JACS
JOURNAL OF THE AMERICAN CHEMICAL SOCIETY
Communication
pubs.acs.org/JACS

Biosynthesis of F_0, Precursor of the F_{420} Cofactor, Requires a Unique Two Radical-SAM Domain Enzyme and Tyrosine as Substrate

Laure Decamps,[†,‡,#] Benjamin Philmus,[§,#] Alhosna Benjdia,[⊥] Robert White,[∥] Tadhg P. Begley,*[,§] and Olivier Berteau*[,†,‡]

[†]Institut National de la Recherche Agronomique, UMR 1319 Micalis, F-78350 Jouy-en-Josas, France
[‡]AgroParisTech, UMR Micalis, F-78350 Jouy-en-Josas, France
[§]Department of Chemistry, Texas A&M University, College Station, Texas 77843, United States
[⊥]Department of Biomolecular Mechanisms, Max-Planck Institute for Medical Research, Jahnstrasse 29, 69120 Heidelberg, Germany
[∥]Department of Biochemistry, Virginia Polytechnic Institute and State University, Blacksburg, Virginia 24061, United States

Supporting Information

ABSTRACT: Cofactors play key roles in metabolic pathways. Among them F_{420} has proved to be a very attractive target for the selective inhibition of archaea and actinobacteria. Its biosynthesis, in a unique manner, involves a key enzyme, F_0-synthase. This enzyme is a large monomer in actinobacteria, while it is constituted of two subunits in archaea and cyanobacteria. We report here the purification of both types of F_0-synthase and their *in vitro* activities. Our study allows us to establish that F_0-synthase, from both types, uses 5-amino-6-ribitylamino-2,4(1*H*,3*H*)-pyrimidinedione and tyrosine as substrates but not 4-hydroxylphenylpyruvate as previously suggested. Furthermore, our data support the fact that F_0-synthase generates two 5′-deoxyadenosyl radicals for catalysis which is unprecedented in reaction catalyzed by radical SAM enzymes.

F_{420} is a deazaflavin derivative used as an essential cofactor by enzymes involved in energy metabolism, antibiotic biosynthesis, and DNA repair in methanogenic archaea,[1] actinobacteria,[2,3] cyanobacteria,[4] and eukaryotes.[5] Nevertheless, numerous F_{420}-dependent enzymes likely remain to be uncovered.[6] In *Mycobacterium* species, F_{420} reduction plays an important role in preventing nitrosative stress in macrophages and is also critical for the activation of bicyclic nitroimidazoles, which are promising antitubercular drugs.[7,8] The F_{420} biosynthetic pathway has thus emerged as a promising target against *Mycobacterium tuberculosis*. While the redox role of F_{420} is now well understood,[9] the mechanism of formation of its deazaflavin core (F_0) is unknown and has not yet been reconstituted in a biochemically defined system. This represents one of the last major unsolved problems in cofactor biosynthesis.

The biosynthesis of the F_0 deazaflavin chromophore of F_{420} is outlined in Figure 1. Labeling studies suggest that 4-hydroxyphenylpyruvate (**1**) or tyrosine (**2**) and 5-amino-6-ribitylamino-2,4(1*H*,3*H*)-pyrimidinedione (diaminouracil, **3**) are the precursors.[10,11] Compound **1** is derived from the metabolism of **2**, and **3** is a precursor to riboflavin.[12] As this oxidative coupling occurs under anaerobic conditions, a simple oxygen-dependent mechanism can be excluded. Sequence analysis of the gene coding for the enzyme responsible for F_0 biosynthesis, F_0-synthase, suggests that it contains two subunits in archaea and cyanobacteria (CofG/CofH), while it is a single large bifunctional enzyme in actinobacteria (FbiC).[13] The enzyme possesses two predicted radical SAM (or radical AdoMet) domains (CX_3CX_2C motif)[14,15] suggesting that it coordinates two [4Fe-4S] centers and is likely to generate two 5′-deoxyadenosyl radicals (5′-dA•)[4] during F_0 formation. In this communication, we report the successful *in vitro* reconstitution and preliminary biochemical characterization of the F_0-synthase catalyzed reaction, poising the system for future inhibition and mechanistic studies.

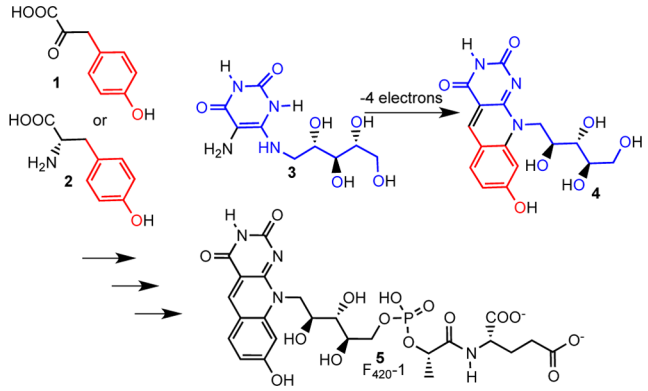

Figure 1. Biosynthesis of the deazaflavin chromophore of F_{420} (F_0, **4**). The structure of F_{420} shown contains a single glutamic acid (F_{420}-1). This number is variable.[3]

Attempts to heterologously express the F_0-synthase gene from *M. tuberculosis* gave very low yield of pure protein despite using a construct with optimized codon usage. We thus searched for a close relative with improved stability and selected the F_0-synthase from a thermophilic organism (*Thermobifida fusca*), with 60% sequence identity to the *M. tuberculosis* enzyme. As shown in Figure 2A, we successfully obtained pure enzyme which exhibits the typical brownish color of radical

Received: August 5, 2012
Published: October 16, 2012

   dx.doi.org/10.1021/ja307762b | *J. Am. Chem. Soc.* 2012, 134, 18173−18176

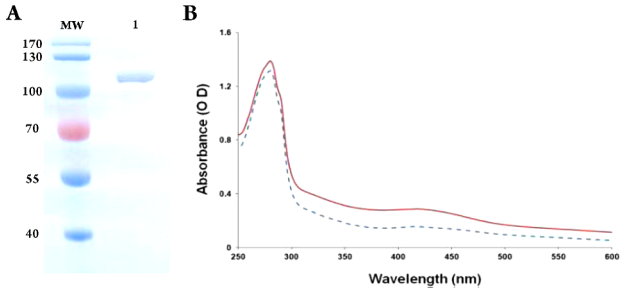

Figure 2. Analysis of purified F_0-synthase (*T. fusca*). (A) SDS-PAGE analysis of the protein lane 1; MW: molecular weight markers in kDa. (B) UV−vis spectra of the "as isolated" (dashed blue line) and the reconstituted (solid red line) enzyme.

SAM enzymes and a UV−visible spectrum with the characteristic shoulders at 330 and 420 nm (Figure 2B, blue trace).[16,17] This enzyme contains 1.2 ± 0.8 mol of Fe per mole of polypeptide. After reconstitution under anaerobic conditions, the enzyme exhibits increased absorbance at 420 nm (Figure 2B, red trace) and contains 9.5 ± 1.2 mol of Fe per mole of polypeptide consistent with the presence of two [4Fe-4S] centers in F_0-synthase. Two additional shoulders could also be seen around the absorbance maximum at 280 nm, suggesting that the isolated enzyme copurified with bound metabolites.

The reconstituted F_0-synthase was reduced with sodium dithionite and assayed for the production of 5′-deoxyadenosine (5′-dA) from *S*-adenosyl-L-methionine (SAM). As shown, the enzyme generated a new product with retention time of 13.6 min (Figure 3A). This product was identified as 5′-dA by

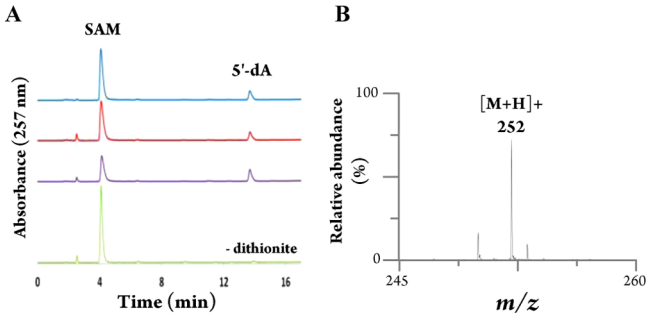

Figure 3. (A) HPLC analysis of the F_0-synthase reaction mixture containing the enzyme and various components after 3 h of incubation. Blue trace: SAM + dithionite; red trace: SAM + **1** + **3** + dithionite; purple trace: SAM + **2** + **3** + dithionite; green trace: SAM without dithionite. (B) MALDI-TOF MS analysis of the compound eluting at 13.6 min.

comigration with an authentic standard, its absorption maximum at 257 nm, and by mass spectrometry (Figures 3B and S1 and S2). The formation of 5′-dA did not require the presence of the potential substrates (**1**, **2**, or **3**), a common feature of radical SAM enzymes.[17]

HPLC analysis of the reaction mixture revealed the formation of trace amounts of a product when F_0-synthase was incubated in the presence of SAM and dithionite (Figure 4A, blue trace). The product eluting at 18.5 min had the same retention time and UV−vis spectrum as authentic F_0 (Figures 4A and S3 and S4), and MS analysis confirmed the expected mass of 363 Da (Figure 4B). The dithionite-reduced enzyme produced low levels of 5′-dA and F_0, without the requirement of addition of substrates and SAM, indicating that all the

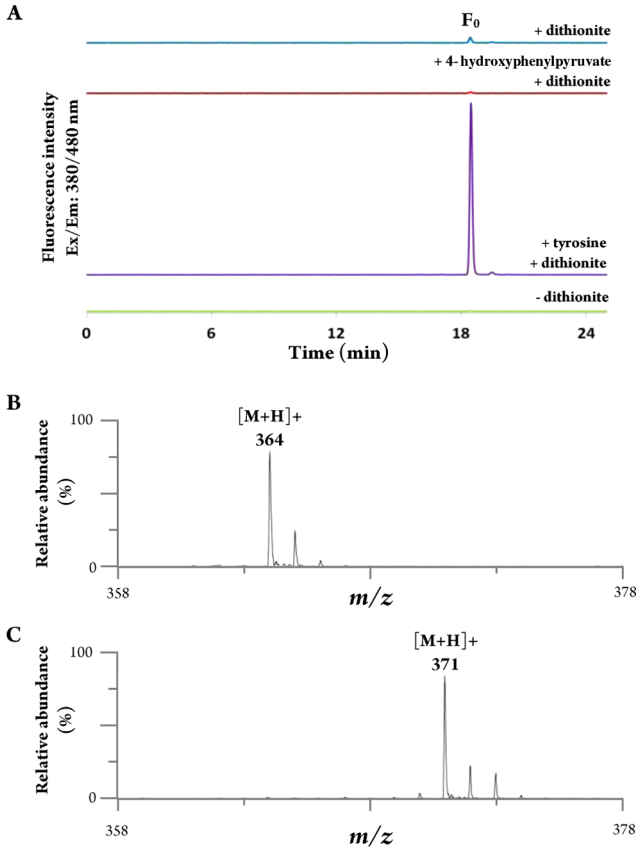

Figure 4. (A) HPLC analysis of the F_0-synthase reaction mixture containing the enzyme and various components. Blue trace: SAM + dithionite; red trace: SAM + **1** + **3** + dithionite; purple trace: SAM + **2** + **3** + dithionite; green trace: SAM + **2** + **3** without dithonite. MALDI-TOF MS analysis of: (B) the compound eluting at 18.5 min and (C) the product of the SAM + [U-^{13}C]-**2** + **3** + dithionite reaction mixture.

substrates were present, at low levels, in the purified enzyme. This result was consistent with the unusual UV−vis spectrum of the enzyme, which exhibited a shoulder around 290 nm. Surprisingly, addition of **1** and **3** did not increase F_0 formation (Figure 4A, red trace).

In vivo labeling studies were unable to differentiate between **1** and **2** as F_0 precursors because these compounds are readily interconverted due to the high level of tyrosine transaminase activity in the cell.[11] As addition of **1** did not result in efficient F_0 synthesis, **2** was a logical choice as an alternative substrate. HPLC analysis of this reaction mixture showed a 77-fold increase in F_0 synthesis over the reaction using **1** as substrate (Figure 4A, purple trace). When the reaction was repeated using fully ^{13}C-labeled **2** ([U-^{13}C]-**2**), MS analysis of the resulting product showed the expected 7 Da mass increase (Figure 4C), confirming **2** as the source of the atoms shown in red in F_0 (**4**) structure in the Figure 1.

Quantitative analysis showed that for each mole of F_0 produced, 3.6 ± 0.8 mol of 5′-dA was formed when dithionite was used as the reductant. As dithionite-reduced radical SAM enzymes are generally more prone to generate 5′-dA independently of the primary enzymatic product, we repeated this experiment using the physiological system flavodoxin/flavodoxin reductase/NADPH to reduce the [4Fe-4S] cluster. Under these conditions, the reaction was slower, and the ratio of 5′-dA to F_0 was close to 2.5, consistent with the abstraction of two H-atoms during each catalytic cycle (Figure 5).

dx.doi.org/10.1021/ja307762b | J. Am. Chem. Soc. 2012, 134, 18173−18176

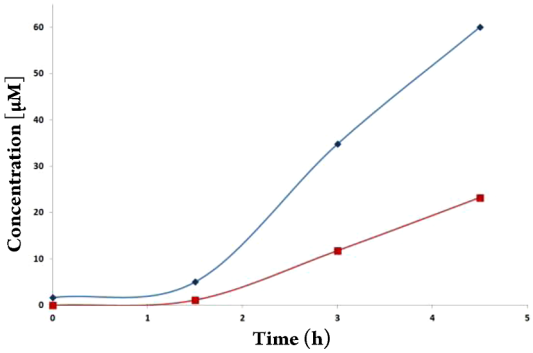

Figure 5. Analysis of 5′-dA (blue trace) and F_0 (red trace) production by F_0-synthase incubated anaerobically with SAM, **2**, and **3** in the presence of flavodoxin/flavodoxin reductase/NADPH.

The reaction catalyzed by F_0-synthase is clearly a complex transformation. To probe the function of the two distinct radical SAM domains, we overexpressed, separately, CofG and CofH in *Escherichia coli*. The *Methanococcus jannaschii* CofG overexpressed well, giving large quantities of soluble protein that were determined to contain 1.7 mol of Fe per mole of polypeptide and exhibited the characteristic absorbance of [4Fe-4S] enzymes (Figure S5A).

The *Nostoc punctiforme* PCC 73102 CofH was more difficult to overexpress but gave good amounts of soluble protein with 1.8 mol of Fe per mole of protein when expressed in the presence of the *suf* operon of *E. coli* (Figure S5B).

When either CofG or CofH were incubated with SAM and dithionite, 5′-dA was seen in the reaction mixture (Figure S6). This suggests that both proteins have the ability to form the 5′-dA•, which they then use for initiation of chemistry. Incubation of both CofG and CofH with SAM, **2** and **3** generated a fluorescent product eluting at 11.5 min, only in the presence of sodium dithionite (Figure 6A, red trace). This product had a UV−vis and mass spectrum consistent with F_0. The ratio of 5′-dA to F_0 was higher than that observed for the fused enzymes (4:1 early in the reaction and increased at later times). The F_0-synthase activity required **2**, dithionite, SAM, CofG, and CofH but not **3** (Figure 6A, blue trace). The activity observed in the absence of **3** suggested that this substrate copurified with either CofG or CofH. Denaturation of CofH with urea followed by derivatization of **3** with diacetyl in trichloroacetic acid resulted in a fluorescent peak that coeluted with a reference sample of 6,7-dimethyl-8-ribityllumazine.[18] No bound metabolites could be identified in denatured CofG. This suggests that **3** is a substrate for CofH and that CofH is therefore likely to catalyze the early steps in F_0 formation and CofG the later steps.

To determine if CofG and CofH functioned independently and released a diffusible intermediate, each protein was separately incubated with SAM, reduced methyl viologen (or sodium dithionite), **2**, and **3** for 5−6 h. After protein removal by ultrafiltration, the small molecule pool from each reaction mixture was then incubated with SAM, **2**, **3**, reduced methyl viologen (or sodium dithionite), and the protein missing from the first incubation. Only in the sequential reaction of CofH followed by CofG was F_0 synthesis observed (Figure 6B). This suggests that CofH produces a stable intermediate that is a substrate for CofG and required for F_0 synthesis. This is also consistent with the detection of trace amounts of **3** associated with purified CofH described above.

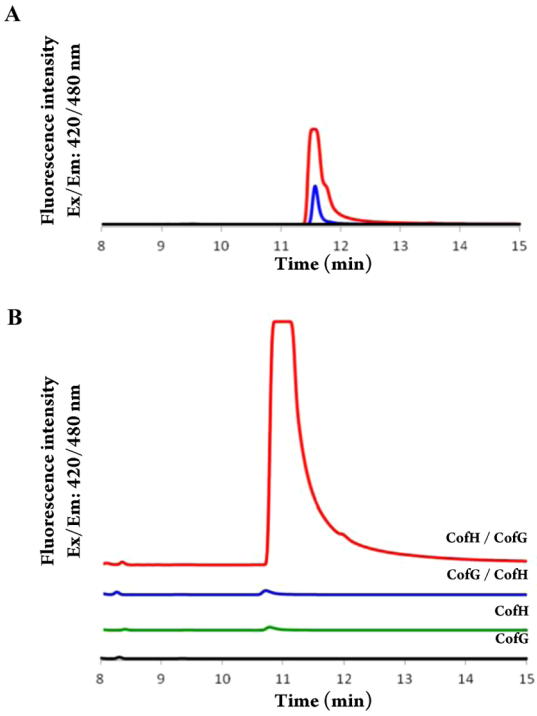

Figure 6. HPLC analysis of the CofG and CofH catalyzed reactions with fluorescence monitoring. (A) Red trace: Chromatogram of the full reaction mixture (CofG + CofH + SAM + **2** + **3** + dithionite); blue trace: chromatogram of the full reaction mixture lacking **3**; black trace: chromatogram of the full reaction mixture lacking CofG and CofH. (B) Red trace: chromatogram of the deproteinized CofH reaction mixture (CofH + SAM + **2** + **3** + reduced methyl viologen) after treatment with CofG; blue trace: chromatogram of the deproteinized CofG reaction mixture (CofG + SAM + **2** + **3** + reduced methyl viologen) after treatment with CofH; green trace: chromatogram of the deproteinized CofH reaction mixture (CofH + SAM + **2** + **3** + reduced methyl viologen); black trace: chromatogram of the deproteinized CofG reaction mixture (CofG + SAM + **2** + **3** + reduced methyl viologen). The chromatography conditions used here were different from those used in Figure 4 (see SI).

While the use of two separate adenosyl radical generating sites on a radical SAM enzyme has not been previously reported, variations on this motif have been observed. MoaA is an enzyme involved in molybdopterin biosynthesis and also utilizes two [4Fe-4S] clusters at its active site.[19,20] Structural studies however demonstrate that the second cluster is involved in GTP binding and does not catalyze adenosyl radical formation. AlbA is an enzyme involved in subtilosin A biosynthesis. Here the second cluster has been proposed to serve as an electron acceptor during thioether formation.[21]

Recently two radical SAM enzymes using **2** as substrate have been identified and provide some insight into the mechanism of F_0-synthase. One (ThiH) is part of the biosynthetic pathway to thiamin,[22,23] and the other (HydG) is involved in the complex biosynthesis of the metal cofactor of [Fe-Fe] hydrogenases.[24] The C-terminal part of F_0-synthase in actinobacteria and the CofH enzyme (from archaea and cyanobacteria) exhibit significant homologies with these two enzymes (Figure S7). Both ThiH and HydG form *p*-cresol as a byproduct resulting from initial hydrogen atom abstraction of the phenolic hydrogen of **2**. It is therefore possible that CofH catalyzes a similar H-atom abstraction leading to the formation of a quinone methide (**6**, Figure 7). Addition of **3** would give **7**. A

dx.doi.org/10.1021/ja307762b | J. Am. Chem. Soc. 2012, 134, 18173−18176

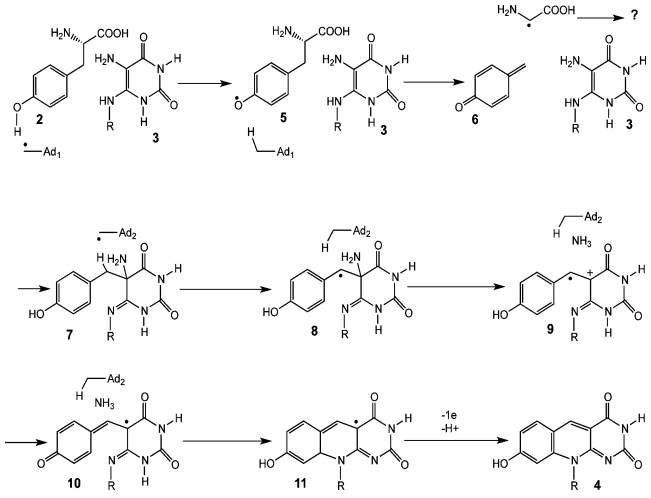

Figure 7. Mechanistic proposal for the reaction catalyzed by F_0-synthase.

second hydrogen atom abstraction at the CofG site would give **8**. Loss of ammonia and deprotonation would give **10**. Cyclization to **11** and a final electron transfer back to the oxidized [4Fe-4S] cluster would complete the formation of the deazaflavin chromophore. Several variations on this proposal are possible, and experiments are currently underway to identify the product of the CofH-catalyzed reaction and to test and refine this hypothesis.

In conclusion, F_0-synthase is a novel radical SAM enzyme that generates the adenosyl radical at two separate sites to mediate the oxidative coupling of **2** and **3** to give the deazaflavin chromophore (**4**). Here we report the successful reconstitution of the holoenzyme as well as its separate domains and develop the system to the point where it is now ready for detailed mechanistic studies. We propose a mechanism for F_0 formation, which will guide these studies.

■ ASSOCIATED CONTENT

Supporting Information

Methods and details regarding enzyme assays and analytical methods. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

Olivier.Berteau@jouy.inra.fr; begley@chem.tamu.edu

Author Contributions

[#]These authors contributed equally.

Notes

The authors declare no competing financial interests.

■ ACKNOWLEDGMENTS

This work was supported by a Ph.D. grant from INRA, AlimH department to L.D., the Robert A. Welch Foundation (to T.P.B., A-0034) and by the National Science Foundation grant MCB0722787 (to R.W.).

■ REFERENCES

(1) Cheeseman, P.; Toms-Wood, A.; Wolfe, R. S. *J. Bacteriol.* **1972**, *112*, 527−31.

(2) Kuo, M. S. T.; Yurek, D. A.; Coats, J. H.; Li, G. P. *J. Antibiot.* **1989**, *42*, 475−8.

(3) Bair, T. B.; Isabelle, D. W.; Daniels, L. *Arch. Microbiol.* **2001**, *176*, 37−43.

(4) Graham, D. E.; Xu, H.; White, R. H. *Arch. Microbiol.* **2003**, *180*, 455−64.

(5) Glas, A. F.; Maul, M. J.; Cryle, M.; Barends, T. R. M.; Schneider, S.; Kaya, E.; Schlichting, I.; Carell, T. *Proc. Natl. Acad. Sci. U.S.A.* **2009**, *106*, 11540−11545.

(6) Selengut, J. D.; Haft, D. H. *J. Bacteriol.* **2010**, *192*, 5788−5798.

(7) Purwantini, E.; Mukhopadhyay, B. *Proc. Natl. Acad. Sci. U.S.A.* **2009**, *106*, 6333−6338.

(8) Singh, R.; Manjunatha, U.; Boshoff, H. I. M.; Ha, Y. H.; Niyomrattanakit, P.; Ledwidge, R.; Dowd, C. S.; Lee, I. Y.; Kim, P.; Zhang, L.; Kang, S.; Keller, T. H.; Jiricek, J.; Barry, C. E., III *Science* **2008**, *322*, 1392−1395.

(9) Walsh, C. *Acc. Chem. Res.* **1986**, *19*, 216−21.

(10) Jaenchen, R.; Schoenheit, P.; Thauer, R. K. *Arch. Microbiol.* **1984**, *137*, 362−5.

(11) Reuke, B.; Korn, S.; Eisenreich, W.; Bacher, A. *J. Bacteriol.* **1992**, *174*, 4042−9.

(12) Bacher, A.; Eberhardt, S.; Eisenreich, W.; Fischer, M.; Herz, S.; Illarionov, B.; Kis, K.; Richter, G. *Vitam. Horm. (San Diego, CA, U.S.)* **2001**, *61*, 1−49.

(13) Choi, K.-P.; Kendrick, N.; Daniels, L. *J. Bacteriol.* **2002**, *184*, 2420−2428.

(14) Frey, P. A.; Hegeman, A. D.; Ruzicka, F. J. *Crit. Rev. Biochem. Mol. Biol.* **2008**, *43*, 63−88.

(15) Sofia, H. J.; Chen, G.; Hetzler, B. G.; Reyes-Spindola, J. F.; Miller, N. E. *Nucleic Acids Res.* **2001**, *29*, 1097−106.

(16) Benjdia, A.; Leprince, J.; Guillot, A.; Vaudry, H.; Rabot, S.; Berteau, O. *J. Am. Chem. Soc.* **2007**, *129*, 3462−3463.

(17) Benjdia, A.; Subramanian, S.; Leprince, J.; Vaudry, H.; Johnson Michael, K.; Berteau, O. *J. Biol. Chem.* **2008**, *283*, 17815−26.

(18) Richter, G. F., M.; Krieger, C.; Eberhardt, S.; Lüttgen, H.; Gerstemschläger, I.; Bacher, A. *J. Bacteriol.* **1997**, *179*, 2022−8.

(19) Hänzelmann, P.; Schindelin, H. *Proc. Natl. Acad. Sci. U.S.A.* **2004**, *101*, 12870−5.

(20) Hänzelmann, P.; Schindelin, H. *Proc. Natl. Acad. Sci. U.S.A.* **2006**, *103*, 6829−34.

(21) Flühe, L. K., T. A.; Gattner, M. J.; Schäfer, A.; Burghaus, O.; Linne, U.; Marahiel, M. A. *Nat. Chem. Biol.* **2012**, *8*, 350−7.

(22) Kriek, M.; Martins, F.; Challand, M. R.; Croft, A.; Roach, P. L. *Angew. Chem., Int. Ed.* **2007**, *46*, 9223−9226.

(23) Challand, M. R.; Martins, F. T.; Roach, P. L. *J. Biol. Chem.* **2010**, *285*, 5240−5248.

(24) Pilet, E.; Nicolet, Y.; Mathevon, C.; Douki, T.; Fontecilla-Camps, J. C.; Fontecave, M. *FEBS Lett.* **2009**, *583*, 506−511.