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Research Paper

Role of type II thioesterases: evidence for removal of short acyl chains produced by aberrant decarboxylation of chain extender units

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Abstract

Background: Modular polyketide synthases (PKSs) function as molecular assembly lines in which polyketide chains are assembled by successive addition of chain extension units. At the end of the assembly line, there is usually a covalently linked type I thioesterase domain (TE I), which is responsible for release of the completed acyl chain from its covalent link to the synthase. Additionally, some PKS clusters contain a second thioesterase gene (TE II) for which there is no established role. Disruption of the TE II genes from several PKS clusters has shown that the TE II plays an important role in maintaining normal levels of antibiotic production. It has been suggested that the TE II fulfils this role by removing aberrant intermediates that might otherwise block the PKS complex.

Results: We show that recombinant tylosin TE II behaves in vitro as a TE towards a variety of *N*-acetylcysteamine and *p*-nitrophenyl esters. The trends of hydrolytic activity determined by the kinetic parameter k_{cat}/K_M for the analogues tested indicates that simple fatty acyl chains are effective substrates. Analogues that modelled aberrant forms of putative tylosin biosynthetic intermediates were hydrolysed at low rates.

Conclusions: The behaviour of tylosin TE II in vitro is consistent with its proposed role as an editing enzyme. Aberrant decarboxylation of a malonate-derived moiety attached to an acyl carrier protein (ACP) domain may generate an acetate, propionate or butyrate residue on the ACP thiol. Our results suggest that removal of such groups is a significant role of TE II.

Keywords: Decarboxylation; Editing enzyme; Polyketide synthase; Tylosin biosynthesis; Type II thioesterase

1. Introduction

Polyketides, such as the macrolide antibiotics erythromycin A and tylosin, constitute a large and structurally diverse class of natural products that exhibit many important biological activities. Their biosynthesis is achieved by repeated condensations of simple carboxylic acid precursors, activated as CoA thioesters, in a manner that closely parallels fatty acid biosynthesis. Chain assembly takes place on modular polyketide synthases (PKSs) that consist of giant multifunctional proteins, with discrete catalytic domains responsible for each successive cycle of chain elongation. Many PKSs contain a terminal thioesterase (TE) domain that is believed to catalyse the release of the fully processed polyketide chain. Alternatively, a specialised cyclase or transferase may be used which forms an amide bond, as in the pipecolate incorporating enzyme thought to be associated with the rapamycin PKS [1], or the amide synthetase found in the rifamycin PKS [2,3]. In other PKS clusters, such as the type I iterative PKS responsible for 6-methylsalicylic acid biosynthesis [4] and the dissociable type II iterative PKS responsible for actinorhodin biosynthesis [5], the mechanism of product release remains a mystery.

Based on the synthase sequences published to date, the type I TE (TE I) mechanism seems to be the preferred method of release in type I systems. For example, the domain sequence of the animal fatty acid synthase (FAS) system terminates with a TE as shown in Fig. 1a, as does the final module (module 6) of the modular type I erythromycin PKS (also known as 6-deoxyerythronolide B synthase or DEBS) shown in Fig. 1b. In the FAS, the cycle of chain extension cycles is repeated iteratively until a fatty acid chain of the required length is reached. At this

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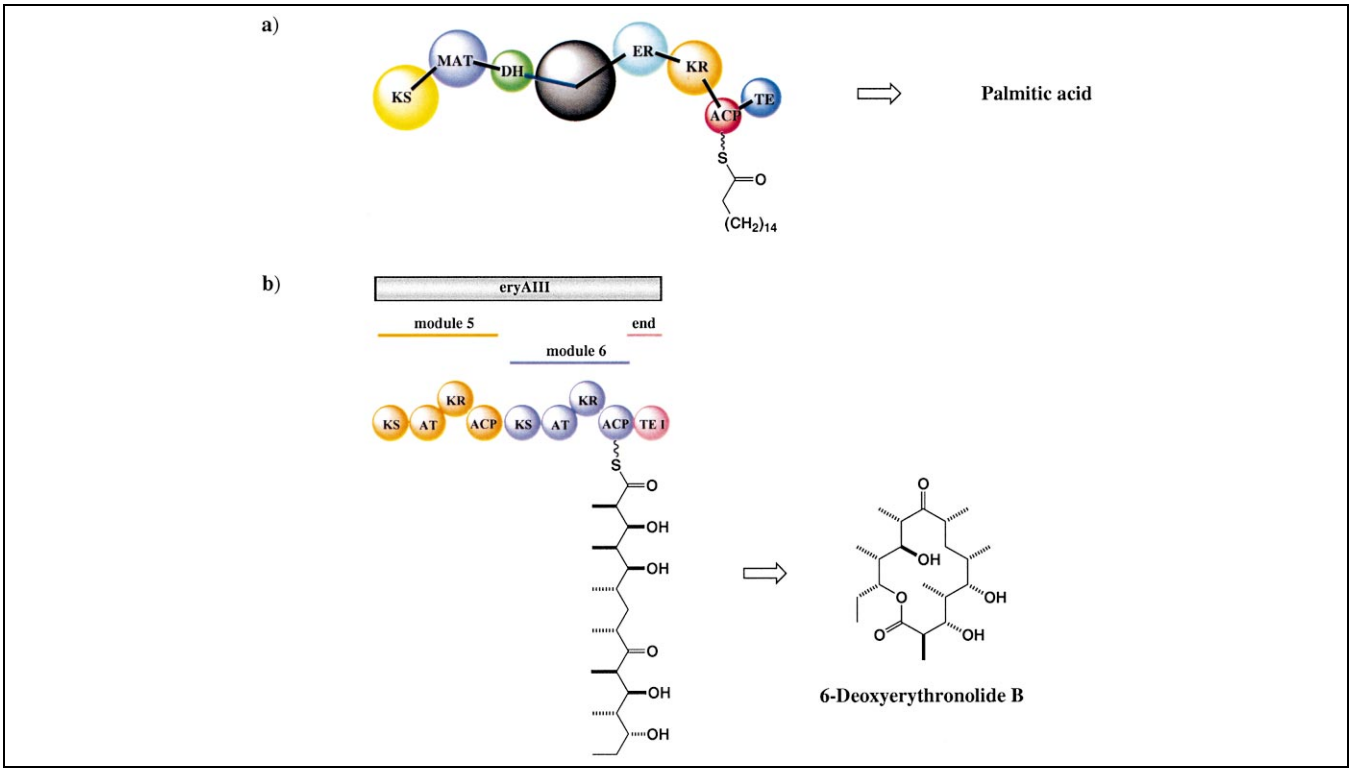


Fig. 1. Chain release catalysed by TE domains in (a) FAS and (b) PKS systems.

point, the TE intervenes by recognising an intermediate of the appropriate length, so that instead of being further extended, the fatty acyl chain is released either by hydrolysis or by transfer to a suitable nucleophile. The TE of the erythromycin PKS, in contrast, meets the growing chain only after it has reached the appropriate stage of development at the end of the synthetic operations in module 6. The TE then fulfils its role by releasing the polyketide chain as a macrolactone. In this context, the TE I does not control chain length by substrate recognition and it has been shown to release intermediates at various stages of chain extension when it is relocated to earlier modules [6–9].

The mechanism of operation of the TE domains associated with PKS and FAS enzymes is closely related to that established for proteolytic enzymes such as chymotrypsin. In the first step, the full-length fatty acyl or polyketide chain is transferred from the thiol group of the phosphopantetheine arm of the acyl carrier protein (ACP) onto the hydroxyl residue of a serine residue at the active site of the TE. The resulting oxyester is then cleaved in an 'off-loading' operation by a suitable nucleophilic species. This would be water in the case of hydrolysis, an external carbinol hydroxyl in the formation of an ester, and a suitable distal hydroxyl group of the polyketide chain in the formation of a lactone. The mode of action of the TE I from the erythromycin PKS has been established by in vitro experiments with a truncated version of DEBS 3, comprising the ACP6-TE bidomain, which was overexpressed in *Escherichia coli* [10–12]. In these experiments, the substrate specificity of the TE I was explored using various synthetic substrates [11,12]. The formation of an acyl–enzyme intermediate was demonstrated for all substrates by electrospray ionisation-mass spectrometry (ESI-MS) [10–12], consistent with the reaction mechanism shown in Fig. 2. The very relaxed substrate specificity of the TE I evident in these in vitro experiments would not be a handicap in the context of the

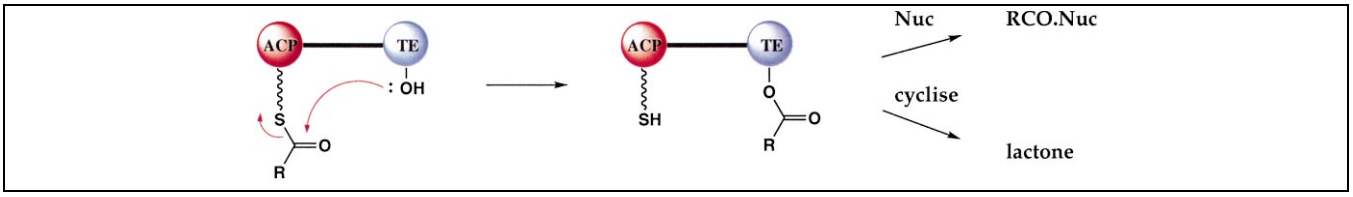


Fig. 2. Mechanism of action of TEs to form an acyl–enzyme intermediate. The resultant tetrahedral intermediate is released by nucleophilic attack of an external nucleophile or an internal nucleophile to form a lactone.

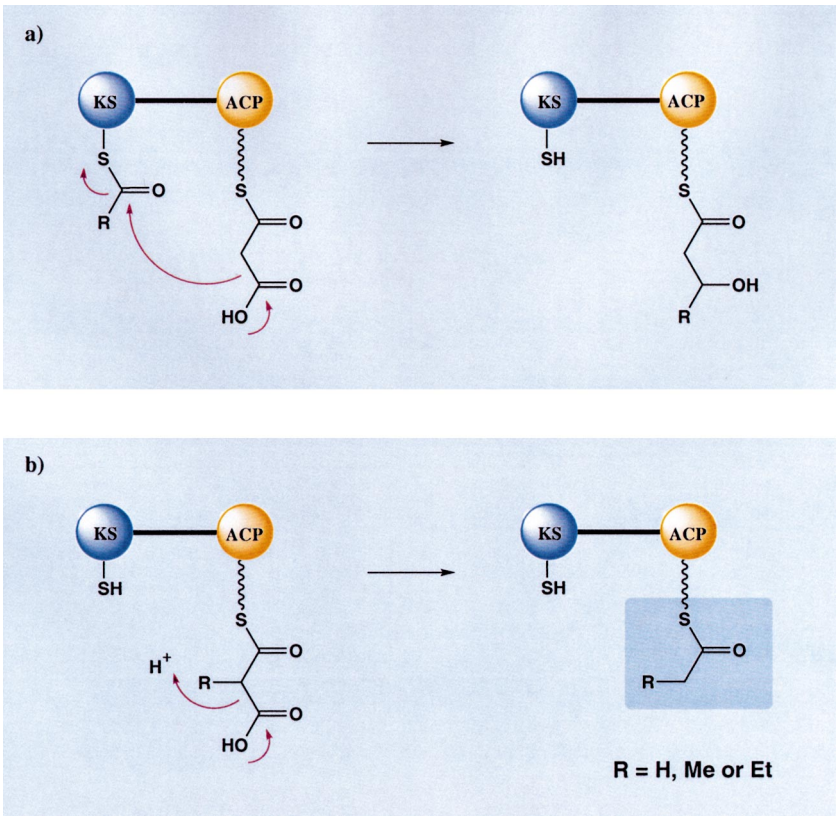


Fig. 3. (a) A typical decarboxylative chain elongation step in FASs and PKSs. (b) Aberrant decarboxylation by the KS domain to give an acetate, propionate or butyrate residue attached to the ACP.

DEBS system because the enzyme does not have to select its substrate from the full set of intermediates on the synthase.

The role of the integrated TE I domains at the carboxyl termini of modular PKSs, therefore, has been clearly established as one of acyl chain release. Furthermore, experiments carried out both in vivo and in vitro have shown that these TE Is are self-sufficient and do not require ancillary activities to carry out their normal function [11–13]. Therefore, it is puzzling that many PKS clusters contain additional TE genes (so-called type II TEs or TE II) in the vicinity of the PKS genes, for which there is no obvious role. So far, investigations into the putative roles of TE IIs in polyketide biosynthesis have been based on gene disruption strategies. Deletion of the TE II gene from the pikromycin cluster of *Streptomyces venezuelae* led to a mutant that produced less than 5% of the expected 12- and 14-membered macrolide products [14]. A similar result was obtained with the tylosin producer *Streptomyces fradiae*, where it was shown that only 10% of wild-type antibiotic production was obtained when the tylosin TE II gene (*ORF5*) was disrupted [15]. Deletion of the analogous TE II gene, *rifR*, in the rifamycin PKS cluster of *Amycolatopsis mediterranei* also established that this additional TE activity is required for normal accumulation of the ansamycin antibiotic rifamycin B [16]. Together, these studies suggest that TE IIs play a beneficial role during polyketide biosynthesis, but are not essential for polyketide production by modular PKSs.

To explain the outcome of their pikromycin TE II disruption experiments Xue et al. proposed that TE IIs may behave as chain terminators and/or cyclases, allowing the simultaneous production of polyketides of different chain lengths [14], as is the case with TE IIs in FAS systems [17]. Experiments where the pikromycin PKS (PikAI–PikAIV) was heterologously expressed in *Streptomyces lividans*, however, have ruled out the possibility that the pikromycin TE II domain is responsible for alternative chain termination, since both 12- and 14-membered ring products accumulated [18]. Rather these experiments suggest that the ability to produce polyketides of different chain lengths is an inherent property of the pikromycin PKS itself. A recent report in the literature has revealed the presence of an alternative translational start site within the *pikAIV* gene, which generates a truncated form of pikromycin module 6 under certain growth conditions, leading to the production of 10-deoxymethynolide [19].

Cundliffe et al. have suggested that type II TEs play an editing or corrective role during polyketide biosynthesis by purging the PKS of abnormal polyketide intermediates formed by the aberrant action of the biosynthetic machinery of the PKS [15]. Schneider et al. have also speculated that the free TE activity in peptide synthetase systems might clear modules that are blocked by the presence of

aberrant intermediates [20]. Removal of the TE II in either system could seriously delay the restoration of activity to blocked modules, and ultimately could result in cessation of polypeptide/polyketide biosynthesis in the absence of an alternative clearance mechanism.

There are a number of possible ways in which the TE II could carry out its editing activity during chain extension. Firstly, it could behave as an indiscriminate chain release enzyme, whereby any polyketide intermediate is removed if it has a long enough half-life and is exposed to attack. Alternatively, it could have the intrinsic ability to differentiate between normal polyketide intermediates and those containing mistakes and only remove incorrectly processed polyketide chains.

Molecular mistakes might arise in a chain extension module via a number of different mechanisms. Most obviously, the chain elongation process in a given module may take a wrong course, so that a keto group is reduced to the wrong oxidation level or methyl/hydroxyl branching groups with the wrong stereochemistry are produced. The ACP of that module would then carry an aberrant product, which could be cleared by further processing by downstream domains to give an aberrant PKS product, or by TE II, as will be explained.

In this paper, we propose an alternative mechanism for generating aberrant acyl intermediates, in which mistakes arise because of occasional malfunctioning of ketosynthase (KS) domains. During normal chain extension, the acyltransferase (AT) domain transfers a chain extension unit (e.g. methylmalonate) to the ACP. This is subsequently bound to the KS active site (non-covalently), where it normally undergoes concerted decarboxylative condensation with the acyl species attached to the KS active site cysteine thiol (Fig. 3a). Occasionally, as an unwanted side reaction, the KS may catalyse the decarboxylation of the chain extender unit in the absence of a suitable acyl thioester bound to the active site thiol of the KS. Protonation of the resulting enolate would leave an acetate, propionate or butyrate residue attached to the ACP (Fig. 3b). Here, we describe in vitro experiments carried out with the TE II from the tylosin-producing PKS of *S. fradiae*. By establishing a substrate specificity profile for tylosin TE II, we have gained valuable insights into the mechanism and function of TE IIs in polyketide biosynthesis.

2. Results

2.1. Overexpression and purification of the tylosin TE II

The expression host of choice was *E. coli*, following the successful overexpression of the ACP6-TE bidomain from the carboxyl terminus of DEBS 3 [10] and the TE Is and TE IIs associated with the rat FAS [21]. Following amplification of the *ORF5* gene by polymerase chain reaction (PCR) with mutagenic primers, designed to introduce an *Nde*I restriction site spanning the first codon of the TE II domain and a *Bam*HI site at the 3′ end of the gene, the *ORF5* gene was introduced into pUC18 and the accuracy of amplification verified by DNA sequencing. The tylosin TE II (*ORF5*) gene was subsequently cloned into the *E. coli* expression plasmid pET-28b(+) to give pMLH27, so that the TE II gene could be expressed as an N-terminal His_6-tagged protein. This plasmid was then used to transform *E. coli* BL21(λDE3)pLysS. The total cell extracts from induced and non-induced cells were analysed by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE). A band corresponding to 30 kDa (Fig. 4) was detected in induced cells carrying the expression plasmid pMLH27, but was absent from induced cells carrying the parent pET vector with no insert. The TE II protein was purified to homogeneity by affinity chromatography using Ni-NTA superflow resin (Qiagen), followed by an anion exchange step (Fig. 4). Typically, 10 mg of protein was obtained from 1 g of cells (wet weight).

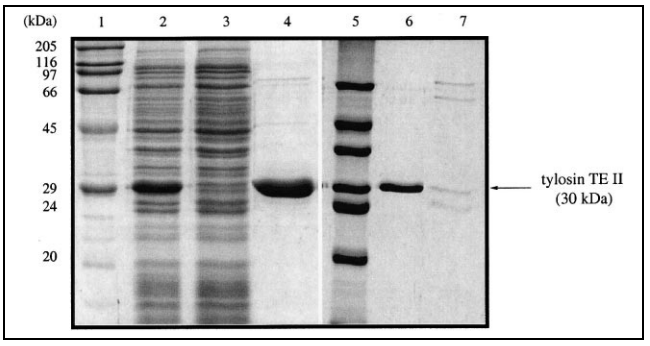


Fig. 4. Purification of the heterologously expressed tylosin TE II domain. Samples of the recombinant protein were analysed by SDS–PAGE at different stages of a typical purification procedure. Gels were visualised by staining with Coomassie brilliant blue. Lane 1, molecular weight markers; lane 2, soluble fraction from induced cells carrying pMLH27; lane 3, Ni-NTA column flow-through; lane 4, purified tylosin TE II after affinity chromatography; lane 5, molecular weight markers; lane 6, purified tylosin TE II after anion exchange; lane 7, contaminant proteins eluted after anion exchange.

2.2. Molecular weight determination for tylosin TE II

The recombinant tylosin TE II protein was analysed by ESI-MS according to the methods used previously by others for analysis of the rat mammary gland TE II [22] and the ACP6-TE bidomain from the erythromycin PKS in *Saccharopolyspora erythraea* [10,12]. The TE II mass species from tylosin TE II produced a characteristic bell-shaped distribution of multiply charged ions, with each adjacent major peak in the spectrum differing by one charge. Spectra were subjected to a normal entropy transform to give a single peak. Analysis of the purified tylosin TE II protein by ESI-MS revealed a major species with a molecular mass of $29\,679.6 \pm 1.3$ Da. This corresponds to the molecular mass predicted for the recombinant protein

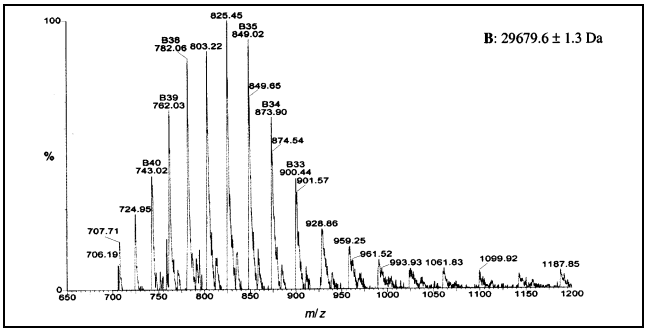


Fig. 5. ESI-MS spectrum of the recombinant tylosin TE II protein. The expected molecular mass of the enzyme from which the N-terminal methionine has been removed is $[M+H]^+$, 29 679.4 Da.

from which the N-terminal methionine has been removed (Fig. 5).

The TE activity of various type I FASs is inhibited by the well-characterised serine proteinase inhibitor phenylmethylsulphonyl fluoride (PMSF) [17,23]. Therefore purified tylosin TE II was treated with PMSF and analysed by ESI-MS. The spectra revealed that about 30% of the recombinant protein had an increase in mass of 155 ± 2.0 Da (data not shown), suggesting that a single reactive serine residue was being specifically modified. Complete modification of the TE II enzyme was not observed presumably due to the occurrence of acid-catalysed elimination of the PMSF group, a phenomenon that has been observed before with PMSF-treated ACP6-TE [10] and PMSF-treated chymotrypsin [24].

2.3. Native molecular mass determination for tylosin TE II

The TE Is [25] and TE IIs [26] from the rat FAS are monomeric, whereas the TE Is associated with modular PKSs are dimeric [27,28]. The quaternary structure of the recombinant tylosin TE II protein was investigated using a combination of gel filtration and analytical ultracentrifugation. The native molecular mass of the purified tylosin TE II protein was estimated by gel filtration chromatography on a calibrated Superdex 200 column. Tylosin TE II eluted with an apparent molecular weight of 26 kDa (calculated mass 30 kDa), suggesting that tylosin TE II also exists as a monomer. This was confirmed by ultracentrifugation studies over a range of protein concentrations and at different rotor speeds. The data for tylosin TE II were fitted to various models, but the best fits were found with a model in which only the monomer was present under any of the conditions used. This is in complete contrast to the type I TE associated with the erythromycin PKS [27].

2.4. Substrate specificity of tylosin TE II

TE-catalysed cleavage of an acyl thioester is believed to involve two discrete steps which may have significantly different structure specificity profiles, transfer of the acyl chain from the thiol of the phosphopantetheine arm of the ACP to the serine hydroxyl group in the active site of the TE, and the subsequent cleavage of this intermediate acyl ester by attack of a nucleophilic species (see Fig. 2). To explore the acylation step, *N*-acetylcysteamine (NAC) thioester derivatives are a logical choice, since they are good structural mimics of the phosphopantetheine moiety of the ACP. They therefore serve as close surrogates for the natural ACP-bound acyl chain, which one might reasonably surmise is the natural substrate for the TE II. For demonstrating the formation of an acyl–enzyme intermediate and studying the deacylation step there is ample precedent for preferring the more reactive *p*-nitrophenolate esters [11,12]. *p*-Nitrophenyl esters are less appropriate structural analogues of the ACP, but they are well-characterised substrates for serine proteinases and have been used routinely in studies on the TEs from animal FAS [29] and the erythromycin PKS [11,12].

To investigate the substrate specificity of tylosin TE II, purified enzyme was incubated with the NAC thioesters **1**–**4** and *p*-nitrophenyl esters **5**–**8** of acetate, propionate, butyrate and pentanoate (Fig. 6a). The compounds **9** and **10** were also tested (Fig. 6b). These substrates were chosen to represent incorrectly processed intermediates generated by mistakes occurring during the chain elongation operations of modules 1 and 2 of the tylosin PKS (intermediates **11** and **12**, respectively, Fig. 6b).

The assay conditions used for monitoring the TE-catalysed hydrolysis of the NAC thioesters **1**–**4** and **9** were adapted from experiments reported by Tai et al. with the rat TE II [30] and Gokhale et al. with the DEBS TE I domain [28]. The release of free thiol was followed by addition of Ellman's reagent (5,5′-dithio-2-nitrobenzoic acid, DTNB). DTNB reacts at neutral pH with sulfhydryl groups to liberate the yellow compound 5-thio-2-nitrobenzoic acid which can be monitored by UV spectroscopy ($\lambda_{max} = 412$ nm, $\varepsilon = 13\,600$ M^{-1} cm^{-1}). Each substrate was tested at various concentrations by incubation with tylosin TE II (1.3 μM) at 30°C in assay buffer (200 mM KPi (pH 7.5), 2.5 mM Tris–HCl (pH 7.5), 50 μM EDTA) in the presence of DTNB (0.2 mM) and 3% (v/v) dimethylsulphoxide to aid substrate solubility. All assays were carried out in duplicate, and the rate of reaction corrected for the background rate of chemical hydrolysis in the absence of enzyme.

A plot of substrate turnover against the substrate concentration and the specificity constants (k_{cat}/K_M) for the NAC thioesters **1**–**4** and **9** are shown in Fig. 7. It was not practical to establish a substrate specificity profile by comparing k_{cat} and K_M values, since they could only be estimated for substrates **2** and **3**. The remaining substrates were not sufficiently soluble in the assay buffer to allow kinetic measurements under substrate-saturating conditions. Therefore comparisons between substrates were made by calculation of the k_{cat}/K_M values at low substrate

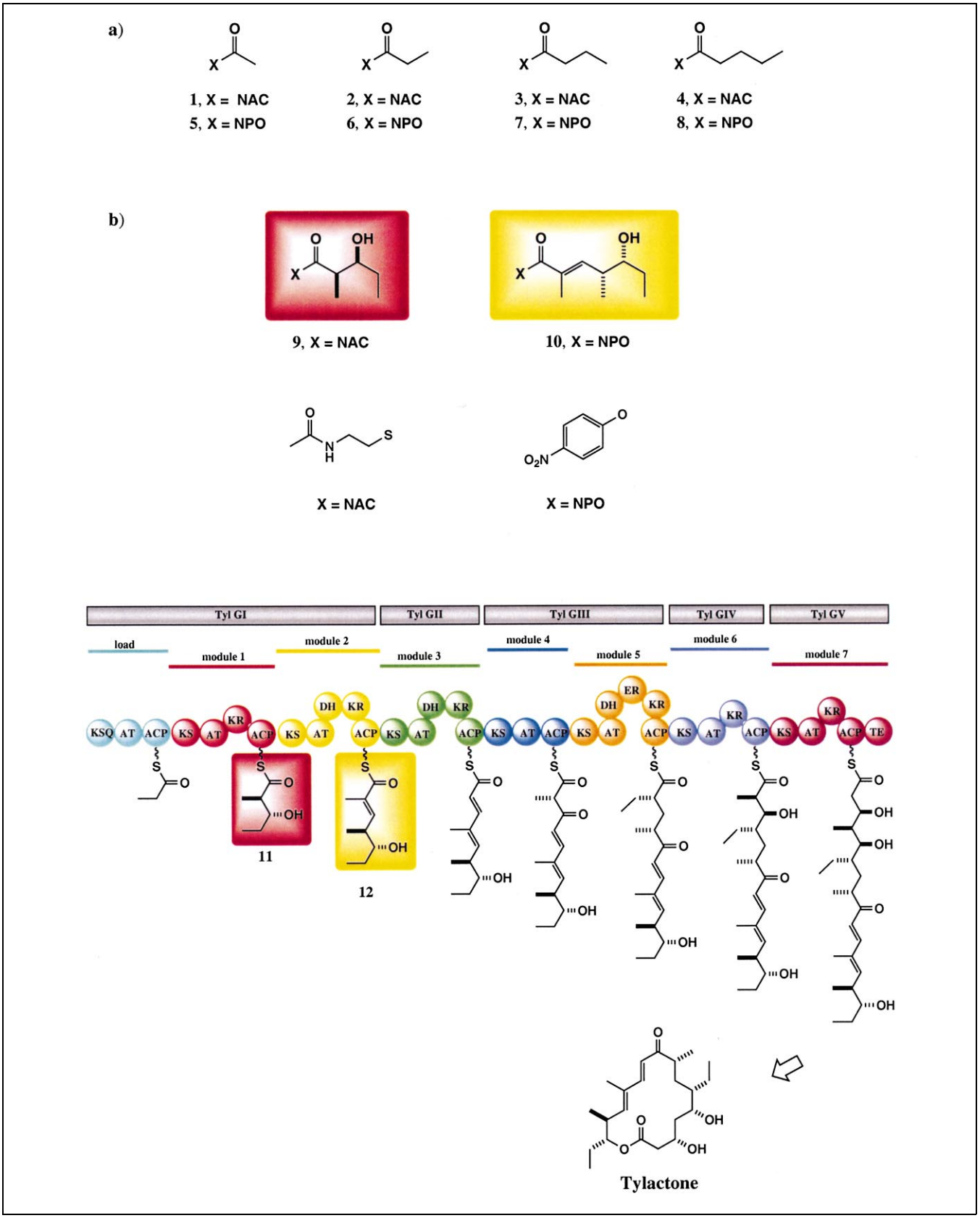
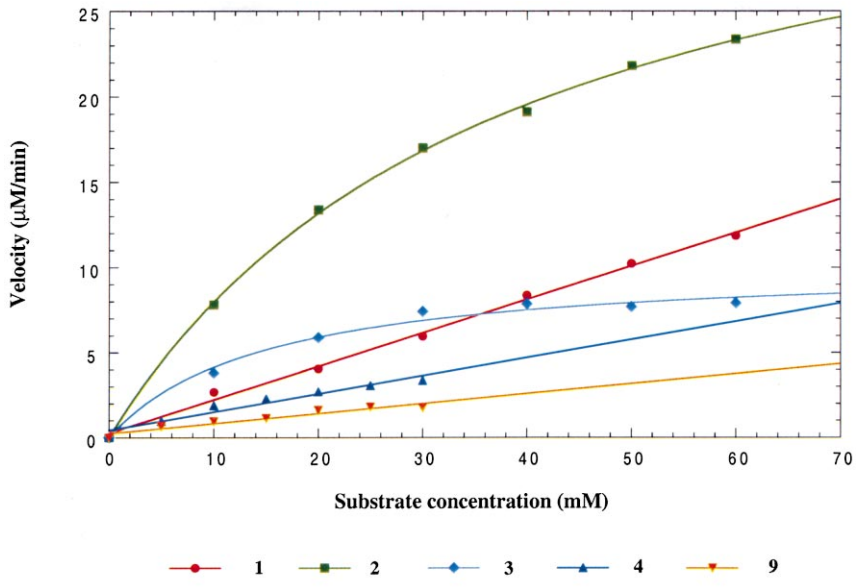


Fig. 6. Substrates chosen for investigation of the specificity of tylosin TE II in vitro. (a) Substrates **1**–**8** were designed to test the effect of chain length on TE II specificity. (b) The ketide analogues **9** and **10** were modelled on the diketide **11** and triketide **12** biosynthetic intermediates of the tylosin PKS.



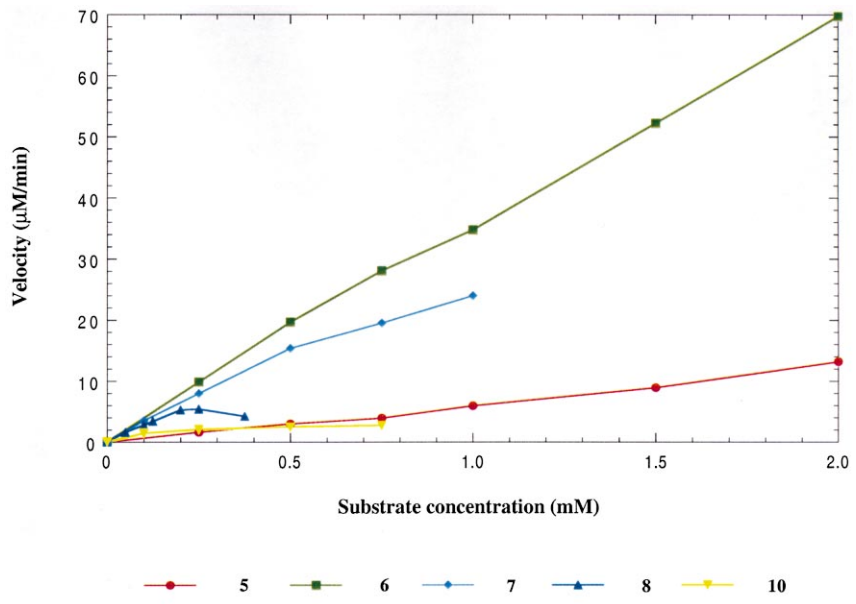
Substrates	k_{cat} (min^{-1})	K_M (mM)	k_{cat}/K_M ($M^{-1}s^{-1}$)
1	-	-	2.5
2	29.2	37.9	12.9
3	11.0	28.2	6.5
4	-	-	1.7
9	-	-	0.9

Fig. 7. Rate versus concentration plots for the hydrolysis of acyl NAC thioester substrates **1**–**4** and **9** by recombinant tylosin TE II. The k_{cat}/K_M values for substrates **1**, **4** and **9** were calculated from the linear slope of each plot at low substrate concentration, whereas the k_{cat}/K_M values for substrates for substrates **2** and **3** were estimated using a Michaelis–Menten curve fit (Kaleidagraph software) or using an Eadie–Hofstee plot.

concentration. All the NAC thioester substrates tested were hydrolysed by tylosin TE II at a significant rate. The trend in reactivity of each of the NAC thioesters, in terms of the specificity constants, is in the order: propionate **2** > butyrate **3** > acetate **1** > pentanoate **4** > diketide **9**. These results indicate that tylosin TE II is capable of removing simple fatty acyl chains produced by aberrant decarboxylation of chain extender units. The fact that there is a 14-fold difference between the k_{cat}/K_M values for propionate **2** and the diketide **9** suggests that the TE II enzyme may play a significant but lesser role in removing incorrectly processed ketide intermediates.

In parallel studies, the *p*-nitrophenolate derivatives **5**–**8** and **10** were incubated with tylosin TE II (1.3 μM) at 30°C in assay buffer (200 mM KPi (pH 7.5), 2.5 mM Tris–HCl (pH 7.5), 50 μM EDTA) in the presence of 3% (v/v) dimethylsulphoxide to aid substrate solubility. The rate of cleavage of the *p*-nitrophenyl esters **5**–**8** and **10** was monitored at various substrate concentrations using UV spectroscopy, by following the rate of formation of the *p*-nitrophenolate anion ($\lambda_{max} = 400$ nm, $\varepsilon = 8570$ M^{-1} cm^{-1}). As before, all assays were carried out in duplicate, and the rate of reaction corrected for the background rate of chemical hydrolysis in the absence of enzyme.

A plot of the initial rates of *p*-nitrophenol production (i.e. substrate turnover) against the substrate concentration and the calculated values of k_{cat}/K_M for each substrate are given in Fig. 8. Unfortunately, due to limits in the solubility of the substrates **5**–**8** and **10** in the aqueous assay buffer, substrate-saturating conditions were unattainable and, therefore, the apparent K_M and k_{cat} values for each substrate could not be calculated with any reliability. As before, all substrates were hydrolysed at significant rates by the tylosin TE II. The high specificity constants observed for the *p*-nitrophenyl esters **5**–**8** and **10** relative to the NAC thioesters **1**–**4** and **9** presumably reflect the high reactivity of *p*-nitrophenol as a leaving group. Comparison of the specificity constants for sub-



Substrates	k_{cat}/K_M ($M^{-1}s^{-1}$)
5	83
6	439
7	306
8	284
10	39

Fig. 8. Rate versus concentration plots for the hydrolysis of acyl *p*-nitrophenyl substrates **5**–**8** and **10** by recombinant tylosin TE II. The k_{cat}/K_M for each substrate was calculated from the linear slope of each plot at low substrate concentration.

strates **5**–**8** and **10** suggests that the substrate specificity of the TE II enzyme is in the order: *p*-nitrophenyl propionate **6** > *p*-nitrophenyl butyrate **7** > *p*-nitrophenyl pentanoate **8** > *p*-nitrophenyl acetate **5** > triketide **10**.

The different order of substrate specificity observed for the *p*-nitrophenyl esters and the NAC thioesters (i.e. the acetate (**1**, **5**) and pentanoate esters (**4**, **8**)) can be attributed to the fact that NAC thioesters are better structural models of the natural acyl–ACP intermediate than *p*-nitrophenyl derivatives. *p*-Nitrophenyl esters are useful, however, since they have the potential to demonstrate the formation of acyl–enzyme intermediates.

2.5. Studies of the acyl–enzyme intermediate

To confirm that the TE-catalysed hydrolysis reaction progressed via an acyl–enzyme intermediate for all the analogues tested, the reaction of tylosin TE II with substrates **1**–**10** was followed by ESI-MS. Prior to ESI-MS analysis, a solution of the substrate in acetonitrile (150 mM) was added to a solution of the enzyme in aqueous buffer (50 mM Tris–HCl, pH 7.4; 1 mM EDTA) and incubated at 30°C for 5 min. The acylation reaction mixture was subsequently diluted with a 1:1 solution of acetonitrile/water, acidified with formic acid and analysed by liquid chromatography-mass spectrometry (LC-MS).

These experiments were expected to reveal the extent of acyl–enzyme formation and consequently whether acylation or deacylation is the rate-limiting step during TE-catalysed cleavage of the NAC thioesters of poor substrates. For activated esters like *p*-nitrophenyl derivatives, deacylation of the acyl–enzyme intermediate is usually the rate-determining step but, for more weakly activated esters (e.g. NAC thioesters), acylation becomes at least partly rate-determining. In the experiments with the NAC thioester substrates **1**–**4** and **9**, only small quantities of the acyl–enzyme (< 5%) were formed at steady state. The signal to noise ratio was too high for confident identification.

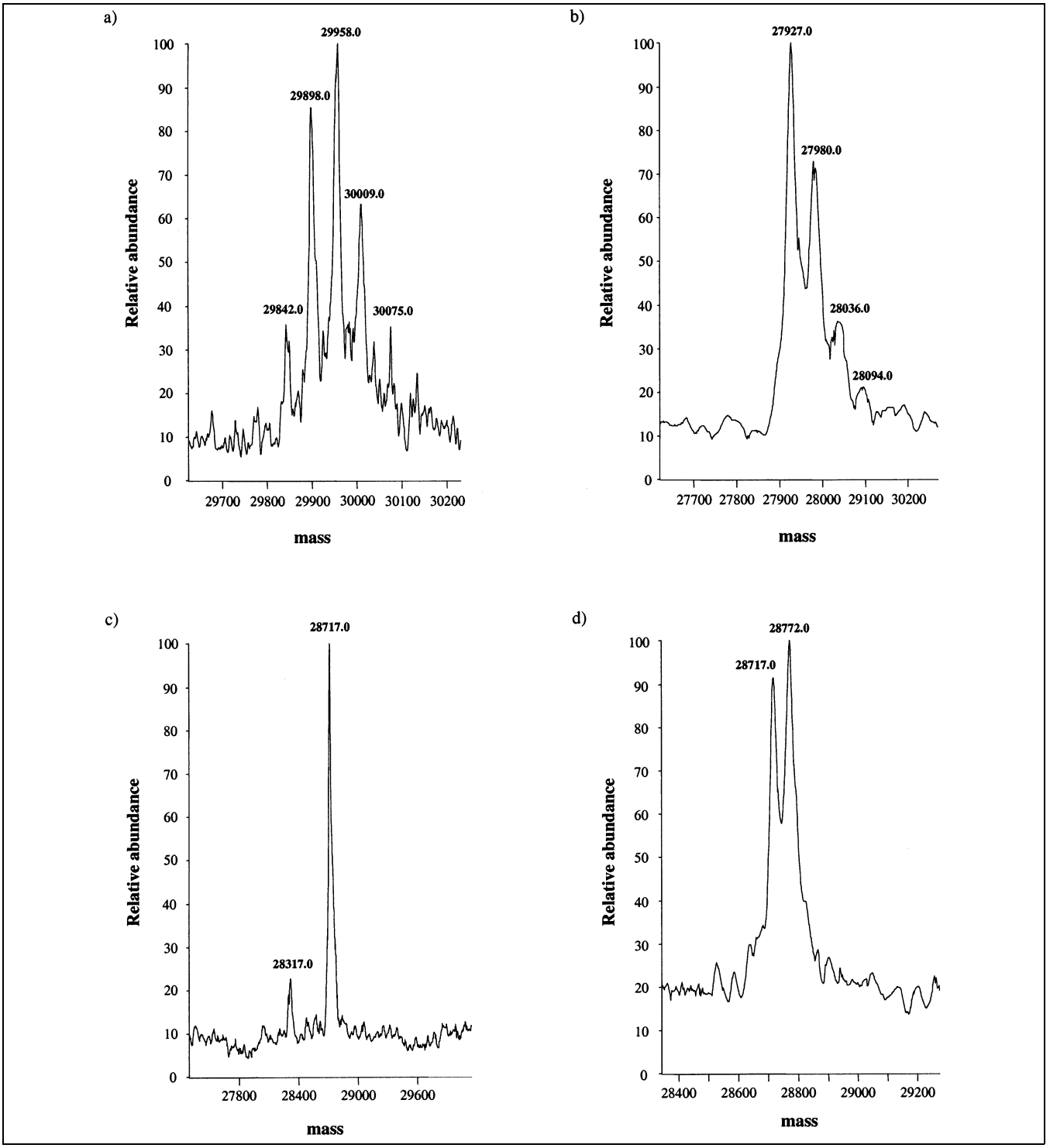


Fig. 9. Analysis of acylation experiments with tylosin TE II and *p*-nitrophenyl propionate **6** by ESI-MS. (a) Acylated tylosin TE II (with His_6 tag). (b) Acylated tylosin TE II (no His_6 tag). (c) Tylosin TE II (no His_6 tag)+DTNB in the absence of *p*-nitrophenyl propionate **6**. (d) Acylated tylosin TE II (no His_6 tag)+DTNB.

Incubation of the *p*-nitrophenyl esters **5**–**8** and **10** with tylosin TE II resulted in multiple acylation (up to 10-fold) of the enzyme (see Fig. 9a for example). *p*-Nitrophenyl esters are extremely reactive, so it seemed likely that they were acylating other nucleophilic residues as well as the active site serine. To ascertain the location of the additional acyl groups, the His_6 tag was removed by thrombin cleavage, and the resulting untagged protein re-incubated with substrates **5**–**8**. LC-MS analysis again showed the presence of multiply acylated peaks (Fig. 9b). Inspection of the peptide sequence of tylosin TE II revealed that there were four cysteine residues present. These would be ideal

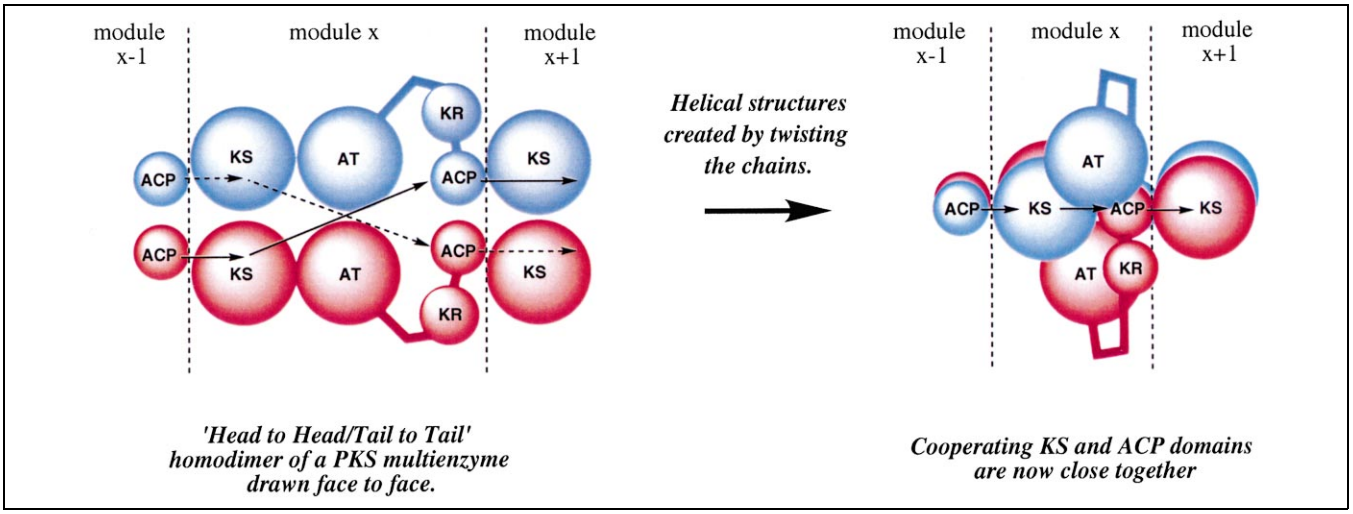


Fig. 10. A three-dimensional representation of a typical type I modular PKS multienzyme, based on the helical model. The complementary polypeptide chains are coloured pink and blue, with the enzymatic domains shown as spheres. The arrows show the independent paths of the two growing polyketide chains. In this arrangement, the KS of one polypeptide chain can only interact with the ACP of the opposing chain, as shown. If blockage of one of the ACP domains occurs, the rate of passage of the polyketide chain through that module will be halved.

candidates for additional acylation sites because of the nucleophilic character of the thiol group. Addition of the thiol-modifying reagent DTNB to the untagged protein gave a molecular species with an average molecular mass of 28 717.0 Da, which is in excellent agreement with that predicted for the enzyme with four modified cysteines. Subsequent incubation of *p*-nitrophenyl propionate **6** with the DTNB-derivatised protein gave a peak corresponding to a single acylation of the enzyme, suggesting that the *p*-nitrophenyl ester substrates in the preliminary studies had acylated four cysteine residues in addition to the active site serine of the TE in the earlier experiments. To ensure that these four cysteine residues did not influence the observed turnover rate for the *p*-nitrophenyl esters, hydrolytic assays were performed with tylosin TE II (with and without a His_6 tag) and the series of simple fatty acyl *p*-nitrophenyl derivatives in the presence of 0.2 mM DTNB. These assays confirmed that acylation of the cysteine residues and/or His_6 tag did not affect the overall rate of hydrolysis of the *p*-nitrophenyl esters. These cysteine residues would, of course, be similarly derivatised by the DTNB used in assaying the hydrolysis of the acyl NAC thioesters.

3. Discussion

When considering the consequences of mistakes made during polyketide biosynthesis, it is important to recognise that modular PKSs are homodimeric and therefore that chain extension modules occur in pairs. This is illustrated in Fig. 10 with the two complementary polypeptide chains arranged head-to-head and tail-to-tail. A combination of complementation and cross-linking studies [27,31] has shown that within a given complementary pair of modules, the KS of one chain co-operates with the ACP in the complementary chain. The growing polyketide chain passes back and forth between one protein subunit as it is passed down the synthase (see Fig. 10). A structural model consistent with all the available data has the core domains of each module twisted in a double helical array, based on the tetrahedral arrangement of the KS and AT domains. This allows the ACP domain in each module to fit between the AT pair so that it is in close proximity to its KS partner in the *opposing* chain. In this arrangement, each KS can co-operate with only one of the ACP domains in the homodimeric module. Therefore, blockage of a particular ACP would be expected to halve the rate of passage of intermediates through that module and, hence, through the synthase as a whole. Consequently, a low frequency of error over all the ACPs would be sufficient to reduce the overall rate of polyketide production to the serious extent observed in previous studies.

To maintain the optimum rate of accumulation of polyketide products the PKS requires a mechanism for removing aberrant structures from blocked ACPs. This could be achieved without the intervention of an external agent if the aberrant intermediate could be transferred to the KS of the downstream module and further extended to produce a polyketide product with a mistake in its structure due to the action of the malfunctioning module. However, this mode of recovery might be relatively inefficient with the result that polyketide production is significantly reduced for an extended period. The TE II, where present, offers a more efficient and immediate way of restoring activity to blocked modules by purging the affected ACP domain directly of its aberrant substrate. This would involve the direct interaction of the TE II with the incorrectly acylated ACP so that the acyl group is transferred

to the active site serine of the TE II. The ACP would then be free to resume its normal operations.

As a corrective agent in the context of a modular PKS, the TE II may not necessarily require the inherent ability to distinguish between normal polyketide intermediates and those containing mistakes. In the normal operation of a chain extension module, the correctly processed intermediates will be strongly bound by the domains which are responsible for their further elaboration and, therefore, will be protected from the TE II, apart from a brief period of exposure during transfer from one domain to the next. An abnormal substrate, on the other hand, might not be so tightly bound and consequently, may be more exposed to attack. The TE II could, therefore, function efficiently by removing only projecting ketide chains from the PKS, much as a hedge trimmer clips projecting shoots from an untidy hedge.

In this paper, we propose that PKS modules may become blocked by the presence of short fatty acyl thioesters attached to the ACP, produced by aberrant decarboxylation of the malonate-derived residue intended for chain extension in downstream chain extension modules. A low rate of KS-catalysed non-condensative decarboxylation of methylmalonate moieties to give propionate can be inferred from experiments with DEBS 1+TE and DEBS 3, presumably due to the aberrant action of KS1 and KS5, respectively. Cell-free extracts containing DEBS 3 were shown to catalyse the synthesis of the expected triketide lactone product by decarboxylation of an enzyme-bound methylmalonyl extender unit [32]. KS-catalysed decarboxylation of methylmalonyl-CoA was also reported to account for the biosynthesis of triketide lactone in vitro by DEBS 1+TE in the absence of propionyl-CoA [33], although it was subsequently shown that this can be largely attributed to contamination of the commercial methylmalonyl-CoA preparation with small quantities ($\sim$5%) of propionyl-CoA [34]. Experiments carried out by Katz et al. in vivo also suggest that initiation of chain formation by the KS1-catalysed decarboxylation of methylmalonate moieties without carbon–carbon formation is not a significant contributor to the flux intermediates on the erythromycin PKS [35]. Nevertheless, the occurrence of an aberrant decarboxylation event at low frequency in module 1 cannot be ruled out completely.

Further insight into the possible significance of KS-catalysed decarboxylation has come from experiments with FAS. Native yeast FAS has been shown to catalyse the decarboxylation of malonyl-ACP [36], although only to a very small extent (1–2% of total FAS activity). However, when the active site cysteine is bound by either an acyl group (as in the normal condensation reaction) or an alkyl group (such as iodoacetamide), decarboxylation is stimulated. In the case of iodoacetamide-treated FAS, the onset of decarboxylation coincides with the abolition of fatty acid synthesis. Similarly, the KSQ domains located in the loading modules of modular PKSs (such as the tylosin and monensin PKSs, for example) and the 'CLF' (KS-like) subunits of aromatic PKSs catalyse the decarboxylation of malonate moieties during the chain initiation process [37]. In both these KS-like domains, the active site cysteine is replaced by a glutamine. Significantly, when the active site cysteine of the FAS KS is treated with iodoacetamide, it is converted into a carboxamidomethylcysteine residue, which is very similar in size, shape and polarity to glutamine. Normal KSs can also be converted into very efficient decarboxylases by replacement of the active site cysteine with glutamine [37,38]. All these examples suggest that there is a fine balance between the operation of the normal decarboxylative condensation reaction and decarboxylation without condensation. Therefore, it is reasonable to suppose that a relatively low rate of aberrant decarboxylation of malonate-derived units by KSs may be a widespread property of modular PKSs. The necessary removal of these unwanted acetyl, propionyl or butyryl groups from the multienzyme complex could be the primary role of the TE II.

The experiments described in this paper show that the TE II of the tylosin cluster is catalytically competent as a TE in vitro. It was able to catalyse the hydrolytic cleavage of a range of NAC thioesters and *p*-nitrophenyl esters. The NAC thioesters, in particular, were recognised as good model substrates in a variety of systems for acyl chains bound to the phosphopantetheine arm of the ACP. It is significant that the best substrate analogues for tylosin TE II with this form of derivatisation are the short acyl chains acetate, propionate and butyrate, which would be formed by aberrant decarboxylation of chain extenders in the various chain extension modules of the tylosin PKS. Representative polyketide chains are also cleaved at a significant rate, indicating that tylosin TE II could also remove aberrant intermediates arising from incorrect operation of chain extension processes. However, recent studies on the rifamycin PKS have demonstrated that the analogous TE II RifR is not responsible for the premature release of the various ketide intermediates found in fermentations of *A. mediterranei* S699 wild-type and *rifF* mutant strains [3,39].

It remains to be established whether the hypothetical function deduced for tylosin TE II is applicable to all TE IIs of PKS origin. Experiments with an *ORF5*-disrupted strain of the tylosin producer *S. fradiae* suggest that TE IIs associated with polyketide biosynthesis may have a common function, as the equivalent TE II gene *nbmB* from *Streptomyces narbonensis* was able to complement the *ORF5* mutant and substantially restore polyketide production [15].

4. Significance

High yield is crucially important in fermentation processes to produce natural products. The earlier demonstra-

tion that the TE II associated with the type I PKS responsible for tylosin biosynthesis in *S. fradiae* helps to maintain high levels of macrolide production therefore deserved investigation. Based on evidence that KSs can catalyse the decarboxylation of malonate moieties to prime polyketide biosynthesis on DEBS 3 or to initiate chain elongation in the monensin and tylosin clusters (KSQ domain), we propose that occasionally, decarboxylation of chain extender units in the absence of a suitable acyl group attached to the KS thiol can occur as an unwanted side reaction, leaving an unreactive acetate, propionate or butyrate residue attached to the ACP. Such an aberrant decarboxylation event, even at low levels, may be sufficient to severely inhibit polyketide biosynthesis, unless the acyl group can be removed. The experiments presented in this paper suggest that tylosin TE II is an ideal candidate for an editing enzyme, which functions by clearing the PKS of short fatty acyl chains. In genetically engineered PKSs aberrant decarboxylation may be more prevalent, therefore co-expression of a TE II may help to increase polyketide production. A better understanding of this aspect of PKS productivity could have significant benefits for many commercially important fermentation processes.

5. Materials and methods

5.1. Materials

Culture medium components were supplied by Difco. Reagents for assays and for chemical synthesis were obtained from Aldrich in the highest purity available and used without further purification. PMSF was obtained from Sigma. The NAC esters **1**–**4** were prepared by reacting the appropriate acid chloride with NAC in the presence of 4-dimethylaminopyridine. The unreacted NAC present in the preparations was removed by elution from a silica gel column impregnated with copper sulphate [40]. Syntheses of the diketide **9** and triketide **10** have been reported previously [41].

5.2. Bacterial strains and plasmids

E. coli DH10B was used as the standard cloning host. Expression of proteins from T7-controlled pET vectors was performed in *E. coli* BL21(λDE3)pLysS [42]. The T7-derived expression vector pET-28b(+) was obtained from Novagen.

5.3. Overexpression of the TE II domain

The tylosin *ORF5* gene was overexpressed in *E. coli* as a His_6-tagged protein. Amplification of *ORF5* by PCR under standard conditions, was achieved using cosmid DNA containing the *tylBA* region as a template, according to the method of Saiki et al. [43]. The forward primer, [5′-d(GG**CATATG**TGGCTGCGTTGCTACGCCCCGGTCCCCGGCAC)-3′], was designed to introduce an *Nde*I site overlapping the start codon, whereas the reverse primer, [3′-d(GG**GGATCC**TCAGTGAGCACTTTTGCTCCCATGGCGGAGT)-3′], was designed to introduce a *Bam*HI site downstream of the stop codon (restriction sites shown in bold type). The PCR product was cloned into pUC18, digested with *Nde*I and *Bam*HI and subsequently cloned into the *Nde*I and *Bam*HI sites of the expression vector pET-28b(+). The resultant plasmid pMLH27 was used to transform *E. coli* BL21(λDE3)-pLysS for expression of the TE II gene. One liter cultures of *E. coli* BL21(λDE3)pLysS/pMLH27 were grown in 2 l flasks using LB medium containing kanamycin (30 μg ml^{-1}) and chloramphenicol (34 μg ml^{-1}) at 28°C. Protein expression was induced at $A_{600} = 0.6$ by addition of isopropyl thio-β-D-galactoside to a final concentration of 1 mM. Cells were harvested after 3 h by centrifugation at $12\,000 \times g$.

5.4. Purification of the TE II domain

All purification procedures were carried out at 4°C. Induced cells (5 g wet weight) were resuspended in 20 ml 50 mM phosphate buffer (pH 8.0) containing 300 mM NaCl, 10 mM imidazole, benzamidine/HCl, soybean trypsin inhibitor, DNase (5 μg ml^{-1}) and RNase (10 μg ml^{-1}). The cells were broken by one pass through a French press at 16 000 psi and insoluble material was sedimented from the lysate by centrifugation at $40\,000 \times g$ for 30 min at 4°C. The lysate was then mixed with previously equilibrated Ni-NTA superflow resin, allowed to bind for 1 h, then packed into a column for the washing and elution steps. The column was washed with 50 mM phosphate buffer (pH 8.0) containing 300 mM NaCl, 20 mM imidazole, and the TE II eluted with the same buffer containing 250 mM imidazole. Elution of the proteins was monitored by recording the absorbance of the eluate at 280 nm. Peak fractions were analysed by SDS–PAGE. The fractions containing the recombinant protein were pooled and desalted using a Hi-Trap desalting column (Pharmacia) into 50 mM Tris–HCl (pH 7.4) containing 1 mM EDTA. The sample was then applied to a Hi-Trap Q anion exchange column (Pharmacia) at a flow rate of 1 ml min^{-1}. Under these conditions, tylosin TE II was found in the column flow-through, whilst the contaminant proteins bound to the column. Typically, 10 mg purified protein was obtained from 1 g wet cells.

5.5. Treatment of purified protein with PMSF

Stock solutions of 100 mM PMSF in dimethyl formamide were used. The purified protein, typically 0.8–1 mg ml^{-1} in 5 mM Tris–HCl (pH 7.4) containing 1 mM EDTA, was incubated at 0°C for 1 h in the presence of 1 mM PMSF. Control protein samples were treated with an equivalent volume of dimethyl formamide.

5.6. ESI-MS of tylosin TE II

The methods used for ESI-MS were based on those used previously for analysis of the TE I domain of the erythromycin PKS. A Liquid Chromatography-Quattro mass spectrometer (Micromass, UK) was used, consisting of an electrostatic spray ion source operating at atmospheric pressure, attached to a triple

quadrupole mass analyser. Protein samples in 5 mM Tris–HCl (pH 7.4), 1 mM EDTA were mixed with an equal volume of acetonitrile and 0.1 vol of formic acid and introduced into the ion source at a flow rate of 4 μl min^{-1} (the mobile phase was 50:50 acetonitrile:water). Spectra were scanned from *m/z* 600 to *m/z* 1500 at 10 s/scan, and processed using the Mass Lynx NT data system. The mass scale was calibrated with horse heart myoglobin (average molecular mass 16951.5 Da.

5.7. Determination of the kinetic parameters for TE-catalysed hydrolysis

Hydrolysis of the NAC thioesters **1**–**4** and **9** was followed spectrophotometrically by observation of the formation of 3-thio-5-nitrobenzoate at (λ_{max} = 412 nm, ε = 13 600 M^{-1} cm^{-1}). Each assay contained (in a total of 1 ml) 200 mM potassium phosphate (pH 7.4), 1.3 μM tylosin TE II, 10 μl of a 20 mM solution of DTNB in 200 mM potassium phosphate (pH 7.4) and a variable amount of substrate dissolved in DMSO. Stock solutions of **1**–**3** were 2 M in DMSO, whereas stock solutions of **4** and **9** were 1 M in DMSO. In all cases, the total content of DMSO in the reaction was adjusted to 30 μl. Reactions were carried out in duplicate and the rate of hydrolysis calculated from the initial linear portion of the curves. In all cases, the rates were corrected for the background rate of chemical hydrolysis in the absence of enzyme. Hydrolysis rates were measured over the concentration range 10–60 mM for substrates **1**–**3**, and 5–30 mM for **4** and **9**.

Hydrolysis of the *p*-nitrophenyl derivatives **5**–**8** and **10** was monitored by following the rate of formation of the *p*-nitrophenolate anion (λ_{max} = 400 nm, ε = 8570 M^{-1} cm^{-1}) at 30°C. Each assay contained (in a total volume of 1 ml) 200 mM potassium phosphate buffer (pH 7.4), 1.3 μM tylosin TE II, and a variable concentration of substrate in DMSO. Stock solutions of **5**–**6** were 100 mM in DMSO, whereas stock solutions of **7**–**8** and **10** were 50 mM in DMSO. In all cases, the total content of DMSO in the reaction was adjusted to 30 μl. Reactions were carried out in duplicate and the rate of hydrolysis calculated from the initial linear portion of the curves. In all cases, the rates were corrected for the background rate of chemical hydrolysis in the absence of enzyme. Hydrolysis rates were measured over the concentration range 0.1–2.0 mM.

5.8. Acylation of tylosin TE II

The substrate (1 μl of 150 mM solution in acetonitrile) was added to an aqueous solution of tylosin TE II (10 μl of 1.0 mg ml^{-1} solution in 50 mM Tris–HCl, pH 7.4; 1 mM EDTA) and incubated at 30°C for 5 min. The reaction mixture was then diluted with acetonitrile (20 μl) and water (20 μl) and acidified with formic acid (1 μl). The resulting solution was subjected to LC-MS analysis (Finnigan MAT LCQ, UK) using a Vydac protein C4 column (250×4.60 mm; 300 Å). Elution was achieved at 1 ml min^{-1} with a gradient of 30–40% solvent B for 10 min followed by a gradient of 40–50% solvent B for 20 min. Solvent A: milli-Q water plus 0.1% TFA; solvent B: acetonitrile plus 0.1% TFA. Spectra were processed using Finnigan BioWorks version 1.0 software.

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