The Role of MbtH-like Proteins in the Adenylation of Tyrosine during Aminocoumarin and Vancomycin Biosynthesis*

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Background: L-tyrosine adenylation is a key step in aminocoumarin antibiotic and vancomycin biosynthesis.

Results: Several, but not all tyrosine-adenylating enzymes require MbtH-like proteins for activity, forming heterotetrameric complexes. A single L→M mutation creates an MbtH-independent enzyme.

Conclusion and Significance: MbtH-like proteins are essential tools in the combinatorial biosynthesis of antibiotics.

SUMMARY

MbtH-like proteins consist of approximately 70 amino acids and are encoded in the biosynthetic gene clusters of non-ribosomally formed peptides and other secondary metabolites derived from amino acids. Recently, several MbtH-like proteins have been shown to be required for the adenylation of amino acid in non-ribosomal peptide synthesis. We now investigated the role of MbtH-like proteins in the biosynthesis of the aminocoumarin antibiotics novobiocin, clorobiocin and simocyclinone D8 and of the glycopeptide antibiotic vancomycin. The tyrosine-adenylating enzymes CloH, SimH and Peca361.18, involved in the biosynthesis of clorobiocin, simocyclinone D8 and of the glycopeptide antibiotic vancomycin. The tyrosine-adenylating enzymes CloH, SimH and Peca361.18, involved in the biosynthesis of clorobiocin, simocyclinone D8 and vancomycin, respectively, required the presence of MbtH-like proteins in a 1:1 molar ratio, forming heterotetrameric complexes. In contrast, NovH involved in novobiocin biosynthesis showed activity in the absence of MbtH-like proteins. Comparison of the active centers of CloH and NovH showed only one amino acid to be different, i.e. L383 versus M383. Mutation of this amino acid in CloH (L383M) indeed led to MbtH-independent adenylation activity. All investigated tyrosine-adenylating enzymes exhibited remarkable promiscuity for MbtH-like proteins from different pathways and organisms. YbdZ, the MbtH-like protein from the expression host E. coli, was found to bind to adenylation enzymes during expression and to influence their biochemical properties markedly. Therefore, the use of ybdZ-deficient expression hosts is important in biochemical studies of adenylation enzymes.

The adenylation of amino acids is a key step in the biosynthesis of many antibiotics (e.g. vancomycin, daptomycin), immunosuppressants (e.g. cyclosporine A), siderophores (e.g. enterobactein, mycobactein) and other bioactive molecules (1). The activated amino acids can be assembled to peptides by non-ribosomal peptide synthases (NRPSs), leading e.g. to the backbone of vancomycin, or can serve as precursors of non-peptidic antibiotics like novobiocin, clorobiocin and simocyclinone D8 (Fig. 1). Approximately half of the biosynthetic gene clusters for non-ribosomally formed peptides, as well as the gene clusters for clorobiocin and simocyclinone D8, contain so-called mbtH-like genes. These small genes are named after mbtH contained in the gene cluster for the siderophore mycobactin in Mycobacterium tuberculosis which codes for a 71 aa protein. The function of mbtH-like genes has remained enigmatic for many years. First proof that these genes are essential for secondary metabolite production was provided by a gene inactivation and complementation study of our group in clorobiocin biosynthesis (2) and by a similar study in the biosynthesis of coelichelin and calcium-dependent antibiotic (3). In vivo investigations were complicated by the fact that
The role of MbtH-like proteins in the adenylation of tyrosine

many bacterial genomes contain several mbtH-like genes which can functionally replace each other. The importance of a specific mbtH-like gene for the biosynthesis of a secondary metabolite can only be assessed after all other mbtH-like genes in the genome have been inactivated (2,3). However, in vivo studies could not define the precise physiological function of the mbtH-like genes, e.g. in catalysis, regulation, transport or protein-protein interactions. The three-dimensional structures of two MbtH-like proteins have been experimentally determined (4-5), but again this did not allow to recognize their function.

The first biochemical evidence for the function of MbtH-like proteins in non-ribosomal peptide biosynthesis has recently been provided in two rapid reports by Felnagle et al. (6) and Zhang et al. (7). Additional data were presented as part of a study on glidobactin biosynthesis (8). These reports showed that MbtH-like proteins interact with adenylylating enzymes which are part of NRPSs. In vitro, the adenylation activity of these enzymes was strongly stimulated by addition of MbtH-like proteins. Out of the five adenylation domains which activate the different amino acids required for capreomycin biosynthesis, three were dependent on the presence of the MbtH-like protein CmnN, while the two others were not. The reason for this difference is unknown (6). The heterologous expression of adenylylating enzymes in E. coli was found to be difficult or even impossible unless the respective MbtH-like protein was co-expressed simultaneously (6-8). From these data, Imker et al. (8) concluded that MbtH-like proteins act as activators, chaperones or both in NRPS assembly line. MbtH-like proteins form complexes with the adenylylating enzymes, but the stoichiometry of these complexes has remained unclear. After purification of such complexes, the molar ratio of adenylylating enzyme to MbtH-like protein was reported as 1:0.42 by Felnagle et al. (6), and as 1:1.7 by Imker et al. (8). If the adenylylating enzyme CmnO and the MbtH-like protein CmnN were purified separately, a mixture of both in a 1:1 molar ratio showed only low activity. 10-fold higher activity was observed when the MbtH-like protein was added in a 16- to 32-fold molar excess (6). Therefore, the composition of the complex of MbtH-like proteins with adenylylating enzymes is yet obscure.

As pointed out in a recent review on the occurrence and functions of MbtH proteins (9), the requirement of many adenylylating enzymes for MbtH-like proteins implicates that the correct use of mbtH-like genes is a crucial factor for the success of combinatorial biosynthesis experiments. Our group is working extensively on the combinatorial biosynthesis of new aminocoumarin antibiotics (10,11). We were therefore interested to investigate the role of mbtH-like genes in the formation of these antibiotics. The biosynthesis of aminocoumarins involves the adenylation of L-tyrosine, followed by its attachment to a peptidyl carrier protein (PCP) domain and its β-hydroxylation by a cytochrome P450 enzyme (Fig. 2A) (12,13). The same reaction sequence is part of the biosynthesis of vancomycin and related glycopeptide antibiotics (14). In contrast to non-ribosomal peptide biosynthesis, however, the resulting β-hydroxytyrosine (β-OH-Tyr) is not transferred by a condensation domain to a peptide backbone. Rather, in the biosynthesis of vancomycin and the related balhimycin, β-OH-Tyr is liberated from the PCP domain by a thioesterase and subsequently activated by another specific adenylation domain of the vancomycin or balhimycin NRPS (Fig. 2A) (15). In aminocoumarin biosynthesis, β-OH-Tyr is oxidized and cyclized to 4,7-dihydroxy-3-aminoacoumarin, which is liberated from the PCP domain and subsequently connected to an acyl moiety via an amide bond (Fig. 2A). In contrast to non-ribosomal peptide biosynthesis, formation of this amide bond does not involve the intermediary attachment of the acyl moiety to a PCP domain (12,16).
identical reaction sequence in novobiocin biosynthesis does not (2). Given the fact that the tyrosine-adenylating enzyme NovH has the same size (600 aa) as CloH, and both proteins share 83% identity in their amino acid sequence (Fig. S1, Supplemental Data), this difference in the requirement for MbtH-like proteins is puzzling. We decided to express the tyrosine-adenylating enzymes NovH, CloH, SimH and Pzca361.18 as well as the cognate MbtH-like proteins, and to biochemically investigate these enzymes and the complexes formed by them.

**EXPERIMENTAL PROCEDURES**

**Chemicals and radiochemicals.** - Tetrasodium [\(^{32}\)P]pyrophosphate (3.38 TBq mmol\(^{-1}\)) was obtained from Perkin Elmer. L-tyrosine was purchased from Merck.

**Cloning of the genes** novH, cloH simH, cloY, simY, cdaX, pcza361.18 and orf1van - The genes novH, cloH, simH, cloY and simY were amplified from cosmids containing the respective clusters (17-19) by polymerase chain reaction. The gene cdaX was amplified from chromosomal DNA of *S. coelicolor* M512. Primers novH\(_F\)_NdeI (5’-GGG AAT TC CAT ATG TGT TCA ACA CAC GTG CGA ACG-3’), novH\(_R\)_XhoI (5’-GCC CTC GAG TCA CTC CTC CAG GCC GTG CGC TA-3’), cloH\(_F\)_NdeI (5’-GGG AAT TC CAT ATG TGT TCA CTC CCC GAG GGT GC GGC CGC-3’), cloH\(_R\)_XhoI (5’-GCC CTC GAG TCA CTC CTT CAC GCC GTG CGC-3’), simH\(_F\)_NdeI (5’-GGG AAT TC CAT ATG TGT TCA CTC CCC GAG GGT GC GGC CGC-3’), simH\(_R\)_NdeI (5’-GGG AAT TC CAT ATG TGT TCA CTC CCC GAG GGT GC GGC CGC-3’), cloY\(_F\)_NdeI (5’-GGG AAT TC CAT ATG TGT TCA CTC CCC GAG GGT GC GGC CGC-3’), cloY\(_R\)_XhoI (5’-GGG AAT TC CAT ATG TGT TCA CTC CCC GAG GGT GC GGC CGC-3’), cdaX\(_F\)_NdeI (5’-GGG AAT TC CAT ATG TGT TCA CTC CCC GAG GGT GC GGC CGC-3’), cdaX\(_R\)_NdeI (5’-GGG AAT TC CAT ATG TGT TCA CTC CCC GAG GGT GC GGC CGC-3’), were used to amplify novH, cloH, simH, cloY, simY and cdaX. The introduced NdeI and XhoI restriction sites of each primer are highlighted in bold. The thrombin cleavage site is underlined. Expression in *E. coli* was confirmed by sequencing. The resulting plasmids pBB28 (simY), pBB34 and pBB37 (cloH) were transformed into *E. coli* Rosetta2 (DE3). pBB25 (simH), pBB26 (cloH), pBB35 (cloH and cloY), pBB43 (pcza361.18) and pBB44 (novH) were transformed into *E. coli* BL21(DE3) either with or without carrying an ybdZ deletion, and carrying pSU20\(_sfp\), a plasmid containing the gene for the Sfp phosphopantetheinyl transferase from Bacillus subtilis for expression of the holoenzymes (20).

**Generation of *Δ*ybdZ E. coli BL21(DE3) strain** - The *E. coli* BL21(DE3) ΔybdZ mutant was generated in BL21(DE3)/pIJ790 using Red/ET-mediated recombination (21). An apramycin resistance cassette [acc(3)IV] was amplified from plasmid pIJ773 (21). The primers used for PCR were as follows: ybdZ\(_F\) (5’-GGG AAT TC CAT ATG TGT TCA CTC CCC GAG GGT GC GGC CGC-3’), ybdZ\(_R\) (5’-GGG AAT TC CAT ATG TGT TCA CTC CCC GAG GGT GC GGC CGC-3’), simH\(_F\)_NdeI (5’-GGG AAT TC CAT ATG TGT TCA CTC CCC GAG GGT GC GGC CGC-3’), simH\(_R\)_NdeI (5’-GGG AAT TC CAT ATG TGT TCA CTC CCC GAG GGT GC GGC CGC-3’), cloY\(_F\)_NdeI (5’-GGG AAT TC CAT ATG TGT TCA CTC CCC GAG GGT GC GGC CGC-3’), cloY\(_R\)_NdeI (5’-GGG AAT TC CAT ATG TGT TCA CTC CCC GAG GGT GC GGC CGC-3’), cdaX\(_F\)_NdeI (5’-GGG AAT TC CAT ATG TGT TCA CTC CCC GAG GGT GC GGC CGC-3’), cdaX\(_R\)_NdeI (5’-GGG AAT TC CAT ATG TGT TCA CTC CCC GAG GGT GC GGC CGC-3’), were used to amplify novH, cloH, simH, cloY, simY and cdaX. The introduced NdeI and XhoI restriction sites of each primer are highlighted in bold. The amplified products were purified by gel electrophoresis, digested with corresponding restriction enzymes and ligated into pET28a for expression as N-terminally His\(_{6}\)-tagged fusion proteins. The nucleotide sequences of pcza361.18 and orf1van were optimized for expression in *E. coli* and synthesized commercially by Mr. Gene (Regensburg, Germany). The two genes were excised from their vectors with NdeI and XhoI and ligated into vector pET28a using the same restriction sites. For coexpression of CloH and CloY, both genes were ligated in the dual expression vector pETDuet1 (Novagene). The cloH gene was amplified with primers introducing a thrombin restriction site at the N-terminus: cloH\(_F\)_BamHI (5’-CGG GAT CCC GTG TGC CGT GGT TCC TTA AAC ACG GGT CTG AAC AAG GC-3’), cloH\(_R\)_NotI (5’-A TAA GAA TGC GTC TCA CCC GAG GGT CGC-3’). Restriction sites of each primer are highlighted in bold, the thrombin cleavage site is underlined. cloY was amplified with the same primers as before and ligated via the restriction sites NdeI and XhoI, resulting in an untagged protein. The correct DNA sequences of the entire genes were confirmed by sequencing. The resulting plasmids pBB28 (simY), pBB34 and pBB37 (cloH) were transformed into *E. coli* Rosetta2 (DE3). pBB25 (simH), pBB26 (cloH), pBB35 (cloH and cloY), pBB43 (pcza361.18) and pBB44 (novH) were transformed into *E. coli* BL21(DE3) either with or without carrying an ybdZ deletion, and carrying pSU20\(_sfp\), a plasmid containing the gene for the Sfp phosphopantetheinyl transferase from Bacillus subtilis for expression of the holoenzymes (20).
mutants was confirmed by PCR with chromosomal DNA.

Site directed mutagenesis of CloH - Site directed mutagenesis of CloH was carried out by PCR amplification of the template cloH_pGEMT using the QuickChange Site Directed Mutagenesis Kit (Stratagene). Reactions were performed according to the manufacturer’s instructions with primers of cloH_L383M_F (5’-CCC GAC TTG ACC GCG CAG aTG TTC GTG GCC AAC CCG T -3’), and the reverse complement. The base changes are indicated by small letters. The PCR program consisted of an initial denaturation at 94 °C for 2 min followed by 18 cycles of 94 °C for 10 s, 55 °C for 30 s, and 68 °C for 6 min. The template DNA was digested with 10 units of DpnI for 1 h at 37 °C before transformation. The correct DNA sequence of the entire gene was confirmed by sequencing, and the DNA fragment was cloned in pET28a via NdeI and XhoI.

Purification of His-tagged Proteins - 35 ml of an overnight culture in Luria-Bertani medium (50 μg ml⁻¹ kanamycin, 25 μg ml⁻¹ chloramphenicol) of cells harboring the respective expression plasmid were used to inoculate 1 liter of terrific broth (50 μg ml⁻¹ kanamycin, 25 μg ml⁻¹ chloramphenicol). The cells were grown at 37 °C to an OD₆₀₀ of 0.6, cooled to 20 °C, induced with 0.4 mM of IPTG and allowed to grow for an additional 14 h at 20 °C. The cells from each culture were harvested by centrifugation (15 min at 4800×g) and resuspended in 25 ml of buffer A (50 mM Tris–HCl pH 8.0, 0.5 M NaCl, 20 mM imidazole, 5 mM β-mercaptoethanol, 10 % glycerol) per 10 g cells. 1% Tween 20 and 0.5 mg/ml lysozyme were added, resuspended cells were broken by a Branson sonifier and the cell debris was removed by centrifugation (45 min at 35,000×g). The supernatant was applied to a nickel-nitrilotriacetic acid-agarose resin column (GE Healthcare) according to the manufacturer’s instructions, using a linear gradient of 0–60% 250 mM imidazole (in 50 mM Tris-HCl pH 8.0, 500 mM NaCl, 10% glycerol, 10 mM β-mercaptoethanol) in 60 min for elution. Fractions containing the protein were pooled and further purified with a HiLoad 26/60 Superdex 200 column (Amersham Pharmacia Biotech) that had been equilibrated with 20 mM Tris-HCl pH 8.0, 150 mM NaCl and 2 mM dithiothreitol, concentrated using an Amicon Ultra 10000 MWCO centrifugal filter (Millipore) and stored at -80 °C. Concentrations of the purified proteins were measured spectrophotometrically at 280 nm using the calculated extinction coefficients. The N-terminal His-tags of the MbtH-like proteins were removed by incubation with 0.4 units thrombin (Sigma) per milligram of MbtH protein for 8 h at 4 °C before gel filtration. The tyrosine-adenylating enzymes were used without further modifications. The MbtH-like proteins were obtained in the following amounts per liter culture CloY 6.9 mg, SimY 27 mg, CdaX 23.8 mg and Orf1van 30.2 mg. From the ybdZ expression hosts, the tyrosine-adenylating enzymes were obtained in the following amounts: NovH 38.6 mg, CloH 2.83 mg, SimH 9.4 mg, Peza361.18 10.3 mg. From the ybdZ expression hosts, the yields were: NovH 64 mg, CloH 11.3 mg, Peza361.18 21.6 mg. The protein yield for the coexpression of CloH and CloY was 4.8 mg per liter culture using the ybdZ expression host. The mutant protein CloHL383M yielded 1.46 mg per 100 ml culture.

ATP-[^32]P]PP, Exchange Assays - ATP-[^32]P]PP₄ exchange assays (100 μl) contained 95 mM Tris-HCl (pH 8.0), 5 mM MgCl₂, 5 mM tris(2-carboxyethyl)phosphine hydrochloride (TECP), 2 mM ATP, 1.5 mM L-tyrosine, 1 μM of the respective tyrosine-activating enzyme, 1.2 μM of the respective MbtH-like protein (unless other amounts are indicated) and 1 mM[^32]P]pyrophosphate (Perkin Elmer). The reactions were initiated by the addition of the tyrosine-activating enzyme and allowed to proceed for 5 min at 30 °C, and then quenched with 500 μl of a suspension of activated charcoal (1.6% w/v) in quenching buffer (4.5% (w/v) tetrasodium pyrophosphate and 3.5% perchloric acid in water). The charcoal was pelleted by centrifugation, washed with quenching buffer, resuspended in 0.5 ml of water and added to 9 ml scintillation liquid. Radioactivity was quantified in a scintillation counter. Data reported are means of two independent reactions. Activity was expressed in katal (kat) (22). For investigation of enzyme kinetics, nonlinear regression was performed with Graph Pad Prism 5.0 (GraphPad Software Inc., La Jolla, USA).

Analytical gel filtration - Analytical gel filtration was performed using a Superdex 200, 10/300 GL column (GE Healthcare) with a 24 ml bed resin and a buffer system of 20 mM Tris-HCl, pH 8.0, and 150 mM NaCl. A standard curve plotting the log of molecular weight standards versus the calculated Kᵥ was generated using the following protein standards: ribonuclease A, chymotrypsinogen, ovalbulmin,
The role of MbtH-like proteins in the adenylation of tyrosine

albumin, aldolase, catalase and ferritin (GE Healthcare). This standard curve was used to calculate the observed molecular weight of SimH and SimH/SimY complexes.

RESULTS

Expression and purification of tyrosine adenylylating enzymes and MbtH-like proteins - The tyrosine-adenylating enzymes NovH, CloH, SimH and Pcza361.18 as well as the MbtH-like proteins CloY, SimY and Orf1van were expressed in E. coli in form of N-terminally His-tagged proteins and purified by Ni²⁺ affinity chromatography, followed by gel chromatography. The MbtH-like proteins were subjected to thrombin cleavage in order to remove the His-tag before gel chromatography (see Experimental Procedures).

The genome of E. coli contains an mbtH-like gene, ybdZ, in the gene cluster for the siderophore enterobactin. Felnagle et al. (6) have shown that purified YbdZ can activate adenylylating enzymes of different NRPSs, albeit with low efficiency. In order to exclude the possibility that YbdZ co-purifies with the expressed adenylylating enzymes and interferes with subsequent investigations, we deleted the ybdZ gene from the E. coli expression host, utilizing the same Red/ET-mediated recombination strategy as described in another study of our group (11). The expression and purification of the adenylylating enzymes was carried out both using the unmodified and using the ΔybdZ expression host. Notably, the yields of the adenylylating enzymes from the ΔybdZ expression strain were 2-4 times lower than from the unmodified strain (see Experimental Procedures). Using the unmodified strain (see Experimental Procedures).

The results depicted in Fig. 3 were obtained with proteins expressed in the ΔybdZ E. coli strain. If CloH and Pcza361.18 were expressed in an E. coli strain with intact ybdZ, results were markedly different: in this case, tyrosine-adenylating activity of CloH and Pcza361.18 alone could clearly be detected, amounting to 20-30 % of the activity measured after addition of the respective MbtH-like protein. This suggests that YbdZ of E. coli had been copurified with the heterologously expressed adenylylating enzymes. In contrast,
SimH purified from strains with intact ybdZ gene did not show more activity than protein purified from a ΔybdZ strain.

The tyrosine-activating enzyme NovH is active in the absence of MbtH-like proteins - In clear contrast to CloH, SimH and Pcza361.18, NovH purified from the ΔybdZ strain showed activity in the absence of an MbtH-like protein (Fig. 3). This MbtH-independent activity is in agreement with our previous in vivo study which showed that biosynthesis of novobiocin, but not of clorobiocin, can be readily observed in strains which completely lack mbtH-like genes (2). However, activity of NovH was only moderate, and was stimulated markedly upon addition of CloY (Fig. 3). In view of the fact that the novobiocin cluster does not contain an mbtH gene, this result was unexpected.

Enzyme kinetics of the adenylation of L-tyrosine - We determined the K_m and k_cat values for the adenylation of tyrosine catalyzed by either NovH alone or by an equimolar mixture of NovH and CloY, using the pyrophosphate exchange assay. The K_m value for L-tyrosine was nearly identical in both cases, but addition of CloY clearly increased the observed turnover number (Table 1). We noticed, however, that the observed K_m value (275 µM) was more than five times lower than the K_m value of 1390 µM determined for the same enzyme in an earlier study by Chen and Walsh (13). In that study, NovH had been expressed in an E. coli strain with intact ybdZ. This prompted us to repeat the investigation of NovH, this time using a protein expressed in an ybdZ- strain. Indeed, that protein showed a K_m value of 1278 µM for L-tyrosine, very similar to the value previously determined by Chen and Walsh (13). The turnover number of the enzyme from the ybdZ- strain (0.12 s^-1) was 1.6 times higher than that of the AybdZ strain. It appears therefore likely that YbdZ had been copurified with NovH, similar as we observed for CloH and Pcza361.18. These data show that MbtH-like proteins influence both the turnover number of the tyrosine-adenylating enzyme and their K_m value for the amino acid. Although the copurified YbdZ increased the turnover number observed for NovH, it actually decreased the catalytic efficiency (k_cat K_m^-1) due to the increased K_m (Table 1).

As mentioned above, CloH, SimH and Pcza361.18 showed only very low activity in the absence of MbtH. Therefore, no kinetic investigations could be performed with these proteins alone. However, when the cognate MbtH-like protein was added, kinetic data could be readily obtained. The K_m values for L-tyr ranged from 85 to 186 µM and the k_cat values from 0.50 to 1.91 s^-1 (Table 1). For all investigated proteins, the dependency of the reaction velocity on L-tyrosine concentration is depicted in Fig. S3 (Supplemental Data).

Stoichiometry of the SimH/SimY and CloH/CloY complexes - Previous investigations (6-8) had suggested that adenylylating enzymes and MbtH-like proteins form complexes, but the stoichiometry of these complexes had remained unclear (see Introduction). We now investigated the adenylylating activity of SimH and CloH in the presence of different amounts of SimY and CloY, respectively. As depicted in Fig. 4, activity steadily increased with increasing amounts of MbtH-like protein until a molar ratio of 1:1 was reached. Addition of further amounts of MbtH-like protein did not lead to a further increase of activity, suggesting that the active complex contains both proteins in a molar ratio of 1:1.

We subsequently carried out an analytical gel chromatography of SimH alone and of a SimH/SimY mixture. Calibration of the column with reference proteins allowed to determine the apparent molecular weight of the eluted proteins. SimH (theoretical molecular weight 107.3 kDa) aggregated in aqueous solution, showing a dominant peak at approximately 650 kDa (Fig. 5A). In contrast, the mixture of SimH and SimY showed a dominant peak at 208 kDa. This is in reasonable agreement with the calculated molecular weight of a heterotetrameric (SimH)2(SimY)2 complex (229 kDa). We isolated this peak from the analytical column. SDS-PAGE analysis readily showed both SimH and SimY protein as components of the complex (Fig. 5B). Similar results were obtained for CloH and CloY (Fig. S4, Supplemental Data). However, CloH alone formed dimers in solution, and therefore in analytical gel chromatography the difference between CloH alone and the CloH/CloY complex was low.

Coexpression of CloH and CloY - Felnagle et al. (6) had reported that the adenylylating enzymes CmnO and VioO showed moderate activity upon addition of the MbtH-like proteins CmnN and VioN. However, much higher activity could be obtained when CmnO or VioO (as N-terminally his-tagged proteins) were simultaneously expressed with CmnN or VioN (as untagged proteins), followed by copurification of the proteins in form of the...
resulting complexes. From the supplemental data of the publication of Felnagle et al. (6), it can be estimated that the activities of the coexpressed CmnO/CmnN and VioO/VioN were 10-fold and 75-fold higher than the activities of complexes obtained by mixing of separately purified proteins. The reason for this observation is unknown.

We decided to coexpress N-terminally histagged CloH and untagged CloY using a pETDUET1 vector (Novagene). As expected, purification by Ni$^{2+}$ affinity chromatography and by gel chromatography resulted in an active tyrosine-adenylating enzyme, indicating that CloH and CloY had formed a complex resulting in their copurification. However, in contrast to the results of Felnagle et al. (6), this complex was not more active, but 50% less active than a mixture of separately purified CloH and CloY proteins. Addition of CloY to the complex increased the activity but again not to a value higher than observed for a mixture of separately purified CloH and CloY. This indicates that some CloY may have been lost from the complex during purification, causing a loss of activity which could be restored by external addition of CloY.

**Stimulation of tyrosine-adenylating enzymes by cognate and noncognate MbtH-like proteins** - Each of the four tyrosine-adenylating enzymes NovH, CloH, SimH and Pcza361.18 was assayed with each of the MbtH-like proteins CloY, CdaX, SimY and Orf1van in a molar ratio of 1:1.2. As shown in Fig. 6, all enzymes were stimulated by all MbtH-like proteins. In each case, addition of CloY resulted in the highest activity. Therefore, SimH and Pcza361.18 did not show preference for their cognate MbtH-like proteins, i.e. SimY and Orf1van, respectively.

**Generation of an L383M mutant of CloH** - NovH showed tyrosine-activating activity in the absence of an MbtH-like protein. This is in contrast to CloH, which shows 83% sequence identity to NovH. A sequence alignment (Fig. S1, Supplemental Data) shows no conspicuous differences between both proteins. No experimentally determined structure is available for NovH or CloH. We therefore modeled their structure after PheA (PDB ID: 1AMU). PheA is the phenylalanine-adenylating domain of the GrsA protein, which is part of the NRPS responsible for gramicidin S biosynthesis (23).

PheA (556 aa) shows 37 and 39% identity to the adenylation domains of NovH and CloH, respectively. A comparison of the structural models of NovH and CloH models showed that all amino acids which are different between the two proteins are located distantly from the active center, with one single exception: in position 383, which is close to the active center, NovH contains a methionine and CloH a leucine residue. PheA contains a lysine residue in the corresponding position (K396). Modeling suggested that the size and nature of the residue in this position may influence the orientation of the side chains of a conserved tyrosine residue (Y397 in NovH/CloH, Y409 in PheA) and a neighboring glutamate residue (E316 in NovH/CloH and E327 in PheA). These residues assist in the binding of the Mg$^{2+}$ ion in the active center. The positions of these residues were similar in the structure of PheA and the model of NovH, but different in model of CloH.

We carried out a site-directed mutagenesis of CloH, mutating the genuine L383 to M as found in NovH. The resulting mutant protein clearly showed some adenylating activity in the absence of CloY (0.22 nKat mg$^{-1}$), amounting to 33% of the activity of NovH. In the presence of CloY, the mutant protein showed an activity of 2.45 nKat mg$^{-1}$ i.e. 36% of the value determined for the genuine CloH in the presence of CloY. Therefore the L383M mutation was successful in generating an MbtH-independent activity, but it also reduced the optimal activity of the enzyme in the presence of CloY.

**DISCUSSION**

The present study shows that the L-tyrosine-adenylating enzymes CloH and SimH of aminocoumarin antibiotic biosynthesis, and Pcza361.18 of vancomycin biosynthesis, require the presence of MbtH-like proteins for their catalytic activity. In the absence of MbtH-like proteins, their activity is lowered by 99.0-99.8%. This is in accordance with our previous observations that inactivation of all mbtH-like genes in a clorobiocin producer strain lowered production of this aminocoumarin antibiotic by 99.3% (2).

In contrast, the L-tyrosine-activating enzyme NovH of novobiocin biosynthesis showed significant activity also in the absence of any MbtH-like protein. Again, this is in accordance with the in vivo data which showed that even after inactivation of all mbtH-like genes, novobiocin was still produced, in approximately half of the amount formed in an mbtH$^+$ strain (2).
For optimal activity of the adenylating enzyme, the respective MbtH-like protein was required in a molar ratio of 1:1. The two proteins formed complexes with each other which coeluted during chromatographic purification. Analytical gel chromatography indicated that the complex contained two monomers of the adenylylating enzyme. We therefore suggest that the adenylylating enzymes form heterotetrameric complexes with the MbtH-like proteins, i.e., of the type (SimH)2(SimY)2.

We found that the tyrosine-adenylating enzymes SimH and CloH required the MbtH-like proteins SimY and CloY in a molar ratio of 1:1. In contrast, Felnagle et al. (6) found that the β-lysine-adenylating enzymes CmnO and VioO require the MbtH-like proteins CmnN and VioN in 16- to 32-fold molar excess for optimal activity in vitro. In contrast to the enzymes investigated in our study, CmnO and VioO are in vivo part of large NRPS assembly lines, composed of several proteins. The absence of the other proteins of the NRPS may possibly affect their conformation, activity and stability in vitro, influencing also their interaction with MbtH-like proteins. In contrast, NovH, CloH, SimH and Pcz361.18 most likely do not interact directly with NRPS assembly lines, but release their products into solution after enzymatic modification (Fig. 2A). We could obtain these tyrosine-adenylating enzymes in good activity by separate expression and subsequent mixing with the MbtH-like proteins in vitro. In contrast, Felnagle et al. (6) reported that separate expression of CmnO and VioO and subsequent mixing with the MbtH-like proteins CmnN and VioN gave low activity. Coexpression of CmnO with CmnN (or VioO with VioN) gave much higher activities, indicating some misfolding of the proteins during separate expression.

The strong requirement of adenylating enzymes for MbtH-like proteins raises the question whether amino acid residues of MbtH-like proteins are involved in catalysis in the active center of the adenylylating enzyme. We modeled the structure of CloH and NovH after the experimentally determined structure of PheA, a phenylalanine adenylylating domain from the biosynthetic gene cluster of gramicidin S (23). This gene cluster does not contain an mbtH-like gene, and purified PheA shows high activity without addition of an MbtH-like protein (24). The genome sequence of the gramicidin S producer strain is not available, but the closely related strain Brevibacillus brevis NBRC 100599 has been sequenced. It shows an NRPS gene cluster closely related to the gramicidin gene, but no mbtH-like gene in the entire genome. PheA is therefore expected to be an MbtH-independent enzyme. Modeling of NovH and CloH showed that all amino acids expected to be in contact with the substrates ATP and L-tyrosine and with the cofactor Mg2+, were identical in both proteins. It appears therefore unlikely that amino acid residues of CloY form direct contacts with the substrates in the active center of the CloH/CloY complex. Rather, binding of CloY may induce a conformational change in the structure of CloH which enhances activity. Some support for this hypothesis can be derived from our mutational experiment: exchange of Leu383 in CloH for Met (as found in NovH) clearly led to an activity of the enzyme in absence of MbtH-like proteins, suggesting that this amino acid exchange is one of the structural differences which are responsible for the MbtH-independent activity of NovH. M383 does not have direct contacts to the substrates but appears to influence the conformation of other residues in the active center. Conformational changes in adenylylating enzymes have been described previously, especially concerning a rotation of the PCP domain during thioester formation. The catalytic site is formed by the interface between the A and PCP domains, which are connected by a flexible hinge. It includes a highly conserved and functionally important loop similar to the P-loop (25) found in ATPases and GTPases. This loop wraps around the triphosphate of ATP. During the adenylate formation changes in the active center as well as a displacement of the P-loop of ~3.5 Å can be observed (26). The thioester formation includes a major structural change where the PCP domain rotates by ~140° burying the ATP-binding site (27). MbtH-like proteins presumably affect only the adenylate-forming part of the reaction, and the available data do not indicate their involvement in the rotation of the PCP domain.

The present study, in accordance with previous in vivo (2,3) and in vitro studies (6-8), shows that adenylylating enzymes have a remarkable promiscuity for MbtH-like proteins from various pathways and organisms (Fig. 6). E.g., the tyrosine-activating enzymes investigated in our study were efficiently activated by CdaX, the MbtH protein encoded in the biosynthetic gene cluster of the calcium-dependent antibiotic (CDA) from Streptomyces coelicolor A(3)2 (3). The peptide antibiotic CDA
does not contain a tyrosyl residue or a residue derived from tyrosine (28). Therefore, the genuine function of CdaX is most likely related to the adenylation of another amino acid than tyrosine. For experiments in combinatorial biosynthesis involving genes for aminoacyl-adenylate forming enzymes, inclusion of an mbtH-like gene may be crucial, but there is flexibility regarding which mbtH-like gene is chosen.

The biosynthetic gene cluster of novobiocin does not contain an mbtH-like gene, and correspondingly NovH showed activity in the absence of an MbH-like protein. Unexpectedly, however, the activity of NovH was still markedly stimulated by MbtH-like proteins such as CloY (Fig. 6). This finding prompted us to initiate a genome sequencing of the producer strain Streptomyces spheroides NCIMB 11891 (syn. S. niveus) and to search for other mbtH orthologs which may assist in novobiocin biosynthesis in this strain. Completion of the sequence is still in progress, but available data confirm that the novobiocin gene cluster and its immediate vicinity do not contain an mbtH-like gene. However, the genome contains at least two mbtH orthologs situated distantly from the novobiocin cluster. The stimulation of NovH by MbH-like proteins shows that, even when a given gene cluster does not contain an mbtH-like gene, a stimulation of the biosynthesis by mbtH-like genes cannot be excluded. This may need to be considered in the design of combinatorial biosynthesis experiments.

More than 400 mbtH-like genes are currently found in the database. Drake et al. (5) solved the crystal structure of an MbH-like protein from Pseudomonas aeruginosa. The protein displays a new protein fold and is shaped like a thin arrow head, with the point of the arrow formed by the C-terminal α-helix. Sequence comparison of 155 MbH-like proteins showed that the conserved residues, including the three highly conserved tryptophans, all lie on one face of the protein, and the authors suggested that this face may interact with conserved components of NRPSs. Buchko et al. (4) determined by NMR the solution structure of another MbH-like protein from Mycobacterium tuberculosis. The solution structure was similar to the aforementioned crystal structure except for the C-terminus which was highly disordered in solution, despite high sequence conservation of this region in the family of MbH-like proteins.

The authors pointed out that conserved but disordered regions of proteins are associated with binding to multiple partners, and suggested that binding via the disordered C-terminal region may explain the promiscuity of MbH-like proteins for interaction with biosynthetic enzymes from different pathways.

We modeled the structure of our MbH-like proteins after the published structures and tried to dock them to the tyrosine-adenylating enzymes using the Hex Protein Docking Server (http://hexserver.loria.fr). However, this resulted in several possible solutions. Therefore, the structure of these complexes remains speculative until a crystal structure can be determined.

Many biochemical studies have been published on amino acid-adenylating enzymes in secondary metabolism, especially in non-ribosomal peptide synthesis (29-31). In nearly all cases these enzymes have been expressed in E. coli strains containing an intact ybdZ gene. Our data suggest that YbdZ can copurify with adenylation enzymes and that this complex formation can considerably affect the biochemical properties of the purified enzymes. We therefore suggest to use only ΔybdZ expression strains for investigations of amino acid-adenylating enzymes in future, and to carefully re-evaluate previous data for possible interference by YbdZ.

REFERENCES
The role of MbtH-like proteins in the adenylation of tyrosine

FOOTNOTES

*This work has been supported by the Deutsche Forschungsgemeinschaft (SFB 766).

FIGURE LEGENDS

FIGURE 1. Structures of the aminocoumarin antibiotics novobiocin, clorobiocin and simocyclinone D8 and of the glycopeptide antibiotic vancomycin. Parts derived from L-tyrosine are drawn bold.

FIGURE 2. A) Adenylation and β-hydroxylation of tyrosine in the biosynthesis of aminocoumarin antibiotics and vancomycin. B) Genes for the formation of 4,7-dihydroxy-3-aminocoumarin and β-hydroxytyrosine in the biosynthetic gene clusters of novobiocin (nov), clorobiocin (clo), simocyclinone (sim), and vancomycin (pcza).

FIGURE 3. Activities of L-tyrosine-adenylating enzymes in the absence and presence of MbtH-like proteins. The MbtH-like proteins were added in a 1.2-fold molar excess over the tyrosine-adenylating enzymes. Data represents the mean value of two independent reactions. The value for CloH is the mean of six independent reactions.

FIGURE 4. L-tyrosine-adenylating activity of CloH and SimH in the presence of different amounts of the MbtH-like proteins CloY and SimY.

FIGURE 5. A) Molecular weight determination of the SimH/SimY complex by analytical gel chromatography. B) SDS-PAGE of the peak eluting at 208 kDa.

FIGURE 6. Activity of L-tyrosine adenylating enzymes in the presence of different MbtH-like proteins. Activity was determined using the PPi exchange assays.
Table 1: Kinetic parameters of tyrosine-adenylating enzymes in the presence of MbtH-like proteins. Unless indicated otherwise, proteins were expressed in an *E. coli* strain in which the *mbtH*-like gene *ybdZ* has been deleted. Tyrosine-adenylating enzymes and MbtH-like proteins were mixed in a molar ratio 1:1.2. The reaction velocities determined at different tyrosine concentrations and the statistical variation of the parameters are depicted in Fig. S3 (Supplemental Data).

<table>
<thead>
<tr>
<th></th>
<th>$K_m$ for L-tyr [$\mu$M]</th>
<th>$k_{cat}$ [s$^{-1}$]</th>
<th>$k_{cat} K_m^{-1}$ [s$^{-1}$ M$^{-1}$]</th>
</tr>
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<tr>
<td>NovH alone</td>
<td>275</td>
<td>0.079</td>
<td>290</td>
</tr>
<tr>
<td>NovH alone, expressed</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>in <em>ybdZ</em> <em>E. coli</em></td>
<td>1278</td>
<td>0.120</td>
<td>94</td>
</tr>
<tr>
<td>NovH + CloY</td>
<td>277</td>
<td>0.438</td>
<td>1580</td>
</tr>
<tr>
<td>CloH and CloY</td>
<td>186</td>
<td>0.497</td>
<td>2670</td>
</tr>
<tr>
<td>SimH and SimY</td>
<td>164</td>
<td>1.01</td>
<td>6180</td>
</tr>
<tr>
<td>Pcza361.18 and Orf1van</td>
<td>85</td>
<td>1.91</td>
<td>22200</td>
</tr>
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</table>
Figures

The role of MbtH-like proteins in the adenylation of tyrosine

Figure 1

Novobiocin: \( R_1 = -\text{CH}_3 \)  \( R_2 = \)  
Clorobiocin: \( R_1 = -\text{Cl} \)  \( R_2 = \)  
Simocyclinone D8  
Vancomycin
Figures

The role of MbtH-like proteins in the adenylation of tyrosine

Figure 2

A

B
Figure 3

The role of MbtH-like proteins in the adenylation of tyrosine

Figure 3
The role of MbtH-like proteins in the adenylation of tyrosine

Figure 4

![Graphs showing the role of MbtH-like proteins in adenylation of tyrosine](image-url)
Figure 5

A

![Graph showing elution volume and absorbance](image)

- ~650 kDa
- ~208 kDa
- ≤15 kDa

B

![Electrophoresis image](image)

- M
- SimH
- SimY

The role of MbtH-like proteins in the adenylation of tyrosine.
The role of MbtH-like proteins in the adenylation of tyrosine

Figure 6

- NovH
- CloH
- SimH
- Pcza361.18

Relative activity [%]

none
CloY
CdaX
SimY
Orf1van
The role of MbtH-like proteins in the adenylation of tyrosine during aminocoumarin and vancomycin biosynthesis
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