Substrate Specificity of Hybrid Modules from Peptide Synthetases*

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Homologous modules from two different peptide synthetases were analyzed for functionally equivalent regions. Hybrids between the coding regions of the phenylalanine-activating module of tyrocidine synthetase and the valine-activating module of surfactin synthetase were constructed by combining the two reading frames at various highly conserved consensus sequences. The resulting DNA fragments were expressed in Escherichia coli as C-terminal fusions to the gene encoding for the maltose-binding protein. The fusion proteins were purified, and the amino acid specificities, the acceptance of different nucleotide analogues, and the substrate binding affinities were analyzed. We found evidence for a large N-terminal domain and a short C-terminal domain of about 19 kDa within the two modules, which are separated by the sequence motif GELCIGG. The two domains could be reciprocally transferred between the two modules, and the constructed hybrid proteins showed amino acid adenylating activity. Hybrid proteins fused at various consensus motifs within the two domains were inactive, indicating that the domains may fold independently and represent complex functional units. The N-terminal domain was found to be responsible for the amino acid specificity of the modules, and it is also involved in the recognition of the ribosyl and the phosphate moieties of the nucleotide substrate. For tyrocidine synthetase I, we could confine the sites for amino acid specificity to a region of 330 residues. The C-terminal domain is essential for the enzymatic activity and has a strong impact on the specific activity of the modules.

Tyrocidine synthetase and surfactin synthetase are multifunctional peptide synthetases produced by Bacillus brevis and Bacillus subtilis, respectively (1–5). The enzymes belong to a superfamily of adenylate-forming enzymes (6–8). Small peptides like tyrocidine A and surfactin are formed by a non-ribosomal pathway according to the thio-template mechanism (6, 9). Prior to incorporation, amino acids and related compounds are activated as adenylates by cleavage of ATP and release of pyrophosphate. Peptide synthetases exhibit a modular structure with several linked modules of about 100 kDa (1, 10–16). Each module is responsible for recognition, activation, and incorporation of a specific amino acid constituent into the peptide product. Various modules of peptide synthetases have been sequenced, and several highly conserved motifs were found (7, 8, 11, 14, 17–19). Mutagenesis and cross-linking experiments gave evidence for the involvement of most of these motifs in the binding of ATP and the adenylate forming activity (20–23). A serine residue in the highly conserved sequence motif LG(G/H/D)S at the C terminus of the modules was clearly identified as the site for covalent attachment of a phosphopantetheine cofactor (24–26). The deletion of the cofactor attachment site in the phenylalanine-activating modules of tyrocidine synthetase I and gramicidin S synthetase I did not affect the amino acid adenylate forming activity (27, 28). The regions responsible for the amino acid substrate specificity have not been analyzed so far, and they are supposed to be located within the variable regions of amino acid adenylating modules.

The modular structure of peptide synthetases implicates genetic approaches to generate optimized peptide antibiotics. Altered peptides have been produced by recombinant peptide synthetases after the exchange of large regions containing complete amino acid adenylating modules (29). However, nothing is known about the structure or conformation of peptide synthetases and about interactions between specific modules. The closer confinement of active sites and the identification of residues involved in the substrate specificity could be a prerequisite for the construction of recombinant peptide synthetases with no or only little interference with the functional conformation of the altered protein.

We report a genetic approach to further confine the putative substrate binding pockets within peptide synthetase modules. We have chosen tyrocidine synthetase I with specificity for phenylalanine (2, 4) and the valine-activating module of surfactin synthetase as models (15, 30). Both modules are well characterized and can be overexpressed and isolated as active proteins from the heterologous host Escherichia coli (2, 24, 27). Variable regions of the two modules with different substrate specificities were combined reciprocally by genetic recombination. The fusion sites were located within the conserved motifs and were created by introduction of unique restriction sites after silent mutations. With this strategy, the sequence and the distance of the conserved motifs to each other remain unaltered, and variable regions are transferred as complete units to the constructed hybrids. Putative substrate binding pockets located within the variable regions could be transferred to the hybrid protein. We could confine the substrate recognition sites to the N-terminal part of the modules and give first evidence for the presence of two independently transferrable domains in amino acid adenylating units of peptide synthetases.

EXPERIMENTAL PROCEDURES

Strains, Plasmids, and Media—The E. coli strain XL1-Blue (31) was used as host for plasmids and for isolation of proteins. Cells were routinely grown in Luria broth at 37 °C. For the isolation of proteins, 500 ml of LB were inoculated 1:100 from a fresh overnight culture, induced with 0.5 mM isopropyl-1-thio-β-D-galactopyranoside at an A600 of 0.5 and harvested after another 3 h of incubation. The cell pellet was...
immediately used for enzyme purification or stored at −40 °C. For the expression of proteins, the corresponding DNA fragments were cloned into the expression vectors pMalC2 (New England Biolabs) or pQE30 (Qiagen).

**DNA Manipulations and PCR**—DNA techniques like restriction, ligation, DNA isolation, and transformations were performed as described (32). For PCR we routinely used Vent DNA polymerase (New England Biolabs) with a denaturation step at 94 °C for 30 s, an annealing step at 42 °C for 1 min, and a polymerization step at 72 °C for 1 min. 20 cycles were performed with a final polymerization step of 5 min at 72 °C. PCR products were purified with the Jet pure kit (Genomed). Oligonucleotides—Oligonucleotides were purchased from TIB MOLBIOL. The following primers were used for construction of the truncated or hybrid modules (f, forward primer; r, reverse primer; T, template plasmid pG12 (2); S, template plasmid pMALD (30)); His-Srf-2, 1Sf (CCGAATTCATGAGCAAAAAATCGATTCAAAAG) and 3Sf (2Sf = 2Sf); His-Srf-2proteinelutedfromtheheparinwithanNaClgradientat180

**Protein Determination and Electrophoretic Techniques**—Protein concentration was routinely determined using the Bradford reagent (33) with bovine serum albumin as a standard. SDS-PAGE was performed in 10% polyacrylamide gels according to the method of Laemmli (34).

**Activity Assay**—Enzymes were tested by the ATP/PPi exchange reaction essentially as described previously (35). In the standard reaction, compounds were added at the following final concentrations: amino acid, 2 mM; MgCl2, 2.5 mM; ATP, 0.5 mM; PPi, 0.1 mM; MES/HEPES buffer, 10 mM, pH 6.5. 32P-Labeled PPi, was added at a total count rate of 0.5 µCi. Unless otherwise stated, the enzymes were incubated for 15 min at 31 °C.

**RESULTS**

**Expression of Terminal Deleted Modules from Tyrocidine Synthetase and Surfactin Synthetase in E. coli and Characterization of the Purified Proteins**—The coding regions for tyrocidine synthetase I and the first module of surfactin synthetase II were truncated by terminal deletions to confine the minimal sizes responsible for amino acid adenylate forming activity. Several deletions were constructed by PCR or digestion with restriction enzymes (Fig. 1). In construct Tys-2, we deleted 31 amino acids from the C-terminal end to amino acid position 535 relative to the wild type tyrocidine synthetase I (27). Further removal of 53 amino acids including the core motif VI from the C-terminal end results in an inactive protein (Fig. 1). In construct Tys-2, we deleted 31 amino acids from the N-terminal end of construct Tys-1 (Fig. 1). We could not detect any differences in substrate specificity between the two proteins. However, the specificity of Tys-2 was reduced to about 11% when compared to Tys-1 (Table II). This gave evidence that the N-terminal end of the module Tys-1 contributes to an efficient conformation of the protein. With the construct Tys-2, the site of substrate specificity of tyrocidine synthetase I could be confined to a peptide with 504 amino acid residues.

The valine-activating module of surfactin synthetase II was reduced in the construct Srf-2 to an active protein with 975 amino acid residues.
amino acid residues (Fig. 1). The C-terminal end of the protein Srf-2 corresponds exactly to the C-terminal end of the proteins Tys-1 and Tys-2. A N-terminal deletion of protein Srf-2 was constructed, which corresponds to the N terminus of protein Tys-2. The resulting protein Srf-3 contains 506 amino acid residues (Fig. 1) and was completely inactive when valine was provided as a substrate. Therefore, in contrast to Tys-2, residues further located to the N terminus may be essential for the amino acid adenylate formation of the first module of surfactin synthetase II.

**Enzymatic Characterization of the Heterologously Expressed Module Srf-2**—The protein Srf-2 was expressed as N-terminal fusion to the 42 kDa E. coli maltose-binding protein. To determine whether this N-terminal extension has any effects on the activity of the C-terminal amino acid adenylate-forming module, we expressed the protein Srf-2 also with a short N-terminal fusion of 12 amino acids, including a (His)6 tag. The expressed protein was designated His-Srf-2. The proteins Srf-2 and His-Srf-2 were purified and compared with regard to their activities. The two proteins accepted the amino acids valine and isoleucine at a ratio of about 10:1.4 in the amino acid adenylation reaction with their cognate amino acids as substrates. When ATP was replaced by dATP, we found with the module Tys-1 a reduction in the adenylation of phenylalanine to a value of about 42% (Table II). The valine-activating module Srf-2 accepted dATP at a higher ratio of about 79% relative to ATP. With the nucleotide analogue AMP-PNP, the relative activity of module Tys-1 was 66% in contrast to about 45% with module Srf-2. The analogue AMP-PNP was not accepted by the two modules. It was obviously not bound by module Tys-1, as no effects on the activity were detected when AMP-PNP was provided in combination with ATP (Table III). However, the activity of module Srf-2 was reduced to about 80%, indicating a competition with ATP. These results demonstrate that in addition to their amino acid substrate specificities, the two peptide synthetase modules are also different in their acceptance of ATP analogues.

The two hybrid modules TS-V.1 and ST-V were also tested for their nucleotide specificities. With phenylalanine as amino acid substrate, module TS-V.1 showed with dATP an activity of 60% and with ATP;S an activity of 62% in the adenylation reaction when compared to ATP. For the analogue ATP;S, the result resembles closely the specificities of the module Tys-1. The acceptance for dATP was enhanced but the nucleotide was still accepted at a lower extent when compared to the module Srf-2. On the other hand, the values obtained with module ST-V were similar to the values obtained with module Srf-2. Module ST-V adenylates valine with dATP at about 82% and with ATP;S at about 29% when compared to the adenylation with ATP (Table III). Our results indicate that in addition to the amino acid specificity, the regions responsible for the discrimination between ATP, dATP and ATP;S are also located in the N-terminal end of the two peptide synthetase modules.

**Catalytic Efficiency and Substrate Affinity of the Hybrid Modules**—We compared the specific activities of the hybrid modules TS-V.1, TS-V.2, and ST-V with the activities of the corresponding modules Srf-2, Tys-1, and Tys-2, respectively. The activity of module ST-V was reduced to about 0.5% when
Activity with ATP. ND, not determined.

The restriction sites were introduced into the codons of the underlined residues are affected by the introduction of a restriction site. X, variable position.

Relevant amino acid sequence of selected core regions in one-letter code, the codons of the underlined residues are affected by the introduction of the restriction site.

c The restriction sites were introduced into the codons of the underlined amino acids by PCR.

### Table II

| Module    | Fusion site | Phe | Val/Ile | Specific activity |
|-----------|-------------|-----|---------|------------------|
| Srf-1     | None        | −   | +       | 14 ± 7           |
| Srf-2     | None        | −   | +       | 12 ± 6           |
| Srf-3     | None        | −   | −       | −                |
| ST-H      | Motif II    | −   | +       | 18 ± 6           |
| ST-V      | Motif V     | −   | +       | 8.00 ± 0.72 × 10^2 |
| Tys-1     | None        | −   | −       | −                |
| Tys-2     | None        | +   | −       | −                |
| Tys-3     | None        | −   | −       | −                |
| TS-III    | Motif III   | −   | −       | −                |
| TS-V      | Motif IV    | −   | −       | −                |
| TS-V.1    | Motif V     | +   | −       | −                |
| TS-V.2    | Motif V     | +   | −       | −                |
| TS-VI     | Motif VI    | −   | −       | −                |


c Core regions used for constructing hybrid genes.

b Ratio of isoleucine to valine in percent.

c Determined with valine or phenylalanine.

### Table III

| Module | dATP | ATP+SP | AMP-PNP | AMP-PNP + ATP |
|--------|------|--------|---------|---------------|
| Tys-1  | 48 ± 5 | 66 ± 5 | 0 | 100 |
| TS-V.1 | 60 ± 9 | 62 ± 6 | ND | ND |
| Srf-2  | 79 ± 10 | 45 ± 7 | 0.5 | 80 |
| ST-V   | 82 ± 10 | 29 ± 6 | ND | ND |

compared with Srf-2 and with valine and ATP as substrates (Table II). In contrast, the hybrid module TS-V.1 had a 4-fold higher activity than module Tys-1. This indicates that residues C-terminal to motif V contribute considerably to the catalytic effectiveness of amino acid adenylating modules and might be involved in the modulation of the velocity of the enzymatic reaction. The truncation of 31 amino acid residues from the N-terminal end of module TS-V.1 had a strong impact on the specific activity of the resulting module TS-V.2. The activity was reduced to about 0.5%, and the module TS-V.2 was even less active than the module Tys-2 with the identical N-terminal deletion. This effect might indicate cooperative interactions between the N terminus of the Tys region and the C terminus of the Srf region in the hybrid module TS-V.1, which could explain the observed high activity. The relative low specific activity of the hybrid module ST-V might therefore be contributed to the failure of the large N-terminal extension of the Srf region to interact with the C-terminal Tys region.

The binding affinities for the cognate amino acid substrates and for ATP were further analyzed. We determined the kinetic constants (K_{m(apparent)} values from Lineweaver-Burk plots in dependence on the concentration of both reaction partners. Concentrations were between 0.05 mM and 0.5 mM for ATP and between 0.1 mM and 1 mM for the amino acid substrate. The tested modules showed only minor variations in the substrate binding affinities. Modules ST-V and Srf-2 had similar K_{m(apparent)} values for valine of about 1.25 and 1.7 mM. Additionally, the binding affinities for ATP were similar with 0.8 mM for module ST-V and 0.4 mM for module Srf-2. The substrate binding activities for the two modules Tys-1 and TS-V.1 were also in comparable ranges. The K_{m(apparent)} values for phenylalanine were 0.5 mM for module TS-V.1 and 0.3 mM for module Tys-1. The affinities for ATP were also similar and were estimated at 0.8 mM for module TS-V.1 and at 1 mM for module Tys-1. The results indicated that substrate binding in the constructed hybrids might be similar compared to the modules Srf-2 and Tys-1. Thus, the observed differences in the specific activities of the two hybrid modules do obviously not result from major alterations in the substrate binding affinities.

### DISCUSSION

We constructed hybrid modules from amino acid adenylating modules with different substrate specificities and derived from different peptide synthetases. Highly homologous regions within the modules were used as specific sites for the construction of gene fusions by in vitro recombination. We have shown that this approach might be useful in the localization and
analysis of active sites in isolated modules of multifunctional enzymes. The phenylalanine adenylating activity was previously confined by C-terminal deletions to the first 535 amino acid residues of the wild type 1077 residue subunit (27). This truncated module corresponds to our construct Tys-1. A further C-terminal deletion of 55 residues including motif VI results in the completely inactive module Tys-3. These findings agree with previous reports where residues located within this deletion have been identified to possibly interact with the adenine moiety of ATP (22) and might therefore be essential for the adenylating reaction.

The N-terminal deletion of 31 amino acid residues in construct Tys-2 results in a reduction of the specific activity to about 11%. However, the deletion does not affect the substrate specificity, and the substrate binding sites of tyrocidine synthetase I should therefore be located between the amino acid positions 32 and 535. This size of 504 amino acid residues might also come close to the limits by which the amino acid adenylating activity of tyrocidine synthetase I could be confined by terminal deletions. We were able to further confine the sites responsible for substrate specificity only by the construction of hybrid modules. The modules Srf-2 and Tys-1 carried corresponding truncations at the C-terminal ends including the motif VII. The deletion did not affect the amino acid adenylating activity as previously reported for tyrocidine synthetase I (27) and gramicidin S synthetase I (28). When aligned to Tys-1, module Srf-2 shows an N-terminal extension of about 600 amino acid residues with the conserved motif I (8). The extension might be involved in the elongation of the growing surfactin peptide, but detailed knowledge about its significance and function is not yet available. Our construct Srf-3 carries a truncation of this extension to a position corresponding to the N terminus of module Tys-2 and lacks any valine adenylating activity. However, fusions at corresponding sites from five different modules to the N terminus of the seventh module of surfactin synthetase were shown to retain their specificities and resulted in proteins with amino acid adenylating activity (29). Residues further N-terminal to that position seem therefore not to affect the substrate discrimination. We assume that the inability to fold into a functional conformation caused the inactivity of our construct Srf-3 rather than the deletion of residues involved in substrate binding.

By analyzing hybrid modules, we could confine the regions responsible for discrimination the amino acid substrates in both modules N-terminal to motif V. For tyrocidine synthetase I, the amino acid specificity is determined by residues within the amino acid positions 32 and 362. However, the involvement of residues C-terminal to motif V in the recognition of common substrate moieties like the carbonyl or amino groups cannot be excluded. The formation of binding pockets seems to be unaffected since the binding affinities for the cognate amino acid substrates are only slightly modified in the hybrid modules ST-V and TS-V.1.

The acceptance of the ATP analogues dATP and ATPγS was also determined from regions N-terminal to motif V, indicating that the recognition of the ribosyl and the phosphate moieties of ATP might also occur in these parts of the enzymes. Most of the conserved sequence motifs in peptide synthetases seem to be involved in ATP binding and cleavage (8). The sequence of motif III occurs in all peptide synthetase modules and related carbonic acid-activating enzymes (8, 22). It is analogous to glycine-rich P-loops described for other nucleotide-binding proteins (36–39). Substitution of the conserved lysine residue with threonine by site-directed mutagenesis of motif III in tyrocidine synthetase I results in the loss of amino acid adenylating activity (20). The lysine is supposed to be involved in the binding of phosphate moieties from ATP. This agrees with our findings where the selectivity for the sulfur-substituted γ-phosphate group is determined by a region containing motif III. The involvement of further residues located C-terminal in the binding of nucleotides was shown by photoaffinity labeling and site-specific mutagenesis (20–23). Three regions C-terminal to motif V spanning amino acid positions Gly373–Trp383, Leu483–Lys494, and Trp455–Arg458 respectively to the N-terminal end of the module Tys-1 could be labeled with 2-azidoadenosine triphosphate. The first region is close to motif V and starts only 6 residues further C-terminal. The labeling with the ATP analogue may indicate the involvement of these regions in the recognition and binding of adenosine. The binding of the ribosyl moiety should be in close vicinity. We found that discrimination of the 2′-hydroxyl residue was determined by regions N-terminal to motif V, indicating that distantly located residues may coordinate in the three-dimensional structure of the enzymes to form the nucleotide binding site.

Despite similar affinities for substrate binding, the catalytic efficiency of the hybrid modules was considerably altered. We observed a dramatic reduction in the specific activity with module ST-V and a considerable enhancement with module TS-V.1. These differences might be explained by modifications in the three-dimensional structure of our constructs. The positioning of the two substrates relative to each other could be altered, resulting in an enhanced or retarded catalysis. Modifications in the substrate binding affinities of the hybrid modules do not seem to be responsible for the modulation of the catalytic efficiency.

Peptide synthetases are members of a superfamily of adenylate-forming enzymes. The common reaction of all members is the ATP-dependent activation of carboxy group substrates as acyladenylates (6). The conserved sequence motifs described for modules of peptide synthetases are also present in other members of this superfamily (17, 18, 40–42). The recently solved structure of firefly luciferase represents the first three-dimensional model of a member of the adenylate-forming superfamily (43). Luciferase is folded in a large N-terminal domain of about 440 residues and a small C-terminal domain of about 100 residues. The two domains are separated by a wide cleft, the proposed active site of the enzyme. A potential substrate binding site may be located between sequences corresponding to the motifs II and IV of peptide synthetases. Sequences homologous to motif V are located close to the hinge of the two domains within the large N-terminal domain in luciferase. The structure agrees with our results where peptide synthetase modules can be divided at motif V into a large N-terminal domain determining the amino acid specificity and part of the nucleotide binding site and into a smaller C-terminal domain with about 170 residues involved in adenosine binding and substrate positioning. We were unable to construct amino acid adenylating hybrids with the motifs II, III, IV, and VI as fusion sites. They may therefore be located within the two domains of the enzymes, and the construction of hybrids could have affected the functionally active conformation of these regions. The fungal peptide synthetases responsible for the biosynthesis of cyclopiazonic and enniatin are reported to modify certain amino acid substrates by methylation (11, 16). In these cases, the corresponding modules carry an insertion of about 430 amino acid residues with homologies to N-methyltransferases. The insertion was always found to be located between motifs V and VI (11, 16). This further supports our view that parts of the region between these two motifs may serve as spacer between the two postulated domains and even large inserts seem to be compatible with a functionally active conformation of the enzyme.
Acknowledgments—We thank Yon Oek Lee for providing plasmid pGC12 and are grateful to Cornelia Bartsch and Clemens Kieker for technical assistance.

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