for PoyD activity.

In several radical SAM enzymes such as spore photoproduct lyase,^{30–34} PolH,³⁵ NeON²⁷ or YydG,⁵ it has been shown that a cysteine residue is used as a critical H atom donor. However, because of the lack of significant homology between PoyD and these enzymes, we could not identify a putative H atom donor. We thus aligned the sequence of PoyD with several PoyD-homologues recently identified.²⁴ In addition to the three cysteine residues from the radical SAM motif, only one cysteine residue (Cys-372) was conserved among these enzymes (Figure 5d). We further searched for homologs in protein databases and identified 67 homologues (sequence identity > 25%) mostly in Proteobacteria. Sequences alignment confirmed that beside the cysteines from the radical SAM motif, only one cysteine residue (i.e., Cys-372 in PoyD) was conserved among these proteins (Supplementary Figure S14).

To probe for the function of Cys-372, we performed its Cys → Ala replacement and coexpressed the corresponding mutant (C372A mutant) in vivo with PoyA. Interestingly, the C372A mutant failed to epimerize PoyA (Figure 5c). However, analysis of the purified C372A mutant showed that its iron content increased from 0.2 ± 0.1 to 3.5 ± 0.2 mol of Fe per polypeptide after anaerobic reconstitution, similarly to the wild-type enzyme.

We further assayed the activity of the C372A and A3 mutants against peptide 1, in vitro (Figure 5e). As shown, the A3 mutant had no enzymatic activity in-line with the in vivo experiments. In contrast, the C372A mutant proved to produce a novel peptide (peptide 13) eluting at 29.4 min and distinct from peptides 4, 5 and 6, produced by the wild-type enzyme.

LC–MS/MS analysis of this novel peptide showed the penultimate valine residue, Val-9, to be epimerized (Supplementary Figure S15). This result was further confirmed by amino acid analysis which showed the presence of D-valine in

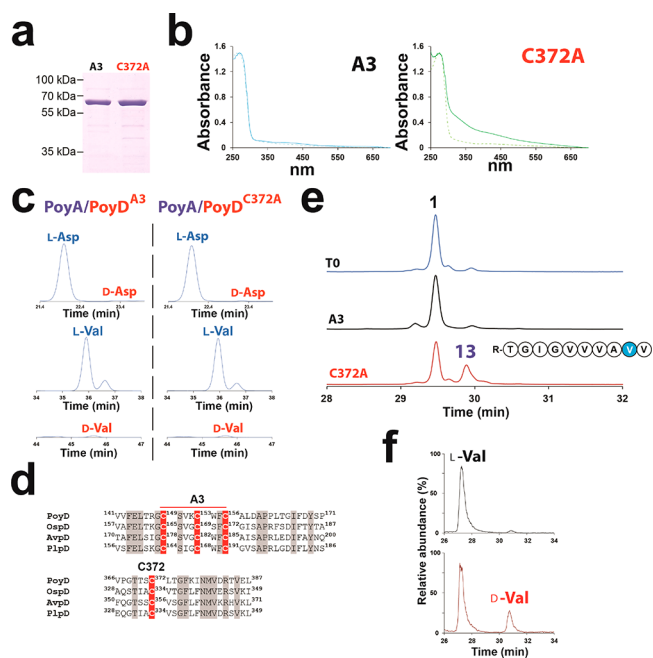


Figure 5. Identification of a potential H atom donor in PoyD. (a) Gel electrophoresis analysis of the A3 and C372A mutants expressed in *E. coli*. (b) UV–visible spectrum of the A3 and C372A mutants before (dotted line) and after anaerobic reconstitution (plain line). (c) HPLC analysis of the amino acid content of PoyA after its in vivo expression in *E. coli* with the A3 or the C372A mutants (left and right panels, respectively). Amino acids were analyzed after acid hydrolysis and derivatization with L-FDVA and their retention times compared with authentic standards. See Supporting Information for experimental conditions. (d) Sequence alignment between PoyD and other proteusins epimerases OspD, AvpD and PlpD. Strictly conserved residues are highlighted in gray or red (cysteine residues). Numbers refers to amino acid residues location in the respective sequences. (e) HPLC analysis of peptide 1 incubated in the presence of the A3 or C372A mutant. Upper trace: HPLC analysis of peptide 1 at $t = 0$. Middle trace: HPLC analysis of peptide 1 after 120 min incubation with the A3 mutant. Lower trace: HPLC analysis of peptide 1 after 120 min incubation with the C372A mutant. The sequence of the product formed by the C372A mutant is indicated. See Supplementary Figure S15 and Supplementary Table S13 for full assignment. (f) LC–MS/MS analysis of the L-/D-Val content of peptides 1 and 13. Upper trace corresponds to peptide 1 and lower trace to peptide 13 produced by the C372A mutant. LC MS/MS experiments were performed using the transition 398 > 352.

peptide 13 with a D-Val/L-Val ratio of ~20%, consistent with the modification of one valine residue out of 5 (Figure 5f). Thus, contrary to in vivo conditions, the C372A mutant is able to catalyze peptide epimerization, in vitro.

Such apparent discrepancies, between in vivo and in vitro activities, have been reported during the investigation of another radical SAM enzyme, the spore photoproduct lyase,³⁶ for which mutation of the H atom donor (i.e., Cys-141)^{30,32,33} has been shown to impair the DNA repair activity in spores but not the ability of the enzyme to repair the spore photoproduct in vitro.^{30,33,37} Altogether, these results support that Cys-372 fulfills an important function likely as a critical H atom donor. However, we cannot rule out that other residues are involved in this process notably tyrosine residues, as shown for carbapenem synthase.³⁸

Processivity of PoyD. The fact that the C372A mutant produced peptide with only one epimerized residue, while the

wild-type enzyme systematically produced peptides with multiple epimerizations, prompted us to assay the activity of PoyD against the monoepimerized **peptide 13**. Indeed, the failure of the C372A mutant to catalyze multiple epimerizations suggested that only peptides containing L-amino acid residues could serve as substrates for PoyD. We thus incubated **peptide 1** with the C372A mutant and purified **peptide 13** (Figure 6a).

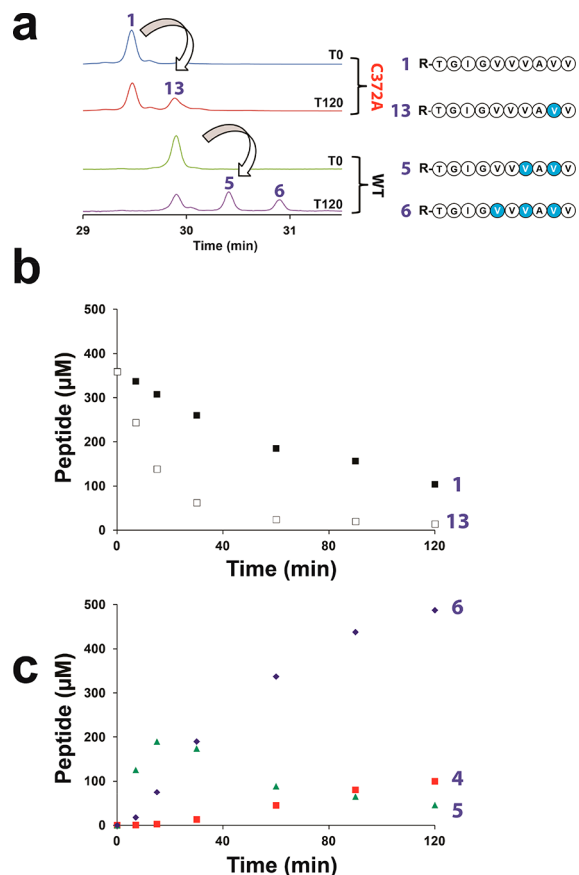


Figure 6. (a) HPLC analysis of peptide 1 after incubation with the C372A mutant and wild-type PoyD. Peptide 1 was incubated with the C372A mutant and analyzed at $t = 0$ (upper blue trace) and $t = 120$ (red trace). After purification, peptide 13 was incubated with PoyD and analyzed by HPLC at $t = 0$ (green trace) and $t = 120$ min (purple lower trace). Numbers refer to the corresponding peptides. (b) Consumption of peptides 1 and 13 during incubation with PoyD. (c) Production of epimerized peptides by PoyD. PoyD was incubated in the presence of peptide 1 under anaerobic conditions with sodium dithionite and SAM. Numbers refer to the corresponding peptides formed.

This peptide was then further incubated with the wild-type enzyme and the reaction analyzed by HPLC and LC–MS. In contrast to **peptide 1**, incubation of **peptide 13** with the wild-type enzyme led to the formation of only two peptides (**peptides 5 and 6**). The implication of this result is 2-fold: it demonstrates that epimerized peptides are substrates for PoyD and, more importantly, that the first epimerization event guides and restricts the following epimerization events in order to preserve the strict 1,3-pattern of epimerization.

To try to discern between processivity and cooperativity, we further performed kinetic experiments in the presence of an equal amount of wild-type (**peptide 1**) and monoepimerized peptide (**peptide 13**). As shown, **peptide 13** was converted

three-times faster than **peptide 1** (Figure 6b). In agreement with this result, **peptide 5**, which was a minor species when PoyD was incubated with **peptide 1** alone (Figure 4c), was the dominant product formed during the first 30 min of the reaction (Figure 6c). As the reaction proceeded, **peptide 5** was then further converted into **peptide 6** containing three D-amino acid residues (Figure 6c). These results demonstrate that PoyD has a better activity on a peptide containing an epimerized residue rather than on a peptide containing only L-amino acid residues. However, in contrast to **peptide 1** which was converted into peptides with different epimerization patterns, **peptide 13** was converted only into **peptide 5** and ultimately **peptide 6** (Figure 6). This suggests that binding and positioning of the substrate is determined, in part, by the presence of epimerized residues.

Interestingly, the transient accumulation of **peptide 5**, in the range of the enzyme concentration ($\sim 150 \mu\text{M}$), is consistent with at least a partial processivity of PoyD, as recently shown for lanthipeptide synthetases.^{39,40} Further studies will be required to definitively address this question.

DISCUSSION

Epimerization reactions were predicted to be catalyzed by radical SAM enzymes more than a decade ago, consecutively to the investigation of the avilamycin A biosynthetic pathway.⁴¹ In vivo studies have shown that a radical SAM enzyme, AviX12, was responsible for a critical C-2 epimerization of a glucose moiety, essential to obtain the active form of this antibiotic. Similarly, radical SAM epimerases have been identified in the biosynthetic pathways of several RiPPs including the bacterial toxin polytheonamide A.^{17,23} However, it is only recently that mechanistic insights have been gained on these novel enzymes. The first radical SAM epimerase characterized at the biochemical level, was the carbohydrate epimerase NeoN which converts neomycin C into neomycin B.²⁷ More recently, while investigating YydG, a radical SAM enzyme of unknown function from *Bacillus subtilis*, we demonstrated this enzyme to be a peptidyl epimerase⁵ converting L-Val and L-Ile into their epimers, during the biosynthesis of the so-called epipeptides.^{2,5}

Interestingly, despite being active on similar hydrophobic amino acid residues, YydG is unrelated, at the sequence level, to PoyD. Notably, YydG is devoid of the RiPP precursor peptide recognition element (i.e., RRE or PqqD-like domain),⁴² characteristic of PoyD and many RiPP modifying enzymes.² In addition, our study shows that PoyD, in contrast to YydG, contains only one [4Fe-4S] cluster.

Epimerized peptides produced in vitro contained modifications only in the C-terminal region strongly supporting a C-to-N directionality for the enzyme, as recently suggested by in vivo experiments. Interestingly, the recent in vitro study of PoyC has demonstrated that it catalyzes methyl transfer to the C-terminal end of a synthetic peptide.¹⁵ We can thus speculate that PoyC, like PoyD, introduces post-translational modifications with a similar C-to-N directionality. Definitive proofs of the directionality of PoyD came from the investigation of the C372A mutant. Indeed, this mutant produced only a monoepimerized peptide. The implications here are 2-fold: first, it unveiled the initiation site of the peptide modification and second, it suggests a processive mode of action of the enzyme. Indeed, we did not evidence the production of other monoepimerized products (i.e., peptides epimerized on other residues) or the formation of peptides with several epimeriza-

tions, as expected in case of the action of several molecules of enzyme.

Interestingly, contrary to recent *in vivo* studies,^{23,43} peptides produced *in vitro* by PoyD contained either the same epimerized residues than the ones found in polytheonamide A (i.e., natural pattern of epimerization: **peptides 3, 4, 9 and 12**) or unnatural epimerizations involving amino acid residues not epimerized in polytheonamide A (i.e., **peptides 5, 6, 8, 10 and 11**).

Of note, we were able to obtain these epimerization patterns using either peptides containing a portion of the leader peptide (i.e., **peptides 1 and 2**) or peptides containing only residues from the core sequence (i.e., **peptide 7**). Hence, the activity of PoyD is largely independent of the leader peptide, as shown for other radical SAM enzymes catalyzing peptide post-translational modifications such as YydG,⁵ Alba⁹ and PoyC,¹⁵ but in contrast to other enzymes such as the KW_cyclase.^{10,12}

On the basis of these results, we can propose the first mechanism for PoyD and proteusin epimerases in general. Following the reductive cleavage of SAM, PoyD generates 5'-dA[•] which abstracts a substrate C_α H atom leading to the formation of 5'-dA and a carbon-centered radical (Figure 7).

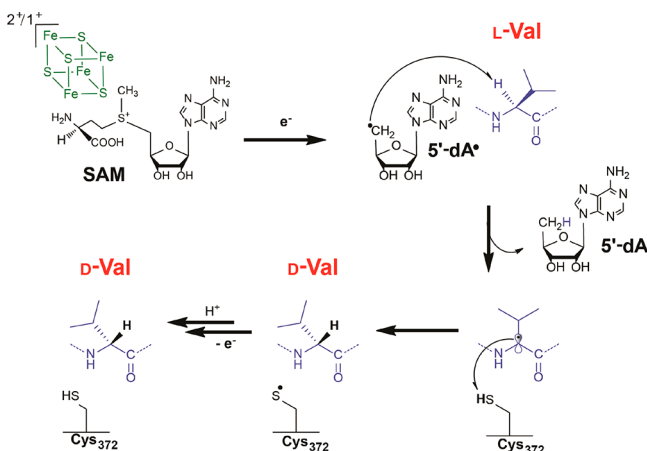


Figure 7. Proposed mechanism for the radical SAM peptide epimerase PoyD. After the reducing SAM cleavage, PoyD generates a 5'-dA[•], which abstracts the amino acid C_α H atom. A carbon-centered radical is formed and quenched by the thiolate H atom of Cys-372 leading to the formation of a D-amino acid residue. Reduction of the thiyl radical is likely assisted by other amino acid residues from PoyD similarly to ribonucleotide reductase or spore photoproduct lyase for the next catalytic cycle.

After the loss of the stereochemistry, one solvent exchangeable H atom is transferred from an H atom donor (likely Cys-372) to the radical intermediate to produce an epimerized amino acid residue and a thiyl radical on the protein.

In the absence of an additional [4Fe-4S] cluster, the most likely hypothesis is that Cys-372 is regenerated by the reduction of the thiyl radical by another cysteine residue like in ribonucleotide reductase.⁴⁴ However, we did not identify an obvious candidate to fulfill this function. Therefore, other residues such as a tyrosine residue^{32–34,37} may also be involved in the regeneration of Cys-372, as suggested for the radical SAM enzyme spore photoproduct lyase.^{30,36,45}

To conclude, our study establishes that PoyD constitutes a distinct group of peptidyl epimerases within the superfamily of radical SAM enzymes. We also demonstrate here that the pattern of epimerization is likely an intrinsic property of the

enzyme that always produces epimerized peptides on the 1,3-positions. Surprisingly, the enzyme is able to recognize a peptide already containing one epimerized residue and to catalyze the next epimerization event, preserving the 1,3 epimerization pattern. The function of the leader peptide, even if not essential, is likely to guide the positioning of PoyA within PoyD active site for the first epimerization event. Further studies should allow to decipher precisely how PoyD interacts with its substrate and what are the molecular determinants of its apparent processivity.

MATERIALS AND METHODS

Expression and Purification of PoyD. The PoyD gene was optimized for expression in *E. coli* and synthesized by Life Technologies. The synthesized PoyD gene was inserted between the NdeI and XhoI restriction sites of a pASK₁₇⁺ plasmid with a Strep-tag fusion. The plasmid was then used to transform *E. coli* BL21 (DE3) star cells. An overnight culture of a single colony of *E. coli* BL21 (DE3)/pASK₁₇⁺-Strep-tag-PoyD was used to inoculate LB medium containing ampicillin (100 μg/L). Cells growth was carried out at 37 °C and 180 rpm until the OD at 600 nm reached ~0.7. Protein expression was performed by adding anhydrotetracycline (400 μM) and iron citrate. Cells were harvested by centrifugation (5000g for 15 min at 4 °C) after an incubation time of 20 h and disrupted by ultrasonication on ice in buffer A (Tris 50 mM, KCl 300 mM, pH 8) supplemented with protease inhibitor cocktail (EDTA-free), 1% Triton 100X. Cells debris were removed by centrifugation at 45 000g for 1.5 h and the protein supernatant was loaded onto a Strep-tactin high capacity gel (IBA) previously equilibrated with buffer A. The gel was washed with 5 column volumes of buffer A and the PoyD protein was eluted with 6 mL of buffer A containing desthiobiotine (3 mM) and dithiothreitol. The purified protein was then concentrated with Amicon concentrator and stored at -80 °C. Protein purity was assayed by SDS-PAGE.

Production of the PoyD Mutant Proteins. The pASK₁₇⁺-Strep-tag-PoyD plasmid served as template for site-directed mutagenesis using this pair of primers: 5'-ACA ACC AGC GCT CTG ACC GGC-3' and 5'-GCC GGT CAG AGC GCT GGT TGT-3' to introduce an alanine at position 372. The triple mutant C149A/C153A/C156A was obtained by two site-directed mutagenesis. First, the C149A mutant was obtained using the pASK₁₇⁺-Strep-tag-PoyD plasmid as template and the primers: 5'-ACC CGT GGT GCT AGC GTT AAA-3' and 5'-TTT AAC GCT AGC ACC ACG GGT-3. The plasmid pASK₁₇⁺-Strep-tag-PoyD-C149A was then used as DNA template to introduce alanine mutations at positions 153 and 156 using the primers 5'-CGT GGT GCT AGC GTT AAA GCT TGG TTT GCT GCA CTG-3' and 5'-CAG TGC AGC AAA CCA AGC TTT AAC GCT AGC ACC ACG-3'. Clones were selected on LB agar plate containing ampicillin (100 μg/L) and DNA sequencing was performed to check the sequence of the mutants. The plasmid was then used to transform *E. coli* BL21 (DE3) star cells for protein expression. The expression and purification of all mutant proteins were conducted in similar conditions to the WT protein.

Enzyme Reconstitution. Reconstitution of the [4Fe-4S] cluster of PoyD and mutants, was achieved in a glovebox under strictly anaerobic conditions (<1 ppm of O₂). Typically, enzymes were reduced with 3 mM DTT for 15 min prior addition of 6 mol equiv of ammonium iron sulfate hexahydrate ((NH₄)₂Fe(SO₄)₂) and sodium sulfide (Na₂S) followed by overnight incubation at 4 °C. Excess unbound iron and sulfur was removed by desalting proteins on Sephadex G25 column against buffer A. Proteins were concentrated with Amicon concentrator and concentration measured on Nanodrop by measuring the absorbance at 280 nm with an extinction coefficient value of 57 870 M⁻¹ cm⁻¹.

Enzyme Assays. Enzyme assays were performed at 25 °C under strictly anaerobic conditions in buffer A. Deuterated buffer was obtained by several cycles of freeze-drying in D₂O. Otherwise stated, 150 μM PoyD protein (WT or mutants after anaerobic reconstitu-

tion), 2 mM SAM and 330 μ M substrate were mixed and the reaction initiated by adding 6 mM sodium dithionite (DTN). For enzyme kinetics reactions, 10 μ L aliquots were sampled overtime and analyzed by HPLC.

Amino Acids Enantiomer Analysis after L-FDVA Derivatization. After reaction with PoyD, peptides were purified by HPLC and hydrolyzed in DCl (or HCl, 6 N) under vacuum conditions at 110 °C for 18 h. Samples were dried using a centrifugal vacuum concentrator and dissolved in 10 μ L Milli-Q water. Reaction mixtures were incubated 1 h at 42 °C after addition of 10 μ L NaHCO₃ 1 M and 25 μ L *N*- α -(2,4-dinitro-5-fluorophenyl)-L-valinamide (L-FDVA). The derivatization reaction was stopped by addition of 10 μ L of HCl 2 N. The mixture was diluted 1/10 in 20% acetonitrile containing 0.1% formic acid before analysis. A similar protocol was used to analyze the amino acid content of PoyA expressed alone or with PoyD and PoyD mutants.

HPLC Analysis. An Agilent 1200 series infinity equipped with a reversed phase column (LiChroCART RP-18e 5 μ m, Merck Millipore) was used to perform HPLC analysis. Samples were diluted 10-fold in 0.1% trifluoroacetic acid solution. The column was equilibrated with solvents A (H₂O, 0.1% TFA) and the solvent B (80% CH₃CN, 19.9% H₂O, 0.1% TFA) was applied as follow: 0–1 min: 0% B; 1–20 min: linear gradient 1.2%/min B; 20–40 min: linear gradient with 3%/min B at a flow rate of 1 mL/min. Detection was performed with a diode array detector at 257, 278, and 340 nm and by fluorescence (ex/em: 278/350 nm).

Liquid Chromatography–Mass Spectrometry Analysis. Mass spectrometry analysis was performed using an LTQ mass spectrometer (Thermo Fisher Scientific) coupled to a nano-HPLC system (Ultimate 3000, Dionex thermo fisher Scientific) with a nanoelectrospray source. Samples were inject onto a Pepmap100 C18 column (0.075 \times 150 mm, 100A, 3 μ m; Dionex) or a Proswift RP4H polymeric nanocolumn (0.1 \times 250 mm, 1000A, Dionex) (for PoyA), at a flow rate of 0.3 μ L/min and 0.45 μ L/min, respectively. The following buffer system was used: Buffer A: formic acid 0.1% and buffer B: 80% acetonitrile, 0.1% formic acid. Several linear gradients were used according the molecules to analyze: 25 to 75% buffer B for peptide analysis; 0 to 20% buffer B for 5'-dA; 30–100% buffer B for L-FDVA derivatives analysis and 0 to 60% buffer B for PoyA analysis. Mass detection was realized in positive enhanced resolution. The doubly charged ions for peptides 1 to 13 were fragmented at 35% NCE and spectra acquired in profile and enhanced resolution mode to locate deuterium incorporation. For L-FDVA derivatives, we extracted the ion current corresponding to the most intense ion daughter detected in MS/MS spectrum (respectively the transition 398 > 352 and 370 > 324 for Val-FDVA and Ala-FDVA were used).

■ ASSOCIATED CONTENT

● Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.7b08402.

Experimental procedures, Figures S1–S15 and Table S1–13 (PDF)

■ AUTHOR INFORMATION

Corresponding Author

*olivier.berteau@inra.fr

ORCID

Olivier Berteau: 0000-0002-3434-5168

Author Contributions

§A.P., A.B., A.G. and X.K. contributed equally.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This work was supported by European Research Council (ERC) grant: Consolidator Grant 617053.

■ REFERENCES

- (1) Arnison, P. G.; Bibb, M. J.; Bierbaum, G.; Bowers, A. A.; Bugni, T. S.; Bulaj, G.; Camarero, J. A.; Campopiano, D. J.; Challis, G. L.; Clardy, J.; Cotter, P. D.; Craik, D. J.; Dawson, M.; Dittmann, E.; Donadio, S.; Dorrestein, P. C.; Entian, K. D.; Fischbach, M. A.; Garavelli, J. S.; Goransson, U.; Gruber, C. W.; Haft, D. H.; Hemscheidt, T. K.; Hertweck, C.; Hill, C.; Horswill, A. R.; Jaspers, M.; Kelly, W. L.; Klinman, J. P.; Kuipers, O. P.; Link, A. J.; Liu, W.; Marahiel, M. A.; Mitchell, D. A.; Moll, G. N.; Moore, B. S.; Muller, R.; Nair, S. K.; Nes, I. F.; Norris, G. E.; Olivera, B. M.; Onaka, H.; Patchett, M. L.; Piel, J.; Reaney, M. J.; Rebuffat, S.; Ross, R. P.; Sahl, H. G.; Schmidt, E. W.; Selsted, M. E.; Severinov, K.; Shen, B.; Sivonen, K.; Smith, L.; Stein, T.; Sussmuth, R. D.; Tagg, J. R.; Tang, G. L.; Truman, A. W.; Vederas, J. C.; Walsh, C. T.; Walton, J. D.; Wenzel, S. C.; Willey, J. M.; van der Donk, W. A. *Nat. Prod. Rep.* **2013**, *30*, 108–60.
- (2) Benjdia, A.; Balty, C.; Bertheau, O. *Front. Chem.* **2017**, *5*, 87.
- (3) Donia, M. S.; Fischbach, M. A. *Science* **2015**, *349*, 1254766.
- (4) Benjdia, A.; Bertheau, O. *Biochem. Soc. Trans.* **2016**, *44*, 109–15.
- (5) Benjdia, A.; Guillot, A.; Ruffie, P.; Leprince, J.; Bertheau, O. *Nat. Chem.* **2017**, *9*, 698–707.
- (6) Hetrick, K. J.; van der Donk, W. A. *Curr. Opin. Chem. Biol.* **2017**, *38*, 36–44.
- (7) Funk, M. A.; van der Donk, W. A. *Acc. Chem. Res.* **2017**, *50*, 1577–1586.
- (8) Flühe, L.; Knappe, T. A.; Gattner, M. J.; Schäfer, A.; Burghaus, O.; Linne, U.; Marahiel, M. A. *Nat. Chem. Biol.* **2012**, *8*, 350–7.
- (9) Benjdia, A.; Guillot, A.; Lefranc, B.; Vaudry, H.; Leprince, J.; Bertheau, O. *Chem. Commun.* **2016**, *52*, 6249–52.
- (10) Schramma, K. R.; Bushin, L. B.; Seyedsayamdost, M. R. *Nat. Chem.* **2015**, *7*, 431–7.
- (11) Barr, I.; Latham, J. A.; Iavarone, A. T.; Chantarojsiri, T.; Hwang, J. D.; Klinman, J. P. *J. Biol. Chem.* **2016**, *291*, 8877–84.
- (12) Benjdia, A.; Decamps, L.; Guillot, A.; Kubiak, X.; Ruffie, P.; Sandstrom, C.; Bertheau, O. *J. Biol. Chem.* **2017**, *292*, 10835–10844.
- (13) Pierre, S.; Guillot, A.; Benjdia, A.; Sandstrom, C.; Langella, P.; Bertheau, O. *Nat. Chem. Biol.* **2012**, *8*, 957–9.
- (14) Benjdia, A.; Pierre, S.; Gherasim, C.; Guillot, A.; Carmona, M.; Amara, P.; Banerjee, R.; Bertheau, O. *Nat. Commun.* **2015**, *6*, 8377.
- (15) Parent, A.; Guillot, A.; Benjdia, A.; Chartier, G.; Leprince, J.; Bertheau, O. *J. Am. Chem. Soc.* **2016**, *138*, 15515–15518.
- (16) Huo, L.; Rachid, S.; Stadler, M.; Wenzel, S. C.; Muller, R. *Chem. Biol.* **2012**, *19*, 1278–87.
- (17) Freeman, M. F.; Gurgui, C.; Helf, M. J.; Morinaka, B. I.; Uria, A. R.; Oldham, N. J.; Sahl, H. G.; Matsunaga, S.; Piel, J. *Science* **2012**, *338*, 387–90.
- (18) Vey, J. L.; Drennan, C. L. *Chem. Rev.* **2011**, *111*, 2487–506.
- (19) Broderick, J. B.; Duffus, B. R.; Duschene, K. S.; Shepard, E. M. *Chem. Rev.* **2014**, *114*, 4229–317.
- (20) Walsby, C. J.; Hong, W.; Broderick, W. E.; Cheek, J.; Ortillo, D.; Broderick, J. B.; Hoffman, B. M. *J. Am. Chem. Soc.* **2002**, *124*, 3143–51.
- (21) Nicolet, Y.; Amara, P.; Mouesca, J. M.; Fontecilla-Camps, J. C. *Proc. Natl. Acad. Sci. U. S. A.* **2009**, *106*, 14867–71.
- (22) Horitani, M.; Byer, A. S.; Shisler, K. A.; Chandra, T.; Broderick, J. B.; Hoffman, B. M. *J. Am. Chem. Soc.* **2015**, *137*, 7111–21.
- (23) Freeman, M. F.; Helf, M. J.; Bhushan, A.; Morinaka, B. I.; Piel, J. *Nat. Chem.* **2017**, *9*, 387–395.
- (24) Morinaka, B. I.; Vagstad, A. L.; Helf, M. J.; Gugger, M.; Kegler, C.; Freeman, M. F.; Bode, H. B.; Piel, J. *Angew. Chem., Int. Ed.* **2014**, *53*, 8503–7.
- (25) Cicchillo, R. M.; Booker, S. J. *J. Am. Chem. Soc.* **2005**, *127*, 2860–1.

- (26) McLaughlin, M. I.; Lanz, N. D.; Goldman, P. J.; Lee, K. H.; Booker, S. J.; Drennan, C. L. *Proc. Natl. Acad. Sci. U. S. A.* **2016**, *113*, 9446–50.
- (27) Kudo, F.; Hoshi, S.; Kawashima, T.; Kamachi, T.; Eguchi, T. *J. Am. Chem. Soc.* **2014**, *136*, 13909–15.
- (28) Benjdia, A.; Subramanian, S.; Leprince, J.; Vaudry, H.; Johnson, M. K.; Berteau, O. *FEBS J.* **2010**, *277*, 1906–20.
- (29) Grell, T. A.; Goldman, P. J.; Drennan, C. L. *J. Biol. Chem.* **2015**, *290*, 3964–71.
- (30) Chandor-Proust, A.; Berteau, O.; Douki, T.; Gasparutto, D.; Ollagnier-de-Choudens, S.; Fontecave, M.; Atta, M. *J. Biol. Chem.* **2008**, *283*, 36361–8.
- (31) Chandor, A.; Berteau, O.; Douki, T.; Gasparutto, D.; Sanakis, Y.; Ollagnier-de-Choudens, S.; Atta, M.; Fontecave, M. *J. Biol. Chem.* **2006**, *281*, 26922–31.
- (32) Benjdia, A.; Heil, K.; Winkler, A.; Carell, T.; Schlichting, I. *Chem. Commun.* **2014**, *50*, 14201–4.
- (33) Benjdia, A.; Heil, K.; Barends, T. R.; Carell, T.; Schlichting, I. *Nucleic Acids Res.* **2012**, *40*, 9308–18.
- (34) Benjdia, A. *Curr. Opin. Struct. Biol.* **2012**, *22*, 711–20.
- (35) Lilla, E. A.; Yokoyama, K. *Nat. Chem. Biol.* **2016**, *12*, 905–907.
- (36) Berteau, O.; Benjdia, A. *Photochem. Photobiol.* **2017**, *93*, 67–77.
- (37) Yang, L.; Nelson, R. S.; Benjdia, A.; Lin, G.; Telsler, J.; Stoll, S.; Schlichting, I.; Li, L. *Biochemistry* **2013**, *52*, 3041–50.
- (38) Chang, W. C.; Guo, Y.; Wang, C.; Butch, S. E.; Rosenzweig, A. C.; Boal, A. K.; Krebs, C.; Bollinger, J. M., Jr. *Science* **2014**, *343*, 1140–4.
- (39) Thibodeaux, C. J.; Ha, T.; van der Donk, W. A. *J. Am. Chem. Soc.* **2014**, *136*, 17513–29.
- (40) Miller, L. M.; Chatterjee, C.; van der Donk, W. A.; Kelleher, N. L. *J. Am. Chem. Soc.* **2006**, *128*, 1420–1.
- (41) Boll, R.; Hofmann, C.; Heitmann, B.; Hauser, G.; Glaser, S.; Koslowski, T.; Friedrich, T.; Bechthold, A. *J. Biol. Chem.* **2006**, *281*, 14756–63.
- (42) Burkhart, B. J.; Hudson, G. A.; Dunbar, K. L.; Mitchell, D. A. *Nat. Chem. Biol.* **2015**, *11*, 564–70.
- (43) Morinaka, B. I.; Verest, M.; Freeman, M. F.; Gugger, M.; Piel, J. *Angew. Chem., Int. Ed.* **2017**, *56*, 762–766.
- (44) Stubbe, J.; Nocera, D. G.; Yee, C. S.; Chang, M. C. *Chem. Rev.* **2003**, *103*, 2167–201.
- (45) Friedel, M. G.; Berteau, O.; Pieck, J. C.; Atta, M.; Ollagnier-de-Choudens, S.; Fontecave, M.; Carell, T. *Chem. Commun.* **2006**, 445–7.