Mutational Analysis of the N-Methyltransferase Domain of the Multifunctional Enzyme Enniatin Synthetase*

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N-Methylcyclopeptides like cyclosporins and enniatins are synthesized by multifunctional enzymes representing hybrid systems of peptide synthetases and S-adenosyl-l-methionine (AdoMet)-dependent N-methyltransferases. The latter constitute a new family of N-methyltransferases sharing high homology within procaryotes and eucaryotes. Here we describe the mutational analysis of the N-methyltransferase domain of enniatin synthetase from Fusarium scirpi to gain insight into the assembly of the AdoMet-binding site. The role of four conserved motifs (I, 2085VLEIGTSGML; II/Y, 2105SYVGLDFS; IV, 2152DLVVFNSVVQYFTPPEYL; and V, 2194ATNGHFLAA-RW) in cofactor binding as measured by photolabeling was studied. Deletion of the first 21 N-terminal amino acid residues of the N-methyltransferase domain did not affect AdoMet binding. Further shortening close to motif I resulted in loss of binding activity. Truncation of 38 amino acids from the C terminus and also internal deletions containing motif V led to complete loss of AdoMet-binding activity. Point mutations converting the conserved Tyr to Val, Ala, and Ser, respectively, strongly diminished AdoMet binding, whereas conversion of this residue to Phe restored AdoMet-binding activity to ~70%, indicating that Tyr is important for AdoMet binding and that the aromatic Tyr may be crucial for AdoMet binding in N-methylpeptide synthetases.

N-Methylated peptides like cyclosporins and enniatins constitute a class of pharmacologically interesting compounds. They are synthesized by a special class of enzymes representing hybrid systems of peptide synthetases and integrated N-methyltransferase domains (1). Like other peptide synthetases, N-methylcyclopeptide synthetases follow a so-called thiol template mechanism, in which the substrate amino acids are activated as thioesters mediated by enzyme-bound 4'-phosphopantetheine residues (1–3). Enniatin synthetase was the first N-methylcyclopeptide synthetase to characterized (4). Sequencing of the enniatin synthetase-corresponding gene (esyn1) from Fusarium scirpi revealed that the enzyme is one single polypeptide chain of 847 kDa (5). It consists of the two modules EA and EB containing the two catalytic binding sites for the substrates D-hydroxyisovaleric acid and the branched-chain l-amino acid, respectively (5). The 55-kDa N-methyltransferase portion M of the enzyme is located within the EB module. N-Methylation takes place after covalent binding of the amino acid on the surface of the corresponding peptide synthetase prior to peptide bond formation (4, 6). S-Adenosyl-l-methionine (AdoMet) serves as the methyl donor (4, 7). The mechanism of formation of N-methylated peptides has been elucidated in the case of the cyclodepsipeptides enniatin (8), beauvericin (9), and cyclosporin (10) and in actinomycin biosynthesis (11).

Biochemical investigations of the N-methyltransferase function of enniatin synthetase (4) revealed that, similar to other methyltransferases, S-adenosyl-l-homocysteine (AdoHcy) and sinefungin are potent inhibitors of the AdoMet-dependent reaction. Sinefungin acted as a competitive inhibitor with respect to AdoMet, whereas AdoHcy exhibited an inhibition pattern characteristic for a partial competitive inhibitor, suggesting a discrete binding site for this inhibitor. Like other methyltransferases, the N-methyltransferase domain of enniatin synthetase can be affinity-labeled by UV irradiation in the presence of AdoMet labeled at the methyl group (4). The photoreaction was shown to be site-specific, and a binding stoichiometry of one methyl group/enzyme molecule was observed (4). It could be shown that AdoHcy diminished photolabeling of enniatin synthetase with [methyl-14C]AdoMet or [methyl-3H]AdoMet; but even in the presence of excess AdoHcy (100 μM), it was not able to totally prevent the photoreaction, as did sinefungin; in contrast, the enzyme-bound radioactivity reached a reduced but constant level of 40% of the uninhibited control (4). This indicates that AdoHcy does not directly compete with AdoMet, but binds to a discrete inhibitory site. AdoHcy reduces the affinity of AdoMet for the enzyme, but, even at infinite high inhibitor concentrations, allows formation of an enzyme-AdoMet-AdoHcy complex, which still yields product. Furthermore, neither sinefungin nor AdoHcy affected substrate activation (adenylation and subsequent thioacylation). Interestingly, AdoHcy inhibited the formation of the unmethylated depsipeptide formed in the absence of AdoMet. In contrast, sinefungin exhibited no influence on the synthesis of demethylleniatin (4). These findings confirmed the assumption that two different binding sites for the inhibitors must be present. This was supported by recent experiments on N-methylation in cyclosporin synthetase. In this case, AdoHcy acted as a noncompetitive inhibitor with respect to AdoMet, indicating the presence of two binding sites in the N-methyltransferase domains of this multi-enzyme (12).

Amino acid sequence comparison of the N-methyltransferase domain of enniatin synthetase with methyltransferases from

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1 The abbreviations used are: AdoMet, S-adenosyl-l-methionine; AdoHcy, S-adenosyl-l-homocysteine; PCR, polymerase chain reaction; mAb, monoclonal antibody.
primary metabolism showed no significant sequence similarities except for one conserved motif (motif I) (2, 5). A conserved phenylalanine in the glycine-rich motif I was found to be crucial in positioning the adenine ring of AdoMet to the Hxal DNA methyltransferase (13). However, the corresponding position of enniatin synthetases is occupied by the first glycine of motif I methyltransferase (13). However, the corresponding position of enniatin synthetases is occupied by the first glycine of motif I.

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Table I

| Deletion variants | Insert construction | Vector construction |
|-------------------|---------------------|--------------------|
| pM2Δ1–135        | PCR amplification of nucleotides 405–705 of pSK1.6BP (primers 1 and 2) | Cleavage of pSK1.6BP with BamHI and SmaI |
| pM3Δ1–151        | Partial cleavage of pSK1.6BP with EcoRV and cleavage with PstI | Cleavage of pUC8 DNA with PstI and HindIII |
| pM4Δ499–558      | PCR amplification of nucleotides 555–1491 of pSK16.BP (primers 3 and 4) | Cleavage of pSK1.6BP with KpnI and EcoRI |
| pM5Δ386–558      | Partial cleavage of pSK1.6BP with HaeII/blunt ends and cleavage with BamHI | Cleavage of pSK1.6BP with BamHI and EcoRV |
| pM5Δ291–497      | Partial cleavage of pSK1.6BP with BglII/blunt ends and partial cleavage with AciI/blunt ends, then ligation | Cleavage of pSK1.6BP with BamHI and EcoRI |
| pM7Δ413–475      | Partial cleavage of pSK1.6BP with DdeI/blunt ends and partial cleavage with HincII, then religation | Cleavage of pSK1.6BP with EcoRI and SmaI |
| pM8Δ526–558      | PCR amplification of nucleotides 555–1578 (primers 3 and 5) of pSK1.6BP and in the process of construction of an EcoRI restriction site at the 3′-end, cleavage with EcoRI and SmaI | Cleavage of pSK1.6BP with BamHI and SmaI |

| Overview of the construction strategies of the deletion variants derived from pSK1.6BP (19) |

Polyacrylamide Gel Electrophoresis—SDS-polyacrylamide gel electrophoresis was performed as described by Laemmli (20). Gels contained 12.5% acrylamide. The relative molecular masses of the enzymes were determined from their mobilities related to those of the standard proteins (Sigma).

Protein Determination—Protein concentrations were determined using the dye binding method of Bradford (21) using bovine serum albumin as a standard.

Plasmids, Bacterial Strains, and Culture Conditions—Escherichia coli strain XL1-Blue (22) was used as a host for plasmids derived from Bluescript (Stratagene, Amsterdam, The Netherlands) and pUC8 (23). The strains were grown in Luria-Bertani broth (1% NaCl, 0.5% yeast extract, and 1% Bacto-Tryptone) at the specified temperature. Tag DNA polymerases, nucleotides, and restriction enzymes were purchased from Life Technologies, Inc. (Karlsruhe, Germany). Plasmid purification kits were purchased from QIAGEN (Hilden, Germany).

DNA Manipulations—DNA manipulations and PCR were performed using standard methods (24). PCR was performed using a Biometra UNO-Thermoblock. Automated DNA sequencing was performed using the ABI Prism 373 genetic analyzer at Firma Martin Meinzer (Berlin).

Oligonucleotides—The oligonucleotides were synthesized by TIB MOLBIOL (Berlin) and metabion (München, Germany). Oligonucleotide primers for deletion variants were as follows: primer 1, ATGGAT-CCTCAGTGGACTGCTGCA; primer 2, GAACCTGACCTGCTGACT; primer 3, GTTGGACGGCGCATTTAC; primer 4, CGGAGTTCCGCGATCTCAGGAGT; and primer 5, CGGAATTCGGGACCGTGGTGGGGAAGCTG (restriction endonuclease recognition sequences are indicated in italic type). Oligonucleotide primers for site-directed mutagenesis were as follows: primer 1A, CTCACAGCGTGTCCTCCTGCTGATG (introducing Val and Ala), and primer 2A, ATTAACCTCCTAAGGG; primer 1B, ATCAAGACCAAGG/AG/AAGCTGTTGAG (introducing Val and Ala) and primer 2B, ATAGGGCCATGGGTA; primer 3A, CTCACAGCGTGTCCTCCTGCTGATG; primer 3B, CTCACAGCGTGTCCTCCTGCTGATG, and primer 4B, ATCAAGACCAAGG/AG/AAGCTGTTGAG (introducing Phe and Ala); and primer 4A, CTCACAGCGTGTCCTCCTGCTGATG. This permits tertiary structure prediction of the N-methyltransferase domain of enniatin synthetase and other AdoMet-dependent methyltransferases. The motifs (II, IV, and V) of enniatin synthetase are conserved in the sequences of AdoMet-dependent methyltransferases.

In a previous work on enniatin biosynthesis, Pieper et al. (17) investigated the role of motifs I, II, Y, IV, and V in AdoMet binding.

Experimental Procedures

Materials—Chemicals were of the highest purity commercially available. [methyl-3H]AdoMet (dilute sulfuric acid solution (pH 2.5–3.5), 57 Ci/mmol), [ methyl-3H]AdoMet (dilute sulfuric acid solution (pH 2.0–2.5), 80 Ci/mmol), and [ethylene-3H]AdoMet (dilute sulfuric acid solution (pH 2.5–3.5), 25 Ci/mmol) were purchased from Amersham Pharmacia Biotech (Braunschweig, Germany). Other chemicals used were purchased from Sigma (Deisenhofen, Germany), Merck (Darmstadt, Germany), and Fluka (Neu-Ulm, Germany). Whole molecule alkaline phosphatase-conjugated anti-mouse IgG and 4-nitrophenyl phosphate were obtained from Sigma.
FIG. 1. Amino acid sequence alignment of 14 N-methyltransferase domains from peptide synthetases of prokaryotic and eucaryotic origin. The alignment was made using the Clustal W alignment program in the GeneDoc package. The following sequences with their GenBank/EBI Data Bank accession numbers were used: ESYNscir, enniatin synthetase from *F. scirpi* (amino acids 2014–2447; Z18755) (5); ESYNsamb, enniatin synthetase from *F. sambucinum* (Z48743) (18); CYSYN, cyclosporin synthetase from *T. niveum* (Z28383) (29); MCYA, microcystin synthetase A from *Microcystis* sp. (AB019578) (30); APMC, actinomycin synthetase II from *S. chrysomallus* (AF204401) (31); SNBDEpri and SNBDEvir, pristinamycin synthetase from *S. pristinaespiralis* (Y11548) and from *S. virginiae* (Y11547), respectively (32). Identical amino acid residues are shown in black. More than 80% sequence similarities are shown in dark gray; >60% sequence similarities are shown in light gray. Sequences showing local similarities (motifs I, II, Y, IV, and V) to methyltransferases are boxed, using the 3D-PSSM web-based method (27, 28).
cloned into pBluescript SK.

PCR-amplified fragments were cleaved with the restriction enzyme (5). B, amino acid sequence alignment of conserved motifs of Tyr223

Site-directed Mutagenesis—Site-directed mutagenesis was performed using the method of Higuchi (25) with two subsequent PCRs. Site-directed mutants Tyr"→ Ala and Tyr"→ Val were constructed from pSK1.6BP as a template by PCR mutagenesis using primer pairs 1A/2A and 1B/2B. For mutants containing Tyr"→ Phe, we used primer pairs 3A/2A and 3B/2B; and for Tyr"→ Ser, primer pairs 4A/2A and 4B/2B were used. The first two PCRs were carried out with primers each differing in two base positions from the wild-type N-methyltransferase domain nucleotide sequence (positions 668 and 669 of pSK1.6BP corresponding to positions 6318 and 6319 of esyn1) and two homologous primers located in the 5'- and 3'-regions, respectively, of the template pSK1.6BP. The two PCR-amplified products (0.7 and 1.0

Expression and Purification of Recombinant Proteins—E. coli strain XL-1 Blue cells harboring the expression vectors were grown in Luria-Bertani medium supplemented with 100 μg/ml ampicillin at 37 °C and 250 rpm until A595 reached 0.3 (UVICONT 930 spectrophotometer) was reached. The cells were induced with 1 mM isopropyl-β-D-thiogalactopyranoside and cultivated for additional 4–6 h. The cells were harvested by centrifugation and stored at −80 °C. Wild-type and recombinant proteins of the deletion variants and mutants were purified as follows. All operations were carried out at 4 °C. Frozen cells from 1–1.5 g of partially purified enniatin synthetase (Fusarium semitectum) were irradiated in an ice-cooled polystyrene microtiter plate at a distance of 2 cm for 60 min with short-wave UV light (254 nm) using a 45-watt mercury lamp (I max = 0.2 A). The reaction was stopped by addition of 1 ml of 7% trichloroacetic acid. Further sample treatment was done as described (4).

Enzyme-linked Immunosorbent Assay—Polystyrene microtiter plates were coated with 100 μl of protein (10 μg/ml of buffer A) overnight at 4 °C. After saturation with 1% bovine serum albumin for 2 h at room temperature, the wells were incubated for 2 h at room temperature with appropriate dilutions of the monoclonal antibody (mAb 28.7 or mAb 25.34) (26) and alkaline phosphatase-conjugated rabbit anti-mouse IgG. After addition of the substrate 4-nitrophenyl phosphate (1 mg/ml), the reaction was monitored by measuring the absorption at 405 nm after 10, 20, and 30 min.

Sequence Analysis—Computer programs supplied as part of the PC Gene sequence analysis package (University of Wisconsin, Madison, WI) were used for sequence analysis and comparison. For structure-guided alignments, the web-based 3D-PSSM method was used (27, 28).

RESULTS

Sequence Analysis of N-Methyltransferase Domains of Peptide Synthetases

During the last years, an increasing number of genes from eucaryotic and procaryotic organisms encoding N-methylpeptide synthetases have been analyzed. Fig. 1 shows a sequence alignment of N-methyltransferase domains of peptide synthetases. These are the domains of enniatin synthetase from F. scirpi (ESYNecir) (5) and F. sambucinum (ESYNsamb) (18) and the seven domains of cyclosporin synthetase from the fungus Tolypocladium niveum (CYSYNd2–5, CYSYNd7, CYSYNhd8, and CYSYNd10) (29). Furthermore, the corresponding sequences of bacterial synthetases are given: microcystin synthetase A from Microcystis sp. (MCYA_mm1) (30), actinomycin synthetase II from Streptomyces chrysomallus (ACM_ms2 and ACM_ms3) (31), and pristinamycin synthetase from Streptomyces pristinaespiralis (SNBDEpri) and Streptomyces virginae (SNBDelec) (32). The N-methyltransferase domains of the enniatin synthetases of Fusarium share high similarity (50–60%) with the other N-methyltransferase domains of eucaryotic origin (cyclosporin synthetase), but lower similarity (20–25%) with the procaryotic systems.

As shown in Fig. 1, the glycine-rich motif I, which is conserved in most methyltransferases (14, 16), can also be found in peptide synthetase N-methyltransferase domains (18). Although no extensive sequence similarities exist among methyltransferases, the tertiary structures of the AdoMet-binding domains of these methyltransferases are strikingly similar to each other, suggesting that many methyltransferases may have a common structure (14, 15). Burmester et al. (18) located the sequence GALDA/VI/F (showing similarity to motif II of Kagan and Clarke (16)) at the C terminus of the N-methyltransferase domains of peptide synthetases. However, structure-guided sequence alignments of the N-methyltransferase domains of peptide synthetases with methyltransferase revealed possible additional conserved motifs, as shown in Fig. 2A (14, 15, 33). Motif II from DNA methyltransferases can also be found in peptide synthetase N-methyltransferase domains, designated motif II/Y (18), similar both in sequence and in location relative to motif I (Figs. 1 and 2, A and B) (13, 34). Motif II/Y has been discussed to be involved in AdoMet binding (17) and is also highly conserved in all N-methyltransferase domains shown in Fig. 1. As shown in Fig. 2A, motifs I, II/Y, and IV are arranged in the same relative order in each type of methyltransferase. Furthermore, the highly conserved sequence NSV/V/A/QYPFPXXXYL (corresponding to positions 2159–2171 in enniatin synthetase from F. scirpi) can be found C-terminal to motif II/Y in all N-methyltransferase domains of peptide synthetases (Fig. 1). This sequence corresponds to motif IV in DNA methyltransferase nomenclature (14, 33), which is similar to motif II of Kagan and Clarke (16). Amino terminus-proximal downstream of motif IV, the sequence 2194ATNG-HFLAAR is found in F. scirpi enniatin synthetase, which...
shows similarity to motif V of DNA methyltransferases, e.g. TaqI methyltransferase (M.TagI) (14). This motif contains a phenylalanine that is absolutely conserved in the N-methyltransferase domains of peptide synthetases (Fig. 1).

The methyltransferases differ in the relative linear order of three regions: the AdoMet-binding region, the catalytic region, and the target recognition region (14). In Fig. 2B, the possible arrangement of the conserved motifs of the N-methyltransferase domain of enniatin synthetase, TaqI methyltransferase, and glycine N-methyltransferase (rat) are shown. The latter two show structural similarities to the N-methyltransferase domain of enniatin synthetase and other N-methyltransferase domains of peptide synthetases. Our results reveal that the N-methyltransferase domain of enniatin synthetase is more closely related to other methyltransferases than was expected.

**Mutational Analysis of the N-Methyltransferase Domain of Enniatin Synthetase**

**Cloning and Expression of Deletion Variants**—We have previously demonstrated that the N-methyltransferase domain of enniatin synthetase could be functionally expressed in E. coli (19) and therefore is suitable for mutational analysis. For further characterization of the N-methyltransferase domain of enniatin synthetase, seven deletion variants were constructed. The corresponding proteins were expressed, partially purified after renaturation, and tested for their ability to bind the cofactor [methyl-14C]AdoMet. The results of Haese et al. (19) provided evidence that motif I is necessary for AdoMet binding. However, the size of the truncated part of the gene was rather large, not allowing a more detailed analysis. Therefore, we completed this study by construction of additional variants to obtain more knowledge about the role of four motifs in cofactor binding. This is shown in Fig. 3B. Two variants (pM2Δ1–135 and pM3Δ1–151) were constructed that differed in the size of the diminished part of the N terminus, both including the four conserved motifs shown in Fig. 2B. Variants pM4Δ499–558, pM5Δ386–558, and pM8Δ526–558 are deleted at the C terminus, all including motifs I–V. Furthermore, variants pM6Δ291–497 (containing a deletion of motifs IV and V) and pM7Δ413–475 (including a deletion of 64 amino acids near the C terminus) were constructed to study the role of this region in AdoMet binding. All deletion variants were derived from plasmid pSK1.6BP, containing the N-methyltransferase domain of *esyn*1 and designated as wild-type N-methyltransferase (see "Experimental Procedures"). Site-directed mutagenesis was used to replace Tyr223 in motif II/Y by Ala, Val, Ser, and Phe.
Expression of recombinant proteins was carried out in E. coli and yielded insoluble protein in inclusion bodies. Therefore, it was necessary to renature these proteins with a denaturation/renaturation method. Denaturation with 8 M urea, renaturation via dialysis, and subsequent DEAE chromatography yielded partially purified native protein (see “Experimental Procedures”).

Control of Refolding by Enniatin Synthetase N-Methyltransferase-specific Monoclonal Antibodies—Billich et al. (26) showed that the enniatin synthetase-directed monoclonal antibodies mAb 28.7 and mAb 28.34 are able to recognize the N-methyltransferase domain of enniatin synthetase in its native state, but not in the denatured form. These antibodies provided a suitable tool to follow the denaturation/renaturation process. Therefore, we used them to monitor the refolding of the overexpressed proteins in an enzyme-linked immunosorbent assay. This could be demonstrated in the case of variant pMVal, pMAla, and pMSer showed a significant loss of AdoMet-binding activity. However, the mutation variant pMPhe showed without loss of binding activity (pM3Δ1–135). The refolded protein gave a signal (absorption, 0.35) comparable to that of the renatured wild-type N-methyltransferase (0.42), whereas denatured protein pM2Δ1–135 gave only a low response (0.07). The other expressed N-methyltransferase variants behaved identically in the enzyme-linked immunosorbent assay (data not shown).

Photolabeling of Recombinant N-Methyltransferase Variants—The cofactor-binding ability of recombinant proteins was measured by photoincorporation of [methyl-14C]AdoMet. Under the conditions described (see “Experimental Procedures”) a linear increase in photolabeling was observed up to 20 min, reaching a plateau of ~0.3 mol of methyl group/mol of wild-type enzyme (data not shown). Our results are shown in Fig. 3B. As shown, variants pM2Δ1–135 and pM3Δ1–151, both truncated up to 21 amino acid residues at the N terminus, were still active. pM3Δ1–151 exhibited an even higher AdoMet-binding activity than the wild-type N-methyltransferase. In contrast, removal of short stretches of the C terminus, as in variants pM4Δ498–558 and pM8Δ526–558, resulted in a loss of AdoMet-binding activity. Variants pM6Δ291–497 and pM7Δ413–475 were found to be inactive with respect to cofactor binding. Variant pM6Δ291–497, containing a deletion of motif V, showed no AdoMet-binding activity in the photolabeling test, as expected. But also variant pM7Δ413–475, in which only a small part in a less conserved region of the N-methyltransferase domains of peptide synthetases was deleted, showed no binding activity, indicating that this sequence may be essential for AdoMet binding.

The deletion variant pM3Δ1–151 (containing the biggest deletion at the N terminus) and the wild-type N-methyltransferase were analyzed by SDS-polyacrylamide gel electrophoresis after photolabeling. Subsequent visualization by autoradiography showed labeled proteins with the expected molecular masses of 45 kDa for the deletion variant pM3Δ1–151 and 62 kDa for the wild-type protein (Fig. 4).

To determine whether the conserved tyrosine in motif II/Y is directly involved in AdoMet binding, four mutants in which the tyrosine was replaced by alanine, serine, valine, and phenylalanine, respectively, were constructed by site-directed mutagenesis (see Fig. 3C). The resulting proteins were expressed, partially purified, and tested via photoincorporation. The mutation variants pMVal, pMAla, and pMSer showed a significant loss of AdoMet-binding activity. However, the mutation variant pMPhe showed ~70% of the wild-type N-methyltransferase AdoMet-binding activity.

**Fig. 4.** Autoradiogram of photoaffinity-labeled wild-type N-methyltransferase pSK1.68BP and the deletion variant pM3Δ1–151. Separation of recombinant proteins was performed by SDS-polyacrylamide gel electrophoresis (12.5%). The molecular masses of the standard proteins are indicated.

**DISCUSSION**

The integrated N-methyltransferases of peptide synthetases are highly conserved (Fig. 1) (18) and differ from other methyltransferases with respect to substrate recognition and binding. In contrast to other methyltransferases, the substrate is presented by the 4'-phosphopantetheine group of the peptide synthetase; and therefore, the substrate recognition of the amino acid/growing peptide is simplified. The process of initial substrate recognition and binding occurs in the adenylation domain of the peptide synthetase (2).

Structural analysis of the N-methyltransferase domain of enniatin synthetase revealed, besides the previously described motifs of Burmester et al. (18), three additional known conserved motifs (II, IV, and V) that are also found in, for example, TaqI methyltransferase and glycine N-methyltransferase from rat (Fig. 2B). This work describes the mutational analysis of the N-methyltransferase domain of enniatin synthetase from F. scirpi to obtain more insight into the structural arrangement and the role of motifs I, II/Y, IV, and V in cofactor binding. Expression of the N-methyltransferase variants in E. coli, however, yielded insoluble protein. Therefore, a denaturation/renaturation method had to be used to obtain native protein. The question arose why photolabeling of the recombinant wild-type N-methyltransferase yielded only 0.3 mol of methyl group/mol of enzyme, whereas cross-linking of native Esyn led to a maximum incorporation of 1 mol of labeled methyl group/mol of protein. The reason for this might be the structural features of the multidomain character of Esyn playing a role in the regulation of the integrated N-methyltransferase function. Interestingly, all renatured recombinant proteins, irrespective of their capability to bind AdoMet or not, were recognized by the enniatin synthetase-specific monoclonal antibodies mAb 28.7 and mAb 28.34. These antibodies are directed against native enniatin synthetase and recognize discontinuous epitopes of the N-methyltransferase domain (26). The results show that all the variants containing an internal deletion must possess a similar three-dimensional arrangement as the wild-type enzyme.

Binding studies with [methyl-14C]AdoMet showed that part of the 21 amino acid residues of the N terminus could be deleted without loss of binding activity (pM3Δ1–151). Therefore, this part of the protein can be excluded to participate in the assem-
ably of the AdoMet-binding pocket. In the case of EcoRII methyltransferase, deletion of 97 amino acids (two amino acids prior to motif I) resulted in a decrease in enzyme activity, whereas further deletions caused a complete loss of activity (34). In a previous study, Haese et al. (19) reported that further deletion of motifs I and IIY abolished AdoMet-binding activity. This shows that at least one of the motifs is involved in AdoMet binding. The glycine-rich motif I is a common element in the sequences of AdoMet-dependent methyltransferases (4, 14, 16). X-ray structure determination of different methyltransferases revealed a considerable flexibility concerning the location of motif I in the polypeptide chains. In the case of the TaqI and HhaI DNA methyltransferases, motif I is located near the N terminus, forming the AdoMet-binding site, whereas the DNA-binding site is C-terminal (13, 35–37). In contrast, in the case of the PvuII DNA methyltransferase, motif I is located closer to the C terminus (38). The AdoMet-binding site of glycine N-methyltransferase from rat liver is located near the C terminus, buried in an additional S domain (39), similar to chemotaxis receptor methyltransferase CheR from Salmonella typhimurium, where the AdoMet-binding pocket is formed by the C-terminal domain (40). As shown in the case of catabol O-methyltransferase, motif II (corresponds to motif IV in DNA nomenclature) interacts with neither the substrate nor the cofactor (41). A similar finding was observed in the case of CheR from S. typhimurium. Truncation of a small part of the C terminus (38 amino acids) of the N-methyltransferase domain of enniatin synthetase and also deletions of internal sequences containing motifs I, IIY, IV, and V (REP-pSKL16BPAA65) (19) as well as only motif V (pM6291–497), which contains an absolutely conserved Phe (Fig. 1), yielded inactive protein. The highly conserved Asn in motif IV (Fig. 1) of the N-methyltransferase domains of peptide synthetases is also found in methyltransferases such as TaqI and glycine N-methyltransferase from rat (35, 36, 42). Cheng et al. (13) have suggested that the side chain of Asn acts as a donor in a hydrogen bond to the target adenine and therefore allows direct transfer of the activated methyl group of AdoMet. Interestingly, the deletion variant pM74413–476, with an internal deletion at the C terminus, gave inactive protein, too. From this result, we conclude that AdoMet binding also depends on structural elements downstream of motif V. Further work is necessary to study the role of the C-terminal region in cofactor binding.

There is some evidence that motif IVY of the N-methyltransferase domain of enyn1 also plays a role in cofactor binding (17). This is supported by the fact that motif IVY shows high identity to cyan1 methyltransferase domains (see Fig. 1). A tyrosine (Tyr<sup>136</sup>) has been reported to form an adduct with AdoMet after photolabeling of rat guanidinoacetate methyltransferase (43). Tyr<sup>136</sup> is located 66 amino acid residues C-terminal to a conserved sequence (E<sub>64</sub>L/D/E(V/L)UGGXXG) that shows high similarity to motif I. This led us to the assumption that the tyrosine in motif IVY is also part of the binding pocket of the cofactor. To elucidate this assumption, we constructed four point mutants in which the tyrosine was replaced by Ala, Val, Ser, and Phe, respectively. Replacement of tyrosine by the amino acids with aliphatic side chains yielded proteins with strongly reduced binding capacity (19–28% compared with the wild-type), whereas the Phe mutant restored ~70% of AdoMet-binding activity. Therefore, we conclude that the tyrosine in motif IVY is crucial for the ability of the enzyme to renature properly or for binding AdoMet. It should be noted that the aromatic ring of the tyrosine might be critical for the N-methyltransferase AdoMet-binding activity and that the hydroxyl moiety is dispensable. Work is in progress to express the whole enyn1 gene and to test the influence of N-methyltransferase variants on the overall reaction of the multienzyme.