Characterization of Tyrocidine Synthetase 1 (TY1): Requirement of Posttranslational Modification for Peptide Biosynthesis†

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ABSTRACT: Tyrocidine synthetase 1 (TY1), produced by *Bacillus brevis* ATCC 8185, consists of a single multifunctional polypeptide chain catalyzing the activation, thioesterification, and epimerization of phenylalanine. Because we were concerned about possible posttranslational issues, a comparative study between the wild-type isolate and the in *Escherichia coli* overexpressed protein was performed. Analysis by matrix assisted laser desorption mass spectrometry (MALDI) provided a molecular mass of 122 516 ± 120 Da for the recombinant protein, which is in agreement with the value of 122 590 Da calculated from the gene sequence. MALDI analysis of the tryptic fragments revealed that in the recombinant TY1 the putative 4′-phosphopantetheine binding site (^{562}Ser) is not modified by the cofactor. The substrate specificity profiles of the amino acid dependent ATP[^{32}P]PP$_i$ exchange reactions were identical, including activation of L-phenylserine, L-tyrosine, and L-methionine. However, the rates of the reverse adenylation reaction for the recombinant protein were only 22% relative to those of the wild-type enzyme. The aminoacylation levels of about 60% for TY1 from *Bacillus brevis* reduced to 1.4% in the overexpressed protein. A similar distribution of the D- and the L-isomer was detected at the thioester attachment site. The p*I* values of the wild-type and expressed TY1 are 4.9 and 5.0, respectively. In conclusion, it could be established that apo- and holo-TY1 differ in their amino acid activating properties. Posttranslational modification by 4′-phosphopantetheine is an essential requirement for aminoacylation, epimerization, and thus the functioning of the multienzyme in peptide synthesis.

Peptide synthetases constitute a family of multienzyme systems involved in the biosynthesis of a variety of bioactive natural products, some with important applications as antibiotics (gramicidin S, tyrocidine, penicillins), immunomodulators (cyclosporin), extracellular surfactants (surfactin), or siderophores (enterobactin, ferrichrome). Prokaryotes as well as lower eukaryotes, like the filamentous fungi, have been shown to contain such complex biosynthetic pathways for which the term *non-ribosomal systems* has been introduced (Kleinkauf et al., 1992). Contrary to the ribosomal pathway, the amino acid sequence in the peptide product is not determined directly at the gene level *via* nucleic acid interactions, but rather by the protein structure of the respective enzyme system.

Much of the present knowledge about the non-ribosomal biosynthesis of peptides is based on the results from experiments on peptide antibiotics from bacilli. The multienzymes involved in the biosynthesis have been partially characterized, and the mechanism has been studied in some detail (Kleinkauf & von Döhren, 1990). Generally, the initial step is the activation of amino acids or non-protein constituents, like hydroxyl and D-amino acids, as acyladenylates. Peptide synthetases, however, often do not display unique substrate specificity, as observed in aminoacyl-tRNA synthetases, allowing the synthesis of a variety of analogous peptide products. The activated amino acids bind covalently to the protein template by aminoacylating a specific site in the activation center. The chemical features of the reactive intermediates are consistent with the existence of a thioester bond (Kleinkauf et al., 1971). The alignment of available peptide synthetase sequences indicated the absence of generally conserved cysteines which might serve as aminoacylation targets. Instead, a pantetheine attachment motif, LGG(H,D)SL, shared by acyl carrier proteins (ACP) of fatty acid synthetases (Schlumbohm et al., 1991; Stein et al., 1995), has been found positionally conserved in all peptide synthetase modules, suggesting the aminoacylation site to be the cysteamine moiety of the cofactor. This motif also strongly resembles sites from polyketide-forming enzyme systems known to carry 4′-phosphopantetheine as cofactor (van Liempt et al., 1991). On the other hand, enzymes of the acyl-CoA synthetase family, such as luciferase and 4-coumaryl-CoA ligase, which are related to the peptide synthetases and transfer their active carboxyls to the cysteamine end of CoA, do not contain the respective motif (MacCabe et al., 1991; Babbit et al., 1992; Guilvout et al., 1993; Pavela-Vrancic et al., 1994a). The highly conserved serine residue has been identified as the site of covalent attachment by affinity labeling of gramicidin S synthetase 2 (GS2) with L-[^{14}C]valine and L-[^{14}C]leucine (Schlumbohm et al., 1991). Recently, the presence of 4′-phosphopantetheine has been confirmed within the active site peptide of the valine domain of GS2 by mass spectrometric analysis (Stein et al., 1994) and gramicidin S synthetase 1 (Stein et

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[] Abbreviations: ACV, δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine; ATP, adenosine triphosphate; DTE, dithioerythritol; EDTA, ethylenediaminetetraacetic acid; PMSF, phenylmethanesulfonyl fluoride; SDS, lauryl sulfate.

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al. 1994). These findings together with the thioester like properties of the aminoacyl intermediates led to the revision of the thiotemplate mechanism, as previously proposed (Kleinkauf & von Döhren, 1990), to a model involving multiple phosphopantetheine cofactors, one for each amino acid activating domain, associated with the peptide synthetase complex and unidirectional substrate transfer between adjacent modules (Schlumbohm et al., 1991; von Döhren et al., 1993). D-Amino acids in the peptide product either originate from the respective L-forms by epimerization or are directly accepted. Epimerization occurs either at the aminoacyl stage or at the peptidyl-intermediate stage (Stindl & Keller, 1993). Both types of reactions have been associated with the site of covalent attachment. The respective peptide product is released from the enzyme system either by cyclization, terminal modification, or hydrolysis; however, these terminating processes have not been investigated.

By sequence alignment it has been established that peptide synthetases are composed of repeating functional units containing highly conserved motifs within 600−700 amino acid residues (Díez et al., 1990; Smith et al., 1990; MacCabe et al., 1991; Gutiérrez et al., 1991; Turgay et al., 1992; Marahiel, 1992; Pavela-Vrancic et al., 1994a). Each of these regions contributes one aminoacyl residue to the peptide. The functional domains are arranged in order of the amino acid sequence in the peptide product and are followed by a second functional unit of about 400−500 amino acid residues presumably involved in amino acid modification and peptide bond formation. So far more than 40 peptide synthetase modules have been sequenced, and the consensus sequence, comprising a new class of sequence motifs, could be established (Pavela-Vrancic et al., 1994a). The conserved sequence motifs have been proposed to be associated with partial catalytic activities (Marahiel, 1992), including ATP binding and acyladenylate formation (Pavela-Vrancic et al., 1994b,c; Gocht & Marahiel, 1994), thioester formation (Schlumbohm et al., 1991), and possible modification reactions like epimerization (Fuma et al., 1993; Gocht, 1994) or *N*-methylation (Haese et al., 1993).

In order to elucidate the key mechanisms of operation, amino acid selection and activation, and elongation of the peptide chain, as well as residue and chain modification, structure−function studies on model systems are essential. TY1 is a representative example of a single module containing all functions associated with adenylate formation, aminoacylation, and epimerization. It is the first multifunctional component of the biosynthetic system catalyzing the formation of the cyclic decapeptide tyrocidine. This multienzyme system is composed of at least three interacting multifunctional enzymes, tyrocidine synthetase 1, 2, and 3 (TY1, TY2, and TY3). TY1 catalyzes the activation of L-phenylalanine prior to epimerization into its D-isomer according to the following scheme (eqs 1−3):

$$\text{L-Phe} + \text{ATP} + E_1 \rightleftarrows (\text{L-Phe} \sim \text{AMP})E_1 + PP_i \quad (1)$$

$$(\text{L-Phe} \sim \text{AMP})E_1\text{-}S^1H \rightleftarrows \text{L-Phe}\text{-}S^1\text{-}E_1 + \text{AMP} \quad (2)$$

$$\text{L-Phe}\text{-}S^1\text{-}E_1 \rightleftarrows \text{D-Phe}\text{-}S^1\text{-}E_1 \quad (3)$$

$$\text{D-Phe}\text{-}S^1\text{-}E_1 + \text{Pro}\text{-}S^2\text{-}E_2 \rightarrow \text{D-Phe}\text{-}\text{Pro}\text{-}S^2\text{-}E_2 + E_1\text{-}S^1H \quad (4)$$

Peptide elongation is initiated by the transfer of the aminoacyl group to TY2 (eq 4), which in turn catalyzes the incorporation of Pro, L-Phe, and D-Phe into the peptide chain. TY3 activates the remaining six constituent amino acids (Asn, Gln, Tyr, Val, Orn, Leu) with subsequent release of tyrocidine by cyclization.

Previous attempts to investigate the enzymology and mechanism of biosynthesis have been hampered by the inability to obtain sufficient material for the in vitro studies. TY1 was the first peptide synthetase to be cloned and expressed to high levels in *Escherichia coli* (Marahiel et al., 1985; 1987). With the exception of the *entF* gene product (Rusnak et al., 1991), no other peptide synthetase has been expressed and produced in high quantities for extensive enzymological studies. However, it remains to be shown that the multienzyme generated by expression of the *tycA* gene in *E. coli* can substitute for the natural enzyme. We here describe the isolation and purification of TY1 from *B. brevis* and the respective protein obtained by overexpression in *E. coli* and provide data on their physical and catalytic properties. The obtained results have shown significant differences between the investigated enzymes, substantiating the assumption that posttranslational modification is required for their proper functioning in the biosynthetic system.

MATERIALS AND METHODS

Chemicals and Materials. ATP and dithioerythritol (DTE) were purchased from Sigma, [^{32}P]PP$_i$ was a product of DuPont, and ^{14}C-labeled amino acids were purchased from Amersham/Buchler. AcA 34 was from Serva, phenyl-Sepharose CL-4B and MonoQ 5/5 were purchased from Pharmacia LKB Biotechnology Inc., and Fractogel TSK TMAE 650 in a Superformance 150-10 column was obtained from Merck.

Bacterial Strains, Growth Conditions, and Transformation. *Bacillus brevis* ATCC 8185 was maintained on 1.5% agar slants of CM medium (10 g of bactopepton, 10 g of yeast extract, 5 g of NaCl, and 0.5 g of $MgSO_4$ per 1 L of distilled water, pH 7.2). Fermentations were performed according to Fujikawa et al. (1968) in 10−80-L Biostat fermentation tanks (Braun, Germany) containing 9.5−75 L of medium.

The *E. coli* strain XL1-blue (Bullock et al., 1987) was used as a host for plasmid pGC12. The plasmid pGC12 contains a 4.6-kb *Hin*cII fragment (containing the *tycA* coding region) from pBT2 (Marahiel et al., 1985) under the control of the lac promoter from pUC18 (Czekay, 1987). The *E. coli* cells were grown in Luria−Bertani medium or on agar plates supplemented with ampicillin (50 μg/mL). Transformation into *E. coli* cells was performed according to Dagert and Ehrlich (1979).

Isolation and Purification of TY1 from B. brevis ATCC 8185. All operations were carried out at 2−4 °C. The cells were suspended in a 3-fold volume of 100 mM Tris/HCl, pH 7.4, containing 0.2 M NaCl, 5 mM DTE, 1 mM $MgAc_2$, 0.1 mM PMSF, 0.3 mg/mL lysozyme, 2 μg/mL DNAse, and 10% (v/v) glycerol. The suspension was stirred for 1 h, followed by cell disruption by sonication and ammonium sulfate (40−60%) precipitation. The protein pellet was dissolved in a minimal volume of 50 mM Tris/HCl, pH 7.4,

containing 0.2 mM NaCl, 1 mM DTE, 1 mM EDTA, and 10% glycerol (w/v), and dialyzed against the same buffer. The crude extract was applied to an Ultrogel AcA 34 (50/100) column and eluted with the above mentioned buffer. The active fractions were pooled, and NaCl was added to give a final concentration of 1 M. In the following purification step the protein solution was applied to a phenyl-Sepharose column (4/12), previously equilibrated with 1 M NaCl in 50 mM Tris/HCl, pH 7.4. The enzyme was eluted in a single step with water and applied to a Fractogel TSK TMAE-650 column previously equilibrated with 50 mM Tris/HCl, pH 7.4, and 1 mM EDTA. Elution was carried out with a linear gradient of 0−0.5 M NaCl in 50 mM Tris/HCl, pH 7.4, and 1 mM EDTA. The protein was finally purified to homogeneity by high-resolution anion-exchange chromatography on MonoQ 5/5 using a linear gradient of 0−0.4 M NaCl in the same buffer.

Isolation and Purification of Recombinant TY1 from E. coli. Purification of TY1 from transformed *E. coli* cells was achieved by using ammonium sulfate precipitation (0−60%), followed by hydrophobic interaction chromatography on phenyl-Sepharose CL-4B as previously described. The flowthrough, containing high concentrations of unbound enzyme, was reloaded on a phenyl-Sepharose column. The bound protein was eluted with water and applied to a Fractogel TSK TMAE-650 column using the same procedure as previously described.

Enzyme Assays. Adenylate formation was measured using the isotope ATP[^{32}P]PP$_i$ exchange reaction. Activity measurements were performed in an assay mixture containing, in a final volume of 70 μL, 50 mM Tris/HCl, pH 7.4, 0.15 mM ATP, 0.75 mM Mg^{2+}, 1 mM DTE, 0.1 mM EDTA, 0.1 mM PP$_i$, 0.1 μCi [^{32}P]PP$_i$, and 1 mM D-phenylalanine. The reaction was started by addition of enzyme and allowed to proceed for 15 min at 37 °C. The reaction was stopped by addition of 1% activated charcoal in 3% perchloric acid, and the [^{32}P]ATP content was determined. Kinetic measurements were performed using the ATP[^{32}P]PP$_i$ exchange reaction at varying substrate concentrations, 0.01−0.1 mM D-Phe, and 0.05−0.4 mM ATP, and the kinetic constants were estimated from Lineweaver−Burk plots.

The aminoacyl enzyme thioester test was performed according to Keller (1987), with the following modifications. The reaction mixture, containing 8 mM ATP, 40 mM Mg^{2+}, 1 mM DTE, 0.25 μCi of ^{14}C-labeled L-phenylalanine, and 100 μL of enzyme solution in a total volume of 127.5 μL, was incubated for 30 min, and the reaction was stopped by addition of 300 μL of 10% trichloroacetic acid. The precipitated protein was pelleted by centrifugation and washed three times with 10% trichloroacetic acid and once with ethanol to remove the unbound amino acid. The pellet was dried by evaporation and subsequently suspended in 100 μL of formic acid. The sample was divided into two fractions. To one fraction was added 20 μL of H_2O, and 20 μL of H_2O_2 was added to the other, producing performic acid in the latter case. Both samples were incubated at room temperature for 1 h. The solvent was evaporated, and the residue was taken up in 50 μL of ethanol and separated by thin-layer chromatography. The filter binding assays were carried out as described. The pH dependence of the aminoacylation reaction was established by measuring thioester formation, as previously described, at pH 2.8−14. The pK_a values were estimated using the Grafit enzyme kinetics program.

Thin-Layer Chromatography (TLC). Silica Gel 60 plates (Merck) and Chiralplates (Machinerey-Nagel) were used with the solvent systems $C_4H_{10}O/CH_3COOH/H_2O$ (80/20/20) and $CH_3OH/H_2O/CH_3CN$ (50/50/30), respectively.

SDS−PAGE and Western Blot Analysis. Gels were prepared according to Laemmli (1970) containing 7.5% or 10% acrylamide. Proteins were visualized by staining with Coomassie blue R-250 (Sigma). Western blot analysis and staining were performed according to Towbin (1979) and Blake (1984) using TY1 antibodies (Pfeifer, 1994).

Isoelectric Focusing. The p*I* of the enzyme was determined on Servalyt Precotes (pH 3−10) and Servalyt PreNets (pH 4−6), both from Serva. The purified enzyme preparations were previously desalted by dialysis.

Determination of Protein Content. The protein concentration was determined either by the method of Warburg and Christian (1941) or by the method of Bradford (1976) with bovine serum albumin as standard.

Tryptic Digestion. A lyophylized sample of TY1 from *E. coli* was redissolved in 8 M urea in 0.4 M ammonium bicarbonate, pH 8.3. Reduction of the sulfhydryl groups was performed by incubation with 4 mM DTE for 1 h at 37 °C, followed by alkylation with 8 mM iodoacetamide for 1 h at room temperature. The sample was diluted with water to yield a 2 M urea concentration and subjected to tryptic digestion by incubation with trypsin for 2 h at 37 °C at a trypsin to protein weight ratio of 1:20. Tryptic digestion was followed by SDS−polyacrylamide gel electrophoresis as described.

Laser Desorption Mass Spectrometry. A sample of recombinant TY1 from *E. coli* and its tryptic digest were subjected to mass spectrometric analysis with a Reflex laser desorption mass spectrometer (Bruker-Franzen Analytic GmbH, Bremen, FRG) using sinapinic acid as matrix and operating in a linear mode with 10-kV acceleration and positive ionization.

Sequence Alignment. Sequence comparisons were carried out on a Silicon Graphics Personal Iris, using the program CLUSTAL. Sequences were further aligned by hand to minimize gaps in regions of low similarity. Amino acid identities or similarities for each position were scored by a GENCONS program written for this purpose. The one-letter code is used to indicate conserved amino acids, a conserved residue being defined as one which occurs in all sequences minus one. The conservation of the similar amino acids, (L,I,V,M), (K,R,H), (D,E), (S,T), and (F,Y,W), is displayed by a lowercase letter code of the most frequently occurring residue.

RESULTS

Purification of TY1. TY1 from *B. brevis* was purified to homogeneity using a series of standard chromatographic steps as described under Materials and Methods. Two assays were employed to follow TY1 during purification: the D-phenylalanine-dependent ATP[^{32}P]PP$_i$ exchange reaction and SDS−PAGE complemented by Western blot analysis (data not shown). Gel electrophoretic analysis of the individual purification steps is shown in Figure 1A, lanes 1 and 2. The protein was subjected to N-terminal sequencing which after 16 successive steps (H_2N-MLANQANLIDNKRELE) showed

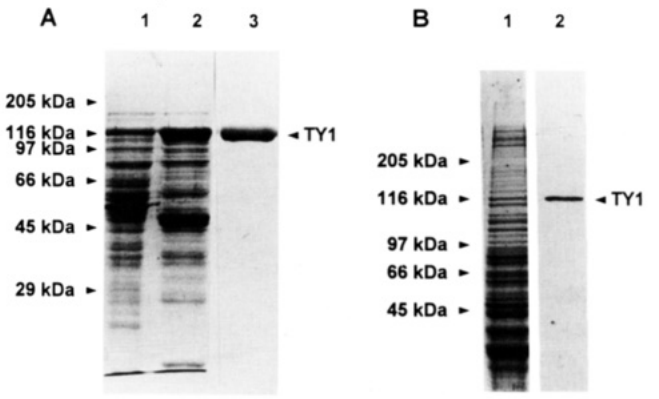

FIGURE 1: (A) SDS-PAGE analysis of TY1 overexpressed in *E. coli*: lane 1, crude extract; lane 2, after purification by chromatography on phenyl Sepharose; lane 3, after purification by ion-exchange chromatography (10% SDS−PAGE). (B) SDS−PAGE analysis of TY1 from *B. brevis*: lanes 1 and 2, after purification by gel filtration and ion-exchange chromatography, respectively (7.5% SDS−PAGE).

the protein to have complete identity to the sequence deduced from the structural gene (Weckerman et al., 1988).

E. coli cells bearing plasmid pGC12, containing the *tycA* coding region, produce large quantities of TY1 in the late stationary phase. In accordance with the results obtained by Czekay (1987) about 20% of the total cellular protein represents the *tycA* gene product. Most of the material, however, was deposited as inclusion bodies; studies were carried out with the soluble fraction (Pfeifer, 1994). Due to overproduction, several steps in the standard purification procedure could be omitted. By using hydrophobic interaction chromatography it was possible to separate the recombinant TY1 from other hydrophobic proteins, and thus obtain a flowthrough containing high concentrations of the enzyme. The protein was further purified by a successive run on a phenyl-Sepharose column, followed by ion-exchange chromatography on Fractogel TSK TMAE (Figure 1B) yielding 130 mg of soluble, homogeneous enzyme from a 10-L culture. The enzyme activity was monitored by the D-phenylalanine-dependent ATP[^{32}P]PP$_i$ exchange reaction.

Characterization of the Wild-Type and Recombinant TY1. (A) Adenylation. In order to compare the features of the adenylation site and gain further insight into the specificity of the amino acid substrate, amino acid activation and adenylate formation were tested by measuring the ATP[^{32}P]-PP$_i$ exchange reaction using a series of amino acids including D-phenylalanine and an analogue of the natural substrate, L-phenylserine. No difference in specificity was observed, as shown in Table 1. Both enzymes preferentially activate D- and L-phenylalanine; however, a higher rate is obtained with the natural substrate, L-phenylalanine. Identical binding constants for L-Phe (K_d = 0.04 mM) were obtained with both enzymes. Aromatic amino acids, like L-tyrosine and L-phenylserine, were activated to a lesser extent. Surprisingly, methionine was activated as well, although it has not been found to occur in any of the so far known peptide synthetase products. The catalytic properties of peptide synthetases with respect to the nucleotide substrate have been previously investigated by enzyme kinetic studies (Pavela-Vrancic et al., 1994a). Both enzymes show the same kinetic pattern. A significant difference in ATP recognition and binding affinity could not be established (data not shown). Further characterization of the wild-type TY1 and recombinant TY1

Table 1: Amino Acid Specificity of Tyrocidine Synthetase 1 (TY1) from *B. brevis* and the Gene Product of *tycA* Expression in *E. coli*

	activity[a] (%)	
amino acid	native TY1	recombinant TY1
L-Phenylalanine	100	100
D-Phenylalanine	68	74
L-Phenylserine	22	23
L-Methionine	12	9
L-Tyrosine	8	6

[a] Activity was calculated relative to the values obtained with L-phenylalanine.

was approached by determining the turnover number, which was calculated using the ATP[^{32}P]PP$_i$ exchange reaction from the amount of [^{32}P]PP$_i$ incorporated into ATP. The wild-type enzyme showed a 4.5-fold higher turnover rate (k_{cat} = 2.57 s^{-1}) than the enzyme produced in *E. coli* (k_{cat} = 0.57 s^{-1}).

(B) Aminoacylation. In peptide synthetases aminoacyl adenylates are cleaved by the action of an enzyme thiol, resulting in formation of a thioester bond between the enzyme and the amino acid. This reaction was monitored by measuring the incorporation of L-[^{14}C]phenylalanine into the enzyme. After a 30-min incubation at 37 °C and removal of the free amino acid, the enzyme-bound intermediate was treated with either formic or performic acid. Previously it has been shown that formic acid selectively hydrolyzes esters, contrary to performic acid, which is specific for thioesters (Gevers et al., 1969). Treatment with performic acid released phenylalanine, which could be identified by TLC on chiral plates, confirming a thioester linkage between the amino acid and TY1. The aminoacylation activity of the enzyme obtained by overexpression in *E. coli* is in marked contrast to the activity of the wild-type enzyme. The filter binding assay showed that 60% of the wild-type enzyme was charged by phenylalanine. Surprisingly, the expressed protein showed only 1.4% thioester formation, indicating incomplete processing and modification of the polypeptide chain. A symmetrical bell-shaped profile with a pH optimum of 9.2 (pK_a 7.84, pK_b 10.45) was obtained by measuring the pH dependence of the aminoacylation reaction, implying the involvement of a thiolate in ester formation. The observed differences were further substantiated by isoelectric focusing; the p*I* of the wild-type enzyme was determined to be 4.9, whereas the p*I* for the expressed protein showed a value of 5.0 (Figure 2). Several minor additional bands with slightly increasing p*I* were also detected, which all cross reacted with TY1 antibodies (result not shown).

(C) Epimerization. In order to show more conclusively that epimerization takes place at the thioester site, L-[^{14}C]-Phe was used as a substrate. The thioester bond was cleaved by treatment with performic acid, and the released amino acid was applied to a chiral plate, showing that the amino acid bound to the wild-type enzyme, as well as to the expressed protein, occurs both in the L- and D-form, at absolute D-Phe:L-Phe ratios of 63:37 and 75:25 for the wild-type and the recombinant protein, respectively.

To further characterize similarities within peptide synthetases, an alignment of sequences following the pantetheine

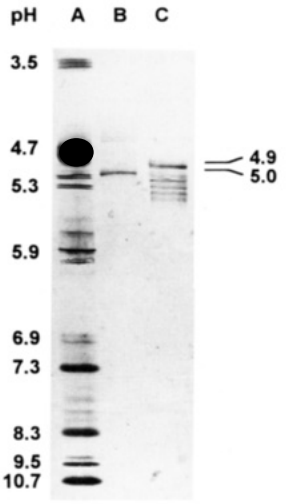

FIGURE 2: Isoelectric focusing: lane A, marker (pH 3−14); lane B, TY1 overexpressed in *E. coli*; lane C, TY1 from *B. brevis*.

binding motif has been performed. A comparison of the consensus sequences was obtained by alignment of seven modules from bacilli, not showing epimerization and cyclization activity, and ten modules comprising an epimerization function (Pfeifer, 1994). In epimerizing peptide synthetases including TY1 seven additional sequence motifs (K−Q) could be established, extending the consensus sequence beyond the cofactor attachment site. The observed differences and similarities, respectively, allowed interpretation of the data with respect to the epimerizing and peptide-bond forming activities (Table 2).

(D) Mass Spectrometric Analysis. A sample of TY1 expressed in *E. coli* was analyzed by matrix assisted laser desorption mass spectrometry. Calibration was performed with BSA (66 266 Da) and the respective dimer. The mass was determined to be 122 516 ± 120 Da (Figure 3). We failed, however, to determine the molecular mass of the wild-type enzyme from *B. brevis* for yet unknown reasons. Under the conditions employed only a broad mass peak was detected (data not shown).

The results obtained from the measurement of the turnover number and thioester formation implicated a significant disparity in the aminoacylation domains of the compared proteins. In order to assess this assumption, the recombinant protein was digested with trypsin and the digest was subjected to mass spectrometric analysis (Table 3). Almost 40% of the expected 75 fragments with mass values above 600 Da, comprising 50% of the entire protein sequence (Figure 4), could be identified, including peptide ^{553}DNFYSLGGDSIQAIQVVAR, which has been found to represent the site of 4′-phosphopantetheine binding (Schlumbohm et al., 1991; van Liempt et al., 1991; Vollenbroich et al., 1993). The molecular peak of 2054.6 corresponds to the mass value of the respective peptide unmodified by the cofactor. The mass deviation of the experimentally determined values was within 0.1% of those predicted from the sequence. In addition, two molecular peaks with mass values of 7146 and 1258 corresponding to fragments ^{306}Tyr−Arg^{372} and ^{886}Glu−Arg^{896}, respectively, were identified, confirming two of four recently detected sequence errors (Gocht, 1994); in addition, the peptide ^{756}DLATGYAQALAGQAISLPEK (*m/z* 2017) confirms one of the two proposed frameshift errors (^{756}DLATGYAQALAGQAISLPEKTDSFQSWSQWL; Bobby Baum, personal communication).

DISCUSSION

Isolation and Identification of TY1. Both native and recombinant TY 1 have been purified to apparent homogeneity, introducing hydrophobic interaction chromatography as a new effective step. Overexpression was conducted in *E. coli* with the pUC derivative pGC12 (Marahiel, 1985, 1987). By matrix assisted laser desorption mass spectrometric analysis of recombinant TY1 a mass value of 122 516 ± 120 Da was obtained, corresponding to the mass value estimated from the gene sequence (122 590 Da). However, sequence comparison with GS1 indicates two still unproven frameshifts leading, after correction, to a new mass value of 122 539 Da (Bobby Baum, personal communication), which shows an even better correlation to the measured molecular mass. This mass indicates already the lack of the expected posttranslational modification by 4′-phosphopantetheine, which would lead to an increase of 339 Da. Attempts to measure the wild-type size were so far unsuccessful.

Catalytic Properties of the Wild-Type and Recombinant Proteins. A comparative analysis of partial reactions has been carried out, especially in view that the apoenzyme would not be aminoacylated. Amino acid specificities of adenylate formation measured by the amino acid dependent ATP[^{32}P]PP$_i$ exchange reaction were identical. Both enzymes catalyzed the activation of L- and D-phenylalanine, L-phenylserine, L-tyrosine, and surprisingly L-methionine to the same extent (Table 1). However, the turnover number of the exchange reaction for the wild-type enzyme was 4.5-fold higher.

Upon measurement of the aminoacylating activity an even more pronounced difference between the two compared proteins could be established. The native enzyme was charged up to 60% with ^{14}C-labeled L-phenylalanine, contrary to expressed TY1 which showed a striking reduction in thioester formation to only 1.4%. Cleavage at the aminoacylation site after exposure to performic acid confirmed the thioester nature of the respective bond. The low level of aminoacylation of TY1 expressed in *E. coli* has recently also been reported by Gocht and Marahiel (1994). Reduced aminoacylating activity was also observed upon expression of other peptide synthetase genes in *E. coli*. The first domain of ACV synthetase from *Streptomyces clavuligerus* expressed in *E. coli* showed only 1.5% thioester formation (T. Schwecke, personal communication). Expression of EntF, which is involved in serine activation in enterobactin biosynthesis in *E. coli*, leads to a partially active enzyme, only catalyzing adenylation (Rusnak et al., 1991).

Generally the stability of the aminoacyl thioester intermediates under the conditions employed should permit complete labeling of the available thiols (Gadow et al., 1983; Schlumbohm, 1987). Half-lifetimes of intermediates at 3 °C are in the range of 0.5 to approximately 100 h, depending on their structure, the pH, and the presence of thiols. If a peptide synthetase is already aminoacylated, not all intermediates may be hydrolyzed during purification, and only partial labeling can be achieved. Thus aminoacylation can serve as a rough estimation of the pantetheine content in peptide synthetases.

The failure to detect significant charging of the recombinant enzyme suggests that the majority of the enzyme

Table 2: Conserved Sequence Motifs in Peptide Synthetase Modules[§]

	Box	Sequence motif	Suggested function	References
Adenylation unit	A	LtYREL----N-lA--L		
	B	iV-ilgvLKAG-vPiDP		
	C	lAYi-YTSGtTG-PKGV	AMP binding Adenylate formation	Bairoch, 1990 Gocht & Marahiel, 1994
	D	N-YGPtE		
	E	GEL-i-G-Gv-RGY-N-P-LT-E-F	Adenine binding	Pavela-Vrancic et al., 1994c
	F	mYrTGDl-RWL	ATPase Motiv ATP binding	Gocht & Marahiel, 1994
	G	iEflGR-D-QVKiRG-RIE-GEIE	Adenylate formation ATP binding	Tokita et al., 1993 Pavela-Vrancic at al., 1994b,c
	H	LP--MvP	Adenine binding	Pavela-Vrancic at al., 1994c
	I	LT-NGK--r--LP	ATP binding	Pavela-Vrancic et al., 1994b
Cofactor unit	J	GGDSI--l-l[a] GGHSL-aM-l[b]	Cofactor binding	Stein et al., 1994
Peptide bond forming unit	K	--l-PIQ-WF---[a] YPsV--Q-RMyil[b]	Peptide bond formation	this paper
	L	l---HD--[a] Li-RHE-L[b]	Peptide bond formation	this paper
	M	--HH--vD-vSW-Il[a] DMHHII-DG-S--I-[b]	Spacer motif Peptide bond formation	Fuma et al., 1993 this paper
	N	----------v--EGHGRE[a] LSK-GQ-DIi-GtP-AGR-[b]	Epimerization ?	Fuma et al., 1993
	O	TvGWFT---P--l[a] i-GMFVNT-LAlR[b]	Epimerization ?	Fuma et al., 1993
	P	P--G-GY[a] -	Epimerization	this paper
	Q	v-FNYLG[a] -	Epimerization	Fuma et al., 1993

[§] The consensus motifs were derived by alignment of seven modules from bacilli, not showing epimerization and cyclization activity, and ten modules comprising an epimerization function. The consensus sequence of the nonepimerizing domains was derived by alignment of seven peptide synthetases from *Bacillus*: gramicidin S synthetase 2 (*B. brevis*), modules 1, 2, and 3 (Turgay et al., 1992); surfactin synthetase 1 (SrfAA) (*B. subtilis*), modules 1 and 2; surfactin synthetase 2 (SrfAB) (*B. subtilis*), modules 1 and 2 (Fuma et al., 1993). The consensus sequence for the epimerizing domain was derived by comparison of the following modules: HC-toxin synthetase (*Helmithosporium carbonum*), module 1 (Scott-Craig et al., 1992); surfactin synthetase 1 (SrfAA) (*B. subtilis*), module 3 (Fuma et al., 1993); surfactin synthetase 2 (SrfAB) (*B. subtilis*), module 3 (Fuma et al., 1993); tyrocidine synthetase 1 (*B. brevis*) (Weckermann et al., 1988); gramicidin S synthetase 1 (*B. brevis*) (Hori et al., 1989; Krätzschmar et al., 1989); ACV synthetase (*Lysobacter lactamgenus*), module 3 (Kimura et al., 1990); ACV synthetase (*Penicillium chrysogenum*), module 3 (Díez et al., 1990; Smith et al., 1990); ACV synthetase (*Aspergillus nidulans*), module 3 (MacCabe et al., 1991); ACV synthetase (*Acremonium chrysogenum*), module 3 (Gutiérrez et al., 1991); ACV synthetase (*Nocardia lactamdurans*), module 3 (Coque et al., 1991). [a] Epimerizing domains. [b] Nonepimerizing domains.

molecules may be unmodified. The inability of the recombinant protein to covalently bind the aminoacyl group may be due to enzymatic or hydrolytic cleavage of the ester linkage or insufficient posttranslational modification with 4′-phosphopantetheine, as has been established with ACP of polyketide synthases cloned and expressed in *E. coli* (Caffrey et al., 1991; Shen et al., 1992). Apo-ACP was not post-translationally modified, or it was modified to a lesser extent, by the *E. coli* holo-ACP synthetase, which transfers the 4′-phosphopantetheine prosthetic group from CoA to ACP. Since peptide synthetases and bacterial ACPs have similar consensus sequences in the 4′-phosphopantetheine attachment site, the cofactor could be loaded by the same type of transferase. A CoA:4′-phosphopantetheine protein transferase was postulated to be involved in the posttranslational modification of peptide synthetases (von Döhren et al., 1993).

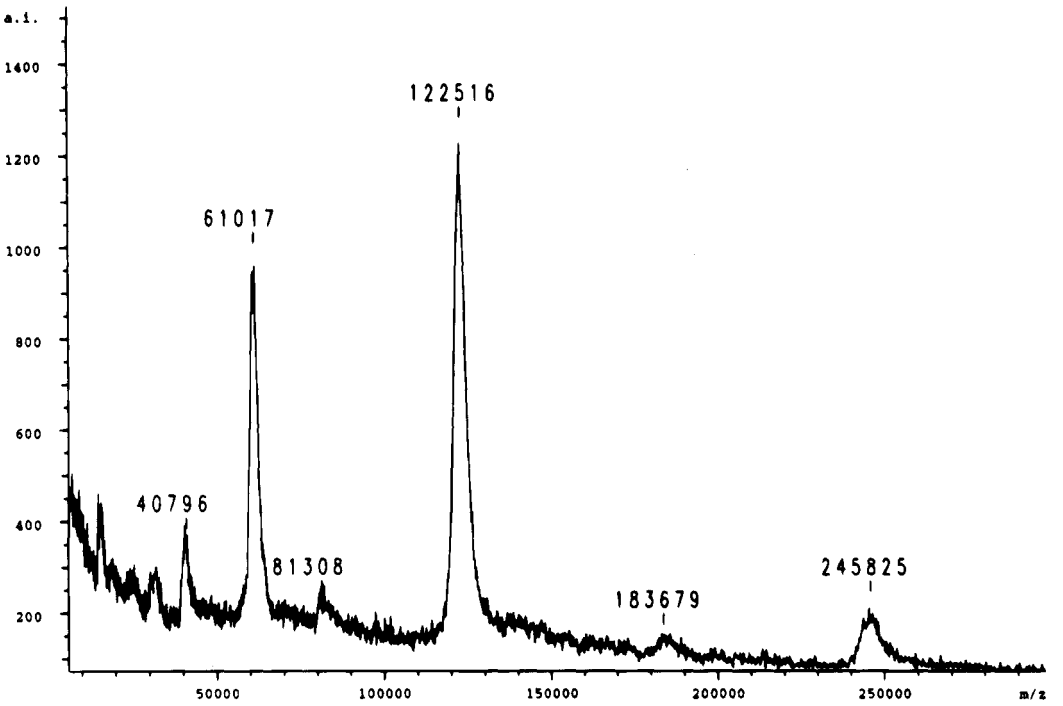

FIGURE 3: Mass spectrometric analysis of recombinant TY1 from *E. coli*. The spectrum was measured by matrix-assisted laser desorption mass spectrometry as described in Materials and Methods.

Table 3: Identified Peaks of Tryptic Peptides from TY1 with Matrix Assisted Laser Desorption/Ionization Mass Spectrometry (MALDI)

peptide	no. of amino acids	calcd *m/z*	obsd *m/z*
^{306}YINAYGPTETS/.../ELCIGGVGLAR	67	7142	7146
^{244}QTIHDFAAFEH/.../PTYLTHLTPER	35	4113	4113.7
^{681}GLGGQLYDFFS/.../VQQAIEAETQR	30	3361	3360
^{846}HANQAYQTEINDLLLAALGLAFAEWSK	27	2988	2988.3
^{133}SVSQLVHDVGYSGEVVVLDEEQLDAR	26	2844	2845.1
^{428}IELGEIESVLLAHEHITEAVVIAR	24	2642	2643.6
^{626}NFTNTGHWNQSSVLYRPEGFDPK	23	2695	2696.5
^{1069}GLQMEEMDDIFELLANTLR	19	2239	2239
^{510}ALPEPDLTANQSQAAYHPPR	21	2176	2176
^{530}TETESILVSIWQNVLGIEK	19	2159	2161.1
^{957}VQPEVTFNYLGQFDADMR	18	2130	2131.9
^{756}DLATGYAQALAGQAISLPEK	20	2017	2015
^{553}DNFYSLGGDSIQAIQVVAR	19	2053	2054.6
^{28}SIHQLFEEQAEAFPDR	16	1917	1918.2
^{666}MVYQHENGNVVQHNR	15	1825	1827.1
^{14}ELEQHALVPYAQGK	14	1583	1583.4
^{100}AGGAYVPIDIEYPR	14	1521	1521.5
^{373}GYWNRPDLTAEK	12	1450	1449.6
^{873}LAQIVIHLEGHGR	13	1443	1443.2
^{405}WLTDGTIEFLGR	12	1408	1408.3
^{886}EDIIEQANVAR	11	1257	1258
^{53}LSYQELNR	8	1022	1022.9
^{44}VAIVFENR	8	947	947.4
^{649}VIQSVMDK	8	919	919.5
^{477}DYAAQK	6	695	695.3
^{399}TGDLAK	6	604	603
^{811}NVSLPK	6	657	655.9
^{621}WFFGK	5	684	683.7
^{579}LETK	4	490	492.8
^{923}LTK	3	360	358
^{396}MYR	3	469	471
^{827}SVR	3	360	358
^{931}IPR	3	384	386.8

Two enzymes, ACP-synthetase and ACP-hydrolase, are responsible for the interconversion of holo- and apoacyl carrier proteins in *E. coli* by the removal or the addition of the prosthetic group, 4′-phosphopantetheine (Vagelos & Larrabee, 1967; Elovson & Vagelos, 1968). It has been established that ACP genes from diverse species are expressed in *E. coli* exclusively or predominantly in their holoenzyme form. Expression of the *Saccharopolyspora erythraea* ACP (Revill & Leadlay, 1991) and spinach ACP genes (Guerra et al., 1988) in *E. coli* gave a enzymatically active proteins, indicating that the ACPs are substrates of the *E. coli* holo-ACP synthetase. However, ACP of the tetracenomycin C polyketide synthase of *Streptomyces glaucescens* isolated from *E. coli* was present only to a small extent in the holoenzyme form (Shen et al., 1992). The recombinant ACP-thioesterase domain of chicken fatty acid synthase expressed in *E. coli* showed partial pantothenylation of the active serine (Pazirandeh et al., 1989). Although *E. coli* ACP synthetase has been reported to have relatively broad substrate specificity (Beremand et al., 1989; Geiger et al., 1991), the poor yield of holo-TY1 from *E. coli* may have resulted from poor recognition by the respective enzyme. Alternatively, it could be due to a deficiency of the transferase relative to the quantity of overexpressed protein, insufficient intracellular concentration of the biosynthetic precursor to the TY1 prosthetic group, or a rapid degradation by the *E. coli* phosphodiesterases, as has been suggested also for holo-ACP (Shen et al., 1992). It has been found that ACP phosphodiesterase of *E. coli* catalyzes the hydrolytic cleavage of the 4′-phosphopantetheine residue of ACP (Fischl & Kennedy, 1990).

Both TY1 and GS1 have been assayed numerous times with microbiological tests for the presence of pantothenate (Pugh & Wakil, 1965). It had been generally accepted that they do not contain the 4′-phosphopantetheine cofactor; however, the reliability of the assay had to be questioned since lactobacilli respond to pantothenic acid, while 4′-phosphopantetheine is offered in the assay. Proof of phosphopantetheine content in the case of EntF has been carried out by β-alanine incorporation, followed by hydrolytic

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MLANQANLID NKRELEQHAL VPYAQGKSIH QLFEEQAEAF PDRVAIVFEN RRLSYQELNR    60
KANQLARALL EKGVQTDSIV GVMMEKSIEN VIAILAVLKA GGAYVPIDIE YPRDRIQYIL   120
QDSQTKIVLT QKSVSQLVHD VGYSGEVVVL DEEQLDARET ANLHQPSKPT DLAYVIYTSG   180
TTGKPKGTML EHKASPICNP FSKIRLASPS KTGSGFLPAC RSTHPFGKCS WLCCLAPRVH   240
PSKQTIHDFA AFEHYLSENE LTIITLPPTY LTHLTPERIT SLRIMITAGS ASSAPLVNKW   300
KDKLRYINAY GPTETSICAT IWEAPSNQLS VQSVPIGKPI QNTHIYIVNE DLQLLPTGSE   360
GELCIGGVGL ARGYWNRPDL TAEKFVDNPF VPGEKMYRTG DLAKWLTDGT IEFLGRIDHQ   420
VKIRGHRIEL GEIESVLLAH EHITEAVVIA REDQHAGQYL CAYYISQQEA TPAQLRDYAA   480
QKLPAYMLPS YFVKLDKMPL TPNDKIDRKA LPEPDLTANQ SQAAYHPPRT ETESILVSIW   540
QNVLGIEKIG IRDNFYSLGG DSIQAIQVVA RLHSYQLKLE TKDLLNYPTI EQVALFVKST   600
TRKSDQGIIA GNVPLTPIQK WFFGKNFTNT GHWNQSSVLY RPEGFDPKVI QSVMDKIIEH   660
HDAVRMVYQH ENGNVVQHNR GLGGQLYDFF SYNLTAQPDV QQAIEAETQR LHSSMNLQEG   720
PLVKVALFQT LHGDHFFLAI HHLVVDGISW RILFKIWQPD TRRHLQGKRS VCPKKRILFK   780
AGHNGCKNNA NEADLLSEIP YWESLESQAK NVSLPKDYEV TDCKQKSVRN MRIRLHPEET   840
EQLLKHANQA YQTEINDLLL AALGLAFAEW SKLAQIVIHL EGHGREDIIE QANVARTVGW   900
FTSQYPVLLD LKQTAPLSDY IKLTKENMRK IPRKGIGYDI LKHVTLPENR GSLSFRVQPE   960
VTFNYLGQFD ADMRTELFTR SPYSGGNTLG ADGKNNLSPE SEVYTALNIT GLIEGGELVL  1020
TFSYSSEQYR EESIQQLSQS YQKHLLAIIA HCTEKKEVER TPSDFSVK GLQMEEMDDIFE  1080
LLANTLR
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FIGURE 4: Identification of tryptic peptides from recombinant TY1 by mass spectrometric analysis (MALDI). The identified peptides are displayed by shading the respective amino acid sequence.

cleavage and identification of pantetheine (Rusnak et al., 1991). In a recent study the incorporation of β-alanine in TY1 has been established (Gocht & Marahiel, 1994). The direct proof for modification of GS1 has recently been achieved by thiol labeling and electrospray mass spectrometry (Stein et al., 1995).

TY1 activates the amino acid in the form of an aminoacyl adenylate:enzyme complex with release of PP_i. The adenylate either reacts in a transfer reaction with an enzyme thiol acceptor, in the reverse reaction with $MgPP_i^{2+}$, or is hydrolyzed by H_2O. Upon aminoacylation, a second adenylate is formed, which due to occupancy of the thioester site cannot react further. A ratio of bound amino acid to AMP of 2:1, measured in the tyrocidine system (Roskoski et al., 1970), provided evidence in favor of this hypothesis. Amino acid activating domains of peptide synthetases lacking the aminoacylation site have been reported to show varying effects on aminoacyl adenylate formation. A fragment of the proline-activating domain in GS2, not containing the aminoacylation motif, was found to catalyze the activation of proline (Hori et al., 1991; Kurotsu, 1991). A kinetic study of this fragment has shown a change of the K_d value for proline from 0.3 to 2.5 mM. Substitution of Ser by Ala at the thioester site of the first four amino acid activating domains of surfactin synthetase showed opposing effects on aminoacyl adenylate formation. The three domains of E1A eliminate aminoacylation with extensive reduction of adenylate formation, while absence of the thioester site in the first domain of E1B had little effect on the Val-dependent ATP[^{32}P]PP_i exchange activity (Vollenbroich et al., 1993, 1994). Due to the low selectivity of the amino acid activating domains, valine might have been activated by an E1B domain containing an unmodified thioester site. The same mutation in the aminoacylation motif of TY1 has been reported to have no effect on the rate of ATP[^{32}P]PP_i exchange relative to the expressed unmodified protein, but no data is provided as to the activation rate of the wild-type enzyme (Gocht & Marahiel, 1994). The large extent of structure preservation in the amino acid activating and adenylate forming domain of recombinant TY1 is, however, indicated by the retention of substrate specificity and binding affinity. Hence, we may presume that the overall structure of the active site has not been affected; however, minor structural changes may have occurred, as might be expected from the altered microenvironment caused by lack of a thiol acceptor. This is in agreement with the characterization of TY1 fragment devoid of the aminoacylation site displaying similar kinetic properties (Dieckmann et al., 1995).

TY1 accepts both the L- and the D-configuration of phenylalanine in the activation reaction; however, only D-Phe is incorporated into the peptide product. It has been established that epimerization takes place at the thioester stage, and a base has been suggested to function as a proton donor and acceptor during epimerization (Kanda et al., 1989). The epimerized amino acid is released by performic acid treatment. Acid hydrolysis of the radioactive product, obtained after using L-[^{14}C]Phe as a radiolabel, yielded a mixture of D- and L-Phe, as established by chromatographic analysis. In connection with reduced thioester formation in the recombinant TY1, a reduction of epimerization was also observed. Hori et al. observed a 90% reduction in epimerase activity of GS1 expressed in *E. coli*; however, they did not

present data for thioester formation (Hori et al., 1989). Biochemical analysis of the epimerization reaction catalyzed by GS1 suggested that a sulfhydryl was required for the reaction (Kanda et al., 1984). However, absence of a conserved cysteine residue further supports the concept of a 4′-phosphopantetheine cofactor as the site of covalent attachment (Hori et al., 1994). Since epimerase activity requires the presence of the thioester attached substrate, the obtained results indicate that posttranslational modification takes place only incompletely in *E. coli*.

The distinction between the compared enzymes was further substantiated by the differences in their isoelectric points. A similar disparity of 0.1 pH unit was observed between the p*I* values of holo-ACP and the Ser-to-Thr mutant in the pantetheine attachment site of ACP (Jaworski et al., 1989). An upward shift in the isoelectric point could be anticipated due to reduction of the total number of acidic groups in the absence of the cofactor. Further evidence for the lack of phosphopantetheine was provided by mass spectrometric analysis of the tryptic digest of the recombinant protein (Figure 4), revealing a molecular peak corresponding to the mass of the active site fragment ^{553}DNFYSLGGDSIQAIQV-VAR unmodified by the cofactor. This technique was shown to provide a powerful and convenient means of detecting recombinant and posttranslational modifications of proteins when expressed in a heterologous bacterial host. It has already been applied in monitoring the posttranslational modification of apo-ACP (Revill & Leadlay, 1991), isopenicillin N synthetase (Aplin et al., 1990), and the valine domain of GS2 (Stein et al., 1994) and to study the thioesterase activity associated with the erythromycin-producing polyketide synthase (Caffrey et al., 1991). The exact molecular mass measurements also provide a reliable test for sequence data checking.

Alignment of this sequence and nine other available modules with epimerization activity shows highly conserved motifs within the region following the thioester site (Pfeifer, 1994), thus providing an extension of the previously derived consensus sequence (Pavela-Vrancic et al., 1994a) beyond this position. In Table 2 we present the conserved sequence motifs obtained by alignment of ten epimerizing and seven nonepimerizing peptide synthetase domains, including the core sequences of the adenylation and cofactor units. The pantetheine attachment consensus motif (J) contains an aspartic acid adjacent to serine. Generally, in peptide synthetases with nonepimerizing activity, histidine is found in this position. An additional motif, K, has been identified next to the cofactor region. It contains the PIQ core sequence, unique to the epimerizing domain of peptide synthetases. Motif M, which has been termed the "spacer motif" (Fuma et al., 1993), may however be involved in the condensation reaction, because the known pH dependence of the peptide bond formation suggests the involvement of a His residue in proton transfer. Since in the course of the condensation reaction with D-Phe selection of the D-isomer occurs, it may be expected that the respective motifs partially differ from those in the nonepimerizing domains. Motifs K, L, and M are positionally conserved in domains of peptide synthetases catalyzing the elongation reaction, and thus we propose their involvement in peptide bond formation. Motifs N, O, and Q have been suggested to be involved in epimerization (Fuma et al., 1993); however, motif O is also present in a slightly altered form in nonepimerizing domains. Considering the functional requirements for epimerization, motif N could be involved in proton transfers. Additionally, motif P has been observed in all compared modules.

In conclusion, the comparison of TY1 from *B. brevis* and the protein generated by expression of the *tycA* gene in *E. coli* provides data on significant differences in catalytic efficiency of the individual domains responsible for partial reactions performed by the enzyme. Although amino acid binding and adenylation are comparable, our data indicate fractional posttranslational modification of TY1 by phosphopantetheine addition when expressed in *E. coli*, as evidenced by extremely low levels of aminoacylation detected. The analysis implies that the presence or absence of the cofactor is irrelevant for substrate binding, suggesting that the adenylate domain is capable of folding independently to form an active enzyme. Multifunctional proteins appear to be composed of independently folded and compact domains, connected by linker regions, which retain activity after limited proteolysis (Aparicio et al., 1993; Pazirandeh et al., 1989). However, the reverse rate of adenylate formation is reduced in the absence of phosphopantetheine, indicating a conformational change in the protein. This change could originate from aminoacylation, since an isolated adenylate-forming domain displays similar kinetics (Dieckmann et al., 1995).

Due to maintenance of the partial reactions associated with adenylate formation, in vitro analysis of the amino acid activating mechanism by site-directed mutagenesis or chemical modification should provide an accurate reflection of the in vivo situation. The recombinant enzyme has been successfully used in the analysis of partial functions, such as ATP binding and adenylate formation, by affinity labeling studies (Pavela-Vrancic et al., 1994b,c). On the other hand, investigation of the aminoacylation site requires both previous analysis of the enzymology of modification by the postulated CoA:4′-phosphopantetheine-protein transferase and determination of the pantetheine content. Work on resolving this problem is currently in progress.

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