JACS
JOURNAL OF THE AMERICAN CHEMICAL SOCIETY

Communication

pubs.acs.org/JACS

Two [4Fe-4S] Clusters Containing Radical SAM Enzyme SkfB Catalyze Thioether Bond Formation during the Maturation of the Sporulation Killing Factor

Leif Flühe, Olaf Burghaus, Beata M. Wieckowski, Tobias W. Giessen, Uwe Linne, and Mohamed A. Marahiel*

Department of Chemistry/Biochemistry, Philipps-Universität Marburg, Hans-Meerwein Straße 4 and LOEWE-Center for Synthetic Microbiology, D-35043 Marburg, Germany

Supporting Information

ABSTRACT: The sporulation killing factor (SKF) is a 26-residue ribosomally assembled and posttranslationally modified sactipeptide. It is produced by *Bacillus subtilis* 168 and plays a key role in its sporulation. Like all sactipeptides, SKF contains a thioether bond, which links the cysteine residue Cys4 with the α-carbon of the methionine residue Met12. In this study we demonstrate that this bond is generated by the two [4Fe-4S] clusters containing radical SAM enzyme SkfB, which is encoded in the *skf* operon. By mutational analysis of both cluster-binding sites, we were able to postulate a mechanism for thioether generation which is in agreement with that of AlbA. Furthermore, we were able to show that thioether bond formation is specific toward hydrophobic amino acids at the acceptor site. Additionally we demonstrate that generation of the thioether linkage is leader-peptide-dependent, suggesting that this reaction is the first step in SKF maturation.

The sporulation killing factor (SKF, Figure 1A) is a circular bacteriocin, produced by the *skf* operon of *Bacillus subtilis* Py79[1] and *B. subtilis* 168. It plays an important role during sporulation: when a *B. subtilis* population starts to experience nutritionally limiting conditions, the regulatory protein Spo0A activates the expression of the *skf* and *sdp* operons, which leads to the production of SKF and the sporulation delay protein (SDP), respectively. Both compounds are exported, and sibling cells with inactive Spo0A begin to lyse. This behavior releases additional nutrients for the *B. subtilis* subpopulation possessing active Spo0A, enabling them to delay the main sporulation event.[1−3]

In addition to its head-to-tail circular peptide backbone, SKF contains one unusual thioether bond, which links the cysteine residue Cys4 with the α-carbon of the methionine residue Met12, and one disulfide bridge.[3] The presence of the thioether bond puts SKF in the newly established class of sactipeptides.[4] The *skf* operon (Figure 1B) consists of the precursor gene *skfA*; the genes *skfB* (radical SAM enzyme), *skfC* (putative CAAX protease), and *skfH* (putative thioredoxin), which are required for the maturation of the 55-amino acid-containing precursor peptide (Figure S1); the genes *skfE* and *skfF*, which are required for export and immunity toward SKF; and the gene *skfG*, with unknown function.[1,3]

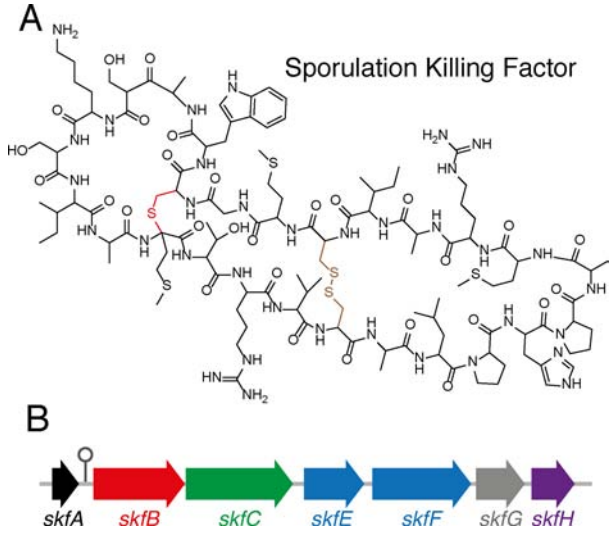


Figure 1. (A) Structure of the sporulation killing factor, with the thioether bond in red and the disulfide bond in brown. (B) Illustration of the *skf* operon of *B. subtilis* 168. The hairpin symbol indicates a stem-loop structure on the mRNA level.[1]

It was shown for the sactipeptide subtilosin A that the radical SAM enzyme AlbA catalyzes thioether bond formation.[5] We hypothesized that its homologue SkfB may have the same function during SKF maturation. To verify this assumption, we first characterized SkfB as a radical SAM enzyme with two [4Fe-4S] clusters. Afterward we were able to show that it catalyzes thioether bond formation in a leader-peptide-dependent manner and that the generation of the thioether linkage is specific toward the acceptor site.

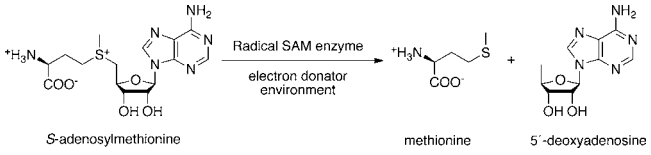
Radical SAM enzymes commonly share a characteristic CXXXCXXC motif, which coordinates a [4Fe-4S] cluster required for the reductive cleavage of *S*-adenosylmethionine (SAM) into methionine (Met) and a 5′-deoxyadenosyl (5′-dA) radical. The generated 5′-dA radical is then responsible for the wide variety of transformations catalyzed by this enzyme class.[6−8] In the absence of a second substrate, 5′-dA is

Received: October 25, 2012
Published: January 2, 2013

   dx.doi.org/10.1021/ja310542g | *J. Am. Chem. Soc.* 2013, 135, 959−962

generated through unspecific proton abstraction of the 5′-dA radical from the environment.[5,9,10] Hence, a protein could be confirmed as a radical SAM enzyme if it catalyzes the aforementioned reductive cleavage of SAM (Scheme 1) and contains at least one [4Fe-4S] cluster.

Scheme 1. Reductive SAM Cleavage Reaction Catalyzed by Radical SAM Enzymes[a]



[a]In the absence of the substrate, the generated 5′-deoxyadenosyl radical reacts with the environment, yielding 5′-deoxyadenosine.

The *skfB* gene was amplified from *B. subtilis* 168 chromosomal DNA and cloned into a pET-28a(+) expression vector. After purification by Ni-NTA affinity chromatography (Figure S2) and subsequent concentration of the elution fractions, the brownish SkfB was reconstituted using lithium sulfide and ammonium iron citrate, resulting in a dark brown solution, typical for Fe-S cluster-containing enzymes. Subsequently the SAM cleavage activity was investigated (Figure 2). To ensure reductive conditions, dithionite was added to the assay.

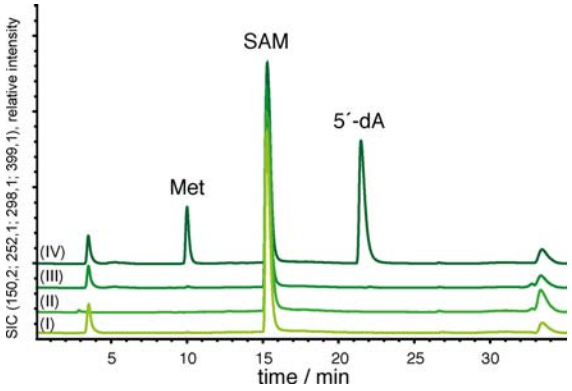


Figure 2. Single-ion chromatograms of different assay conditions: (I) control without SkfB; (II) assay without dithionite; (III) assay with nonreconstituted SkfB; and (IV) assay with reconstituted SkfB under reductive conditions.

Only in its reconstituted state and under reductive conditions was SkfB able to cleave SAM into Met and 5′-dA, as is characteristic for radical SAM enzymes. Subsequent analyses aimed to confirm the presence of iron−sulfur clusters in SkfB employing different spectroscopic techniques. The UV−vis and EPR spectra (Figure S2) of reconstituted SkfB and its iron and sulfur contents of 8.19 ± 0.07 and 8.36 ± 0.14 equiv, respectively, indicated the presence of at least one [4Fe-4S] cluster. To investigate the presence and nature of a second Fe-S cluster, an SkfB C117A-C121A-C124A triple alanine mutant was generated by SLIM mutagenesis.[11] The EPR and UV−vis spectra as well as the SAM cleavage activity of the mutant SkfB are shown in Figure S3. Because the recorded spectra are typical for [4Fe-4S] cluster-containing enzymes[10,12,13] and the iron content of the mutant is 4.5 ± 0.2 equiv, we conclude that SkfB is a radical SAM enzyme that contains two [4Fe-4S] clusters.

To further investigate the SkfB-catalyzed reaction, the precursor peptide substrate SkfA had to be produced first. Therefore, the *skfA* gene was amplified from *B. subtilis* 168 chromosomal DNA and cloned into the pET-48b(+) vector. Subsequent SLIM mutagenesis[11] was used to exchange the existing HRV-3C with the TEV protease cleavage site. After purification, cleavage of the Trx-Tag by the TEV protease, and final purification by HPLC, a SkfA derivative with an additional serine residue at the N-terminus (SerSkfA) could be obtained (Figure S4). A typical precursor peptide modification assay contained 200 μM SerSkfA, 20 μM reconstituted SkfB, 1 mM SAM, and 1 μM sodium dithionite and was carried out in a strictly anaerobic environment. In the control SkfB was omitted from the reaction mixture. Prior to HPLC-MS analysis of the reaction mixture (Figures 3, S8, and S9), the assay and control reaction were exposed to aerobic conditions, so as to allow disulfide bond formation.

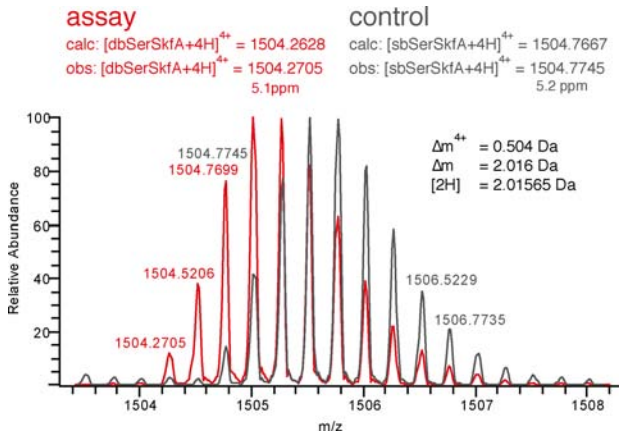


Figure 3. MS spectra from the HPLC-MS analysis of the assay and control reaction with SerSkfA. In the control reaction a singly bridged SerSkfA derivative (sbSerSkfA) is formed, whereas in the assay a doubly bridged SerSkfA derivative (dbSerSkfA) is observed. The mass difference between the two compounds is 2.016 Da.

Due to exposure to oxygen before HPLC analysis, disulfide bridge formation occurs in the control reaction, resulting in the observed sbSerSkfA species. In the assay a dbSerSkfA species is observed. The mass difference between sbSerSkfA and dbSerSkfA is in exact agreement with the mass difference expected for the formation of a thioether bond (calcd 2.01565 Da; obsd 2.016 Da). Further assays that were quenched by the addition of iodoacetamide while still under anaerobic conditions confirmed the formation of a thioether linkage (Figures S8 and S9). Similar to the AlbA-catalyzed reaction,[5] all three cysteine residues could be carbamidomethylated in the control reaction, whereas only two of them could be modified in the presence of SkfB.

To study the specificity of SkfB toward the donor and acceptor amino acids, we generated 13 SerSkfA derivatives (Table S5, Figures S5−S7): two Cys4 mutants, the Cys4-to-Met12 exchange mutant, and 10 Met12 mutants. As expected, SkfB is not able to modify the SerSkfA C4A derivative (Figure S10). The SerSkfA C4S species is also not modified by SkfB (Figure S11), indicating that the enzyme is not able to catalyze ether bond formation. The same behavior was observed for AlbA as well,[5] which could suggest a similar reaction

dx.doi.org/10.1021/ja310542g | J. Am. Chem. Soc. 2013, 135, 959−962

mechanism. With the generation of the SerSkfA C4M M12C mutant, we intended to address the question of whether SkfB is capable of generating a thioether linkage with inverted directionality. Because no modification could be observed, we conclude that the positions of the donor and acceptor amino acids play important roles in SkfB-catalyzed thioether formation (Figure S12).

To investigate the substrate specificity of SkfB toward the acceptor amino acid, the following SerSkfA derivatives were generated: M12A, M12S, M12T, M12N, M12Y, M12F, M12Q, M12K, M12E, and M12L. The first six mutations were chosen due to the presence of the respective substitute amino acids in other known sactipeptides like subtilosin A,[14] thurincin H,[15] and thuricin CD.[16,17] The last four amino acids were selected due to their structural similarity to methionine. The results of the assays are shown in Figures S13−S22. Interestingly, SkfB possesses broad substrate specificity toward the acceptor site. All investigated hydrophobic and aromatic amino acids were tolerated as acceptors for thioether bond generation, showing product formation amounts similar to that observed with the natural methionine residue (Table S7). The small hydrophilic amino acids Ser, Thr, and Asp were also incorporated into a thioether bond but with a lower yield compared to that with Met. No thioether bond formation was observed when using the larger hydrophilic amino acids Glu, Gln, and Lys as acceptors. This behavior points toward a hydrophobic binding pocket at the acceptor site where hydrophobic and aromatic amino acids are recognized best, small hydrophilic amino acids are still tolerated, and larger hydrophilic amino acids not tolerated at all. This broad substrate tolerance also indicates that radical generation at the α-carbon is independent of the acceptor amino acid. To elucidate whether thioether bond formation is leader-peptide-dependent, we assayed a leaderless SkfA derivative (Figure S23). No modification could be observed, suggesting that the generation of the thioether linkage may be the first step in SKF maturation, as was shown for subtilosin A.[5] Thus, our data are in agreement with the SKF maturation mechanism proposed by Liu et al.[3] (Figure S27).

Finally, we intended to further elucidate the mechanism of thioether bond formation and specifically the location and role of the second Fe-S cluster. Therefore, an alignment containing several radical SAM enzymes involved in sactipeptide biosynthesis was created (Figure S24). As in AlbA, three cysteine residues are conserved in the C-terminal region of SkfB that could function in the coordination of the second [4Fe-4S] cluster. To test this hypothesis, a SkfB C380A-C385A-C387A triple alanine mutant was generated via SLIM mutagenesis. After purification and reconstitution, its iron content was determined to be 4.2 ± 0.1 equiv, pointing toward a loss of one [4Fe-4S] cluster. The UV−vis and EPR spectra as well as the measurement of the SAM cleavage activity (Figure S25) demonstrated that the [4Fe-4S] cluster, which is coordinated by the CXXXCXXC motif, is still active. Hence it follows that Cys380, Cys385, and Cys387 constitute the coordination site of the second [4Fe-4S] cluster. To show that this cluster is crucial for the synthesis of the thioether bond, as was shown for AlbA before,[5] we carried out precursor peptide modification assays with the SkfB C380A-C385A-C387A mutant (Figure S26). As expected, the SerSkfA peptide was not modified, indicating that the second [4Fe-4S] cluster is essential for thioether bond formation. Thus, a mechanism similar to that of AlbA in subtilosin A biosynthesis can be proposed (Figures 4 and S28). In the first step, a reducing agent transfers an electron to the

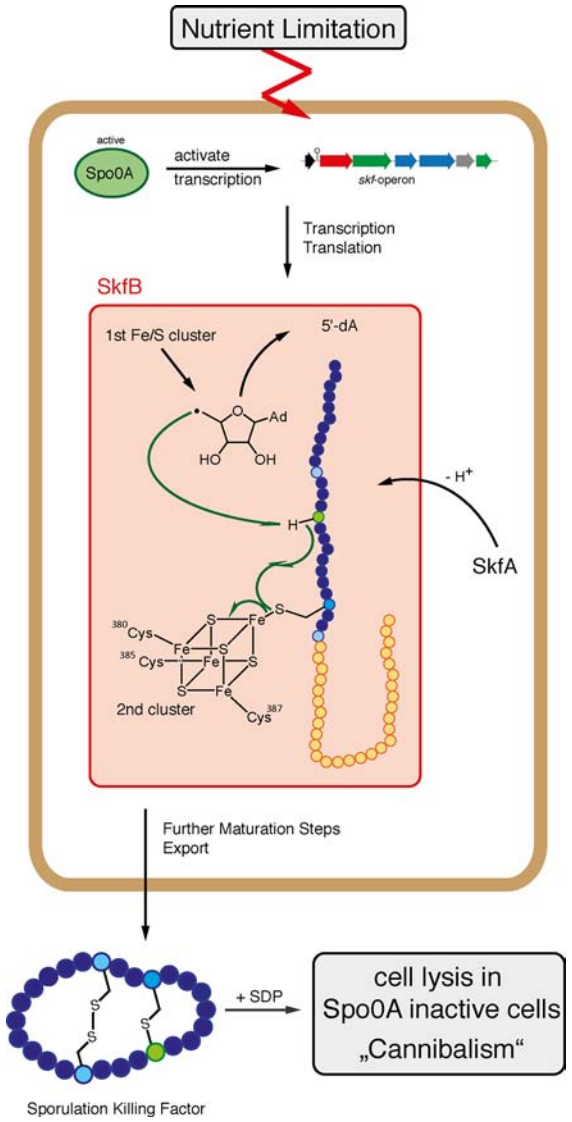


Figure 4. Role of SkfB in a *B. subtilis* subpopulation with active Spo0A, leading to cannibalistic behavior.

[4Fe-4S] cluster coordinated by the CXXXCXXC motif. Subsequently, bound SAM is reductively cleaved into Met and a 5′-dA radical. The generated 5′-dA radical then abstracts a hydrogen atom from the α-carbon of the methionine residue of SkfA, which is bound to the second [4Fe-4S] cluster through the thiol group of Cys4. In the last step, the generated carbon-centered radical forms a thioether bond with the coordinated sulfur atom of Cys4, while the second Fe-S cluster accepts an electron and gets reduced. The electron may then be transferred via an intramolecular mechanism to the first Fe-S cluster, allowing the enzyme to regenerate or be transferred to an external electron acceptor. This mechanism is also in agreement with the results of the SerSkfA C4S and SerSkfA C4M M12C assays. Interactions of oxygen with iron greatly differ from sulfur−iron interactions. Oxygen has a much higher affinity toward iron, which would inhibit subsequent ether bond formation. In the case of the SerSkfA C4M M12S derivative, the inverted arrangement of donor and acceptor amino acids would prevent the coordination of the cysteine residue by the second [4Fe-4S] cluster, due to spatial separation.

dx.doi.org/10.1021/ja310542g | J. Am. Chem. Soc. 2013, 135, 959−962

In conclusion, we have demonstrated that SkfB-catalyzed thioether bond formation most likely follows the same mechanism as that of AlbA. Additionally, we gained new insights into the substrate specificity of SkfB regarding the donor and acceptor amino acids. As expected, the donor amino cannot be varied, whereas the acceptor site shows broader substrate tolerance. Those results establish SkfB as a key component of the physiological response of *B. subtilis* to nutrient limitation, ultimately leading to cell lysis and subsequent cannibalistic behavior (Figure 4). Furthermore, we have established a system that generates only one specific thioether bond, making it ideal for future investigations aimed at the detailed mechanistic elucidation of thioether bond formation in sactipeptides.

■ ASSOCIATED CONTENT

Supporting Information

Experimental methods, supplementary figures, and results. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

marahiel@staff.uni-marburg.de

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank N. Fritzler and J. Bamberger for practical realization of the HPLC-MS measurements. Especially we thank Thomas A. Knappe for fruitful discussions and initiation of the project. This work has been supported by the Deutsche Forschungsgemeinschaft and the LOEWE Center for Synthetic Microbiology (SYNMIKRO).

■ REFERENCES

(1) González-Pastor, J. E.; Hobbs, E. C.; Losick, R. *Science* **2003**, *301*, 510.
(2) Engelberg-Kulka, H.; Hazan, R. *Science* **2003**, *301*, 467.
(3) Liu, W.-T.; Yang, Y.-L.; Xu, Y.; Lamsa, A.; Haste, N. M.; Yang, J. Y.; Ng, J.; Gonzalez, D.; Ellermeier, C. D.; Straight, P. D.; Pevzner, P. A.; Pogliano, J.; Nizet, V.; Pogliano, K.; Dorrestein, P. C. *Proc. Natl. Acad. Sci. U.S.A.* **2010**, *107*, 16286.
(4) 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.; Göransson, U.; Gruber, C. W.; Haft, D. H.; Hemscheidt, T. K.; Hertweck, C.; Hill, C.; Horswill, A. R.; Jaspars, 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.; Müller, R.; Nair, S. K.; Nes, I. F.; Norris, G. E.; Olivera, B. M.; Onaka, H.; Patchett, M. L.; Piel, J.; Reaney, M. J. T.; Rebuffat, S.; Ross, R. P.; Sahl, H.-G.; Schmidt, E. W.; Selsted, M. E.; Severinov, K.; Shen, B.; Sivonen, K.; Smith, L.; Stein, T.; Süssmuth, 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.
(5) Flühe, L.; Knappe, T. A.; Gattner, M. J.; Schäfer, A.; Burghaus, O.; Linne, U.; Marahiel, M. A. *Nat. Chem. Biol.* **2012**, *8*, 350.
(6) Sofia, H.; Chen, G.; Hetzler, B.; Reyes-Spindola, J.; Miller, N. *Nucleic Acids Res.* **2001**, *29*, 1097.
(7) Frey, P. A.; Hegeman, A. D.; Ruzicka, F. J. *Crit. Rev. Biochem. Mol. Biol.* **2008**, *43*, 63.
(8) Duschene, K. S.; Veneziano, S. E.; Silver, S. C.; Broderick, J. B. *Curr. Opin. Chem. Biol.* **2009**, *13*, 74.
(9) Duschene, K. S.; Broderick, J. B. *FEBS Lett.* **2010**, *584*, 1263.
(10) Chatterjee, A.; Li, Y.; Zhang, Y.; Grove, T. L.; Lee, M.; Krebs, C.; Booker, S. J.; Begley, T. P.; Ealick, S. E. *Nat. Chem. Biol.* **2008**, *4*, 758.
(11) Chiu, J.; March, P. E.; Lee, R.; Tillett, D. *Nucleic Acids Res.* **2004**, *32*, e174.
(12) Hagen, K.; Watson, A. *J. Am. Chem. Soc.* **1983**, *105*, 3905.
(13) Ugulava, N. B.; Gibney, B. R.; Jarrett, J. T. *Biochemistry* **2000**, *39*, 5206.
(14) Kawulka, K. E.; Sprules, T.; McKay, R. T.; Mercier, P.; Diaper, C. M.; Zuber, P.; Vederas, J. C. *J. Am. Chem. Soc.* **2003**, *125*, 4726.
(15) Sit, C. S.; van Belkum, M. J.; McKay, R. T.; Worobo, R. W.; Vederas, J. C. *Angew. Chem., Int. Ed.* **2011**, *50*, 8718.
(16) Rea, M. C.; Sit, C. S.; Clayton, E.; O'Connor, P. M.; Whittal, R. M.; Zheng, J.; Vederas, J. C.; Ross, R. P.; Hill, C. *Proc. Natl. Acad. Sci. U.S.A.* **2010**, *107*, 9352.
(17) Sit, C. S.; McKay, R. T.; Hill, C.; Ross, R. P.; Vederas, J. C. *J. Am. Chem. Soc.* **2011**, *133*, 7680.