Structural and Functional Organization of the Surfactin Synthetase Multienzyme System*

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By gel filtration of a crude extract of Bacillus subtilis ATCC 21332 and OKB 105, the multienzyme system that forms the lipopeptapeptide surfactin was separated into three enzyme fractions, $E_1$, $E_2$, and $E_3$. $E_1$, which appeared near the exclusion limit of the column, activates all amino acid components of surfactin as aminoaaclyldeacylates and thioesters according to the thioester mechanism. In addition, a leucine-activating enzyme ($E_2$) and an acyltransferase ($E_3$) were detected that show molecular masses of ~160 and 40 kDa, respectively. The surfactin synthetase multienzyme system was reconstituted by complementation of all three enzyme fractions, yielding high rates of lipopeptide formation. $E_1$ is composed of two multifunctional polypeptides ($E_{1A}$ and $E_{1B}$) with molecular masses of 460 and 435 kDa, respectively, that can be separated by high-resolution anion-exchange chromatography on Pharmacia Mono Q. $E_{1A}$ binds L-Glu and L-Leu in a molar ratio of 1:2, whereas $E_{1B}$ incorporates L-Val, L-Asp, and L-Leu in a molar ratio of 1:1:1. The hydroxy fatty acid moiety is contributed by the acyltransferase accepting the hydroxy fatty acid coenzyme A thioester as substrate. The transfer of the hydroxy fatty acid to $E_1$, and the formation of the hydroxyacyl-L-glutamate intermediate are the initiation steps in the biosynthesis of surfactin. The amino acid-activating enzyme components $E_{1A}$, $E_{1B}$, and $E_2$ have been highly purified and partially characterized.

Lipopeptides represent a class of microbial secondary metabolites that appear in manifold variation in microorganisms. Many of them show attractive therapeutic and biotechnological properties. Only limited information is yet available on the biosynthesis of such compounds on the molecular level (1). In particular, knowledge is still lacking on the structural and functional organization of the multienzyme systems that are involved in these processes as well as on the gene structures that code for these proteins. Our research is concentrated on the biosynthesis of a family of lipopeptides that are produced by various strains of Bacillus subtilis (1, 2). A representative of these agents is surfactin, which is one of the most efficient biosurfactants so far known (3).

\[
\begin{align*}
C_{16-17}CH(CH_2)_nCOO^- &\rightarrow L-Glu \rightarrow L-Leu \rightarrow D-Leu \\
&\rightarrow L-Leu \rightarrow D-Leu \rightarrow L-Asp \rightarrow L-Val
\end{align*}
\]

**STRUCTURE 1**

It is a cyclic lipopeptapeptide (4-9) that appears as a complex mixture of several species that show variations both in their β-hydroxy fatty acid component (8, 9) and in their peptide ring (10). Its lipid portion is a mixture of several β-hydroxy fatty acids with chain lengths of 13-15 carbon atoms. The main component is 3-hydroxy-13-methyltetradecanoic acid (5).

Previously, we reported on the de novo biosynthesis of surfactin in cell-free extracts of B. subtilis ATCC 21332 and OKB 105. A multienzyme fraction was partially purified that activates the amino acid components of surfactin in two steps as aminoaaclyldeacylates and thioesters (11). In this paper, we investigate the structural and functional organization of the surfactin synthetase multienzyme system. It is demonstrated that at least four enzyme components, which have been purified and functionally characterized, are involved in the biosynthesis of surfactin. By complementation of these proteins, the entire multienzyme system could be reconstituted, yielding high rates of surfactin formation.

**EXPERIMENTAL PROCEDURES**

**Materials**

14C-Labeled amino acids were purchased from Amerham/Buchler (Braunschweig, Germany). Tetrasodium [32P]pyrophosphate was from Du Pont-New England Nuclear (Bad Homburg, Germany). Coenzyme A as the lithium salt was obtained from Sigma (Deisenhofen, Germany). AcA 34 was from Serva (Heidelberg, Germany). DE52 was from Whatman (Maidstone, Kent, Great Britain), and Sephacryl S-300 and Mono Q HR 5/5 were from Pharmacia LKB Biotechnology Inc. (Freiburg, Germany).

B. subtilis ATCC 21332 and OKB 105 were cultivated as published previously (11, 12).

**Methods**

**Syntheses**

3-Hydroxytetradecanoic acid and its thioester adduct with coenzyme A were synthesized as reported recently (11). N-(3-Hydroxytetradecanoyl)-L-glutamic acid was prepared using the procedure of Lapidot et al. (13).

**Thin-layer Chromatography**

Silica Gel 60 plates (Merck, Darmstadt, Germany) were used with the following solvent systems: solvent system A, CHCl3/MeOH/H2O (65:25:4, by volume); and solvent system B, CHCl3/MeOH/ErOH/H2O (70:30:35:15, by volume).
**Determination of Protein Content**

After each purification step applied for the preparation of the enzyme components of surfactin synthetase, the protein concentration was measured using the procedures of Warburg and Christian (14) and Bradford (15).

**Assays**

Assays for ATP/PPi exchange measurements, covalent binding of substrate amino acids by surfactin synthetase, and in vitro formation of surfactin have been described in a previous publication (11). Leucine racemase activity was tested using a similar procedure as that described for phenylalanine racemase in the biosynthesis of gramicidin S (16-18).

The acyltransferase enzyme E3 of surfactin synthetase was assayed measuring the hydrolysis of the β-hydroxytetradecanoyl coenzyme A thioester substrate in the absence of the other enzymes and amino acid substrates. The free thiol group of coenzyme A liberated in this reaction was measured spectrophotometrically with 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) following a procedure similar to that reported for the assay of medium-chain fatty acid thioesterases (19). Reaction mixtures contained 0.2 mM DTNB and bovine serum albumin at a concentration of 50 mg/liter in 0.2 M Tris/HCl, pH 8.0, in a total volume of 600 μl. Reaction mixtures were incubated at 25 °C for 1 h. The absorption change at 412 nm was measured with a Perkin-Elmer/Hitachi Model 506 double-beam dual-wavelength spectrophotometer. The rate of hydrolysis was estimated using a molar extinction coefficient of ε = 13,600 mol⁻¹ cm⁻¹.

**Determination of Pantothenic Acid**

Samples of the multienzyme fraction E3 were digested with 1 n KOH for 1 h at 95 °C and, after adjustment at pH 8.0 with Tris/HCl, treated with 5 units of bovine alkaline phosphatase (Sigma) for 2 h at 37 °C. Pantothenic acid liberated from the peptide was determined microbiochemically using Lactobacillus plantarum ATCC 8014 as the test organism (20, 21).

**Enzyme Purification**

All operations were carried out at 0-4 °C with the exception of the final FPLC step, which was performed at room temperature in a minimum of time. The same purification procedure was used for preparation of the multienzyme E3 and the leucine-activating enzyme E2.

**Step 1**—A cell lysate was prepared from B. subtilis OKB 105 as described previously (11). Nucleic acids were removed by precipitation with 1% streptomycin sulfate. The precipitate formed in the range of 30-70% saturation was dissolved in a minimum volume of 50 mM Tris/HCl, pH 7.5, 5% dithioerythritol, 0.25 mM EDTA, and 10% (w/v) sucrose (buffer A) and dialyzed against the same buffer. This solution (crude extract) was taken as the reference for the following purification steps.

**Step 2**—The crude extract was applied to a Ultrogel AcA 34 or Sephacryl S-300 superfine column (45 × 2.5 cm). The proteins were eluted with buffer A without dithioerythritol. Here, the thiol-protecting agent was omitted from the equilibration and elution buffer because of interference with the DTNB assay for detection of E3.

After localization of this enzyme, dithioerythritol was added to the enzyme fractions at a concentration of 5 mM. E3 and E2 were detected in the eluted fractions by thioether formation with l-Leu. Then the positions of E2, E3, and E1 were determined carefully using the in vitro assay for surfactin formation always by combination with the two complementary enzymes.

**Step 3**—The active fractions containing either E1 or E2 were combined. Each pool was loaded onto a DE52 anion-exchange column (10 × 3.5 cm) equilibrated with buffer A without EDTA. The proteins were eluted with a linear gradient from 0 to 0.5 M NaCl. The active fractions were pooled, and the proteins were precipitated with ammonium sulfate (70% saturation). The pellets obtained by centrifugation were suspended in a minimum volume of buffer A and dialyzed against the same buffer.

**Step 4**—The concentrated protein solutions obtained from Step 3 were layered onto 5-20% (w/v) sucrose gradients in 50 mM Tris/HCl, 0.25 mM EDTA, and 5 mM dithioerythritol (each 34 ml), which were centrifuged at 27,000 rpm and 4 °C in a Du Pont/Sorvall AH 627 rotor. The centrifugation times were 22 h for E1 and 26 h for E2. After the run, the gradients were fractionated using a Kontron piercing unit. The gradient fractions were tested for surfactin formation and substrate acid activation.

**Results**—Active fractions of the sucrose gradients containing E1 or E2 were combined, and the enzymes were finally purified by high-resolution anion-exchange FPLC on Pharmacia Mono Q HR 5/5 (5 × 0.5 cm) using a linear gradient of 0-0.5 M NaCl in 50 mM Tris/HCl, pH 7.5, and 5 mM dithioerythritol.

**SDS-Polyacrylamide Gel Electrophoresis**

For the electrophoresis experiments, the technique of Laemmli (22) was used. Gels consisted of 5, 7.5, and 10% acrylamide for the enzymes E1, E2, and E3, respectively, were run in 375 mM Tris/HCl, pH 8.8, in the presence of 0.1% (w/v) SDS at room temperature. The N,N-methylenebisacrylamide concentrations were 0.13, 0.2, and 0.26%, respectively. Samples were treated with 1% (w/v) SDS and 2% mercaptoethanol for 5 min at 60 °C. The dimensions of the separation gels were 140 × 90 × 1.5 mm. Electrophoresis was performed for 5-6 h at a constant current of 40 mA. The proteins were visualized with Serva Blue R or by silver staining (23).

The following marker proteins were used for molecular mass determination: for the molecular mass determination of E1, gramicidin-S synthetase 2 (350 kDa), β-hexokinase (185 kDa), and α-galactosidase (115 kDa); for the molecular mass determination of E2, gramicidin-S synthetase 1 (126.7 kDa), phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (45 kDa), cytochrome c (12.5 kDa), and carbonic anhydrase (30 kDa); and soybean trypsin inhibitor (20.1 kDa), and α-lactalbumin (14.4 kDa).

**Western Blotting and NH2-terminal Sequencing of Multienzyme E3 of Surfactin Synthetase**

E3 was purified for NH2-terminal sequencing by preparative SDS-polyacrylamide gel electrophoresis (3.5% (w/v) acrylamide for the resolving gel (140 × 90 × 1.5 mm) and 5% (w/v) acrylamide for the resolving gel (140 × 90 × 1.5 mm)). The gel was run at a constant current of 20 mA for the stacking gel and 40 mA for the resolving gel using the buffer system developed by Laemmli (22). After electrophoresis for 3 h, the gel was equilibrated for 30 min in transfer buffer (192 mM glycine, 25 mM Tris/HC1, pH 8.3) and 20% (v/v) methanol according to Towbin et al. (24). The polyvinylidene difluoride membrane (0.45 mm) was washed with methanol and equilibrated for 1 min in transfer buffer. Electroboblotted was performed at 4 °C for 19 h at 30 V (80 mA), followed by 1 h at 100 V (260-310 mA) onto a double-membrane layer using a Bio-Rad blotting cell. After transfer of the proteins, the polyvinylidene difluoride membrane was washed with quartz-distilled water for 5 min, stained with Coomassie Brilliant Blue R-250 for 5 min, destained as described by Matsudaira (25) for 10 min, and washed again with water for 1 h. The stained protein bands were excised and submitted to sequence analysis using an Applied Biosystems Model 120A Pulsed Liquid-phase Sequencer.

**Molecular Mass Determination of E3 by Gel Filtration**

The leucine-activating enzyme of the surfactin synthetase complex (E3) and a collection of calibration proteins were gel-filtrated on a Sephacryl S-300 superfine column (100 × 1.5 cm) equilibrated with a solution of 50 mM Tris/HCl, pH 7.5, 0.1 M NaCl, and 10% (w/v) sucrose. In the case of E3, 5 mM dithioerythritol was added. The proteins were dissolved in 1 ml of equilibration buffer containing 5% (v/v) glycerol, loaded onto the column, and eluted at a flow rate of 20 ml/h. The positions of the calibration proteins were detected by measurement of the absorbance at 280 nm. The elution position of E3 was determined by the leucine-dependent ATP/PPi exchange assay. The following marker proteins were used: carbonic anhydrase (30 kDa), bovine serum albumin (67 kDa), alcohol dehydrogenase (150 kDa), β-amylase (200 kDa), apoferritin (443 kDa), and thyroglobulin (669 kDa).

**RESULTS**

**Reconstitution of Surfactin Synthetase from Three Enzyme Fractions**—Recently, we developed an in vitro system for the biosynthesis of surfactin. Efficient formation of the lipopep-

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1 The abbreviations used are: DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); PFLC, fast protein liquid chromatography.
tide was detected in cell-free extracts of *B. subtilis* ATCC 21352 and OKB 105 that were obtained by treatment of these organisms with lysozyme and a French press, followed by nucleic acid precipitation and ammonium sulfate fractionation (11). In our previous studies, we prepared a multi-enzyme fraction by gel permeation chromatography that catalyzed ATP/PPi exchange reactions mediated by the amino acid components of surfactin and showed covalent binding of all amino acid substrates via thioester linkages. *De novo* synthesis of surfactin was not achieved with this multi-enzyme. Therefore, the aim of this study was to identify the enzyme components of the surfactin synthetase complex to reconstitute this multi-enzyme system and to study its structural and functional organization.

A crude cell-free extract of *B. subtilis* OKB 105 was fractionated by gel filtration on Ultrogel AcA 34. The eluate was screened for the activation of the constituent amino acids of surfactin, measuring substrate-dependent ATP/PPi exchange reactions and acid-stable incorporation of these substrates into surfactin synthetase. In addition to the multi-enzyme fraction *E*<sub>0</sub>, which activates all four amino acid components of surfactin as reported previously (11), another enzyme (*E*<sub>3</sub>), which activates leucine, was observed in close relationship to *E*<sub>0</sub>. The fractions containing *E*<sub>0</sub> and *E*<sub>3</sub> were tested for racemization of leucine with a technique similar to that used by several authors for the assay of phenylalanine racemase in the biosynthesis of gramicidin S (18-19). *De novo* synthesis could not be detected. The multi-enzyme fraction *E*<sub>0</sub> alone as well as in combination with *E*<sub>3</sub> does not show efficient lipo-peptide formation (Table I). Therefore, we assumed that additional enzyme activities must be involved in this process. We considered that a thioesterase/acyltransferase-like enzyme may accomplish the transfer of the hydroxy fatty acid substrate to the peptide-forming part of surfactin synthetase. To prove this hypothesis, we screened the fractions obtained from the gel filtration experiment for an enzyme activity catalyzing the hydrolysis of the β-hydroxytetradecanoic acid coenzyme A thioester substrate using a technique similar to that applied for the assay of medium-chain thioesterases (19). Indeed, such an activity eluted from the AcA 34 column at ~40 kDa. DTTNB was used as an indicator for the detection of the free sulfhydryl group of coenzyme A that appeared in the hydrolysis process. By complementation of *E*<sub>0</sub> with the enzyme fractions *E*<sub>0</sub> and *E*<sub>3</sub>, efficient surfactin formation was obtained, as demonstrated in Table I. Usually, rates on the order of 10-100 pmol of surfactin per mg of protein and per min were obtained. In this manner, the multi-enzyme system for the biosynthesis of surfactin could be reconstituted from these partially purified enzyme components. Each of them could then be specifically detected using the assay of *in vitro* synthesis of surfactin by complementation with the other two enzyme fractions, as shown in Fig. 1.

**TABLE I**  
*Synthesis of surfactin by complementation of the three component enzyme fractions E<sub>0</sub>, E<sub>3</sub>, and E<sub>0</sub> with surfactin synthetase obtained by AcA 34 gel filtration*  

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Surfactin (cpm)</th>
<th>108</th>
<th>195</th>
<th>195</th>
<th>3,430</th>
<th>468</th>
<th>19,230</th>
</tr>
</thead>
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<tr>
<td><em>E</em>&lt;sub&gt;0&lt;/sub&gt;</td>
<td></td>
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<tr>
<td><em>E</em>&lt;sub&gt;3&lt;/sub&gt;</td>
<td></td>
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</tr>
<tr>
<td><em>E</em>&lt;sub&gt;0&lt;/sub&gt; + <em>E</em>&lt;sub&gt;3&lt;/sub&gt;</td>
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</tr>
<tr>
<td><em>E</em>&lt;sub&gt;0&lt;/sub&gt; + <em>E</em>&lt;sub&gt;3&lt;/sub&gt; + <em>E</em>&lt;sub&gt;0&lt;/sub&gt;</td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Fig. 1.** Gel filtration of crude extract of *B. subtilis* OKB 105 on Ultrogel AcA 34. Three enzyme fractions of surfactin synthetase (*E*<sub>0</sub> (×), *E*<sub>3</sub> (●), and *E*<sub>0</sub> (▲)) were detected by the *in vitro* assay for surfactin formation by complementation with the other two fractions of this multi-enzyme. The column was eluted with buffer A without dithioerythritol. Flow rate was 18 ml/h.
higher mobilities than bands A and B and may be attributed to partially degraded proteins.

Separation of Multienzyme Fraction $E_1$ into Its Protein Components by FPLC on Mono Q—The protein species $E_{1a}$–$E_{1d}$ shown in Fig. 2b can be separated by high-resolution anion-exchange FPLC on Pharmacia Mono Q. $E_{1a}$ appears at the highest ionic strength. The other proteins are eluted at slightly lower NaCl concentrations in the order $E_{1b}$, $E_{1c}$, and $E_{1d}$. In Fig. 3a, $E_{1a}$ and $E_{1b}$ were localized in the elution pattern of the Mono Q column by the thioester formation assay with L-[14C]Leu. It is demonstrated that surfactin formation was detected in both fractions if aliquots were incubated in assay mixtures containing the complementary multienzyme in the presence of $E_2$ and $E_3$.

From Table III, it is apparent that the protein $E_{1a}$, which is eluted at the higher ionic strength, shows thioester formation with L-Leu and L-Glu in a molar ratio of ~2:1. Efficient incorporation of L-[14C]Glu was only achieved in the presence of the acyltransferase enzyme $E_3$ and the fatty acid substrate βHA-CoA. Enzyme $E_{1b}$, which appeared at slightly lower NaCl concentrations, binds L-Asp, L-Val, and L-Leu in a molar ratio of ~1:1:1. $E_{1a}$ shows minor binding effects for L-Val and L-Asp. Presumably, these amino acids are bound to the reaction centers of this multienzyme to some extent because they are structurally related to L-Leu and L-Glu, respectively. In a similar manner, a low amount of L-Glu as a substrate analog of L-Asp may be incorporated into $E_{1b}$.

By SDS-polyacrylamide gel electrophoresis, it has been demonstrated in Fig. 4 that $E_{1a}$ usually appears from the Mono Q column in pure form, whereas $E_{1b}$ may overlap with $E_{1a}$ and/or $E_{1c}$, leading to a shift of the maximum of the leucine incorporation by one fraction in relation to the other thioester formation reactions of $E_{1b}$. However, after higher purification of this multienzyme by a second FPLC run on Mono Q, all activities of $E_{1b}$ were found at the same position, as demonstrated in Fig. 3b. From the rechromatography experiment, it is again apparent that, in particular, the thioester formation of $E_{1b}$ with L-aspartic acid is decreased by prolonged exposure of the enzyme to the gradient material (NaCl). Using SDS-polyacrylamide gel electrophoresis, molecular masses of 460 and 435 kDa were estimated for $E_{1a}$ and $E_{1b}$, respectively.

The multienzyme fraction $E_1$ obtained from the sucrose gradient centrifugation step was separated into the proteins $E_{1a}$ and $E_{1b}$ by preparative SDS-polyacrylamide gel electrophoresis and blotted on polyvinylidene difluoride membranes for NH$_2$-terminal sequencing by Edman degradation. The following sequence was found for $E_{1a}$: Met-Glu-Ile-X-Phe-Tyr-Pro-Leu... ($X$ = amino acid that could not be determined). For $E_{1b}$, NH$_2$-terminal sequences could not be obtained yet.

The leucine-activating enzyme $E_2$ of surfactin synthetase was efficiently purified using the same procedure applied for...
the multienzyme fraction $E_1$, $E_2$, and $E_3$ appeared from the Mono Q column at a lower salt concentration than the components of $E_4$ (Fig. 3a). Additional purification steps are needed to obtain electrophoretically homogeneous preparations of this protein. $E_2$ activates L-leucine as adenylate and thioester, but not the other amino acid substrates of surfactin synthetase. Minor effects have been observed with the substrate analog L-valine partially replacing leucine at its reaction center. $E_5$ does not catalyze racemization of leucine. Obviously, it is not a racemizing enzyme. From gel filtration, a molecular mass of $\sim 160$ kDa was detected for this protein.

**Analysis of Initiation Step of Surfactin Formation**—The acyltransferase enzyme $E_3$ of surfactin synthetase was partially purified after the initial gel filtration step by anion-exchange chromatography on Q-Sepharose fast flow, adsorption chromatography on hydroxyapatite, and finally by high-resolution anion-exchange FPLC on Pharmacia Mono Q. It was assayed by monitoring surfactin formation after complementation with enzymes $E_1$ and $E_2$ and by hydrolysis of the $\beta$-hydroxytetradecanoic acid coenzyme A thioester, which is used as fatty acid substrate in the biosynthesis of surfactin. Using the DTNB assay, the following rates of hydrolysis were obtained for its D-, L-, and DL-forms: 12, 117, and 34 nmol/h, respectively. Obviously, there is a preference for the hydrolysis of the L-form.

The transfer of the $\beta$-hydroxy fatty acid substrate of surfactin from the acyltransferase to glutamic acid bound to the multienzyme $E_1$ in activated form was studied in a crude cell-free extract of *B. subtilis* OKB 105. The results are shown in Table IV. The covalent incorporation of L-$[^{14}C]$glutamic acid into surfactin synthetase was taken as an indicator for the action of the acyltransferase. The complex formation of the multienzyme with L-$[^{14}C]$Glu was not affected by the other amino acid substrates whether they were added to the reaction mixture separately or whether they were applied in combinations. In the presence of the $\beta$-hydroxytetradecanoyl coenzyme A thioester, which is used as fatty acid substrates in the biosynthesis of surfactin. The multienzyme fraction $E_1$, $E_2$, and $E_3$ were separated in the final FPLC step on Pharmacia Mono Q. The covalent incorporation of amino acid substrates into both proteins was measured using reaction mixtures as described (11).

**Table III**

Covalent incorporation of substrate amino acids into the multifunctional enzymes $E_{1A}$ and $E_{1B}$

<table>
<thead>
<tr>
<th>Substrate amino acid</th>
<th>Covalent incorporation into $E_{1A}$</th>
<th>Covalent incorporation into $E_{1B}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-$[^{14}C]$Leucine</td>
<td>9.2</td>
<td>7.6</td>
</tr>
<tr>
<td>L-$[^{14}C]$Valine</td>
<td>1.4</td>
<td>9.3</td>
</tr>
<tr>
<td>L-$[^{14}C]$Aspartic acid</td>
<td>0.9</td>
<td>7.3</td>
</tr>
<tr>
<td>L-$[^{14}C]$Glutamic acid</td>
<td>4.1</td>
<td>1.2</td>
</tr>
</tbody>
</table>

**Fig. 4. SDS-polyacrylamide gel electrophoresis of multienzyme fraction $E_1$ (5% polyacrylamide gels).** Lanes 1 and 3, samples of $E_{1A}$ (460 kDa) and $E_{1B}$ (455 kDa) after separation by FPLC on Mono Q; lane 2, gramicidin-S synthetase 2 as marker protein (530 kDa); lane 4, marker proteins (myosin (subunit, 200 kDa), $\alpha$-galactosidase (subunit, 115 kDa), phosphorylase (94 kDa), and bovine serum albumin (67 kDa)); lanes 5 and 6, samples of $E_{1A}$ and $E_{1B}$ after rechromatography on Mono Q.
formation with L-[14C]Glu in the presence of βHA-CoA to identify the acylated initiation product in the biosynthesis of surfactin. After precipitation with trichloroacetic acid and washing the protein pellet, the bound intermediate was eluted from the enzyme by treatment with performic acid and analyzed by thin-layer chromatography, as shown in Fig. 5. The activation of the amino acid substrates of surfactin synthetase is accomplished in a two-step mechanism involving aminoacyladylates and thioester formation. The biosynthesis of surfactin is initiated by the transfer of the amino acid-activating domains. Obviously, surfactin synthetase is equipped with 4-phospho- acylglutamate formation, which is the first intermediate of surfactin biosynthesis. The entire reaction sequence. In the absence of the other enzyme components of surfactin synthetase, the hydroxy fatty acid coenzyme A thioester substrate is hydrolyzed by the acyltransferase. Analysis of the natural lipopeptide product has shown that the lipid moiety of surfactin is a mixture of several β-hydroxy fatty acids with chain lengths of 13–15 carbon atoms. Obviously, the transpeptidase enzyme shows a broad specificity concerning its fatty acid substrate. In our studies, we used the β-hydroxy-n-tetradecanoyl glutamate in the biosynthesis of surfactin (11). These differences in the rates of hydrolysis of both species imply a possible proofreading function of the acyltransferase enzyme. From our preliminary experiments, we imply that the conversion of L-leucine into its D-form presumably does not take place in the process of leucine activation. This is based on the following observations. We use L-Leu as substrate in the in vitro cell-free extracts of B. subtilis ATCC 21332 and OKB 105. In this paper, we fractionated the proteins in the crude extracts by gel permeation chromatography according to their molecular size and demonstrated that at least four enzymes are involved in the biosynthesis of surfactin. Apparently, the whole multienzyme system is composed of a multienzyme fraction (E1) that activates all amino acid components of this lipopeptide, a leucine-activating enzyme (E2), and an acyltransferase (E3). Combining these enzyme fractions, the entire multienzyme system for surfactin formation could be reconstituted. The relatively small effect observed for the combination of fractions E1 and E3 presumably depends on the presence of certain amounts of the acyltransferase E2 in the E3 fraction.

E1 has been highly purified to almost complete electrophoretic homogeneity. The E1 complex was separated by high-resolution anion-exchange FPLC into two multifunctional polypeptide chains (E1A and E1B), each of them bearing three amino acid-activating domains. Obviously, E1A binds L-Glu and L-Leu in a molar ratio of 1:2, whereas E1B incorporates L-Val, L-Asp, and L-Leu in a ratio of 1:1:1. Occasionally, an additional minor protein pair (E1C and E1D) appeared in variable extent, showing slightly lower molecular masses but similar relationships concerning the electrophoretic mobilities and the pattern of substrate amino acid activation as those of the dominant pair (E1A and E1B). Presumably, E1C and E1D represent partially degraded forms of the two main proteins.

The activation of the amino acid substrates of surfactin synthetase is accomplished in a two-step mechanism involving aminoacyladylates and thioester formation. The multienzyme fraction E1 contains pantetheine as a carrier for the assembly of the growing lipopeptide chain. These characteristics indicate that surfactin is formed according to the thioester mechanism (27–30) that is frequently used in nonribosomal peptide synthesis.

Biosynthesis of surfactin is initiated by the transfer of the β-hydroxy fatty acid substrate from the acyltransferase E2 to the multifunctional polypeptide E1A, followed by β-hydroxyacylglutamate formation, which is the first intermediate of the entire reaction sequence. In the absence of the other enzyme components of surfactin synthetase, the hydroxy fatty acid coenzyme A thioester substrate is hydrolyzed by the acyltransferase. Analysis of the natural lipopeptide product has shown that the lipid moiety of surfactin is a mixture of several β-hydroxy fatty acids with chain lengths of 13–15 carbon atoms. Obviously, the transpeptidase enzyme shows a broad specificity concerning its fatty acid substrate. In our studies, we used the β-hydroxy-n-tetradecanoyl glutamate in the biosynthesis of surfactin (11). These differences in the rates of hydrolysis of both species imply a possible proofreading function of the acyltransferase enzyme. From our preliminary experiments, we imply that the conversion of L-leucine into its D-form presumably does not take place in the process of leucine activation. This is based on the following observations. We use L-Leu as substrate in the in vitro...
assay for surfactin formation. If L-leucine was exclusively applied in a thioester reaction mixture, the same enantiomer was detected after cleavage of the thioester complex of $E_i$ and [14C]leucine with 0.1 N NaOH or by treatment with performic acid and derivatization of the decomposition product with N$^\alpha$-(5-fluoro-2,4-dinitrophenyl)-L-alaninamide (Marfey’s reagent) (31), followed by high pressure liquid chromatography analysis. Therefore, we assume that the conversion of L-Leu into its D-form occurs in the elongation cycle at the stage of the lipotri- and hexapeptide. This is different from the reaction pattern observed in the biosynthesis of gramicidin S and tyrocidine. Here, the light enzymes gramicidin-S synthetase 1 and tyrocidine synthetase 1 accept both L- and D-Phe as substrates and racemize this amino acid in thioester-bound form. (18, 32, 33). Further studies are needed to clarify the mechanism of D-Leu formation in the whole process of the biosynthesis of surfactin. Recently, important progress has been achieved concerning the analysis of the gene structures coding surfactin synthetase and the regulation of surfactin production. Zuber and co-workers (34–36) have identified and characterized three gene loci in the genome of B. subtilis OKB 105 that are involved in surfactin formation. By transposon Tn917 insertion, they detected the srfA locus (36), which shows an operon structure comprising at least 25 kilobases that is divided into several open reading frames. The NH$_2$-terminal sequence that we have obtained for the multifunctional polypeptide E$_{srf}$ corresponds to the beginning of the first open reading frame of the srfA operon defining the start point for the structural genes coding the surfactin multienzyme system (36).

Research is in progress to investigate the sequence of the intermediate steps in the biosynthesis of surfactin by careful analysis of the elongation and initiation processes.

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REFERENCES

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