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Research Paper

Exploring the impact of different thioesterase domains for the design of hybrid peptide synthetases

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Abstract

Background: A large number of pharmacologically important peptides are synthesized by multifunctional enzymes, the nonribosomal peptide synthetases (NRPSs). The thioesterase (Te) domain at the C-terminus of the last NRPS catalyzes product cleavage by hydrolysis or complex macrocyclization. Recent studies with excised Te domains and peptidyl-*S*-*N*-acetyl cysteamine substrate substitutes led to substantial insights in terms of cyclization activity and substrate tolerance of these enzymes. Their use in engineered hybrid NRPSs is an interesting but yet only little explored target for approaches to achieve new structural diversity and designed products.

Results: To study the capability of various Te domains to function in hybrid NRPSs, six different Te domains that catalyze different modes of termination in their natural systems were fused to a bimodular model NRPS system, consisting of the first two modules of tyrocidine NRPS, TycA and ProCAT. All Te domains were active in hydrolyzing the enzymatically generated dipeptide substrate D-Phe-Abu from the NRPS template with, however, greatly varying turnover rates. Two Te domains were also capable of hydrolyzing the substrate D-Phe-Pro and partially cyclized the D-Phe-Abu dipeptide, indicating that in an artificial context Te domains may display hydrolytic and cyclization activities that are not easily predictable.

Conclusions: Te domains from heterologous NRPSs can be utilized for the construction of hybrid NRPSs. This is the first comparative study to explore their influence on the product pattern. The inherent specificity and regioselectivity of Te domains should allow control of the desired product cleavage, but can also lead to other modes of termination potentially useful for generating structural diversity. Our results provide the first data for choosing the proper Te domain for a particular termination reaction.

Keywords: Domain exchange; Nonribosomal peptide synthesis; Peptide antibiotic; Rational protein engineering

1. Introduction

Many pharmacologically important natural products such as the antibiotics vancomycin and erythromycin as well as the immunosuppressive agents cyclosporin and rapamycin are assembled on nonribosomal peptide synthetases (NRPSs) or modular polyketide synthases (PKSs). Both classes of enzymes have in common a modular architecture in which each module contains all catalytic prerequisites to incorporate one monomeric building block, typically an amino acid or acetate and propionate, into the growing chain. The organization of different modules in the multimodular enzymes determines the size and sequence of the stepwise assembled natural products [1,2]. The decapeptide antibiotic tyrocidine A, for example, is synthesized nonribosomally by three NRPSs, TycA, TycB, and TycC [3], that consist of a total of 10 modules, each being responsible for the incorporation of one amino acid into the final product (Fig. 1).

A module can be subdivided into domains, representing the individual enzyme activities that together are necessary for a round of chain elongation [1]. Three domains essential for peptide synthesis line up an elongation module: an adenylation (A) domain responsible for substrate recognition and activation [4], a peptidyl carrier protein (PCP),

Abbreviations: A domain, adenylation domain; C domain, condensation domain; Dhb, dihydroxybenzoic acid; DKP, diketopiperazine; E domain, epimerization domain; HPLC, high performance liquid chromatography; MS, mass spectroscopy; NAC, *N*-acetylcysteamine; NRPS, nonribosomal peptide synthetase; PCP, peptidyl carrier domain; PKS, polyketide synthase; Ppant, 4′-phosphopantetheine; PP_i, inorganic pyrophosphate; T domain, thiolation domain; Te domain, thioesterase domain; TLC, thin layer chromatography

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also referred to as thiolation (T) domain, which binds activated amino acids and elongation intermediates as thioesters at its cofactor 4′-phosphopantetheine (Ppant) and serves as a transport unit [5], and a condensation (C) domain that catalyzes peptide bond formation (compare e.g. module 2 in Fig. 1) [6]. The initiation module, which provides the first amino acid, lacks the condensation domain (e.g. module 1 in Fig. 1). The termination of peptide synthesis is usually facilitated by a thioesterase-like Te domain connected to the last module [7]. Optional domains like epimerization (E) and *N*-methylation (M) domains can be inserted within modules. By modifying the amino acid substrate at the respective position, they are an important means to increase the structural diversity of the resultant products [8,9].

Te domains have recently gained much attention, because they control the last step of nonribosomal peptide synthesis, the effective and regioselective product release by cleaving the thioester bond between the PCP of the last module and the full-length peptide chain [7,10]. To this end, the linear peptide chain is translocated onto the serine residue of the conserved signature sequence GxSxG, which is part of a catalytic triad that operates in chain release [11]. The nucleophile for the cleavage reaction can be either a water molecule leading to linear products or an internal nucleophilic group of the peptide itself yielding cyclic or branched cyclic products (see Fig. 2 for different examples). The resultant cyclic peptides are more resistant towards proteases and the decreased conformational flexibility probably favors the biologically active conformation. The Te domains are thus also an important means to achieve structural diversity and biological activity for this class of natural products. In some cases, Te domains even catalyze multimerization of the peptide chain assembled by the preceding modules. In enterobactin biosynthesis for example (Fig. 2), the dihydroxybenzoic acid (Dhb)-Ser intermediates are transferred sequentially onto the serine side chain and linked by the Te domain until the trimer is intramolecularly cyclized and released [11].

Very recently, a method was described to directly assay isolated Te domains. The excised 28 kDa Te domain of tyrocidine NRPS TycC was found to accept peptidyl-*S*-*N*-acetylcysteamines (NACs) as diffusible substrate substitutes [7]. A decapeptidyl-*S*-NAC that corresponded to the sequence of tyrocidine A was cyclized to yield the peptide antibiotic with a k_{cat} of 59 min^{-1} and a K_m of 3 μM. Also several other decapeptidyl-*S*-NACs, including a nona- and an undecapeptidyl-*S*-NAC, were cyclized, albeit with lower efficiency. This finding of a relatively broad specificity is in agreement with previous studies in which Te domains were fused to internal modules of NRPS templates and resulted in the premature hydrolytic termination of nonribosomal peptide synthesis [12–15].

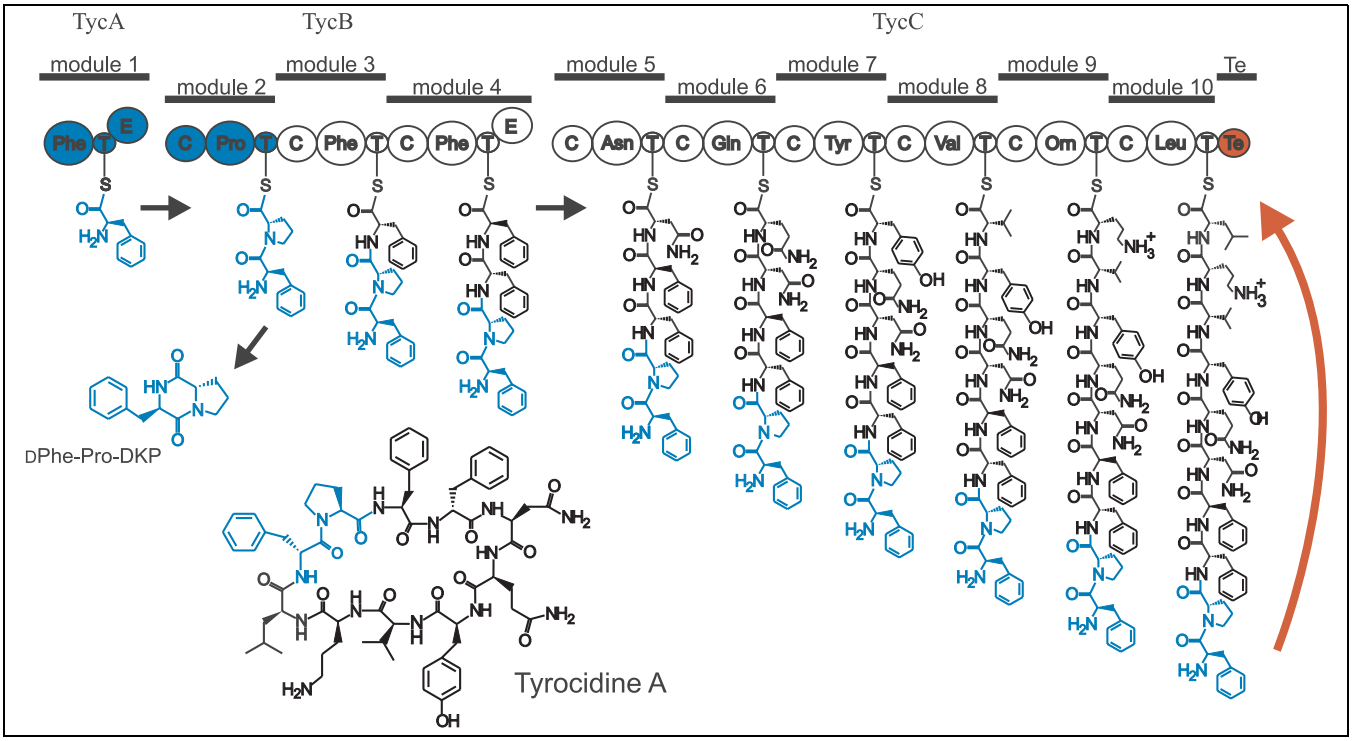

Fig. 1. Nonribosomal assembly of tyrocidine A. Three NRPSs, TycA, TycB, and TycC, act in concert to synthesize the cyclic decapeptide from the amino acid precursors. TycA comprises one module, TycB three, and TycC six modules, each of which is responsible for the incorporation of a cognate amino acid into the growing chain. The Te domain (red) at the last module of TycC catalyzes peptide cyclization and thereby release of the final product. The D-Phe-Pro intermediate bound to the second module is chemically unstable and is released as a side product as the cyclic D-Phe-Pro-DKP. The first two modules of tyrocidine NRPS, TycA and ProCAT (blue), were used in this study as a minimal bimodular system.

In this work, various heterologous Te domains were fused to a simple NRPS model system in order to investigate their activity and selectivity in artificial hybrid systems. The exploration of their amenability and impact on the design of hybrid NRPSs is an important step toward the fine-tuned engineering of more complex systems.

2. Results

2.1. Strategy

The Te domains of the tyrocidine [3], surfactin [16], fengycin [17], enterobactin [18], and yersiniabactin NRPSs [19], as well as the erythromycin PKS [20] were each fused to a bimodular NRPS to assay their activity in simple hybrid systems. These Te domains were selected, because they catalyze different modes of termination reaction in their native systems (see Fig. 2). As bimodular NRPS we chose the well established model context TycA/ProCAT [6], which represents the first two modules of the tyrocidine NRPS (see Fig. 1). These two proteins can be produced in recombinant form in *Escherichia coli* and were shown to reconstitute the initiation reaction in tyrocidine biosynthesis, the formation of the D-Phe-Pro dipeptide bound to the T domain of ProCAT (see Fig. 3). TycA activates, binds, and epimerizes L-Phe to D-Phe. ProCAT, the first module of TycB, activates and binds L-Pro. When both proteins are incubated together, D-Phe is transferred from TycA onto L-Pro bound on ProCAT to give the dipeptide D-Phe-Pro-*S*-Ppant still attached to ProCAT [6]. Due to the high proportion of *cis* conformation in the D-Phe-Pro dipeptide, which is induced by *N*-alkylated amino acids such as Pro, the dipeptide is rapidly released from the enzyme by a non-enzyme-catalyzed reaction to give the cyclic D-Phe-Pro-diketopiperazine (D-Phe-Pro-DKP) (see Fig. 3). Thus, hybrid systems of the kind TycA/ProCAT-Te (where '-' indicates the fusion site) allow the study of the activity of the fused Te domains in hydrolyzing the linear dipeptide D-Phe-Pro in competition with the non-enzyme-catalyzed cyclization reaction. On the other hand, if non-*N*-alkylated amino acids are used as substrates for ProCAT, the resultant dipeptide would provide a stable substrate for the Te domains. As will be shown in this paper, we found that alanine and aminobutyric acid are sufficiently activated by ProCAT to provide this 'molecular switch' to turn off or on the non-enzyme-catalyzed reaction.

Inherent in the construction of hybrid enzymes is always the choice of the artificial fusion site [21]. To fuse the PCP domain of ProCAT and the various Te domains we used the same location relative to the PCP domain as previously described [13]. A putative linker region connecting the PCP domain and the following C or Te domain is a

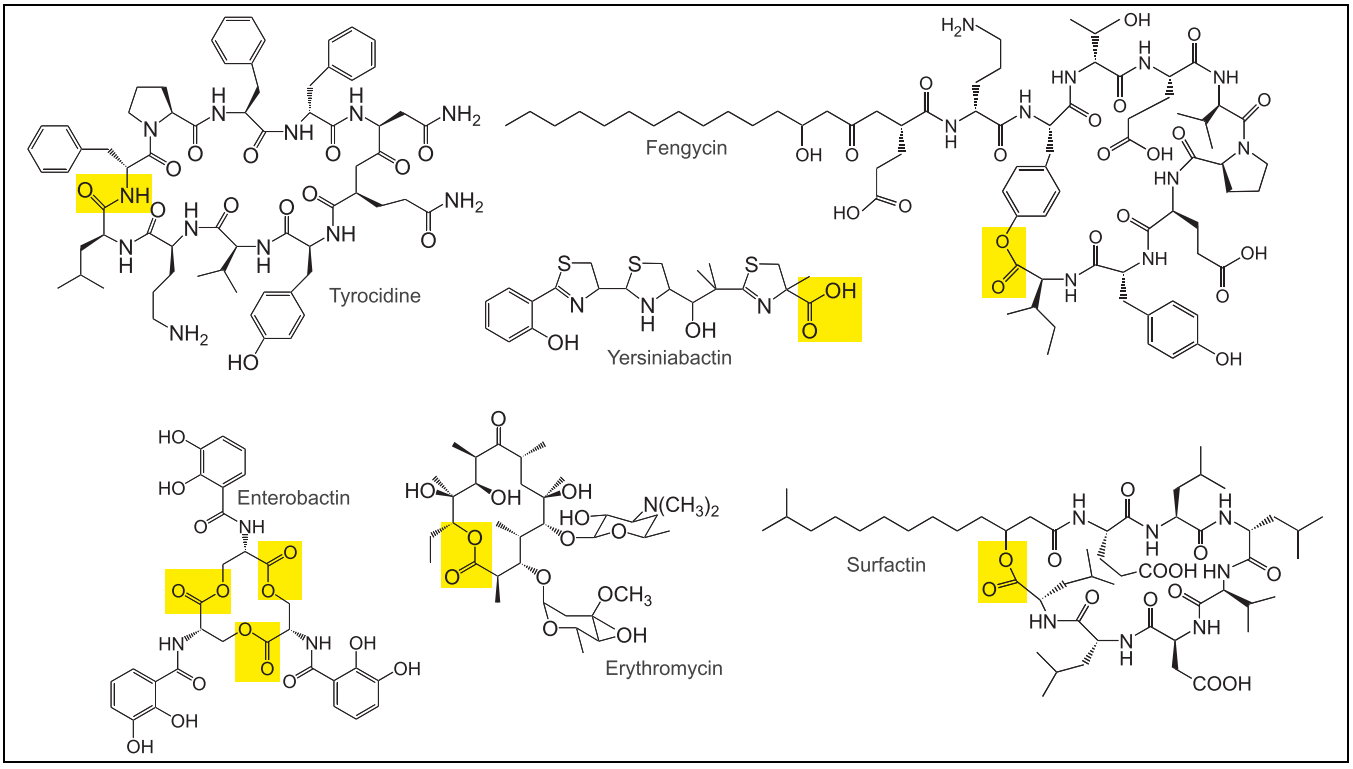

Fig. 2. Natural products synthesized by NRPSs and/or PKSs. The functional group formed by the corresponding Te domain is highlighted in yellow. The tyrocidine Te domain forms a peptide bond, the surfactin Te domain uses a β-hydroxy group of a fatty acid moiety as nucleophile, the fengycin Te domain catalyzes cyclization onto a tyrosyl side chain, the enterobactin Te domain trimerizes and cyclizes the precursor via serine side chain groups, the yersiniabactin Te domain acts as a hydrolase, and the erythromycin Te domain controls macrolactonization onto a hydroxyl moiety.

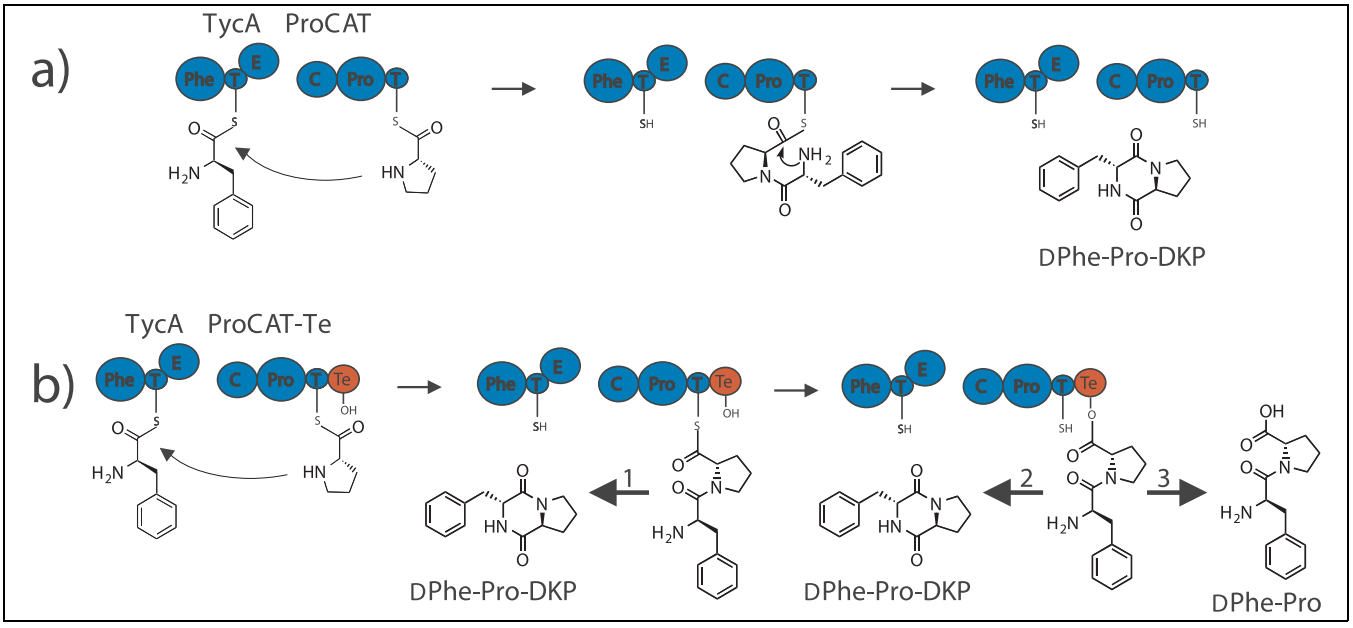

Fig. 3. Expected reactions in the experimental set-up using the initiation reaction of tyrocidine biosynthesis. (a) Dipeptide formation performed by the first two modules of the tyrocidine NRPSs TycA and ProCAT and non-catalyzed release as cyclic D-Phe-Pro-DKP. (b) Alternative product channeling performed by TycA and ProCAT-Te hybrid enzyme. (1) The dipeptide could be released as D-Phe-Pro-DKP without transfer to the fused Te domain. (2) Release as D-Phe-Pro-DKP after transfer to the fused Te domain. (3) Hydrolytic release as linear D-Phe-Pro catalyzed by the fused Te domain.

non-conserved sequence of about 15 amino acids and rich in amino acids typical for loops [13,22]. The fusion site was placed within this linker 38 amino acids downstream of the conserved serine residue of the PCP domain. Since the Te domains are always the last domains of NRPS, they provide a natural C-terminus.

2.2. *Construction, production and biochemical characterization of proteins*

Construction of hybrid enzymes was performed at the DNA level as described in Section 5. For protein overproduction and posttranslational modification with the cofactor Ppant, *E. coli* BL21[pREP4/gsp] was transformed with the plasmids pTycA, pProCAT-Te_{tyc}, pProCAT-Te_{srf}, pProCAT-Te_{fen}, pProCAT-Te_{ent}, pProCAT-Te_{yer}, and pProCAT-Te_{DEBS}. This strain co-expresses the gene *gsp* encoding the Ppant transferase Gsp that ensures conversion of the recombinant proteins into the holo-form [6]. All proteins were overproduced as C-terminal His_6-tagged fusions and purified by Ni^{2+}-NTA chromatography (Fig. 4a).

To test the functionality of the recombinant proteins, we first measured the enzymatic activity of A domains in TycA and the ProCAT-(Te) constructs by the ATP–pyrophosphate exchange assay. To define their substrate selectivity, the assays were performed with all proteinogenic amino acids and selected non-proteinogenic amino acids (Fig. 4b). The A domain of TycA efficiently hydrolyzed ATP in the presence of either L-Phe (97%) or D-Phe (100%). In agreement with previous studies, some side specificities for other aromatic amino acids like L-Trp and L-Tyr as well as for L-Leu and L-Met were detected (see Fig. 4b) [13,23]. All ProCAT-(Te) constructs recognized several *N*-alkylated amino acids as substrates. For example, azetidin-2-carboxylic acid, the four-membered ring derivative of L-Pro, showed an even higher exchange rate (100%) than the natural substrate L-Pro (83%). Sarcosine (*N*-methylglycine) also served as a good substrate (12%). Importantly, we found that the amino acids L-Ala (4%) and L-Abu (aminobutyric acid) (6%) were also significantly activated by ProCAT's A domain and therefore were considered promising candidates for suppression of the non-catalyzed DKP formation.

The functionality of the PCP domains in the recombinant proteins and their modification with the cofactor Ppant was validated by the thioester-binding assay. TycA covalently bound [^{14}C]Phe, while all ProCAT-(Te) constructs bound [^{14}C]Pro.

2.3. *Reaction profiles with L-Pro, L-Ala, and L-Abu as substrates for ProCAT*

In order to determine whether L-Ala and L-Abu could substitute for L-Pro as substrates of ProCAT and if thereby DKP formation would be suppressed, we measured a dipeptide formation profile as reported previously [6]. This assay monitors the transfer of [^{14}C]Phe from TycA onto ProCAT with rapid reloading of TycA and dipeptide release from ProCAT by time-dependent measurement of trichloroacetic acid (TCA)-precipitable radioactivity resulting from enzyme bound labeled amino acids or pep-

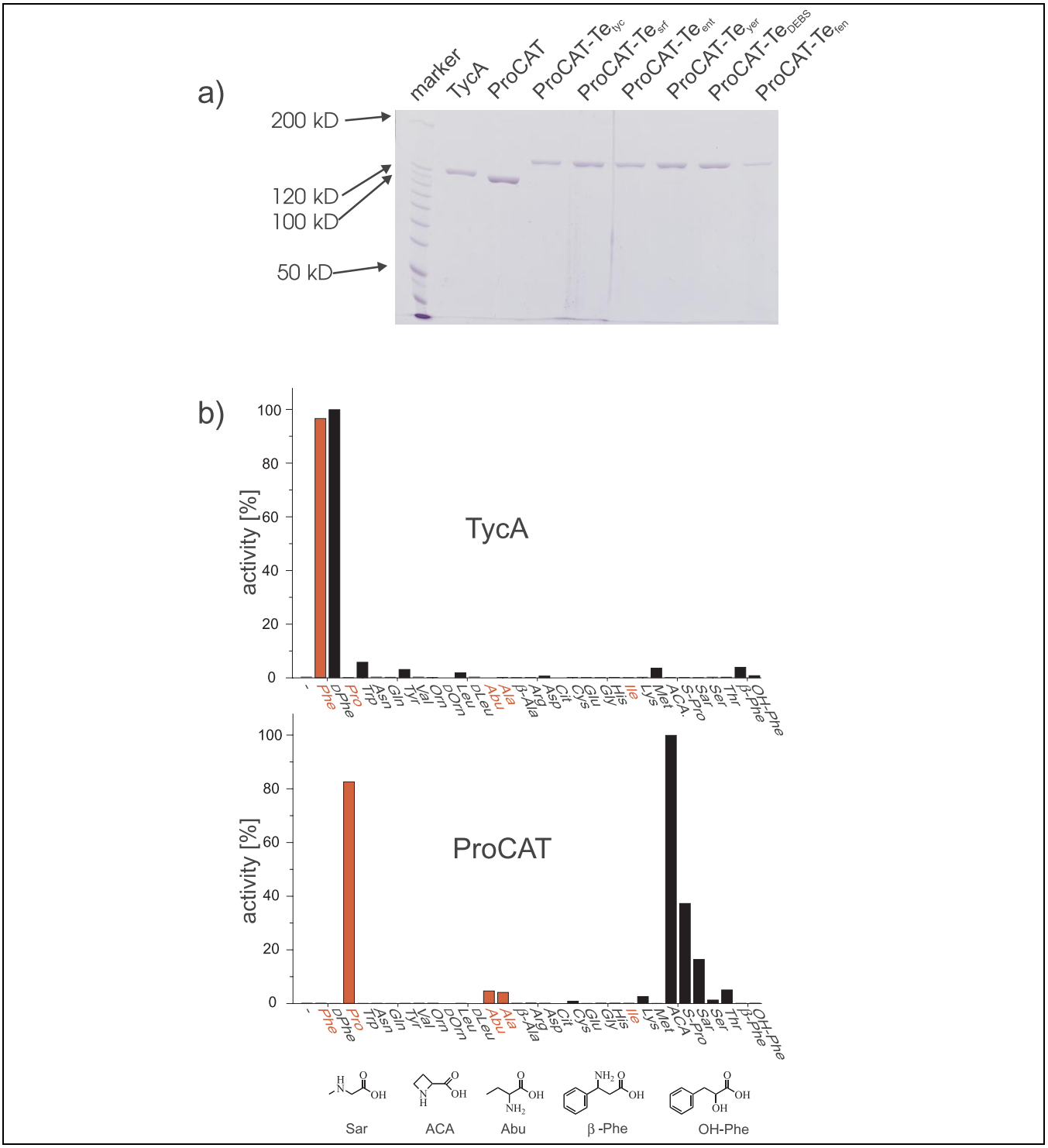

Fig. 4. Recombinant proteins used in this study. (a) SDS–PAGE of the purified proteins. Calculated masses of the proteins: TycA 123.5 kDa; ProCAT 119.7 kDa; ProCAT-Te$_{tyc}$ 148.8 kDa; ProCAT-Te$_{srf}$ 145.8 kDa; ProCAT-Te$_{fen}$ 145.6 kDa; ProCAT-Te$_{ent}$ 147.2 kDa; ProCAT-Te$_{yer}$ 149.1 kDa; and ProCAT-Te$_{DEBS}$ 148.7 kDa. (b) ATP–PP$_i$ exchange rates measured for the A domains of TycA and ProCAT. Amino acids used in further assays are highlighted in red.

tides. The results are illustrated in Fig. 5a. The substrate amino acid for ProCAT was always added after a pre-incubation of TycA with [^{14}C]L-Phe (black line). If a suitable non-labeled substrate for ProCAT is added an elongation reaction can take place resulting in a ProCAT-bound dipeptide containing [^{14}C]L-Phe. Rapid recharging of TycA with [^{14}C]L-Phe leads to a stoichiometric increase of roughly one equivalent of [^{14}C]L-Phe that can be detected by counting of TCA-precipitable radioactivity. When L-Pro was used as substrate (green line), a rapid

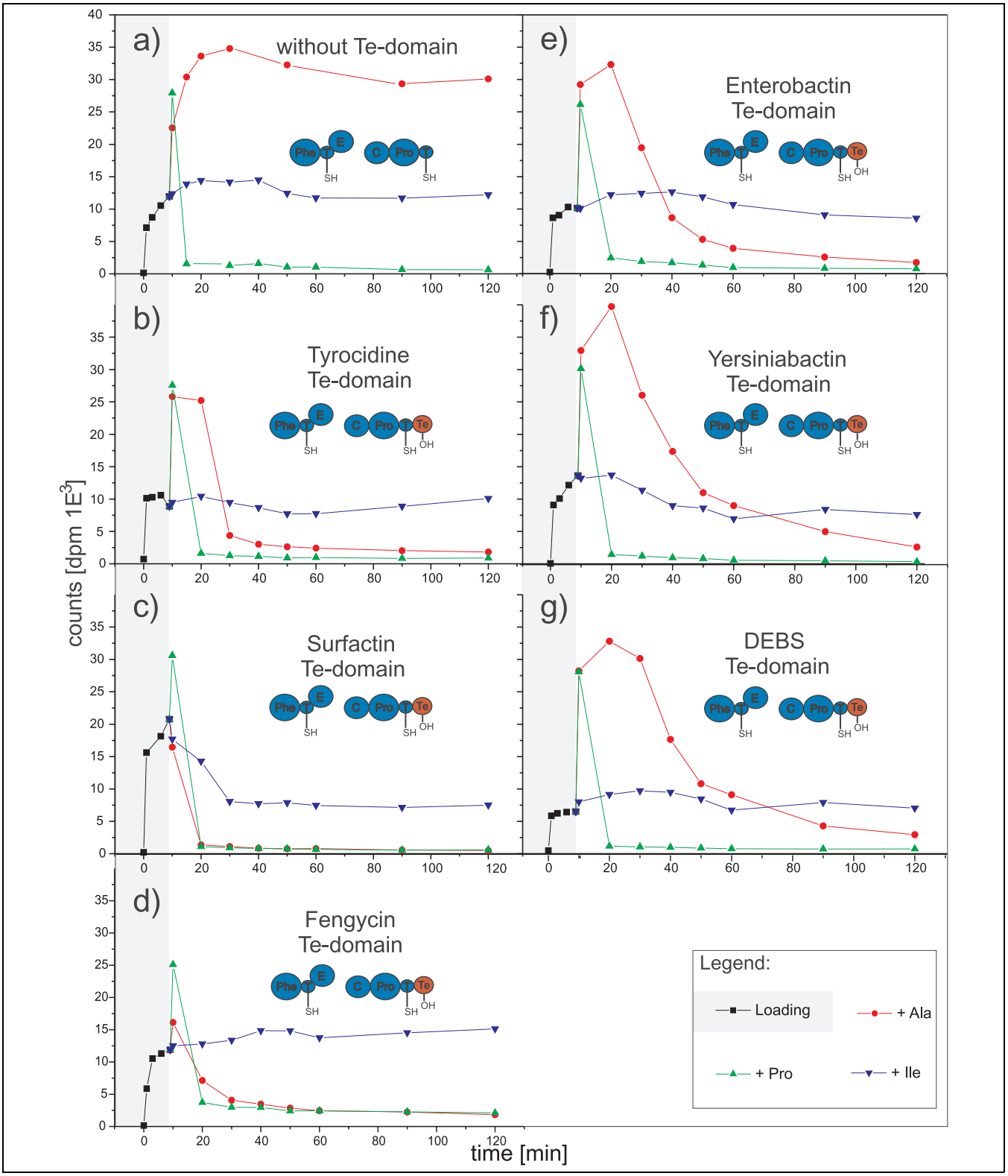

Fig. 5. Reaction profiles of TycA and ProCAT-(Te_x). 0–9 min: charging of TycA with [^{14}C]Phe. After 10 min the reaction mixture was split and the second amino acid was added: L-Pro green line, L-Ala red line, L-Ile blue line. The reaction was monitored for 120 min. Enzymes and enzyme-bound amino acids and peptides were precipitated by the addition of TCA. Increase of TCA-precipitable radioactivity resulted from two equivalents of [^{14}C]L-Phe per NRPS (TycA and ProCAT). Decrease of TCA-precipitable radioactivity resulted from consumption of [^{14}C]L-Phe by incorporation into released dipeptides and therefore a lower stoichiometric ratio of [^{14}C]Phe per NRPS.

increase followed by a decrease of precipitable radioactivity could be observed. The decrease resulted from [^{14}C]D-Phe-Pro-DKP formation causing consumption of [^{14}C]L-Phe which is only in about four-fold stoichiometric excess relative to the enzymes. When L-Pro was substituted by L-Ala (red line), the increase of precipitable radioactivity reached a stationary plateau for the entire time period of 120 min. A similar curve was obtained when L-Pro was replaced by L-Abu (data not shown). L-Ile was added in a control reaction (blue line) showing that the incorporated radioactivity remained constant when a non-cognate amino acid was used. These results indicate that ProCAT can accept L-Ala and L-Abu as substrates for elongation reactions and suggest that the formed dipeptides D-Phe-Ala and D-Phe-Abu are not released as cyclic DKPs, but remain, as expected, attached to ProCAT and can provide a stable substrate for Te domains fused to ProCAT.

2.4. *Reaction profiles of the hybrid systems TycA/ProCAT-Te$_x$*

To study the influence of the six Te domains on product release in the TycA/ProCAT-Te$_x$ hybrid systems reaction profiles were taken (see Fig. 5b–g) and compared with the data of the TycA/ProCAT system without a fused Te domain (Fig. 5a).

The reaction profile for TycA/ProCAT-Te$_{tyc}$ is shown in Fig. 5b. When L-Pro was used as substrate (green line) no difference to the system without fused Te domain was observed. Following the loading of TycA with [^{14}C]L-Phe (black line), the characteristic increase and decrease of enzyme-bound radioactivity could be detected, resulting from formation of [^{14}C]D-Phe-Pro and release of [^{14}C]D-Phe-Pro-DKP. However, using L-Ala as substrate for ProCAT-Te$_{tyc}$ (red line) the curve differs significantly. After addition of L-Ala enzyme-bound radioactivity increases and then drops rapidly to background. The same curve pattern was detected using L-Abu as substrate (data not shown). This indicates that the dipeptides formed, D-Phe-Ala and D-Phe-Abu, are released by the Te$_{tyc}$ domain.

In the case of TycA/ProCAT-Te$_{srf}$ (Fig. 5c) again a similar reaction profile was detected when using L-Pro as substrate, but when using L-Ala the difference to the system without Te domain was even more striking. A decrease of enzyme-bound radioactivity was observed immediately after the addition of the second amino acid. A similar profile was obtained when using L-Abu (data not shown). The system TycA/ProCAT-Te$_{fen}$ (Fig. 5d) behaved similarly, although a slight increase of enzyme-bound radioactivity was observed after the addition of L-Ala.

The reaction profiles of the three other hybrid enzymes ProCAT-Te$_{ent}$, ProCAT-Te$_{yer}$, and ProCAT-Te$_{DEBS}$ are qualitatively similar to the one of ProCAT-Te$_{tyc}$ for L-Pro and L-Ala, indicating that all Te domains are capable of catalyzing release of the dipeptide D-Phe-Ala. In terms of velocity these data suggest the order Te$_{srf}$ > Te$_{fen}$ > Te$_{tyc}$ > Te$_{ent}$ > Te$_{yer}$ > Te$_{DEBS}$ according to their activity in the hybrid systems. The initial increase in precipitable radioactivity observed for all systems except TycA/ProCAT-Te$_{srf}$ when using [^{14}C]L-Phe and L-Ala or L-Abu indicates that activation and covalent binding of these non-cognate amino acids by the ProCAT-(Te) enzymes is not the rate-limiting step.

2.5. *Product identification by HPLC/MS*

To identify the reaction products we performed high performance liquid chromatography/mass spectrometry (HPLC/MS) analysis. For the system TycA/ProCAT, D-Phe-Pro-DKP was the only product detectable from the assay with L-Phe and L-Pro. It eluted with a retention time of 27.0 min and its identity was confirmed by its $[M+1]^+$ peak at *m/z* 245 (see Fig. 6a). When L-Ala or L-Abu were used no dipeptides could be detected under these conditions (Fig. 6b,c).

For TycA/ProCAT-Te$_{tyc}$ with L-Pro as substrate the only product signal at 27.0 min corresponded to D-Phe-Pro-DKP ($[M+1]^+$ at *m/z* 245). No traces of the linear D-Phe-Pro could be detected (see Fig. 6a). Substitution of L-Pro by either L-Ala or L-Abu led to signals of the corresponding linear dipeptides D-Phe-Ala (23.0 min) and D-Phe-Abu (27.8 min) as confirmed by the masses $[M+1]^+$ at *m/z* 237 and $[M+1]^+$ at *m/z* 251, respectively (calculated 237 and 251). No signals with the masses of the cyclic D-Phe-Ala-DKP or D-Phe-Abu-DKP were detected (Fig. 6b,c).

The system TycA/ProCAT-Te$_{srf}$ with L-Pro as substrate revealed the presence of a new product in addition to the cyclic D-Phe-Pro-DKP (see Fig. 6a). This compound eluted at 25.5 min, co-migrated with a chemical standard of linear D-Phe-Pro and could be confirmed by its $[M+1]^+$ signal at *m/z* 263 as the linear D-Phe-Pro. In the assays containing L-Ala, the linear dipeptide D-Phe-Ala was verified as a product (23.0 min, $[M+1]^+$ *m/z* 237). An additional product eluted at 21.7 min and was identified by its $[M+1]^+$ signal at *m/z* 219 as the cyclic D-Phe-Ala-DKP (Fig. 6c). Similarly, when L-Abu was used as substrate (Fig. 6b), the linear D-Phe-Abu and cyclic D-Phe-Abu-DKP were identified (27.7 min, $[M+1]^+$ *m/z* 251, and 23.7 min, $[M+1]^+$ signal at *m/z* 233, respectively). The system TycA/ProCAT-Te$_{fen}$ yielded the same product patterns.

The formation and release of D-Phe-Ala were also confirmed for ProCAT-Te$_{ent}$ and ProCAT-Te$_{yer}$, but this product could not be detected in assays with ProCAT-Te$_{DEBS}$ and L-Ala, probably due to only marginal turnover rates. However, substitution of L-Ala by L-Abu in this system led to a detectable, but still weak product signal for D-Phe-Abu (data not shown). When using L-Pro as substrate for these latter three hybrid enzymes, the only detectable product was the cyclic D-Phe-Pro-DKP.

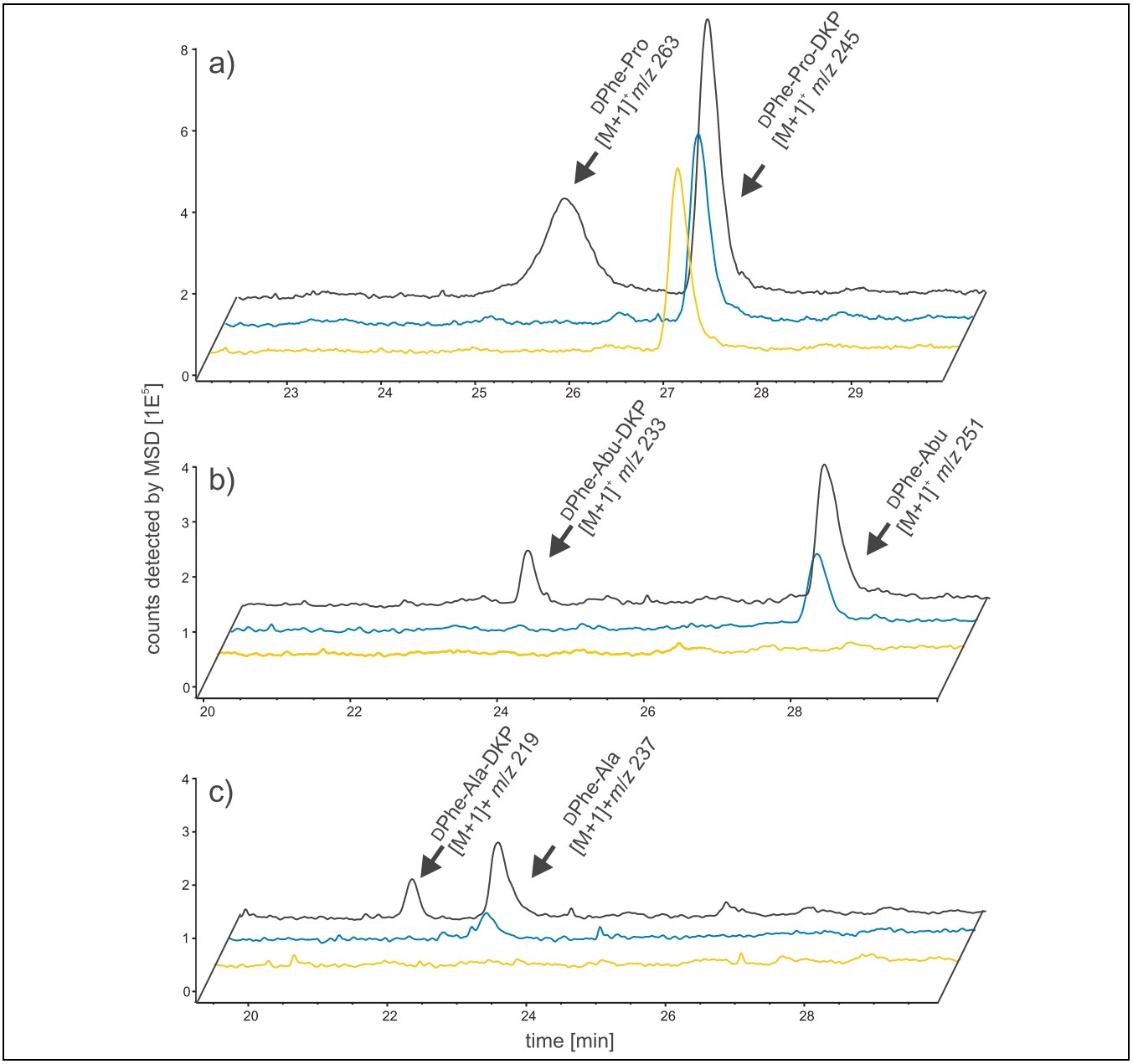

Fig. 6. HPLC/MS analysis of products formed by TycA and ProCAT-(Te_x). (a) Product assays with L-Phe and L-Pro. (b) Product assays with L-Phe and L-Abu. (c) Product assays with L-Phe and L-Ala. Yellow: TycA/ProCAT; blue: TycA/ProCAT-Te_{tyc}; black: TycA/ProCAT-Te_{srf}. The product pattern of the hybrid system with the Te_{ent} domain is qualitatively similar to that of the Te_{tyc} domain, Te_{fen} domain to that of Te_{srf} domain, and Te_{yer} and Te_{DEBS} domains to that of the system without Te domain.

2.6. *Determination of product formation rates by TLC/autoradiography and radioactivity scanning*

To determine the formation rates of the various products we performed radioactive assays with subsequent separation of the products by thin layer chromatography (TLC). Product spots were assigned after autoradiography and formation rates were calculated from radioactive scanning of time-dependent experiments. These results are summarized in Figs. 7 and 8, and in Table 1.

For the system TycA/ProCAT without Te domain, the autoradiographic analysis of the assay containing L-Phe and [^{14}C]L-Pro showed two spots with R_f values of 0.29 and 0.69 that can be assigned to [^{14}C]L-Pro and D-Phe-[^{14}C]Pro-DKP (see Fig. 7, lane 2). No linear D-Phe-Pro was detected (R_f value of the chemical standard is 0.51) in agreement with the HPLC/MS results. Product analysis of the assay with [^{14}C]L-Phe and L-Abu showed only one spot corresponding to [^{14}C]L-Phe (see Fig. 7, lane 1). No linear D-Phe-Abu could be detected. A turnover rate of 3.7 min^{-1} for the formation of D-Phe-[^{14}C]Pro-DKP was determined (see Fig. 8 and Table 1).

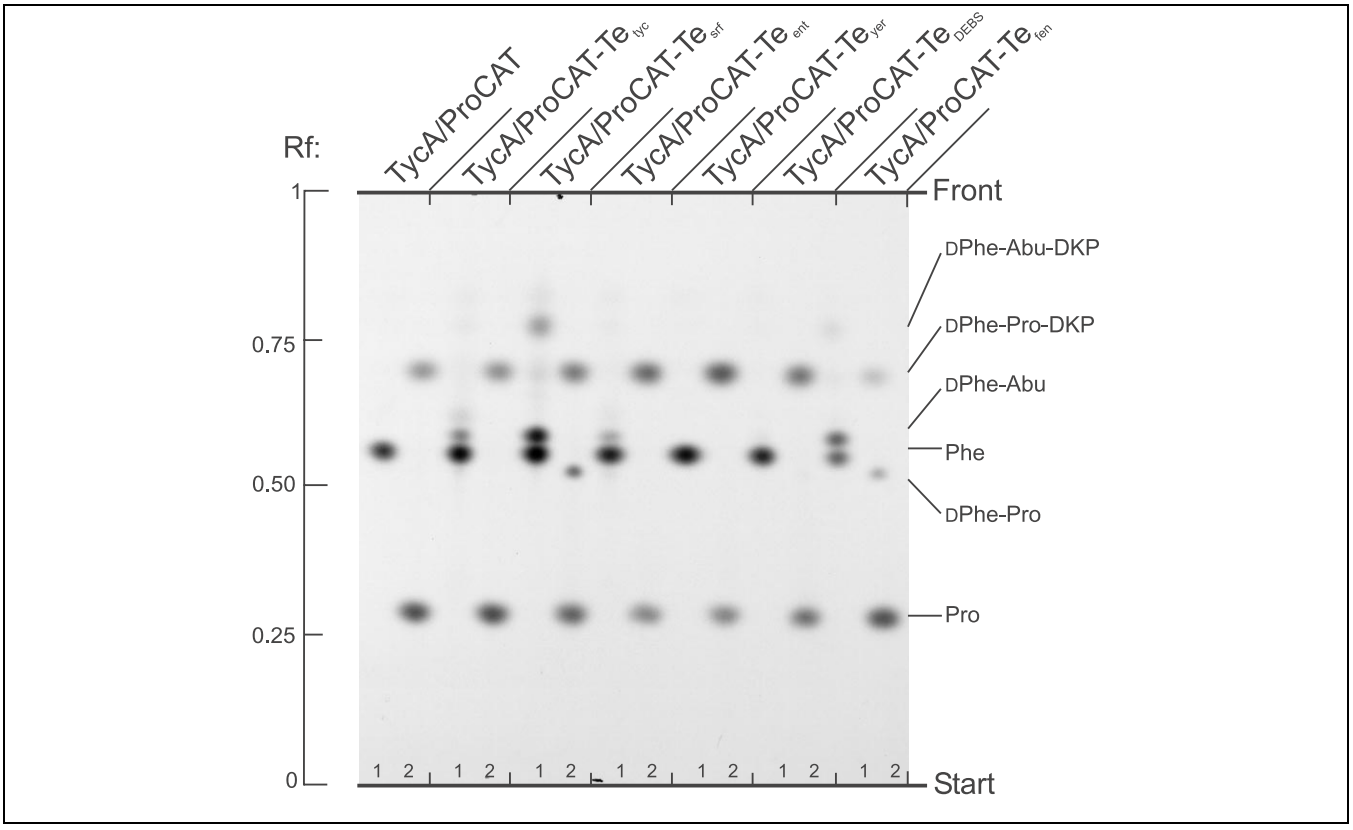

Fig. 7. TLC/autoradiographic analysis of the products formed by TycA and ProCAT-(Te_x). Lane 1: product assays with [^{14}C]L-Phe and L-Abu; lane 2: product assays with L-Phe and [^{14}C]L-Pro.

The autoradiogram of the products formed by TycA/ProCAT-Te_{tyc} when incubated with L-Phe and [^{14}C]L-Pro showed two spots corresponding to [^{14}C]Pro (R_f: 0.29) and D-Phe-[^{14}C]Pro-DKP (R_f: 0.69). Using [^{14}C]L-Phe and L-Abu two spots corresponding to [^{14}C]Phe (R_f: 0.55) and [^{14}C]D-Phe-Abu (R_f: 0.59) were detected (Fig. 7). A standard for D-Phe-Abu was not available, however, comparison with the R_f values of L-Ala (0.33), L-Abu, (0.38), and D-Phe-Ala (0.55) provides good evidence for this assignment. The formation rate of D-Phe-[^{14}C]Pro-DKP was determined at 3.9 min^{-1}, whereas [^{14}C]D-Phe-Abu was synthesized at 0.4 min^{-1} (Fig. 8, Table 1).

For the system TycA/ProCAT-Te_{srf} incubated with L-Phe and [^{14}C]Pro, an additional product spot (R_f: 0.51) was detected that co-migrated with a chemical standard of linear D-Phe-Pro (Fig. 7). The formation of this product had already been confirmed by HPLC/MS (see above) and was determined to have a rate of 1.2 min^{-1}. [^{14}C]D-Phe-Pro-DKP was simultaneously formed at 2.4 min^{-1}. When [^{14}C]L-Phe and L-Abu were used as substrates, [^{14}C]D-Phe-Abu was formed at 2.1 min^{-1}. We also found an additional labeled product exhibiting an R_f value of 0.77 (Fig. 7), presumably the cyclic [^{14}C]D-Phe-Abu-DKP, formed at a rate of 0.8 min^{-1} (Fig. 8, Table 1). Replacing L-Abu with L-Ala resulted in disappearance of this product, but another product with an R_f value of 0.72 was formed (data not shown). These R_f values fit well with those of the characterized linear and cyclic dipeptides. The product pattern of TycA/ProCAT-Te_{fen} was qualitatively similar (Fig. 7), but the products were formed at somewhat lower rates: [^{14}C]D-Phe-Pro-DKP was synthesized at 1.2 min^{-1}, [^{14}C]D-Phe-Pro at 0.6 min^{-1}, [^{14}C]D-Phe-Abu at

Table 1
Product formation rates of the systems TycA/ProCAT-(Te_x)

Product	Product formation rate (min^{-1})						
	TycA ProCAT	TycA ProCAT-Te_{tyc}	TycA ProCAT-Te_{srf}	TycA ProCAT-Te_{fen}	TycA ProCAT-Te_{ent}	TycA ProCAT-Te_{yer}	TycA ProCAT-Te_{DEBS}
D-Phe-Pro	–	–	1.2	0.6	–	–	–
D-Phe-Pro-DKP	3.7	3.9	2.4	1.2	3.4	3.9	1.6
D-Phe-Abu	–	0.4	2.1	1.3	0.2	n.d.	n.d.
D-Phe-Abu-DKP	–	–	0.8	0.2	–	–	–

n.d. = not determined; only very little product formation, insufficient for determination of turnover rates.

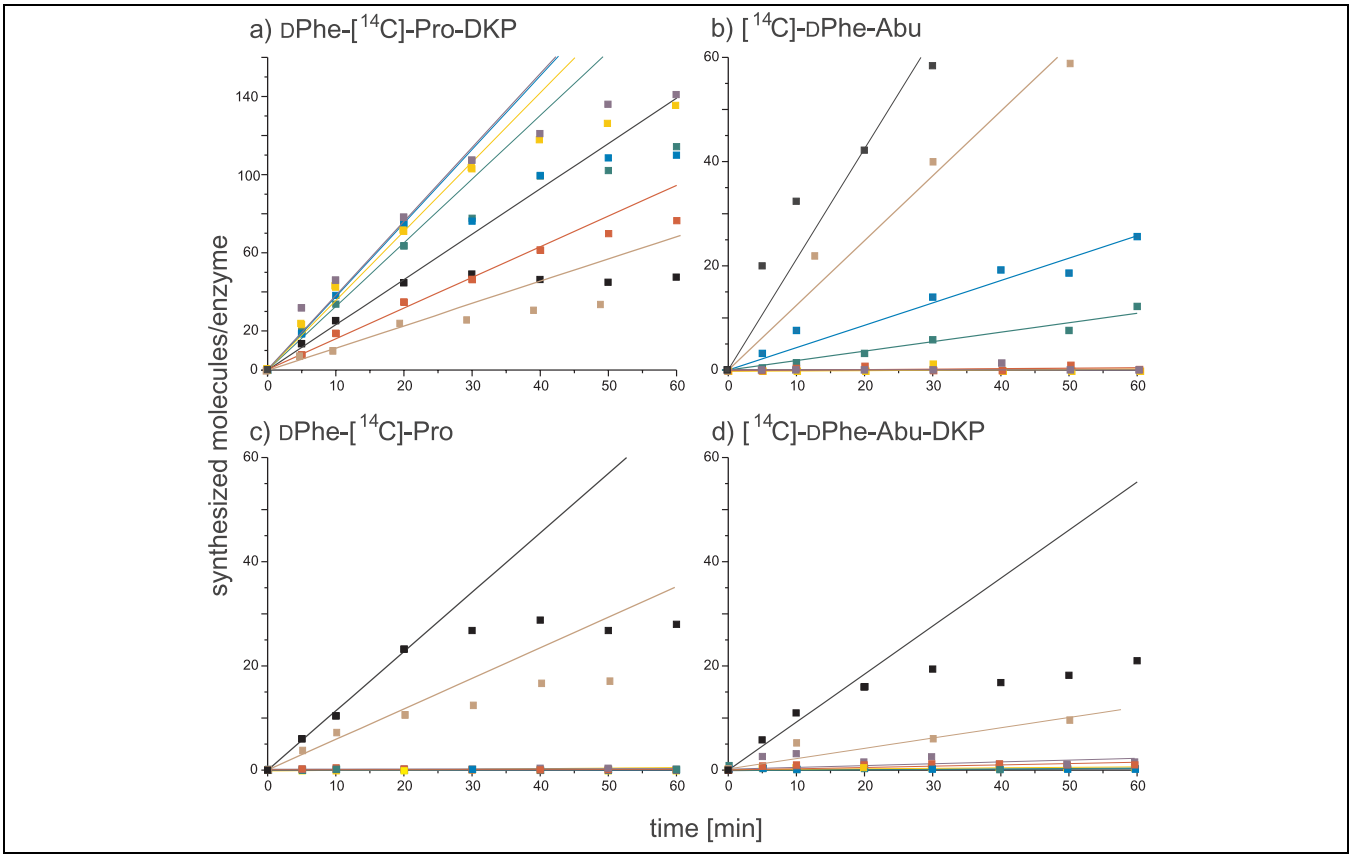

Fig. 8. Time-dependent product formation catalyzed by TycA and ProCAT(-Te_x). Values result from radioactivity scanning of products separated by TLC. (a) D-Phe-[^{14}C]Pro-DKP. (b) [^{14}C]D-Phe-Abu. (c) D-Phe-[^{14}C]Pro. (d) [^{14}C]D-Phe-Abu-DKP. Yellow: TycA/ProCAT; blue: TycA/ProCAT-Te_{tyc}; black: TycA/ProCAT-Te_{srf}; green: TycA/ProCAT-Te_{ent}; violet: TycA/ProCAT-Te_{yer}; red: TycA/ProCAT-Te_{DEBS}; brown: TycA/ProCAT-Te_{fen}. Formation rates were determined from the initial linear turnover (see Table 1).

1.3 min^{-1}, and [^{14}C]D-Phe-Abu-DKP at 0.2 min^{-1} (Fig. 8, Table 1).

TLC analysis and subsequent autoradiography also confirmed the results on the product patterns formed by the hybrid system with the Te domains from the enterobactin synthetase (Fig. 7). The product formation rates for D-Phe-[^{14}C]Pro-DKP and [^{14}C]D-Phe-Abu by the system TycA/ProCAT-Te_{ent} were measured at 3.4 min^{-1} and 0.2 min^{-1} (Fig. 8, Table 1). However, for the hybrids containing the Te domains of the yersiniabactin and erythromycin synth(et)ases, product detection again proved difficult. No detectable amounts of [^{14}C]D-Phe-Abu were formed by TycA/ProCAT-Te_{yer}. The formation rate of D-Phe-[^{14}C]Pro-DKP by this system was measured at 3.9 min^{-1}. TycA/ProCAT-Te_{DEBS} did not release [^{14}C]D-Phe-Abu in detectable amounts and the turnover rate of D-Phe-[^{14}C]Pro-DKP production was determined to be 1.6 min^{-1} (Figs. 7 and 8, Table 1).

3. Discussion

The aim of this study was to investigate the portability of various Te domains for the construction of hybrid NRPSs. Two examples for this kind of experiment have previously been reported. In both cases, however, the Te domain was fused only to other modules of the parent NRPS system. First, the Te domain of the seventh module of surfactin NRPS was fused to the fourth and fifth modules resulting in premature hydrolysis of the predicted lipotetra- and lipopentapeptides [12]. Although the natural lipoheptapeptide product is cyclic, obviously only the linear products, resulting from hydrolysis, were observed in the engineered strains. In the second example, the Te domain from the tyrocidine NRPS was fused to the ninth and fifth modules and also catalyzed hydrolysis of the predicted peptides [13,15].

We have fused six Te domains from different NRPS and PKS systems to a bimodular NRPS model system that consists of the first two modules of tyrocidine NRPS [6]. Depending on the substrate amino acid used a D-Phe-Pro-*S*-Ppant or a D-Phe-Abu-*S*-Ppant substrate is provided for the fused Te domains. The artificial systems of the kind TycA/ProCAT-Te were useful to monitor hydrolytic activity of the Te domains. We observed activity of the Te domains in product release for all six hybrid systems, suggesting that Te domains will be a general tool for catalyzing product release from hybrid biosynthetic templates,

useful either for the manipulation of existing ones or for de novo construction. The Te domains of the tyrocidine NRPS (from *Bacillus brevis*), surfactin NRPS (*Bacillus subtilis*), fengycin NRPS (*B. subtilis*), and enterobactin NRPS (*E. coli*) exhibited reasonable to very good turnover rates in the same order as the NRPS native systems [13,24,25]. In contrast, the Te domains of the yersiniabactin NRPS (*Yersinia pestis*) and the erythromycin PKS (*Saccharopolyspora erythraea*) revealed only little activity (estimated 10–50-fold lower).

An important task for the action of Te domains is the ratio of cyclization versus hydrolysis. As has recently been proven with excised Te domains and peptidyl-*S*-NACs as diffusible substrate substitutes, the Te domains control the complex macrocyclization reaction to yield the cyclic products that are often difficult for the chemist to synthesize [7]. In their natural biological system these cyclases thus must be optimized to efficiently suppress water as the attacking nucleophile in the catalytic center. In fact, for the Te_{tyc} domain cyclase, glycerolysis rather than hydrolysis was the preferred cleavage reaction at glycerol concentration as low as 10 mM in the artificial trimodular hybrid system TycA/ProCAT-LeuCATTe, which was described in [13] (H.D. Mootz, D. Schwarzer and M.A. Marahiel, unpublished results). However, there are also important peptide antibiotics with a linear backbone that result from hydrolysis of the nonribosomally assembled peptide chain from the NRPS complex, e.g. the β-lactam precursor ACV [26] and the vancomycin group antibiotics [27]. In these cases the Te domains must act as hydrolases. It will clearly be an important goal for the design of complex hybrid NRPSs to be able to control regioselectivity in the macrocyclization reaction, i.e. in cases with more than one intramolecular nucleophile being present. Due to its simplicity our system cannot provide peptides of sufficient length and side chain variability to study the chemo- and regioselectivity of the Te domains with cyclization activity. The minimal bimodular NRPS system TycA/ProCAT used is mainly suited to investigate the hydrolytic activity of the fused Te domains. The advantage of our approach over studies with peptidyl-*S*-NACs lies in the simulation of the natural context with a peptidyl substrate bound to the Ppant cofactor of the PCP domain adjacent to the Te domain. It is likely that this context has influence on the activity and selectivity of the Te domain. The fact that all Te domains tested can catalyze hydrolysis is interesting for their mechanistic characterization. All these Te domains are potentially appropriate to cleave peptides assembled on hybrid NRPSs in linear form, although only the yersiniabactin Te domain is a natural hydrolase.

Despite its simplicity, our bimodular TycA/ProCAT-Te system revealed some insights into the specificity of the fused Te domains, since different product patterns were observed: the Te domains of the surfactin and fengycin NRPSs were the only Tes capable of hydrolyzing the D-Phe-Pro-*S*-Ppant substrate. Peptidyl-*S*-Ppant substrates with a C-terminal proline residue are probably particularly demanding because proline is an imino acid. These Te domains may therefore find application in cleaving such peptides. From our results it is not possible to clearly explain why the other Te domains failed to enhance the rate of cleavage of the D-Phe-Pro dipeptide. Given the formation rates of linear D-Phe-Abu in the other hybrid systems, at least those with the Te_{tyc} and Te_{ent} domains, it seems unlikely that these systems could not compete with D-Phe-Pro-DKP formation because of insufficient interaction between the PCP domain and the fused Te domains. The capability of the Te_{srf} and Te_{fen} domains to hydrolyze the D-Phe-Pro-*S*-Ppant substrate may also be a result of their natural specificity. In contrast to the Te_{tyc} domain, they generate an ester instead of an amide bond and should be optimized for an attacking hydroxyl group and not for an amino group. We would therefore expect that water as a hard nucleophile is more efficiently accepted by such a 'macrolactonase' than by a 'macrolactamase'. We believe that this specificity also contributes to the highest activity of the surfactin and fengycin Te domains in cleaving the D-Phe-Ala and D-Phe-Abu peptides (D-Phe-Abu was produced at five- and three-fold higher rates, respectively, compared to the Te_{tyc} system and 10- and six-fold faster, respectively, compared to the Te_{ent} system). Another point that might account for the activity of the Te_{srf} and Te_{fen} domains is the similarity of their natural substrates and the substrates generated by the TycA/ProCAT system with regard to the stereochemical D configuration of the second last amino acid (compare Fig. 2, the last two amino acids in surfactin are –DLeu-Leu–, and in fengycin –DTyr-Ile–).

The Te_{srf} and Te_{fen} domains also displayed another interesting activity. They induced a partial DKP formation of the D-Phe-Ala and D-Phe-Abu dipeptides that was not observed for the other Te domains. To form the cyclic dipeptides, the peptide bond between the two amino acids has to be in *cis* conformation. With exception of Xaa-Pro peptides, however, the *trans* conformation is energetically clearly favored [28] and DKP formation is suppressed. Either the shape of the substrate pockets of the Te_{srf} and Te_{fen} domains lowers the energetic barrier between the *cis* and *trans* conformations of the bound D-Phe-Abu dipeptide or they specifically stabilize the *cis*-peptide conformer. We suppose that this finding reflects the pre-organization of the peptide bond between the last two amino acids in the nascent surfactin and fengycin peptide chains prior to macrolactonization catalyzed by the Te domains. This result shows that the retained inherent specificity of fused Te domains can lead to cyclization reactions that are not easily predictable. On the other hand, this finding offers another source for generating structural diversity in artificial systems. For example, the Te_{srf} and Te_{fen} domains might be a good starting point for an evolved Te domain optimized for the production of DKP-based com-

pounds, which represent a large class of natural products [29]. In this respect, it is interesting to mention that an additional cyclization activity was also found for the excised Te_{tyc} domain that naturally catalyzes the head-to-tail cyclization of a decapeptidyl substrate. This enzyme could also dimerize and then cyclize the pentapeptidyl-*S*-NAC substrates corresponding to the half of the symmetric decapeptide gramicidin S [7].

The Te domain from the enterobactin NRPS was chosen because it represents a special activity in trimerizing the Dhb-Ser monomer followed by cyclization. Since the trimerization occurs via the serine side chain hydroxyl groups we did not expect such multimerization activity in our system. Only hydrolysis of the D-Phe-Ala and D-Phe-Abu dipeptides was demonstrated, while the D-Phe-Pro dipeptide was not processed.

In previous studies the Te_{DEBS} domain and ACP-Te_{DEBS} didomain, excised from the erythromycin PKS, were assayed using simplified *S*-NAC- and *p*-nitrophenyl-coupled ketides. Only hydrolytic product cleavage could be detected for several such substrate substitutes [30,31]. The fusion the Te_{DEBS} domain to the TycA/ProCAT NRPS system was a particularly daring experiment, since the monomers and intermediates in polyketide biosynthesis do not contain α-amino groups. Although this certainly imposes significant substrate recognition problems we could demonstrate that peptidyl transfer into the active site of the Te domain and slow product release by hydrolysis had occurred. The fact that even the formation of D-Phe-Pro-DKP, which occurs independently of a fused Te domain, was affected by a factor of about 2–3 in this system suggests that the Te_{DEBS} domain also interfered with folding or stability of larger parts of the hybrid protein. Te domains from PKS might have potential for cleaving peptides ending with α- or β-hydroxy acids, which can be encountered in many natural products.

4. Significance

In conclusion, we fused various Te domains to a bimodular NRPS model system to test for their capability to release the enzymatically provided dipeptide substrates. We found that the activities of the Te domains in all hybrid systems tested are promising for the potential of Te domains to be used for the construction of engineered hybrid NRPSs. While all Te domains accepted and hydrolyzed the D-Phe-Ala and D-Phe-Abu dipeptides, only the Te domains of the surfactin and fengycin NRPSs could also cleave the D-Phe-Pro substrate. Since Te domains catalyze the last step of nonribosomal peptide synthesis, they process complex substrates making the adequate exploration of their behavior in hybrid NRPS a difficult task. However, our simple bimodular NRPS system already revealed differences in their activity and regioselectivity, underlining the importance of understanding possible alternative termination reactions. Investigating the activity of Te domains in hybrid NRPSs has the advantage that the peptidyl-*S*-Ppant substrates are presented on the adjacent PCP domain, which is the best simulation of the natural context. Such studies will be important to further explore the rules and domain repertoire for fine-tuning the design of more complex hybrid systems.

5. Materials and methods

5.1. Construction of expression plasmids

For all DNA manipulations and *E. coli* transformations standard methods were applied [36]. The construction of the plasmids pTycA, pProCAT, pTe_{tyc}, and pTe_{srf} was described previously [13,32]. The gene fragments encoding the Te_{ent}, Te_{yer}, Te_{DEBS}, and Te_{fen} domains were amplified by PCR from the plasmids pEnt-1293 [11], pPCP3-TeHMWP [33], pACP-TE [34], and the cosmid pFC3-1 [35], respectively, using Vent polymerase (NEB, Schwalbach, Germany). Modified oligonucleotides (MWG Biotech, Ebersberg, Germany) were used to introduce terminal restriction sites (underlined) for cloning (for the Te_{ent} domain, DS99-1: 5′-TAAAGATCTGCTGAAGAAGACAGCAC-3′ and DS99-2: 5′-TAAAAGCTTGGTGGTGGTGGT; for Te_{yer} domain, DS99-3: 5′-ATAAGATCTGCGGCTACGTCAGGAGAG-3′ and DS99-2; for Te_{DEBS} domain, HM98-30: 5′-ATAAGATCTCAGCAGCTCGACAGCG-3′ and HM98-31: 5′-ATAGGATCCTGAATTCCCTCCGCCCA-3′; for Te_{fen} DS0023: 5′-AAACCATGGCTAGATCTCAGTCAGCGGCCGG-3′ and DS0024: 5′-TAAGGATCCAGTCTTATTTGGCAGCAC-3′). The PCR fragments encoding the Te_{ent} and Te_{yer} domains were digested with *Bgl*II and *Hin*dIII and ligated into pQE60 vector (Qiagen, Hilden, Germany) that was digested in the same way, to give the plasmids pTe_{ent} and pTe_{yer}. The amplificate for the Te_{DEBS} domain was digested by *Bam*HI and *Bgl*II and ligated into a *Bgl*II-digested and calf intestinal phosphatase-treated pQE60 vector. The amplificate for Te_{fen} was digested with *Nco*I and *Bam*HI and ligated into a pQE60 vector that was digested in the same way, resulting in the plasmid pTe_{DEBS}. pTe_{tyc}, pTe_{srf}, pTe_{fen}, pTe_{ent}, pTe_{yer}, pTe_{DEBS} and pTe_{fen} were then digested with *Bgl*II and *Xba*I and the excised fragments encoding the Te domains were ligated into the pProCAT vector that was digested in the same way, to give the final plasmids pProCAT-Te_{tyc}, pProCAT-Te_{srf}, pProCAT-Te_{fen}, pProCAT-Te_{ent}, pProCAT-Te_{yer} and pProCAT-Te_{DEBS}.

5.2. Production and purification of recombinant enzymes

E. coli BL21[pREP4-gsp] was used as the heterologous host for recombinant protein production and was transformed with the expression plasmids described above. The plasmid pREP4-gsp carries the *lacI*^{q} gene for efficient repression of transcription before induction to provide a tightly controlled expression of the cloned genes, and the *gsp* gene encoding the Ppant transferase Gsp ensuring the posttranslational in vivo conversion of the produced NRPSs from the inactive apo- into the active holo-form in vivo [6]. Expression and purification by Ni-affinity chromatography of the recombinant proteins was performed as described before [6,13]. Pooled enzyme fractions were dialyzed against assay

buffer (50 mM HEPES, 100 mM NaCl, 1 mM EDTA, 2 mM dithioerythritol, pH 8.0). Protein concentrations were determined using the calculated extinction coefficient for the absorption at 280 nm (for TycA 136 580, ProCAT 92 230, ProCAT-Te_{tyc} 126 060, ProCAT-Te_{srf} 116 650, ProCAT-Te_{fen} 119 210, ProCAT-Te_{ent} 134 410, ProCAT-Te_{yer} 145 110, ProCAT-Te_{DEBS} 134 290).

5.3. Standard NRPS assays

Activities of the enzymes in the adenylation reaction was measured with the ATP–inorganic pyrophosphate (PP_i) exchange assay [6], and their ability to covalently bind amino acids at their PCP domains was monitored with the thioester formation assay [6]. [^{32}P]PP_i was purchased from NEN and ^{14}C-labeled amino acids [^{14}C]L-Phe (450 mCi/mmol) and [^{14}C]L-Pro (246 mCi/mmol) were purchased from Hartmann Analytics, Braunschweig, Germany.

5.4. Reaction profiles for dipeptide formation and product release

A 3.5 ml reaction mixture containing 500 nM TycA and 500 nM of the respective ProCAT-(Te) enzyme, 2.2 μM [^{14}C]L-Phe and 10 mM $MgCl_2$ was preincubated in assay buffer at 37°C. The reaction was initiated by the addition of 5 mM ATP. After 9 min, enough time for TycA to be loaded with its substrate, the reaction mixture was divided into three portions by carefully pipetting equal parts into four cups containing the second amino acid, L-Pro, L-Ala, L-Abu, or L-Ile, at a final concentration of 1 mM. Before and after the division step, 100 μl aliquots were removed at various time points and the reaction was stopped by the addition of 800 μl ice-cold TCA (10%) and 15 μl bovine serum albumin solution (25 mg/100 ml). Following 30 min incubation on ice the samples were centrifuged for 30 min at 13 000 rpm and 4°C to collect the precipitated proteins. The pellet was washed with 800 μl ice-cold TCA (10%), redissolved in 200 μl formic acid and mixed with 3.5 ml liquid scintillation fluid (Rotiscint Eco Plus; Roth) for liquid scintillation counting using a 2100 TR liquid scintillation analyzer (Packard).

5.5. HPLC/MS analysis of the products

For HPLC/MS analysis, product assays were performed in a three-fold scale-up compared to the above described radioactive assay. Radioactive amino acids were replaced by 1 mM nonradioactive amino acids, reaction mixtures were incubated at 37°C for 1 h, stopped by the addition of 150 μl butanol/chloroform (4:1) and evaporated under reduced pressure. The pellet was dissolved in 200 μl 90% buffer A (0.05% formic acid/water, v/v) and 10% buffer B (0.045% formic acid/methanol, v/v). The HPLC method and column were used as described previously [15].

5.6. Determination of products and product formation rates by TLC

For TLC analysis the assays were performed in a total volume of 100 μl at a concentration of 500 nM for both TycA and ProCAT-(Te) with either 2.2 μM [^{14}C]L-Phe, 100 μM L-Phe, 5 mM L-Abu, and 10 mM $MgCl_2$ in assay buffer or 3.4 μM [^{14}C]L-Pro, 100 μM L-Pro, 1 mM L-Phe, and 10 mM $MgCl_2$ in assay buffer. The reaction mixtures were incubated at 37°C. Reactions were initiated by the addition of 5 mM ATP, and stopped after 60 min by the addition of a half volume of 1-butanol/chloroform (4:1, v/v), vortexed, and evaporated under reduced pressure. The pellet was resuspended in 30 μl methanol. 3 μl of this solution was applied onto a silica gel 60 TLC plate (Merck). The plates were developed in H_2O/1-butanol/acetic acid/ethyl acetate (1:1:1:1, v/v), and analyzed by autoradiography. For determination of the time dependence of product formation, the same assays were performed in a larger scale. At various time points, 100 μl aliquots were removed and prepared as described above. The developed TLC plates were then analyzed by radioactivity scanning (counting time 10 or 15 min) using radio scanner Ritastar (Raytest, Straubenhardt, Germany) and the supplied RITA software. Chemical standards were purchased from Bachem (D-Phe-Ala) and Sigma (D-Phe-Pro).

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References

[1] M.A. Marahiel, T. Stachelhaus, H.D. Mootz, Modular peptide synthetases involved in non-ribosomal peptide synthesis, Chem. Rev. 97 (1997) 2651–2673.
[2] D.E. Cane, C.T. Walsh, C. Khosla, Harnessing the biosynthetic code: combinations, permutations, and mutations, Science 282 (1998) 63–68.
[3] H.D. Mootz, M.A. Marahiel, The tyrocidine biosynthesis operon of *Bacillus brevis*: complete nucleotide sequence and biochemical characterization of functional internal adenylation domains, J. Bacteriol. 179 (1997) 6843–6850.
[4] E. Conti, T. Stachelhaus, M.A. Marahiel, P. Brick, Structural basis for the activation of phenylalanine in the non-ribosomal biosynthesis of gramicidin S, EMBO J. 16 (1997) 4174–4183.
[5] T. Weber, R. Baumgartner, C. Renner, M.A. Marahiel, T.A. Holak, Solution structure of PCP, a prototype for the peptidyl carrier domains of modular peptide synthetases, Struct. Fold Des. 8 (2000) 407–418.
[6] T. Stachelhaus, H.D. Mootz, V. Bergendahl, M.A. Marahiel, Peptide bond formation in nonribosomal peptide biosynthesis. Catalytic role of the condensation domain, J. Biol. Chem. 273 (1998) 22773–22781.
[7] J.W. Trauger, R.M. Kohli, H.D. Mootz, M.A. Marahiel, C.T. Walsh, Peptide cyclization catalysed by the thioesterase domain of tyrocidine synthetase, Nature 407 (2000) 215–218.
[8] A. Haese, R. Pieper, T. von Ostrowski, R. Zocher, Bacterial expression of catalytically active fragments of the multifunctional enzyme enniatin synthetase, J. Mol. Biol. 243 (1994) 116–122.

[9] T. Stachelhaus, C.T. Walsh, Mutational analysis of the epimerization domain in the initiation module PheATE of gramicidin S synthetase, Biochemistry 39 (2000) 5775–5787.
[10] A. Schneider, M.A. Marahiel, Genetic evidence for a role of thioesterase domains, integrated in or associated with peptide synthetases, in non-ribosomal peptide biosynthesis in *Bacillus subtilis*, Arch. Microbiol. 169 (1998) 404–410.
[11] C.A. Shaw-Reid, N.L. Kelleher, H.C. Losey, A.M. Gehring, C. Berg, C.T. Walsh, Assembly line enzymology by multimodular nonribosomal peptide synthetases: the thioesterase domain of *E. coli* EntF catalyzes both elongation and cyclolactonization, Chem. Biol. 6 (1999) 385–400.
[12] F. de Ferra, F. Rodriguez, O. Tortora, C. Tosi, G. Grandi, Engineering of peptide synthetases. Key role of the thioesterase-like domain for efficient production of recombinant peptides, J. Biol. Chem. 272 (1997) 25304–25309.
[13] H.D. Mootz, D. Schwarzer, M.A. Marahiel, Construction of hybrid peptide synthetases by module and domain fusions, Proc. Natl. Acad. Sci. USA 97 (2000) 5848–5853.
[14] S. Doekel, M.A. Marahiel, Dipeptide formation on engineered hybrid peptide synthetases, Chem. Biol. 7 (2000) 373–384.
[15] U. Linne, M.A. Marahiel, Control of directionality in nonribosomal peptide synthesis role of the condensation domain in preventing misinitiation and timing of epimerization, Biochemistry 39 (2000) 10439–10447.
[16] P. Cosmina, F. Rodriguez, F. de Ferra, G. Grandi, M. Perego, G. Venema, D. van Sinderen, Sequence and analysis of the genetic locus responsible for surfactin synthesis in *Bacillus subtilis*, Mol. Microbiol. 8 (1993) 821–831.
[17] G.H. Lin, C.L. Chen, J.S. Tschen, S.S. Tsay, Y.S. Chang, S.T. Liu, Molecular cloning and characterization of fengycin synthetase gene *fenB* from *Bacillus subtilis*, J. Bacteriol. 180 (1998) 1338–1341.
[18] A.M. Gehring, I. Mori, C.T. Walsh, Reconstitution and characterization of the *Escherichia coli* enterobactin synthetase from EntB, EntE, and EntF, Biochemistry 37 (1998) 2648–2659.
[19] A.M. Gehring, E. DeMoll, J.D. Fetherston, I. Mori, G.F. Mayhew, F.R. Blattner, C.T. Walsh, R.D. Perry, Iron acquisition in plague: modular logic in enzymatic biogenesis of yersiniabactin by *Yersinia pestis*, Chem. Biol. 5 (1998) 573–586.
[20] J. Cortes, K.E. Wiesmann, G.A. Roberts, M.J. Brown, J. Staunton, P.F. Leadlay, Repositioning of a domain in a modular polyketide synthase to promote specific chain cleavage, Science 268 (1995) 1487–1489.
[21] H.D. Mootz, M.A. Marahiel, Design and application of multimodular peptide synthetases, Curr. Opin. Biotechnol. 10 (1999) 341–348.
[22] R. Dieckmann, M. Pavela-Vrancic, H. von Döhren, H. Kleinkauf, Probing the domain structure and ligand-induced conformational changes by limited proteolysis of tyrocidine synthetase 1, J. Mol. Biol. 288 (1999) 129–140.
[23] M. Gocht, M.A. Marahiel, Analysis of core sequences in the D-Phe activating domain of the multifunctional peptide synthetase TycA by site-directed mutagenesis, J. Bacteriol. 176 (1994) 2654–2662.
[24] K. Fujikawa, Y. Sakamoto, T. Suzuki, K. Kurahashi, Biosynthesis of tyrocidine by a cell-free enzyme system of *Bacillus brevis* ATCC 8185. II. Amino acid substitution in tyrocidine, Biochim. Biophys. Acta 169 (1968) 520–533.
[25] B. Walzel, B. Riederer, U. Keller, Mechanism of alkaloid cyclopeptide synthesis in the ergot fungus *Claviceps purpurea*, Chem. Biol. 4 (1997) 223–230.
[26] D.J. Smith, A.J. Earl, G. Turner, The multifunctional peptide synthetase performing the first step of penicillin biosynthesis in *Penicillium chrysogenum* is a 421,073 dalton protein similar to *Bacillus brevis* peptide antibiotic synthetases, EMBO J. 9 (1990) 2743–2750.
[27] A.M. van Wageningen, P.N. Kirkpatrick, D.H. Williams, B.R. Harris, J.K. Kershaw, N.J. Lennard, M. Jones, S.J. Jones, P.J. Solenberg, Sequencing and analysis of genes involved in the biosynthesis of a vancomycin group antibiotic, Chem. Biol. 5 (1998) 155–162.
[28] M.S. Weiss, A. Jabs, R. Hilgenfeld, Peptide bonds revisited, Nat. Struct. Biol. 5 (1998) 676.
[29] F.G. Healy, M. Wach, S.B. Krasnoff, D.M. Gibson, R. Loria, The *txtAB* genes of the plant pathogen *Streptomyces acidiscabies* encode a peptide synthetase required for phytotoxin thaxtomin A production and pathogenicity, Mol. Microbiol. 38 (2000) 794–804.
[30] K.J. Weissman, C.J. Smith, U. Hanefeld, R. Aggarwal, M. Bycroft, J. Staunton, P.F. Leadlay, Die Thioesterase der Polyketid-Synthase von Erythromycin: Einfluß der Acylkettenstruktur auf den Abkopplungsmechanismus der Substratanaloga von der Acyl-Enzym-Zwischenstufe, Angew. Chem. 110 (1998) 1503–1506.
[31] R.S. Gokhale, D. Hunziker, D.E. Cane, C. Khosla, Mechanism and specificity of the terminal thioesterase domain from the erythromycin polyketide synthase, Chem. Biol. 6 (1999) 117–125.
[32] R.M. Kohli, J.W. Trauger, D. Schwarzer, M.A. Marahiel, C.T. Walsh, Exploring the generality of peptide cyclization catalyzed by isolated thioesterase domains of non-ribosomal peptide synthetases, Biochemistry 40 (2001) 7099–7108.
[33] Z. Suo, C.C. Tseng, C.T. Walsh, Purification, priming, and catalytic acylation of carrier protein domains in the polyketide synthase and nonribosomal peptidyl synthetase modules of the HMWP1 subunit of yersiniabactin synthetase, Proc. Natl. Acad. Sci. USA 98 (2001) 99–104.
[34] P. Caffrey, B. Green, L.C. Packman, B.J. Rawlings, J. Staunton, P.F. Leadlay, An acyl-carrier-protein-thioesterase domain from the 6-deoxyerythronolide B synthase of *Saccharopolyspora erythraea*. High-level production, purification and characterisation in *Escherichia coli*, Eur. J. Biochem. 195 (1991) 823–830.
[35] T.P. Lin, C.L. Chen, L.K. Chang, J.S. Tschen, S.T. Liu, Functional and transcriptional analyses of a fengycin synthetase gene, *fenC*, from *Bacillus subtilis*, J. Bacteriol. 181 (1999) 5060–5067.
[36] J. Sambrook, E.F. Fritsch, T. Maniatis, Molecular Cloning: A Laboratory Manual, 2nd edn., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989.