Initiation of Surfactin Biosynthesis and the Role of the SrfD-Thioesterase Protein†

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ABSTRACT: In this paper, the initiation reactions in surfactin biosynthesis by *Bacillus subtilis* OKB 105 were investigated. Evidence for a specific role of the SrfD protein, the external thioesterase enzyme in surfactin biosynthesis, was obtained for the first time. The action of SrfD was investigated both with the native, but only partially purified, enzyme and the highly purified, His-tagged protein overexpressed in *Escherichia coli*. Surfactin can be formed by the interaction of the three amino acid activating components of surfactin synthetase SrfA, B and C alone. This process is stimulated by SrfD. In the initiation reactions, the β-hydroxy fatty acid substrate is transferred from β-hydroxymyristoyl-coenzyme A to the start enzyme SrfA followed by formation of β-hydroxymyristoyl-glutamate. The same reactions were also observed with the recombinant L-Glu-activating module of surfactin synthetase. Lipopeptide formation can be initiated by these function units alone, but SrfD efficiently supports and stimulates the formation of initiation products. From these results, we infer that SrfD functions as the thioesterase/acyltransferase enzyme in the initiation process previously postulated by Menkhaus et al. [Menkhaus et al. (1993) *J. Biol. Chem. 268*, 7678−7684], thus enhancing surfactin formation.

The biosynthesis of the lipoheptapeptide surfactin by *Bacillus subtilis* is encoded by the *srf*-operon which comprises four open reading frames codifying the protein components SrfA−D of the surfactin synthetase multienzyme system (*1*−*4*). In previous studies, it was demonstrated that cell-free synthesis of this lipopeptide is accomplished by the interaction of three enzyme fractions designated as E1, E2, and E3, which have been obtained by AcA34 gel filtration of a cell-free extract of *B. subtilis* ATCC 21332 or OKB 105 (*4*, *5*). Fraction E1 contains the two large enzymes of surfactin synthetase SrfA and SrfB, each comprising three amino acid activating modules. SrfA thioesterifies L-Glu and two leucine residues, while SrfB incorporates L-Asp, L-Val, and L-Leu as thioesters. SrfC is a one-module enzyme that contributes the C-terminal L-leucine-residue. It was found that these three multifunctional proteins assemble the surfactin lipopeptide chain and produce surfactin when complemented with a low molecular weight protein fraction (*5*, *6*). It was assumed that this fraction contains an acyltransferase enzyme E3 which is involved in the initiation of surfactin biosynthesis by transfer of the β-hydroxy fatty acid component to the L-Glu-activating module of SrfA, in this way starting the elongation process. However, efforts to identify and characterize this acyltransferase have failed so far.

Recently important progress has been provided by several authors (*2*, *7*−*11*) on the termination of surfactin formation. Generally, two thioesterase functions have been detected in the organization of numerous bacterial nonribosomal peptide synthetases (NRPS) (*12*, *13*). An internal thioesterase domain is located at the C-terminal end of the last module of a peptide synthetase. It shows high homologies to thioesterase enzymes of type I involved in fatty acid biosynthesis. In several peptide forming multienzyme systems, like gramicidin S synthetase (*14*), tyrocidine synthetase (*15*), and surfactin synthetase (*2*, *7*), a second, external separate thioesterase enzyme was detected. These proteins show striking homologies to thioesterases of type II encoded by a distinct gene associated with the NRPS cluster.

Schneider and Marahiel (*16*) reported from mutant studies that in surfactin biosynthesis both thioesterase enzymes are essential for the entire process of lipopeptide formation. Deletion of the thioesterase domain at the C-terminus of SrfC and of the thioesterase-like protein SrfD led to a 97 and 84% reduction of in vivo surfactin formation, respectively. Extensive knowledge has been accumulated, in particular, on the role of the internal thioesterase domains (*8*−*11*). Apparently, the internal thioesterase domain is involved in the termination of surfactin production releasing the assembled lipopeptide chain by hydrolysis and stereospecific macrolactone cyclization (*11*). In contrast, the role of the external thioesterase enzyme SrfD in the biosynthetic process was completely unknown until now. SrfD (SrfTE-II) is codified by the fourth open reading frame *srfD* of the *srf*-operon (*2*). Recently, Schwarzer et al. (*17*) proposed a

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general role of TE-IIs. They demonstrated that external thioesterases in nonribosomal peptide biosynthesis, as SrfD, are involved in the regeneration of misacylated peptide synthetases as a result of apo- to holo-conversion of the thiolation domains of such multienzymes by 4′-phosphopantetheinyl transferase enzymes, like Sfp. Such enzymes are able to catalyze the cleavage of NRPS bound amino acids and intermediate peptides as well as of CoA-derivatives, in particular, acetyl-CoA, which is efficiently hydrolyzed.

The aim of this paper is to clarify whether SrfD exerts a specific function of SrfD in the biosynthesis of surfactin. Our results show that SrfD plays an important role in the initiation process. We provide evidence that the N-terminal, L-Glu-activating module of surfactin synthetase is able to bind the β-hydroxy fatty acid substrate and to connect it to the start amino acid L-glutamate, thus initiating surfactin biosynthesis. The SrfD protein efficiently supports this process as a thioesterase/acyltransferase enzyme by mediating the transfer of the fatty acid substrate to the Glu-module and stimulating β-hydroxyacyl-glutamate formation.

MATERIALS AND METHODS

Materials. L-[U-^{14}C]leucine (294 mCi/mmol) and L-[U-^{14}C]glutamic acid (254 mCi/mmol) were purchased from Amersham Biosciences Europe GmbH (Freiburg, Germany). Ultrogel AcA 34 was from BioSepra (Frankfurt a. M., Germany). Q-Sepharose Fast Flow and the FPLC columns Superdex-75 and MonoQ HR5/5 were from Amersham Biosciences. Amylose and coenzymeA were purchased from Sigma (Taufkirchen, Germany). β-Hydroxymyristic acid was obtained from GERBU Biotechnik GmbH (Gaiberg, Germany). The fatty acid substrate of surfactin synthetase, β-hydroxymyristoyl-coenzymeA, was prepared as described in ref *6*. Lysozyme was a product of Sigma. All other chemicals used were reagent grade.

Growth of Organism. *Bacillus subtilis* OKB 105, which was kindly made available to us by P. Zuber (Oregon Graduate Institute School of Science and Engineering, Oregon Health and Science University, Beaverton, USA), was cultivated in the Landy medium (*18*) by shaking cultures at 29 °C for 8 h. *B. subtilis* cells were collected by centrifugation and stored at −20 °C.

Escherichia coli DH 5α and M15 were grown in dyt medium (16 g/L tryptone; 10 g/L yeast extract and 5 g/L NaCl) at 37 °C overnight in a New Brunswick G-25 shaker. The main culture was inoculated with this material and cultivated for 8 h at 37 °C. In the case of the M15 strain protein expression was induced at a final concentration of 1 mM iso-propyl-β-D-thiogalactoside after an OD_{600} value of approximately 0.6 was attained. Cells were harvested by centrifugation and stored at −20 °C.

Determination of Protein Content. After each purification step in the preparation of the components of surfactin synthetase SrfA−D and the isolated glutamic acid activating module of SrfA overexpressed in *E. coli* DH 5α the protein content was measured according to Bradford (*19*).

Enzyme Assays. Assays for substrate specific ATP/PP_i exchange reactions, thioester formation of substrate amino acids with surfactin synthetase and in vitro formation of surfactin were performed as described by Ullrich et al. (*6*). The activity of the acyltransferase enzyme E3 was assayed by its stimulating effect on surfactin formation.

For the detection and analysis of the initiation products of surfactin synthetase assay mixtures contained aliquots of either SrfA or the isolated, heterologously overexpressed L-Glu-activating module of SrfA in the presence of 6.2 μM L-[^{14}C] Glu; 10 mM ATP; 10 mM $MgCl_2$; 10 mM dithioerythritol (DTE)[1] and 1 mM EDTA in 50 mM Tris/HCl buffer, pH 7.4.

Product Analysis. L-[^{14}C] leucine labeled surfactin and L-[^{14}C] glutamic acid containing initiation products were separated and analyzed on silica gel 60 plates (Merck, Darmstadt, Germany) using $CHCl_3$/ MeOH/ H_2O (65:25:4, by vol.) as solvent system A. Radioactive compounds were detected (a) by autoradiography after exposure to a X-ray film (Konica, Japan) for at least four weeks or (b) by radioscanning using a Berthold TLC-Linear Analyser "Tracemaster 20".

In addition, for identification of these products by TLC and mass spectrometric analysis they were separated by reversed phase HPLC on a μRPC column using a Smart microseparation system (Amersham Biosciences, Freiburg, Germany). Products were extracted from assay mixtures with *n*-butanol. The solvent was evaporated in a SpeedVac centrifuge. The dry material was taken up in 100 μL of 0.1% trifluoroacetic acid (solvent A) and applied to the column. Products were eluted with a linear gradient of acetonitrile. Solvent B contained 100% acetonitrile/0.1% trifluoroacetic acid. The flow rate was 100 μL/min. Fraction size: 100 μL.

Enzyme Preparation. The preparation of the components of surfactin synthetase was essentially performed as reported by Menkhaus et al. (*5*). All operations were carried out at 2−4 °C.

Step 1 − Preparation of a Cell-Free Extract. Cell lysates were prepared from *B. subtilis* OKB 105 as described previously (*6*). Nucleic acids were removed by precipitation with 1% streptomycin sulfate. Proteins were salted out at 70% ammonium sulfate (w/v) for 30 min. The precipitate formed was dissolved in a minimum volume of 10 mL 50 mM Tris/HCl, pH 7.5; 0.25 mM EDTA; 5 mM DTE and 10% sucrose (buffer A) and dialyzed against the same buffer.

Step 2 − Gel Filtration on Ultrogel Aca 34. The crude extract was applied to an Ultrogel AcA34 column (50 × 2.5 cm). The proteins were eluted by buffer A. In the obtained fractions (fraction size: 4 mL), SrfA-C were monitored by thioester incorporation with L-[^{14}C]leucine. SrfD was detected by measurement of the SrfD induced stimulation of in vitro surfactin formation by complementation of a reaction mixture containing SrfA, B, and C with low molecular weight fractions of the AcA34 gel filtration. The protein composition in the active fractions was determined by SDS−polyacrylamide gel electrophoresis using 12.5% SDS−polyacrylamide slab gels. The proteins were transferred from the gels to poly(vinylidene difluoride) membranes by electroblotting. SrfD was detected by immune

[1] Abbreviations: DTE, dithioerythritol; SDS, sodium dodecyl sulfate; PMSF, phenylmethylsulfonylfluoride; PVDF, poly(vinylidene difluoride); MBP, maltose binding protein; PAGE, polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography; FPLC, fast protein liquid chromatography; TLC, thin-layer chromatography; MALDI-TOF MS, matrix assisted laser desorption/ionization-time-of-flight mass spectrometry; PSD, post source decay; NRPS, nonribosomal peptide synthetase.

staining with antibodies raised against a maltose binding protein−SrfD fusion protein.

Step 3 − Anion Exchange FPLC of the Components of Surfactin Synthetase SrfA-D on MonoQ HR 5/5. Fractions containing either SrfA and B, SrfC or SrfD were loaded onto a Mono Q HR 5/5 column equilibrated with 50 mM Tris/HCl buffer, pH 7.5, containing 5 mM DTE. In each case, proteins were eluted with a linear gradient of 0−0.5 M NaCl in the same buffer at a flow rate of 1 mL/min. The fraction size was 1 mL. SrfA−C were monitored by thioester incorporation of L-[^{14}C]leucine and surfactin formation. SrfD was assayed by stimulation of surfactin formation and detected by immunestaining.

Purification of SrfD by Gel Electrophoresis Chromatography. For proteinchemical studies SrfD was purified by electrophoresis−chromatography. Fractions in the range of 26−40 of the AcA34 gel filtration of a crude extract of *B. subtilis* OKB 105 (step 2) containing SrfD were concentrated by ultrafiltration and applied onto a preparative discontinuous disc SDS−polyacrylamide gel of 37 mm diameter in a Prep Cell, model 491 which was obtained from BioRad Laboratories GmbH (München, Germany). Proteins were fractionated according to Laemmli (*20*) by preparative electrophoresis chromatography. The heights of the stacking and resolving gels were 3 and 7 cm, respectively. The sample was diluted with sample buffer (62.5 mM Tris/HCl, pH 6.8, containing 1% SDS; 10% glycerol and 5% β-mercaptoethanol) and 2 mL of a saturated solution of bromophenol-Blue (Serva, Heidelberg, Germany) containing 50% (w/v) sucrose. Then 5% methanol was added. Proteins were denatured at 56 °C for 10 min. The sample was layered on the stacking gel. Electrophoresis chromatography was performed at a constant power of 12 W for 20 h. Proteins leaving the separating gel were collected with a streaming buffer (Tris/HCl, pH 8.3; 0.1% SDS; 200 mM glycine). The flow rate was 100 μL/min. Fractions of 2 mL were collected and analyzed by SDS−polyacrylamide gel electrophoresis on a slab gel.

Overexpression and Purification of the SrfD Protein, the Gene Product of the Fourth Open Reading Frame of the srf-Operon. The *srfD* gene encoding the external thioesterase of surfactin synthetase was amplified by PCR using Deep Vent polymerase and chromosomal DNA of *B. subtilis* OKB 105 as template. The purified fragment was cloned into the SpHI- and BglII- sites of vector pQE−70 (Qiagen, Hilden, Germany) and transformed into *E. coli* strain M 15 (Qiagen). The primers used for DNA amplification were

5′-GAGGAGAATTCGCATGCGCCAACTCTTCAAA-TCATTTG (forward primer) and 5′-CGCTTTAGATCTCG-GTTGAATGATCGGATG (reverse primer).

The authenticity of the amplification product was demonstrated by sequence analysis of the entire insert. The SrfD protein was heterologously overexpressed in *E. coli* M15 and tagged with six histidine residues at its C-terminus.

E. coli cells were grown at 37 °C overnight in dyt medium in the presence of 100 μg/mL ampicillin and 25 μg/mL kanamycin. Cells were induced with 1 mM IPTG at an OD_{600} of 0.5. The SrfD protein was purified from a cell-free extract of this *E. coli* strain. Cells were suspended in lysis buffer (50 mM Tris/HCl, pH 8.5; 10 mM β-mercaptoethanol and 1 mM PMSF) and disintegrated by French press treatment at a pressure of 4000 psi. Cell fragments were removed by centrifugation. The supernatant was loaded onto a nickel-NTA-agarose column for affinity chromatography of the His-tagged protein. The column was rinsed with 20 mM Tris/HCl-buffer containing 100 mM KCl; 20 mM imidazole; 10 mM β-mercaptoethanol and 10% (v/v) glycerol to remove proteins weakly bound to the matrix. The His-tagged SrfD protein was eluted with 20 mM Tris/HCl buffer containing 100 mM imidazole, 10 mM β-mercaptoethanol, and 10% (v/v) glycerol. The fraction size was 4 mL.

SrfD was identified by Western blotting and immune staining with a MBP−SrfD antibody followed by N-terminal sequence analysis by Edman degradation.

Overexpression and Purification of the Maltose Binding Protein−SrfD Fusion Protein. The DNA segment comprising the *srfD* gene was amplified by PCR using Taq-polymerase and chromosomal DNA of *B. subtilis* OKB 105 as the template. It was purified by preparative agarose electrophoresis and cloned into the Bam HI and Hind III sites of the pMal-C2 vector (New England Biolabs, Schwalbach, Germany). In this way, the *srfD* gene was fused with the gene of a maltose binding protein integrated into the vector. The primers used for DNA amplification were

5′-GGGATCCATGAGCCAACTCTTC (forward primer) and 5′-AAGCTTCGGTTGAATGATCGGATG (reverse primer).

SrfD was overexpressed as fusion protein with a maltose binding protein in *E. coli* DH 5α. Cells of this strain were transformed with pMAL-C2-srfD and cultivated overnight. In this construct, the maltose binding protein was fused to the N-terminus of SrfD enabling affinity chromatography of SrfD at an amylose matrix in one step. Cells were collected by centrifugation, suspended in 20 mM Tris/HCl buffer containing 200 mM NaCl and 1 mM EDTA (buffer B) and disintegrated by French Press treatment at a pressure of 4000 psi. The cell debris was removed by centrifugation for 30 min at 23000*g*. The supernatant was loaded on an amylose column (1 × 10 cm) equilibrated with buffer B. After intensive washing of the column with buffer B the MBP−SrfD fusion protein was eluted with the same buffer containing 10 mM maltose. The fraction size was 3 mL. Aliquots of the product were stored at −20 °C.

Overexpression and Purification of the Recombinant L-Glu-Activating Module of Surfactin Synthetase. The DNA fragment encoding the L-Glu-activating module of surfactin synthetase was amplified by PCR using Vent polymerase (NEB) and chromosomal DNA of *B. subtilis* ATCC 21322 as a template. The purified fragment was cloned into the NcoI and BamHI sites of vector pQE60 (Qiagen, Hilden, Germany) and transformed into *E. coli* strain M15xpREP4 (Qiagen). The primers used for DNA amplification were

5′-CGGCCATGGAAATAACTTTTTACCCTTTAAACG (forward primer) and 5′-CGGGATCCACGATTGGCCCGT-TCATTCAGTTCACG (reverse primer).

The L-Glu-activating module of surfactin synthetase comprising the first 1540 amino acid residues of SrfA was heterologously overexpressed in *E. coli* DH 5α and tagged with six histidine residues at its C-terminus. It was purified from a cell free extract of this *E. coli* strain. Cells were suspended in lysis buffer (50 mM Tris/HCl, pH 8.5; 10 mM β-mercaptoethanol and 1 mM PMSF) and disintegrated by French press treatment at a pressure of 4000 psi. Cell fragments were removed by centrifugation. The supernatant was loaded onto a nickel-NTA-agarose column. The His-

tagged protein was purified by affinity chromatograpy in a similar way as described for SrfD. Protein containing fractions were pooled, applied to a MonoQ HR 5/5 column and finally purified by anion exchange FPLC until electrophoretic homogeneity. Fractions containing the L-Glu-module were stored at −20 °C.

SDS−Polyacrylamide Gel Electrophoresis. SDS-polyacrylamide gel electrophoresis (SDS−PAGE) was performed according to Laemmli (*20*) in a mini-gel apparatus (Hoefer, San Francisco, USA). 5 and 12.5% polyacrylamide gels were run in Laemmli buffer. The dimensions of the separation gels were 8.0 × 7.5 cm. Electrophoresis was performed for about 1.5 h at a constant current of 10−20 mA. The proteins were visualized with Serva Blue R or by silver staining. Low and high molecular weight standard kits (14.4−97 and 53−220 kDa, respectively) of Amersham Biosciences Europe GmbH (Freiburg, Germany) were used. After SDS−PAGE slab gels were electroblotted onto poly(vinylidene difluoride) membranes (Schleicher & Schüll, Dassel, Germany) in a semidry Novablot apparatus (LKB, Bromma, Sweden) according to the protocol of the manufacturers. The protein transfer was performed for 1 h at 5 mA/cm^2. The blotted proteins were stained with a 0.2% (w/v) Ponceau S solution.

Immunestaining of the SrfD Protein. The SrfD protein was immune stained with an SrfD-directed antibody raised in rabbits by the BioGenes Company (Berlin, Germany) using the maltose binding protein−SrfD fusion protein overexpressed in *E. coli* DH 5α as the antigen. The PVDF blot membrane was treated with 20 mM Tris/HCl buffer, pH 7.3 containing 154 mM NaCl and 1% bovine serum albumin (block buffer). Thereafter, the membrane was incubated for 2 h at room temperature with the MBP−SrfD antibodies which were applied in a dilution of 1:40 000 in block buffer. The residual antibodies were removed by rinsing with 20 mM Tris/HCl buffer, pH 7.3 containing 154 mM NaCl and 0.05% Tween 20 (TBS buffer) followed by incubation with anti-rabbit-IgG-alkaline phosphatase conjugate (1:20 000, v/v) in block buffer for 1 h at room temperature. The excess of antibodies was removed by rinsing with TBS buffer. After treatment of the membrane with 100 mM Tris/HCl buffer, pH 9.5 containing 5 mM $MgCl_2$ (AP buffer) SrfD was visualized by treatment with staining solution (45 μL of a solution of 50 μg of nitrotetrazoliumchloride-Blue, sodium salt and 35 μL of a solution of 50 μg of 5-bromo-4-chloro-3-indolyl-phosphate each per milliliter of 70% (v/v) dimethylformamide in 10 mL of AP buffer). The staining procedure was stopped by rinsing the membrane with bidistilled water.

In Gel Digestion of SrfD with Trypsin. SrfD was purified either by AcA34 gel filtration in combination with anion exchange FPLC on MonoQ or under denaturating conditions by SDS−polyacrylamide gel electrophoresis chromatography, as described above. After SDS−PAGE of the obtained fractions, the Coomassie-stained band at the 30 kDa position containing the SrfD protein was excised from the gel and washed three times with bidistilled water for 20 min. Subsequently, the residual dye was removed by treatment with 50% (v/v) acetonitrile in water for 20 min and with acetonitrile for 1 min. After removal of the solvent, the gel pieces were suspended in 100 μL of 100 mM ammonium carbonate buffer, pH 8.1 and the proteins were digested by trypsin (1 μg of trypsin dissolved in 20 μL of this buffer) for 24 h at 37 °C. Tryptic peptides were extracted twice with 100 μL of 50 and 70% (v/v) acetonitrile in 0.1% trifluoroacetic acid, respectively. Each extraction was performed for 30 min at 37 °C. The combined extracts were dried in a Speed Vac concentrator and dissolved in 100 μL 0.1% (v/v) trifluoroacetic acid. The tryptic peptides were separated by high-resolution reversed phase FPLC on a μRPC SC 2.1/10 column using a Smart microseparation system. They were eluted with a linear gradient of 5−45% acetonitrile (eluent B: 0.1% (v/v) trifluoroacetic acid in acetonitrile) in 100 min with a flow rate of 100 μL/min.

Protein and Peptide Analysis. N-terminal sequencing of SrfD and tryptic peptide fragments of this protein were performed by Edman degradation using an Applied Biosystems Procise protein sequencer (Perkin-Elmer/Applied Biosystems, Weiterstadt, Germany). Samples were dissoved in 100% methanol and adsorbed to a polybrene-coated glass filter treated with trifluoroacetic acid and precycled as described by Hewick et al. (*21*).

Matrix-assisted laser desorption ionization-time-of-flight (MALDI-TOF) mass spectra of peptide fragments were recorded on a Bruker Reflex MALDI-TOF instrument equipped with a 337-nm nitrogen laser for desorption and ionization. Peptide samples were dissolved in 40% acetonitrile/0.1% trifluoroacetic acid (v/v) and analyzed in a saturated α-cyano-4-hydroxy-cinnamic acid matrix solubilized in 40% acetonitrile/0.1% trifluoroacetic acid (v/v). 1 μL aliquots of peptide samples were spotted onto a matrix layer previously crystallized on the target. Positive ion detection and reflector mode were used. Sequence analysis of the peptides was performed by post source decay (PSD)-MALDI-TOF mass spectrometry.

RESULTS

Identification of Enzyme E3 as the External Thioesterase SrfD in Surfactin Biosynthesis. In previous publications, it was reported that the biosynthesis of surfactin is accomplished by cooperation of three enzyme fractions E1, E2, and E3 obtained by gel filtration of a cell free extract of *B. subtilis* OKB 105 on AcA34 (*4*, *5*). Studies with mutant enzymes revealed (*4*) that fraction E1 comprises the two large enzymes SrfA and SrfB of surfactin synthetase, while E2 was assigned to the one-module subunit SrfC. Fraction E3 contained a small enzyme in the molecular mass range of 20−40 kDa, which was ascribed to a putative acyltransferase (*5*). It was assumed that this protein is involved in the initiation process by transfer of the fatty acid substrate to the start enzyme SrfA, but unfortunately it could not yet be identified and characterized. Therefore, the main aim of this paper was to investigate the initiation reactions of surfactin biosynthesis in detail and to clarify the role of the external thioesterase enzyme SrfD, the gene product of the fourth open reading frame of the *srf* operon. Its specific function in the biosynthetic process was hitherto unknown.

Following these objectives, we prepared the enzyme components of the surfactin synthetase multienzyme system essentially as described previously (*4*, *5*). *B. subtilis* OKB 105 was cultivated at 29 °C in the Landy medium (*18*) and harvested after 8 h. For preparation of a cell-free extract, cells were incubated for 20 min at 30 °C with lysozyme and desintegrated by French Press treatment. After streptomycin

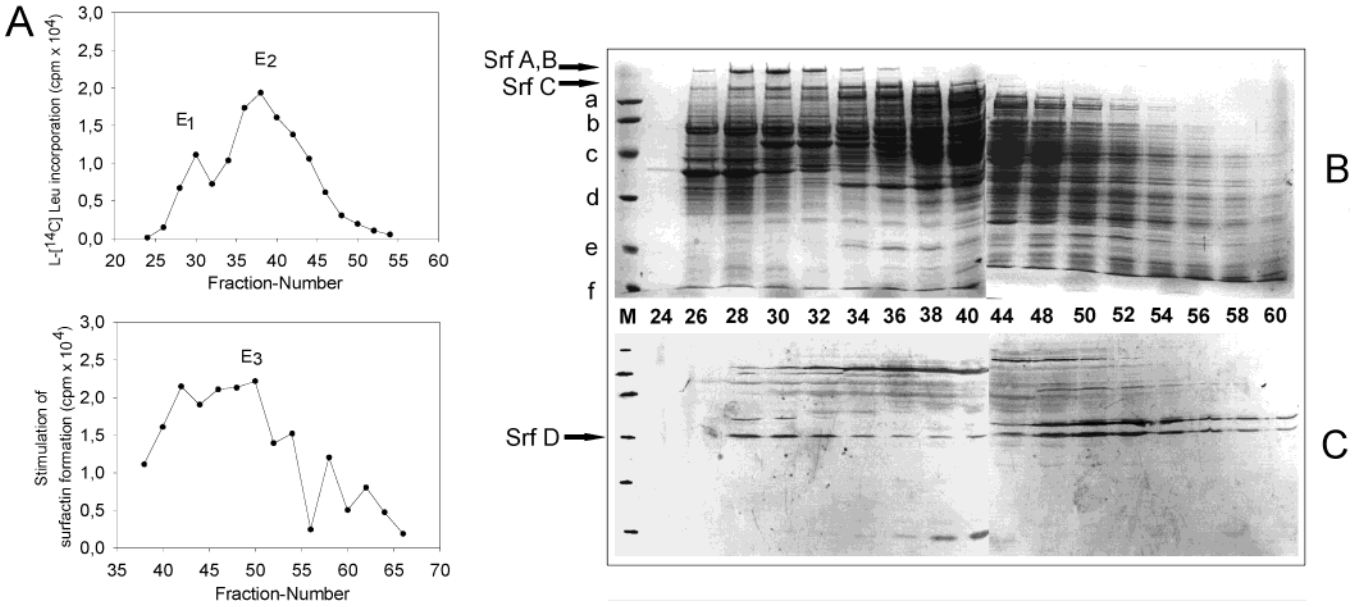

FIGURE 1: Gel filtration of a crude cell-free extract of *B. subtilis* OKB 105 on Ultrogel AcA34. (A) The peptide-forming components of surfactin synthetase SrfA-C were detected by thioester formation with L-[^{14}C]leucine in fractions 24−54. Enzyme E3 was monitored by the in vitro assay for surfactin formation by complementation of fractions 39−60 with aliquots of fractions 30 and 38 containing SrfA/B and SrfC, respectively. (B) SDS−polyacrylamide gel electrophoresis of the proteins in the range of fractions 24−60 of the AcA34 gel filtration (lanes 24−60). The positions of SrfA, B, C, and D are marked by arrows. Lane M: Low molecular weight marker proteins: phosphorylase, 97 kDa (a); bovine serum albumin, 66 kDa (b); ovalbumin, 45 kDa (c); carbonic anhydrase, 30 kDa (d); trypsin inhibitor, 20.1 kDa (e); α-lactalbumin,14.4 kDa (f). 12.5% SDS−polyacrylamide slab gels were used for separation. Proteins were detected by staining with Coomassie. (C) The proteins separated by SDS−polyacrylamide gel electrophoresis in Figure 1B were electroblotted onto a PVDF membrane and exposed to an antibody raised against a maltose binding protein−SrfD fusion protein which was applied in a dilution of 1: 40 000 in 20 mM Tris/HCl buffer, pH 7.3 containing 150 mM NaCl.

sulfate precipitation and salting out proteins at 70% (w/v) ammonium sulfate, the crude extract was fractionated by AcA34-gel filtration, as shown in Figure 1 A−C. The components SrfA−C of surfactin synthetase were monitored by thioester formation with L-[^{14}C]leucine and by in vitro surfactin biosynthesis (Figure 1A). Enzyme E3 was detected by its stimulatory effect on surfactin formation. It was found in fractions 38−60 eluted from the AcA34 gel filtration (Figure 1A). For assaying this enzyme an aliquot of each of these fractions was complemented with aliquots of fractions 30 and 38 containing SrfA/B and SrfC, respectively.

The protein composition of the fractions eluted from the AcA34 column was analyzed by SDS−PAGE, as shown in Figure 1B. The protein bands were electroblotted onto a PVDF membrane. The positions of SrfA, SrfB, and SrfC were marked by arrows. SrfD, the fourth component of the surfactin biosynthetic system, was visualized by immunestaining with an antibody raised against a maltose binding protein−SrfD fusion protein obtained by genetic engineering techniques and overexpression in *E. coli*. The antibody was applied in a dilution of 1:40 000. Immune staining was observed essentially at three positions on the blot membranes corresponding to proteins of molecular masses with approximately 30, 35, and 70 kDa. The immune response at the 30-kDa position was found over a large fraction range of the AcA34 gel filtration (Figure 1C). It appeared both in the high molecular weight part comprising SrfA−C (fractions 26−40) (range I) as well as in the low molecular weight range in fractions 46−60 (range II) containing enzyme E3. To identify the immune-reactive protein in range I the corresponding fractions were pooled, concentrated by ultrafiltration in Centricon PM-10-ultrafiltration cells and loaded onto a Biorad PrepCell. Proteins were separated by preparative gel electrophoresis chromatography, as shown in Figure 2. The protein distribution in the obtained fractions was tested by analytical SDS−PAGE on 12.5% polyacrylamide slab gels (Figure 2A). After blotting onto a PVDF membrane, the protein crossreacting with the SrfD-directed antibody was localized in fraction 34 at a position near the 30-kDa protein marker (Figure 2B).

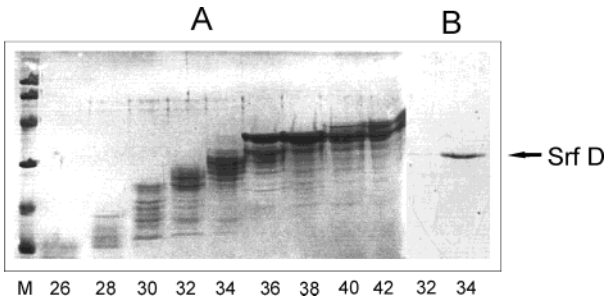

FIGURE 2: Separation of the proteins in the high molecular weight fractions of the AcA34 gel filtration by gel electrophoresis chromatography. (A) Fractions in the range of 26−40 of the AcA34 column were pooled, concentrated by ultrafiltration in Centricon PM-10 ultrafiltration cells (Amicon Bioseparations/Millipore, Eschborn, Germany), and applied onto a Bio-Rad Prep-Cell, model 491. The protein distribution in every second fraction in the range of fractions 26−42 obtained after preparative SDS−polyacrylamide gel electrophoresis was tested by analytical SDS−PAGE on a 12.5% polyacrylamide slab gel (lanes 26−42); lane M: low molecular weight marker proteins (see Figure 1). (B) After electroblotting on a PVDF membrane the protein crossreacting with the MBP−SrfD-directed antibody was localized in fraction 34. The position of SrfD was marked by an arrow.

The material of range II of the AcA34 gel filtration was further purified by anion exchange FPLC on Mono Q HR 5/5 (Figure 3). Enzyme E3 was again detected by its stimulatory effect on surfactin formation (Figure 3A). Figure

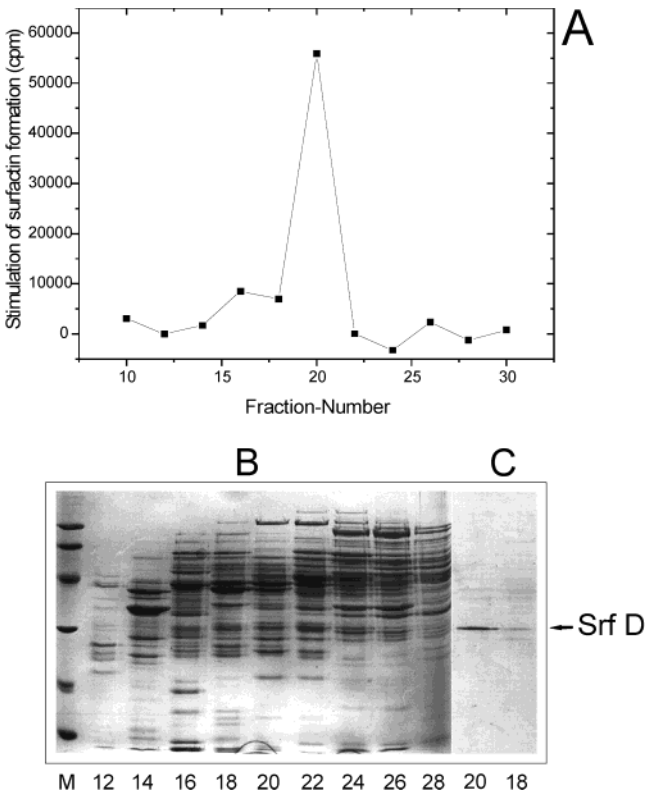

FIGURE 3: Anion exchange FPLC of enzyme E3 (SrfD) on MonoQ HR 5/5. (A) Fractions 44−52 obtained from AcA34 gel filtration were pooled and loaded onto the MonoQ-column. The proteins were eluted by a gradient from 0 to 0.5 M NaCl in 50 mM Tris/HCl buffer pH 7.5 containing 5 mM DTE. In fractions 10−30 eluted from the MonoQ column the stimulatory effect of the E3 protein on surfactin biosynthesis was tested after complementation with aliquots of fractions 30 and 38 of the AcA34 gel filtration containing SrfA, B, and C. (B) Coomassie-stained 12.5% SDS−polyacrylamide slab gel showing the protein composition in each second fraction in the range of fractions 12−28 of the MonoQ-eluate (lanes 12−28); lane M: low molecular weight marker proteins (see Figure 1). (C) After electroblotting of the proteins from the slab gel shown in Figure 3B onto a PVDF membrane the protein crossreacting with the maltose binding protein-SrfD-directed antibody was localized in fractions 18−20. The position of SrfD was marked by an arrow.

3B shows the protein composition in fractions 12−28 of the MonoQ-eluate as determined by SDS−PAGE. After electroblotting onto a PVDF membrane the protein crossreacting with the anti-MBP−SrfD antibody was localized in samples of fractions 18−20 (Figure 3C). It was found near the position of the 30-kDa protein marker. The immune response appeared in the same fractions as the activity of enzyme E3.

To identify SrfD unambiguously in fraction 34 of the electrophoresis chromatography shown in Figure 2B and in fractions 18−20 of the FPLC on MonoQ in Figure 3C, we analyzed tryptic fragments by MALDI-TOF mass spectrometry. For tryptic peptide mapping, an in gel digestion of the immune-reactive protein was performed. The protein band near the 30-kDa position crossreacting with the anti-MBP−SrfD antibody was excised from 12.5% SDS−polyacrylamide gels. They were cut into small pieces which were suspended in 100 μL of 100 mM ammonium carbonate buffer, pH 8.1. Proteins were digested with trypsin overnight (24 h at 37 °C). The obtained tryptic peptides were separated by high-resolution reversed phase FPLC using a μRPC SC 2.1/10 column. In both samples, the same specific tryptic peptide fragments were obtained from which SrfD could be identified by mass spectrometric analysis. In each case, two prominent peptides $[M + H]^+$ with mass numbers of m/z = 2214.2 (peptide a) and 1359.7 (peptide b) were investigated. Their sequence was obtained by post source decay (PSD) MALDI-TOF mass spectrometry from a series of b_n and Y_n ions as shown in Figure 4. The sequence data revealed that these two peptides correspond to the tryptic fragments E102−R121 (peptide a) and E151−R161 (peptide b) of SrfD (*2*, *22*), respectively. The sequence of each peptide was corroborated by proline directed internal fragments which arose from the parent ion by bond cleavage between amino acids 4 and 5; 14 and 15; 15 and 16 as well as 16 and 17. In the same way a C-terminal fragment PSFR ($[M + H]^+ = 506.3$) was detected for peptide b which originated from bond cleavage between amino acids 7 and 8. The sequences of peptides a and b were confirmed by Edman degradation experiments.

The other two immune responses at the 35- and 70-kDa positions observed after the initial AcA34 gel filtration step were unspecific. They were not related to the SrfD protein. The corresponding proteins were removed in the following purification steps. By in gel digestion with trypsin followed by mass spectrometric peptide mapping it was ensured that SrfD-specific fragments were not found in these samples. The nature of these proteins has not yet been identified. From all these results, we infer that the E3 protein, previously reported by us (*5*) to be involved in the initiation of surfactin formation, is identical with the SrfD protein, the fourth component of the surfactin synthetase multienzyme system.

The Effect of SrfD on the Thioester Activation Reactions of Surfactin Synthetase and in Vitro Surfactin Formation. To clarify the specific function of the external thioester enzyme SrfD in the biosynthesis of surfactin we investigated its effect (a) on the thioester activation reactions of surfactin synthetase with its amino acid substrates; (b) on the formation of the end product surfactin; and (c) on the initiation reactions in surfactin biosynthesis.

For this purpose, Srf A−C were further purified by anion exchange FPLC on MonoQ (Figure 5 A−C), as outlined in previous papers (*4*, *5*). The enzymes were eluted from the column with a linear gradient from 0 to 0.5 M NaCl. They were monitored by thioester incorporation of L-[^{14}C]-leucine and by the surfactin formation assay, as described previously (*6*). From Figure 5A, it is apparent that SrfC was well separated from SrfA/B. With increasing NaCl concentrations they appeared in the order SrfC followed by SrfB and SrfA. Maximal thioester formation of SrfC with L-[^{14}C]Leu was found in fraction 25. The maximum of the thioester formation of SrfA and B which overlapped in fractions 35−40 was found in fraction 38. The activity profiles for these proteins match with the staining intensity of the SrfA−C bands in the SDS−polyacrylamide gels (7.5% gels) in Figure 5B. As demonstrated by immune staining with our SrfD-specific antibody (Figure 5C) the SrfA−C containing fractions were completely free of the external thioesterase enzyme SrfD. This enzyme material was used to assay surfactin formation using L-[^{14}C]Leu as tracer. The surfactin product was monitored both by filter binding tests with scintillation counting and by thin-layer chromatography in combination with radioscanning.

Because native SrfD could be obtained only in a low amount and in partially purified form pure recombinant SrfD

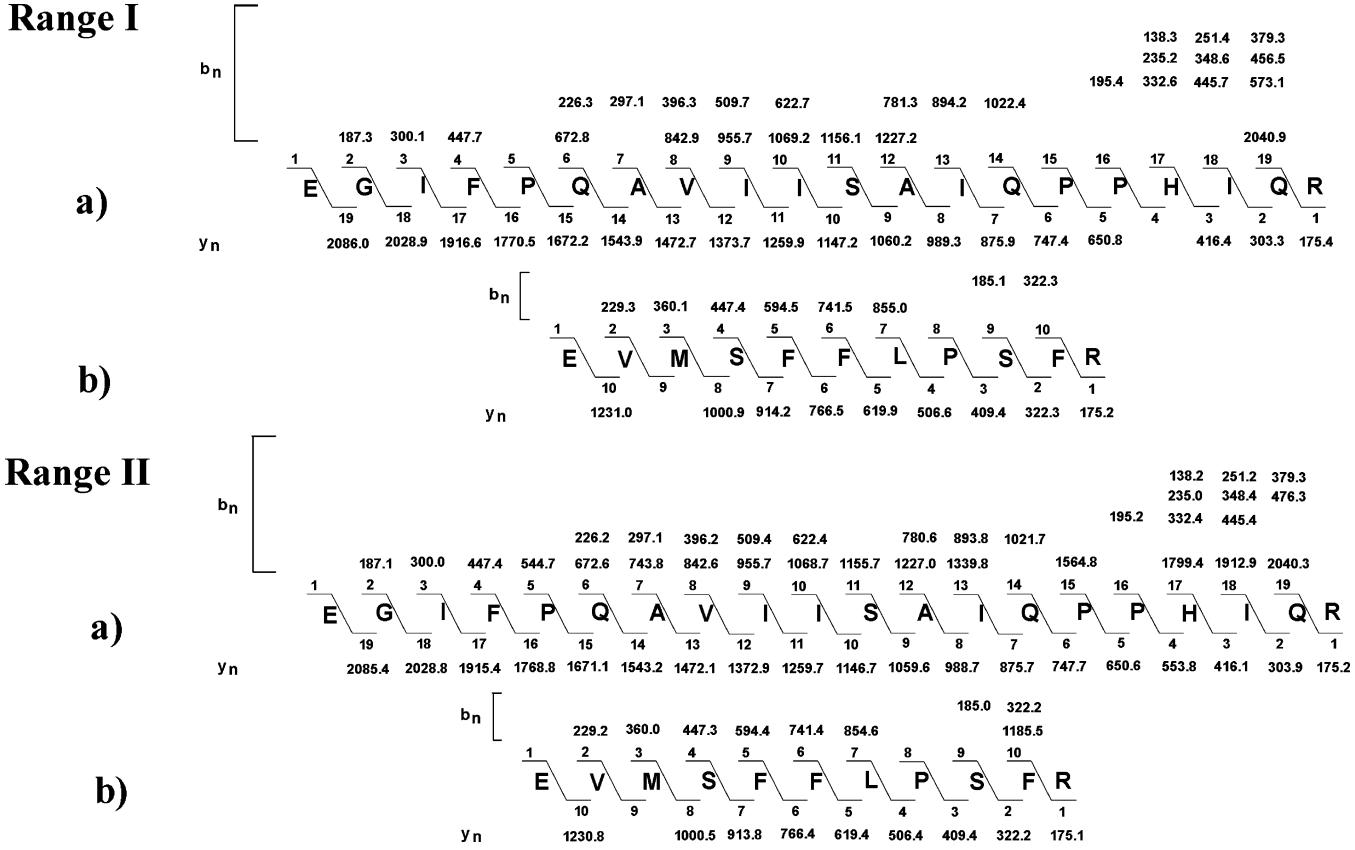

FIGURE 4: Sequence analysis of tryptic peptide fragments of the SrfD protein with mass numbers of $m/z = 2214.24$ (peptide a) and 1359.68 (peptide b) by post-source decay (PSD)-MALDI-TOF mass spectrometry. SrfD was isolated both from the high molecular weight range of the eluate of the AcA34 gel filtration (range I: fractions 26−44) and the low molecular weight range (range II; fractions 46−60). b-Ions indicated for peptide a in lines 1−4 from the top and for peptide b in line 1 correspond to proline directed internal fragments.

protein tagged with six histidines at its C-terminus (SrfDH6) was used to investigate its effect on the thioester activation reactions and product formation of surfactin synthetases Srf A−C. It was purified until homogeneity by affinity chromatography on a nickel-NTA-agarose column, as described under Methods. The high purity of the recombinant SrfDH6 protein is shown in Figure 5B by SDS−PAGE on a 12.5% SDS−polyacrylamide slab gel.

In Figure 6 the thioester formation of (A) SrfA/B with L-[^{14}C]-leucine and (B) of the recombinant L-Glu-activating module with L-[^{14}C] -glutamic acid is shown as a function of time without and in the presence of SrfDH6. Incorporation of the radiolabeled amino acid substrates was not increased by this protein, as would be expected in the case of a mischarge of the thiolation sites according to the proofreading model of Schwarzer et al. (*17*).

In Figure 7 it is demonstrated that surprisingly formation of the end product surfactin could be achieved with the amino acid activating components of surfactin synthetase SrfA−C alone without the participation of an acyltransferase enzyme as previously postulated (*5*); however, biosynthesis of surfactin was stimulated by SrfD. Kinetic studies of surfactin formation by SrfA−C are presented in Figure 7A,B. Assay mixtures were incubated at 37 °C for various time intervals. The surfactin product was extracted from the reaction mixtures with *n*-butanol, separated on silica gel 60 thin layer plates using chloroform/methanol/water 65:25:4 (by vol) as mobile phase (solvent mixture A) and monitored by radioscanning. In Figure 7A surfactin production is shown after an incubation time of 2 h in the presence of and without SrfDH6. In Figure 7B, the integral of the surfactin peak is plotted as a function of the reaction time. The lipopeptide product was identified by MALDI-TOF-MS as the sodium and potassium adducts containing a fatty acid with a hydrocarbon side chain of 14 carbon atoms ($[M + Na]^+ = 1045$ and $[M + K]^+ = 1061$).

Investigation of the Initiation Reactions in the Biosynthesis of Surfactin. Knowledge is still lacking on the initiation processes in nonribosomal peptide biosynthesis. In this paper, we investigated the initiation reactions in surfactin formation by comparing the initiation products formed (a) by the complete start enzyme SrfA and (b) by the separate L-Glu-activating module of this protein obtained by cloning and overexpression in His-tagged form. *E. coli* DH 5α was used as host organism. The Glu-module was highly purified by affinity chromatography on a nickel-NTA-agarose column. It was transformed into its holo-form by 4′-phosphopantetheinylation with Sfp, the *B. subtilis* specific 4′-phosphopantetheine transferase, in the presence of coenzyme A as described by Weinreb et al. (*23*). SrfA and the isolated Glu-module of surfactin synthetase were assayed by thioester formation with L-[^{14}C]Glu. Initiation products formed in the presence of the fatty acid substrate β-hydroxymyristoyl-coenzymeA which were released into the assay medium were investigated. In reference experiments, products were detected which were formed in the absence of the fatty acid substrate. The kinetic studies of initiation product formation by the isolated Glu-module are shown in Figure 8.

Products were extracted from the reaction mixtures with *n*-butanol, concentrated in a SpeedVac centrifuge, and

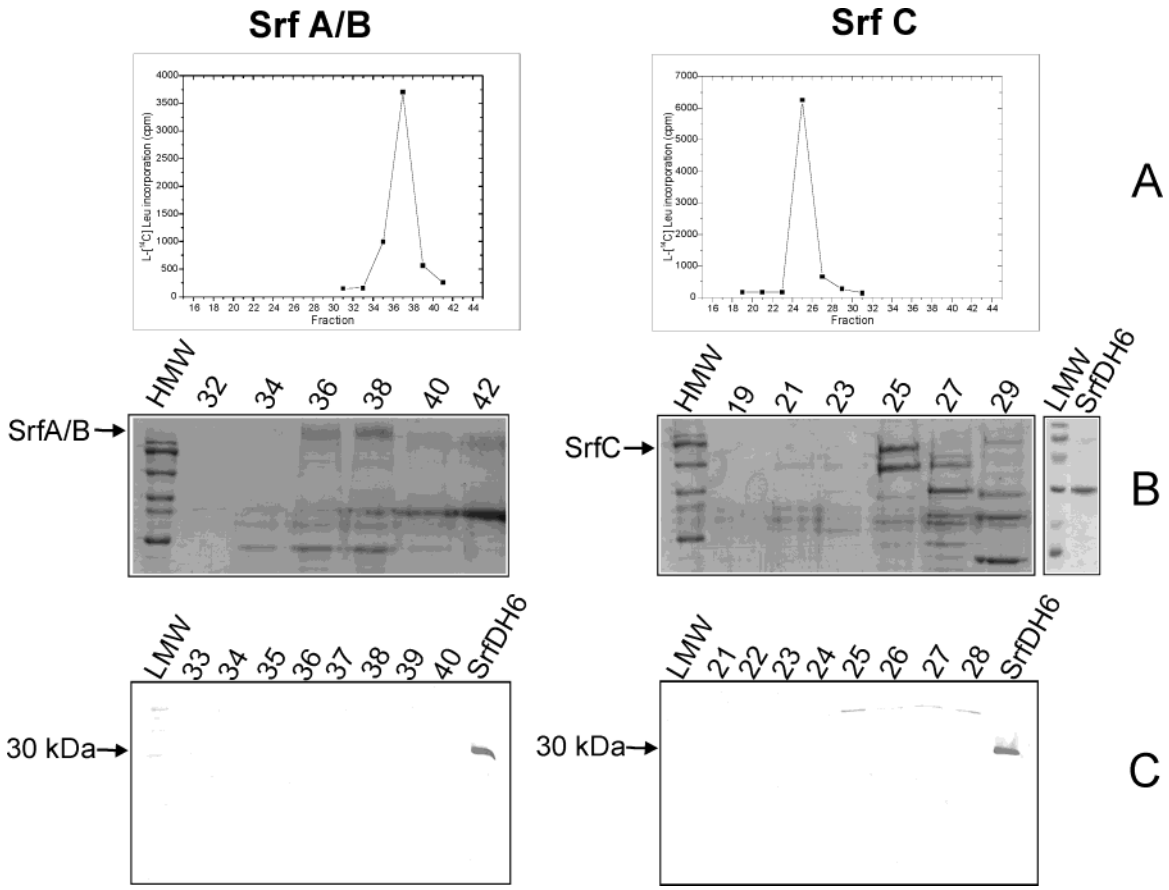

FIGURE 5: Anion exchange FPLC of the component enzymes SrfA-C of surfactin synthetase on Mono Q HR 5/5. (A) SrfA-C containing fractions obtained from AcA 34 gel filtration were loaded onto the MonoQ-column. The proteins were eluted by a gradient from 0 to 0.5 M NaCl in 50 mM Tris/HCl buffer pH 7.5 containing 5 mM DTE and assayed by thioester incorporation of L-[^{14}C] leucine. (B) Coomassie-stained 7.5% SDS−polyacrylamide slab gel showing the protein composition in every second fraction in the range of fractions 19−42 of the Mono Q eluate. The positions of Srf A, B, and C are marked by arrows. Lane HMW: High molecular weight marker proteins: myosin, 220 kDa; α_2-macroglobulin, 170 kDa; β-galactosidase, 116 kDa; transferrin, 76 kDa; glutamic dehydrogenase, 53 kDa. Recombinant SrfD-protein tagged with six histidine-residues (SrfDH6) was purified until homogeneity by nickel-NTA-agarose affinity chromatography. The high purity of SrfDH6 was detected by SDS−PAGE using a 12.5% polyacrylamide gel and staining with Coomassie (right side). Lane LMW: Low molecular weight marker proteins (see Figure 1). (C) The proteins in every second fraction in the range of fractions 21−40 of the Mono Q eluate were separated by SDS-gel electrophoresis on a 12.5% SDS−polyacrylamide slab gel, electroblotted onto a PVDF membrane and exposed to an antibody raised against a maltose binding protein−SrfD fusion protein which was applied in a dilution of 1:40 000 in 20 mM Tris/HCl buffer pH 7.3 containing 150 mM NaCl; lane SrfDH6: recombinant, His-tagged SrfD protein as the reference.

separated by thin-layer chromatography on silica gel 60 plates using solvent mixture A. Samples were taken at various incubation times. The products were detected by autoradiography (Figure 8A) and radioscanning (Figure 8B). Three main initiation products (a, c, d) were observed. Product a was found at an R_f value of 0.16−0.2. Products c and d appeared as a double spot in the range of $R_f = 0.58-0.62$. The same initiation products are formed by SrfA and the isolated recombinant Glu-module. In the absence of the hydroxy-fatty acid substrate another yet unknown compound with an R_f value of 0.26 (b) was found which possibly may be a thioester adduct of glutamic acid with dithioerythitol, because it disappeared, if DTE was removed from the reaction mixture. In previous studies it was shown that product a is the linear form of β-hydroxymyristoyl-glutamate, while c and d are cyclic species (*5*). This was confirmed by MALDI-TOF-mass spectrometry indicating mass numbers of $[M + H]^+ = 374$ and 356, respectively. According to the R_f values previously determined with model compounds product d in Figure 8A should originate by lactone bond formation between the α-carboxyl group of L-glutamic acid and the β-hydroxy group of the fatty acid moiety. The spot with a slightly lower R_f value of 0.59 may possibly be the corresponding product with the carboxyl group in δ-position of L-Glu being involved in the lactone bond, but this hypothesis has still to be clarified.

The concentration of the initiation products a, c, and d increased in the assay medium as a function of time. The obtained results demonstrate that efficient formation of these compounds was achieved in the absence of SrfD corroborating our finding that surfactin can be produced by SrfA, B, and C alone. From these data we infer that the β-hydroxy fatty acid substrate can directly be accepted at the reaction center of the Glu-module forming the β-hydroxyacyl-glutamate initiation products.

The Effect of SrfD on the Initiation Process. In Figure 9 the effect of SrfD on the formation of initiation products by the isolated Glu-module was investigated. Products released into the assay medium were monitored by thin-layer chromatography and radioscanning. In Figure 9A it is shown that addition of an aliquot of the E3-fraction containing native SrfD to an initiation assay stimulated the formation of initiation products by a factor of 2−5. In particular, the amount of product a was increased. In a similar way

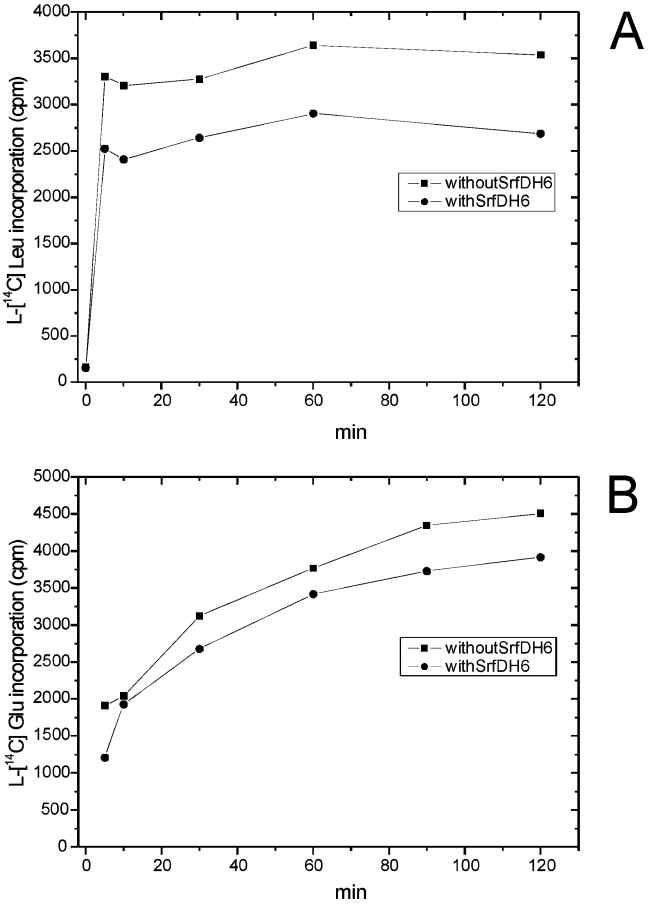

FIGURE 6: The time course of thioester formation of (A) SrfA/B with L-[^{14}C] leucine and (B) of the recombinant L-Glu-activating module of surfactin synthetase with L-[^{14}C] glutamic acid without (■) and in the presence of SrfDH6 (●). The thioester binding assay was performed as reported in ref *6*.

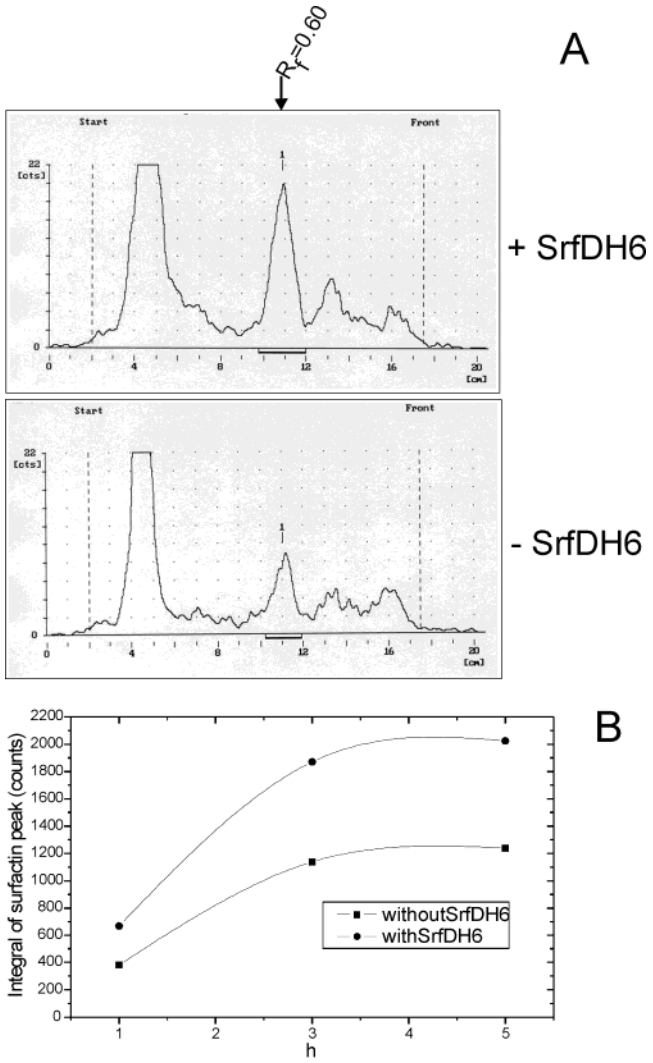

FIGURE 7: Biosynthesis of surfactin catalyzed by Srf A−C in the presence of and without SrfDH6. (A) Surfactin formation was detected by thin-layer chromatography and radioscanning. The assay was run for 6 h in the presence of and without SrfDH6. Using solvent A surfactin appeared at $R_f = 0.60$. (B) Surfactin formation was monitored by determination of the integral of the surfactin peak in the radioscans as a function of incubation time combining aliquots of fraction 25 (Srf C) and 38 (Srf A/B) of the anion exchange FPLC on MonoQ shown in Figure 5A without (■) and in the presence of SrfDH6 (●).

recombinant SrfDH6 efficiently increased the formation of initiation products (Figure 9B). These results give evidence that SrfD is the active component in fraction E3 which is responsible for the stimulation of product formation in surfactin biosynthesis.

In Figure 10A,B the initiation products formed by the L-Glu-activating module were fractionated in a micropreparative manner by reversed phase FPLC on a μRPC column using the Smart microseparation system. They were eluted with a linear gradient from 0 to 100% acetonitrile/0.1% TFA. Comparison of the chromatograms obtained in the absence and the presence of SrfD again manifests the stimulatory effect of this protein on the formation of initiation products. In Figure 10 B their distribution in the fractions obtained in the presence of recombinant SrfDH6 is demonstrated. The linear β-hydroxymyristoyl glutamate (compound a) was found in fractions 24−29 with a maximum at fraction 28, while the cyclic forms with R_f values of 0.59 and 0.61 appeared in fractions 30−32. Here the maximum was found in fraction 32. In Figures 8−10 two other yet unknown compounds with R_f values of 0.31 and 0.33−0.34 appeared which have still to be identified.

DISCUSSION

In this paper, we demonstrate for the first time that surfactin formation can be achieved by the three substrate amino acid activating subunits of surfactin synthetase SrfA−C (fractions E1 and E2) alone. Enzyme E3, the putative acyltransferase involved in the initiation reaction (*5*), is not absolutely required, but stimulates the biosynthetic process. This finding was corroborated by our results from initiation studies. The initiation reaction in the biosynthesis of surfactin is the transfer of its fatty acid component from the fatty acid substrate β-hydroxymyristoyl-coenzymeA to the start enzyme SrfA. This process was investigated using both the complete SrfA start enzyme and its N-terminal L-Glu-activating module which was cloned and overexpressed in His-tagged form in *E. coli* DH 5α. Both proteins formed the same initiation products, i.e., the linear and cyclic forms of β-hydroxymyristoyl-glutamate also in the absence of enzyme E3. These results imply that the β-hydroxymyristoyl residue can be transferred from coenzymeA directly to a specific binding site on the L-Glu-activating module of SrfA without the participation of an auxiliary enzyme, like an acyltransferase, as previously postulated (*5*, *6*), but also the

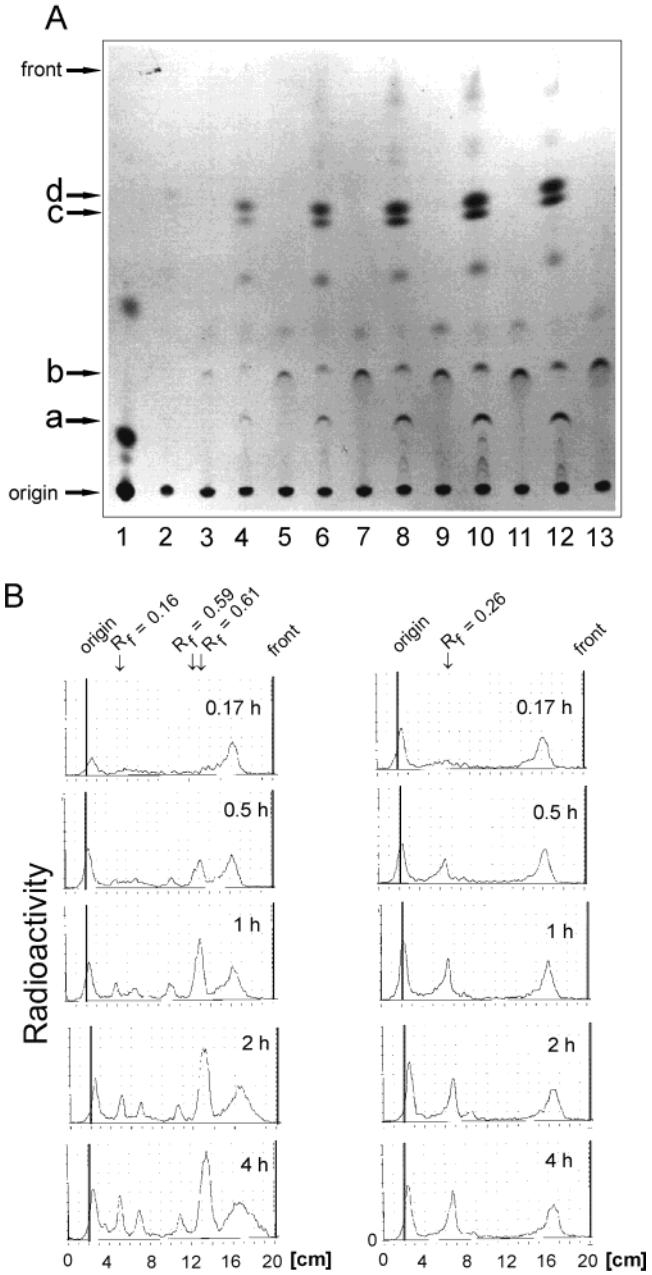

FIGURE 8: Initiation products formed by the overexpressed L-Glu-activating module after various incubation times. (A) Initiation products separated by thin-layer chromatography using solvent A were detected by autoradiography: Lane 1: L-[^{14}C] Glu tracer; lanes 2, 4, 6, 8, 10, and 12: Initiation products formed in the presence of the β-hydroxymyristoyl-CoA substrate after 0.17, 0.5, 1, 2, 4, and 5 h incubation times. Lanes 3, 5, 7, 9, 11, and 13: Reference measurements without the fatty acid substrate at the same incubation times. (B) Initiation products separated by thin-layer chromatography and detected by radioscanning. Left side: measurements at incubation times of 0.17, 0.5, 1, 2, and 4 h in the presence of the β-hydroxymyristoyl-CoA substrate. Right side: Reference measurements in the absence of the fatty acid substrate.

initiation process was efficiently stimulated in the presence of E3.

Another important question that had to be clarified was the nature of enzyme E3, which hitherto could not be characterized in detail. Likewise, a specific function in the biosynthesis of surfactin could not yet be established also for the external thioesterase enzyme SrfD of the surfactin synthetase multienzyme system. This aspect led us to the idea that enzyme E3 might be identical with SrfD. Therefore,

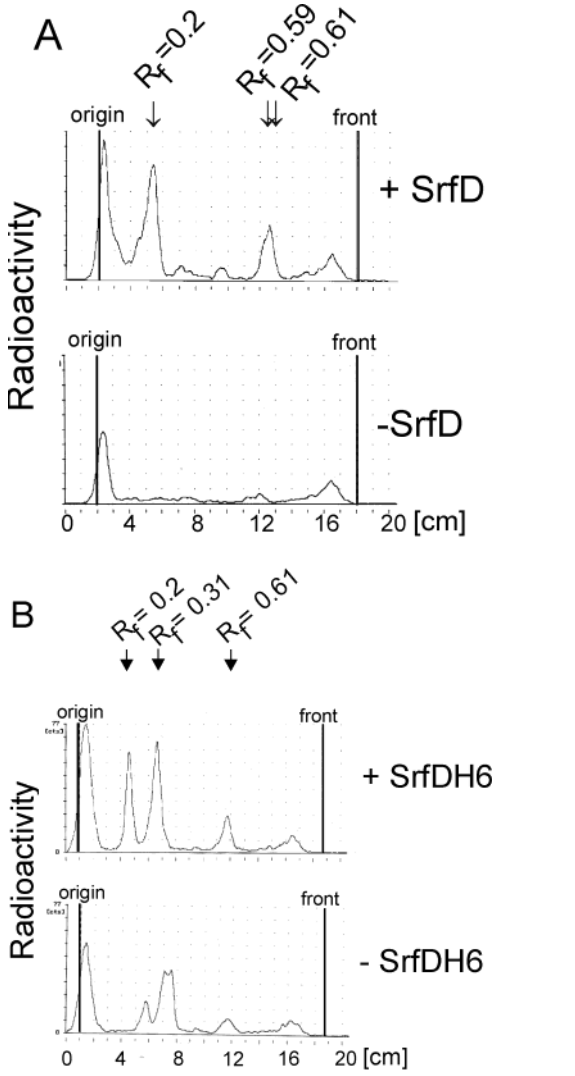

FIGURE 9: Effect of enzyme E3/SrfD on the formation of initiation products catalyzed (A) by enzyme E3 (native SrfD) and (B) by recombinant SrfDH6 cloned and overexpressed in *E. coli* M 15. Initiation products released into the assay medium in the presence and without SrfD were separated by TLC using solvent A and detected by radioscanning. The assay was run for 3 h. Different enzyme preparations have been used for experiments A and B. DTE has been omitted from the reaction mixtures.

we investigated the presence of SrfD in the E3-containing fractions. E3 was detected by its stimulating effect in the biosynthesis of surfactin. By immune staining with SrfD-directed antibodies and by mass spectrometric sequence analysis of SrfD-specific tryptic peptide fragments using PSD-MALDI-TOF-MS SrfD could indeed be identified in these fractions. From these results, we infer that SrfD may function as an acyltransferase/thioesterase enzyme in the initiation of surfactin biosynthesis, but for native SrfD this interpretation was not quite conclusive, because it could be obtained only in a small amount and in partially purified form. To clarify this conclusion definitely, we prepared pure His-tagged SrfD by cloning and overexpression in *E. coli* M15. Recombinant SrfDH6 which was highly purified by affinity chromatography on a nickel-NTA-agarose column also stimulated the initiation process efficiently, as was observed for enzyme E3. In this way, we presented evidence for a specific role of SrfD in the biosynthesis of surfactin for the first time.

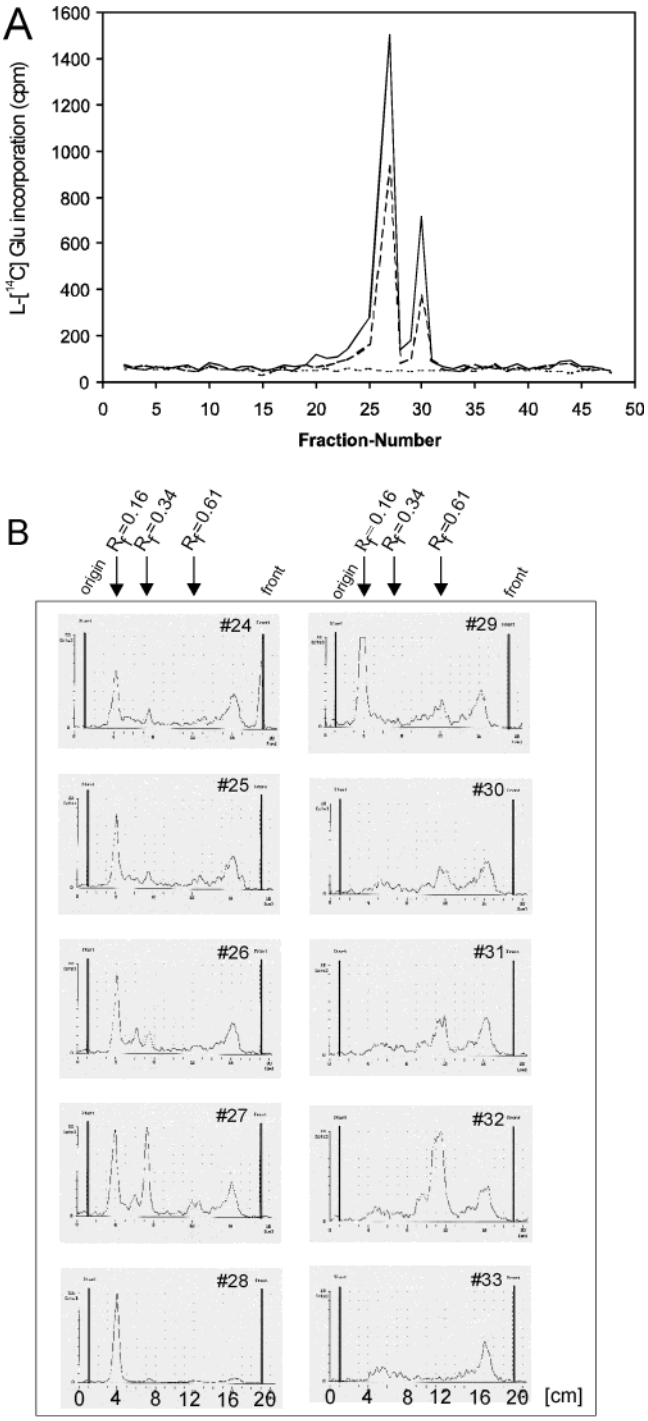

FIGURE 10: Analysis of the initiation products in surfactin biosynthesis. (A) Separation of the initiation products formed by the recombinant L-Glu-activating module by reversed phase FPLC on a μRPC SC 2.1/10 column using a Smart micro-separation system in the presence of recombinant SrfDH6 (solid line) and without SrfDH6 (dotted line), respectively. The assay for initiation product formation was run for 14 h. DTE has been omitted from the reaction mixtures. (B) Identification of the initiation products in the fractions obtained by reversed phase FPLC on μRPC in experiment A by thin-layer chromatography (solvent A) in combination with radioscanning. Initiation products were formed in the presence of recombinant SrfDH6.

In a recent report, Schwarzer et al. (*17*) suggested that external thioesterase enzymes in nonribosomal peptide biosynthesis, like SrfD, generally may be involved in proofreading functions to eliminate incorrect charging of the 4′-phosphopantetheine cofactors at the reaction centers of peptide synthetases. In this paper we clearly demonstrate that in addition to possible editing effects SrfD is involved in the catalysis of the acyl transfer reaction in the initiation of surfactin biosynthesis thus stimulating surfactin formation. The most straightforward way to clarify a mischarge of the thiolation sites of surfactin synthetase is to compare the thioester incorporation of its amino acid substrates without and in the presence of SrfD. In Figure 6, such experiments have been performed for the incorporation of L-[^{14}C]leucine into SrfA/B and of L-[^{14}C]glutamic acid into the recombinant L-Glu-activating module of SrfA. In the case of a partial block of the reaction centers, for example, by an acetyl residue originating from the pool of CoA-derivatives in the cell, an increase of thioester formation would be expected in the presence of SrfD. In our experiments, however, we found a slight, but significant and reproducible decrease, which is consistent with the findings of Schwarzer et al. (*17*) that external thioesterases of NRPS are able to hydrolyze the thioester complexes of these multienzymes with substrate amino acids and peptide intermediates. Therefore, the stimulating effects of SrfD are not due to the provision of additional reactive thiol groups as the consequence of proofreading activities of SrfD. In particular, in the case of the recombinant L-Glu-activating module mischarging effects were not relevant, because 4′-phosphopantetheinylation of the purified protein by Sfp was performed in vitro in the absence of amino acids and other acyl compounds which could block its thiolation site in the cofactor transfer to the apo-form. On the other hand, the stability of thioesters at the thiolation sites of native peptide synthetases is rather low (*24*). Therefore, it is to be expected that a mischarge at a reaction center would disappear in the course of the isolation and purification of such enzymes.

The mechanism of the specific role of SrfD in the initiation of surfactin biosynthesis can be interpreted as an increase of the availability of the fatty acid substrate by a stimulation of the hydrolysis of the acyl-CoA substrate by its thioesterase function and promoting the transfer of the fatty acid to its binding site on the Glu-module of SrfA in a concerted action. Our results point to a general role of SrfD and related enzymes as thioesterase/acyltransferase enzymes in nonribosomal peptide biosynthesis mediating acyl transfer reactions in the corresponding biosynthetic process. To consolidate this suggestion the effect of SrfD on other acyl transfer steps involved in the assembly of surfactin will be tested in forthcoming studies.

Within the *B. subtilis* cell SrfD presumably is a component of a supramolecular complex which comprises all four protein subunits of the surfactin synthetase multienzyme system SrfA-D. First evidence for this conclusion was obtained from our immunological studies. As is apparent from the immune staining tests shown in Figure 1C and from mass spectrometric analysis of SrfD-specific peptide fragments (Figure 4), SrfD was found in several fractions after purification of the enzyme components of surfactin synthetase by AcA34 gel filtration. It appeared both in the high molecular mass range I including SrfA−C as well as in the 20−40 kDa range II (Figure 1D) where it would have been expected according to its molecular mass of 27.621 Da calculated from its gene sequence (*2*, *22*). The detection of SrfD in the high molecular mass range implies that the supramolecular surfactin synthetase complex gradually dis-

sociates into its components after cell disintegration. We assume that a part of this aggregate persisted until the cell free extract was loaded on the AcA34 column. This portion continued to dissociate in the course of the gel filtration process. Therefore, SrfD could be detected in the eluate in free form together with the larger enzymes SrfA−C of surfactin synthetase. This conclusion was corroborated by the observation that a part of SrfD also appeared together with SrfA, B, and C when a crude cell extract was rapidly fractionated by anion exchange chromatography on Q-Sepharose immediately after cell disruption and removal of the cell debris by centrifugation omitting the streptomycin sulfate precipitation and ammonium sulfate fractionation prepurification steps (data not shown). Under such conditions, a significant part of surfactin synthetase was still associated as a supramolecular enzyme complex. This pattern was in contrast to the profiles obtained in the fractionation of the free surfactin synthetase components SrfA−D by anion exchange chromatography. In this case, these proteins were eluted separately with increasing ionic strength. SrfD appears first, followed by SrfC, and finally by SrfB and SrfA, as shown in Figures 3 and 5.

An important task for the future to understand the initiation of surfactin biosynthesis completely is the localization of the fatty acid binding site on the start enzyme SrfA. Information on the mode of binding of the fatty acid substrate to SrfA may be derived from sequence comparison of fatty acyl CoA ligases with adenylation domains of peptide synthetases which both belong to the same class of adenylate forming enzymes using a similar reaction mechanism. For acyl CoA-ligases, a specific consensus motif has been elucidated, the so-called FACS motif, that is involved in fatty acid binding (*25*). This functional domain comprises about 25 amino acids. It contains nine highly conserved and 12 homologous amino acid residues. The function of all these structural elements in fatty acid recognition and binding was investigated by Black et al. (*25*) using site-specific mutagenesis. Sequence comparison of adenylation domains of peptide synthetases revealed a segment in the range of the highly conserved F- and G-motif of these domains which shows a significant homology to the FACS-motif (*26*). From site-specific mutagenesis and affinity labeling experiments (*27*, *28*) it was concluded that the F- and G-motifs of peptide synthetases are involved in the adenylation process. It remains to be clarified by dissection studies and systematic amino acid replacements in the FACS-homologous segments, whether these regions are also relevant for binding of the fatty acid substrates to lipopeptide forming multienzymes.

Work is in progress to clarify the mechanism of the initiation reactions in the biosynthesis of surfactin in detail.

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