Heterologous expression, purification, reconstitution and kinetic analysis of an extended type II polyketide synthase

Robert JX Zawada[1] and Chaitan Khosla[1,2,3]

Background: Polyketide synthases (PKSs) are bacterial multienzyme systems that synthesize a broad range of natural products. The 'minimal' PKS consists of a ketosynthase, a chain length factor, an acyl carrier protein and a malonyl transferase. Auxiliary components (ketoreductases, aromatases and cyclases) are involved in controlling the oxidation level and cyclization of the nascent polyketide chain. We describe the heterologous expression and reconstitution of several auxiliary PKS components including the actinorhodin ketoreductase (*act* KR), the griseusin aromatase/cyclase (*gris* ARO/CYC), and the tetracenomycin aromatase/cyclase (*tcm* ARO/CYC).

Results: The polyketide products of reconstituted *act* and *tcm* PKSs were identical to those identified in previous *in vivo* studies. Although stable protein–protein interactions were not detected between minimal and auxiliary PKS components, kinetic analysis revealed that the extended PKS comprised of the *act* minimal PKS, the *act* KR and the *gris* ARO/CYC had a higher turnover number than the *act* minimal PKS plus the *act* KR or the *act* minimal PKS alone. Adding the *tcm* ARO/CYC to the *tcm* minimal PKS also increased the overall rate.

Conclusions: Until recently the principal strategy for functional analysis of PKS subunits was through heterologous expression of recombinant PKSs in *Streptomyces*. Our results corroborate the implicit assumption that the product isolated from whole-cell systems is the dominant product of the PKS. They also suggest that an intermediate is channeled between the various subunits, and pave the way for more detailed structural and mechanistic analysis of these multienzyme systems.

Addresses: Departments of [1]Chemical Engineering, [2]Chemistry and [3]Biochemistry, Stanford University, Stanford, California 94305-5025, USA.

Correspondence: Chaitan Khosla
E-mail: ck@chemeng.stanford.edu

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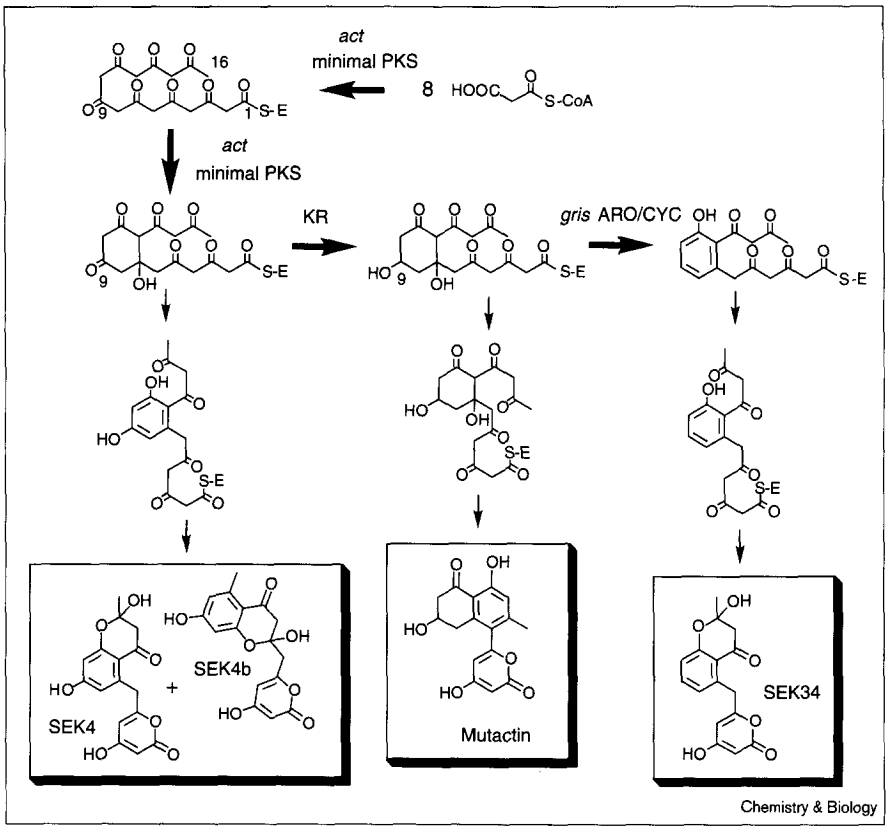
Introduction

Aromatic polyketides are a large family of structurally diverse natural products that have many important antibiotic and pharmacological properties. This class of natural products is synthesized by bacteria that have type II polyketide synthases (PKSs) that are structurally and mechanistically related to bacterial type II fatty acid synthases (FASs) [1–3]. Both classes of synthases are multifunctional enzymes that catalyze repeated decarboxylative condensations between acylthioesters (usually a growing polyketide chain and a malonyl extender unit). However, type II PKSs and FASs differ in the oxidation level of their products, the ability of PKSs to regiospecifically reduce certain β-keto groups, and the ability of PKSs to catalyze regiospecific intramolecular cyclizations on the polyketide backbone.

The structural diversity and medicinal relevance of their products have motivated intensive manipulation and analysis of the molecular recognition features of PKSs. Much of this manipulation was carried out using a host–vector system in *Streptomyces coelicolor* that enabled the efficient construction and expression of recombinant PKS gene sets [4]. These recombinants provided a means to decipher the role(s) of individual subunits of the PKS in the overall catalytic cycle, as well as to dissect their substrate specificity [4–16]. In turn, these insights were used to develop a set of 'design rules' to engineer recombinant PKS gene clusters that generated novel polyketides in a predictable manner. This capability has opened the possibility of generating polyketide libraries through combinatorial biosynthesis [16,17]. More recently, the properties of a class of subunits, the aromatase/cyclases (ARO/CYCs) have been further dissected using gene fusions. Their didomain architecture was established by expressing the domains as separate polypeptides and chain-length specificity was mapped to the amino-terminal domain of these subunits [18].

Although the above genetic studies have led to many new insights into aromatic PKS mechanism and specificity, their utility is largely limited to qualitative analysis of these multifunctional systems. To overcome this shortcoming, *in vitro* systems have also been developed. Cell-free activity of both minimal [19,20] and extended PKSs [20] have been demonstrated. More recently, the components of the minimal PKS have been purified and reconstituted for

Figure 1



Proposed biosynthetic pathway catalyzed by the actinorhodin (*act*) PKS. After biosynthesis of the full polyketide chain by the minimal PKS, which includes a ketosynthase (KS), a chain length factor (CLF), an acyl carrier protein (ACP) and malonyl CoA:ACP transacylase (MAT), the nascent octaketide chain is altered by various downstream subunits. The ketoreductase (KR) reduces the C9 carbonyl. The ARO/CYC aromatizes this reduced first ring. In systems lacking a full complement of these subunits, shunt products are produced. (Biosynthetic intermediates are purely hypothetical.)

both the actinorhodin (*act*) [21]) and tetracenomycin (*tcm*) [22] systems. These minimal PKSs include a ketosynthase–chain length factor (KS–CLF) heterodimer, an acyl carrier protein (ACP), and a malonyl-CoA:ACP malonyl transferase (MAT). (There is some debate about the absolute necessity of the MAT in a reconstituted system; under physiologically relevant concentrations of the ACP, however, the presence of the MAT has a significant impact on the kinetics of this multicomponent system [23,24].) Here we describe the purification, reconstitution and *in vitro* analysis of extended PKS systems composed of the *act* minimal PKS, the *act* ketoreductase (KR) and the didomain griseusin (*gris*) aromatase/cyclase (ARO/CYC). We used the *gris* ARO/CYC here because the *act* ARO/CYC could not be expressed at sufficiently high levels. In addition, the monodomain tetracenomycin (*tcm*) ARO/CYC, *tcm*N, was reconstituted with the *tcm* minimal PKS. Unlike earlier work in which the full-length *tcm*N gene was expressed [22,25], here we only express the ARO/CYC domain of *tcm*N, which we refer to as the *tcm* ARO/CYC in this manuscript.

The *act* KR is involved in reducing the C9 carbonyl group of the nascent polyketide chain [8,26]. The *gris* ARO/CYC is a didomain protein [18,27] that is required for the aromatization of the first carbocycle in polyketide chains that have been reduced at the C9 position by the KR (Figure 1) [13]. The *tcm* ARO/CYC influences the regiospecificity of cyclization of unreduced (but not reduced) polyketide backbones. In its absence, the unreduced nascent polyketide can undergo either a C7/C12 or C9/C14 cyclization; in contrast, in its presence only the C9/C14 cyclization is observed [14,15,25] (Figure 2). The *tcm* ARO/CYC may also be involved in the aromatization of the second ring [14].

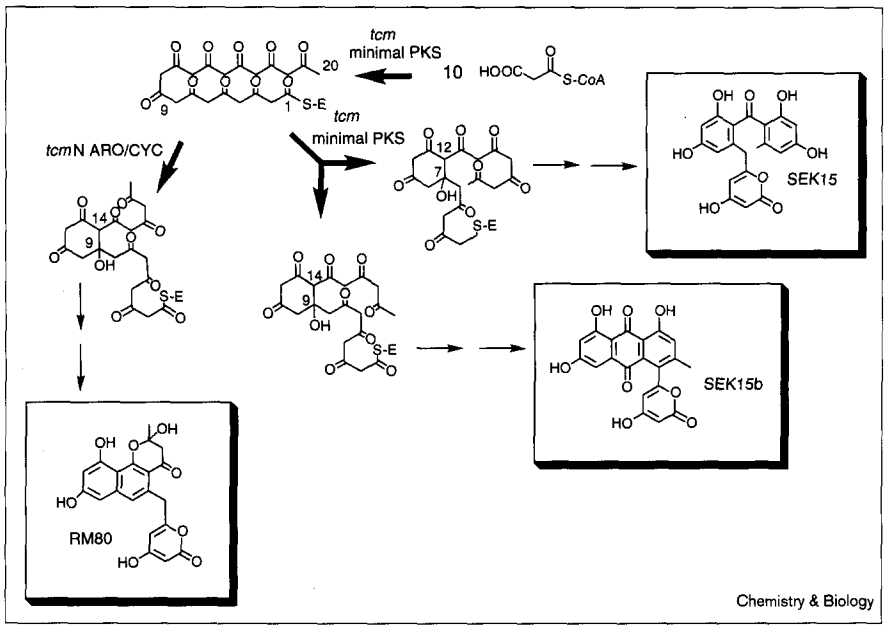
Results

Heterologous expression of the *act* KR, *gris* ARO/CYC and *tcm* ARO/CYC

The *act* KR, *gris* ARO/CYC and *tcm* ARO/CYC genes were cloned into an *Escherichia coli* expression vector (pET21c) generating plasmids pRZ153, pRZ112 and pRZ106, respectively, as described above. Following disruption of the *E. coli* cells and centrifugation, the supernatants were analyzed using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE). The *act* KR and *tcm* ARO/CYC were clearly identifiable in crude extracts at their corresponding molecular masses (27 and 20 kDa respectively); the *gris* ARO/CYC (37 kDa), in contrast, was not obviously detectable (see Figure 3, lanes 2, 8 and 14). In order to verify that these proteins were synthesized in an active form, they were assayed with the minimal PKS. The results of these assays are described below.

Figure 2

Proposed biosynthetic pathway catalyzed by the tetracenomycin (*tcm*) PKS. After biosynthesis of the full polyketide chain by the minimal PKS, which includes a KS, a CLF, an ACP and a MAT, the nascent decaketide chain cyclizes in a number of ways. In the absence of the *tcm* ARO/CYC, the nascent polyketide chain will condense between the C7 carbonyl and C12 methylene or between the C9 carbonyl and C14 methylene leading to the formation of SEK15 and SEK15b, respectively. The *tcm* ARO/CYC catalyzes an aldol condensation between the C9 carbonyl and C14 methylene leading to the production of RM80. The *tcm* ARO/CYC may also be involved in the aromatization of the second ring. (Biosynthetic intermediates are purely hypothetical.)



Purification of the *act* KR, *gris* ARO/CYC and *tcm* ARO/CYC

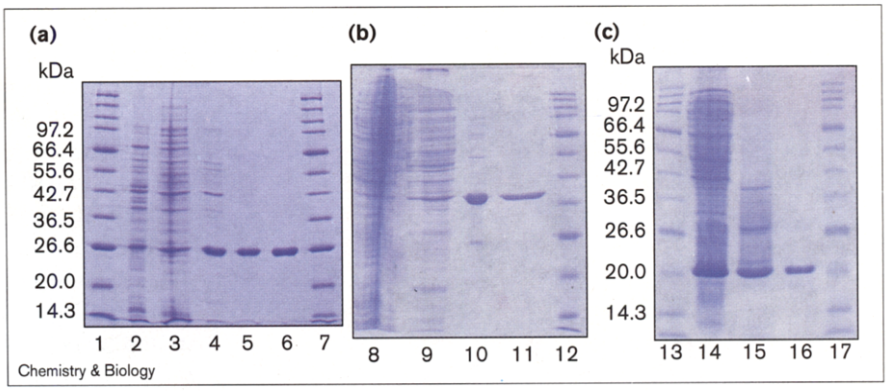
Crude extracts of *E. coli* BL21(DE3)/pRZ106, pRZ112 or pRZ153 were fractionated, and fractions were assayed for their ability to catalyze biosynthesis of the expected polyketides. The *act* KR was applied to butyl sepharose, which gave a 70% enriched preparation of KR. Further purification of this preparation using hydroxyapatite and anion-exchange chromatography resulted in a nearly homogeneous (> 95% pure) sample of *act* KR (Figure 3a). Approximately 10 mg of *act* KR was obtained from each liter of *E. coli* BL21(DE3)/pRZ153 culture.

The *gris* ARO/CYC was applied to a high-performance phenyl sepharose column, which resulted in a 10% enriched preparation of ARO/CYC. This material was then processed using anion-exchange chromatography and gel-filtration chromatography. The final preparation was nearly homogeneous in *gris* ARO/CYC (> 95% pure) (Figure 3b). Each liter of *E. coli* BL21(DE3)/pRZ112 yielded ~1 mg of *gris* ARO/CYC.

The *tcm* ARO/CYC was purified by anion-exchange chromatography followed by cation-exchange chromatography. The anion-exchange column yielded an 80% enriched preparation of ARO/CYC, whereas the cation-exchange column yielded a nearly homogeneous sample (> 95% pure) of the *tcm* ARO/CYC domain (Figure 3c). Each processed liter of *E. coli* BL21(DE3)/pRZ106 yielded ~20 mg of *tcm* ARO/CYC domain.

Figure 3

Purification gels for the *act* ketoreductase (KR), *gris* ARO/CYC and *tcm* ARO/CYC domain. **(a)** Purification gel of the *act* KR. Lanes 1 and 7 are molecular mass markers; lane 2, crude extract of BL21(DE3)/pRZ153; lane 3, extract of BL21(DE3)/pRZ153 following PEI and $(NH_4)_2SO_4$ precipitation; lane 4, *act* KR after Butyl sepharose chromatography; lane 5, *act* KR following hydroxyapatite chromatography; lane 6, *act* KR after Resource Q. **(b)** Purification gel of *gris* ARO/CYC. Lane 8, BL21(DE3)/pRZ112 after PEI and $(NH_4)_2SO_4$ precipitation; lane 9, *gris* ARO/CYC following phenyl sepharose HP chromatography; lane 10, *gris* ARO/CYC after Resource Q; lane 11, *gris* ARO/CYC following size exclusion chromatography; lane 12, molecular weight marker. **(c)** Purification gel for *tcm* ARO/CYC domain. Lanes 13 and 17 are molecular mass markers; lane 14, BL21(DE3)/pRZ106 after PEI and $(NH_4)_2SO_4$ precipitation; lane 15, *tcm* ARO/CYC domain following HiTrap Q; lane 16, *tcm* ARO/CYC domain after HiTrap SP.



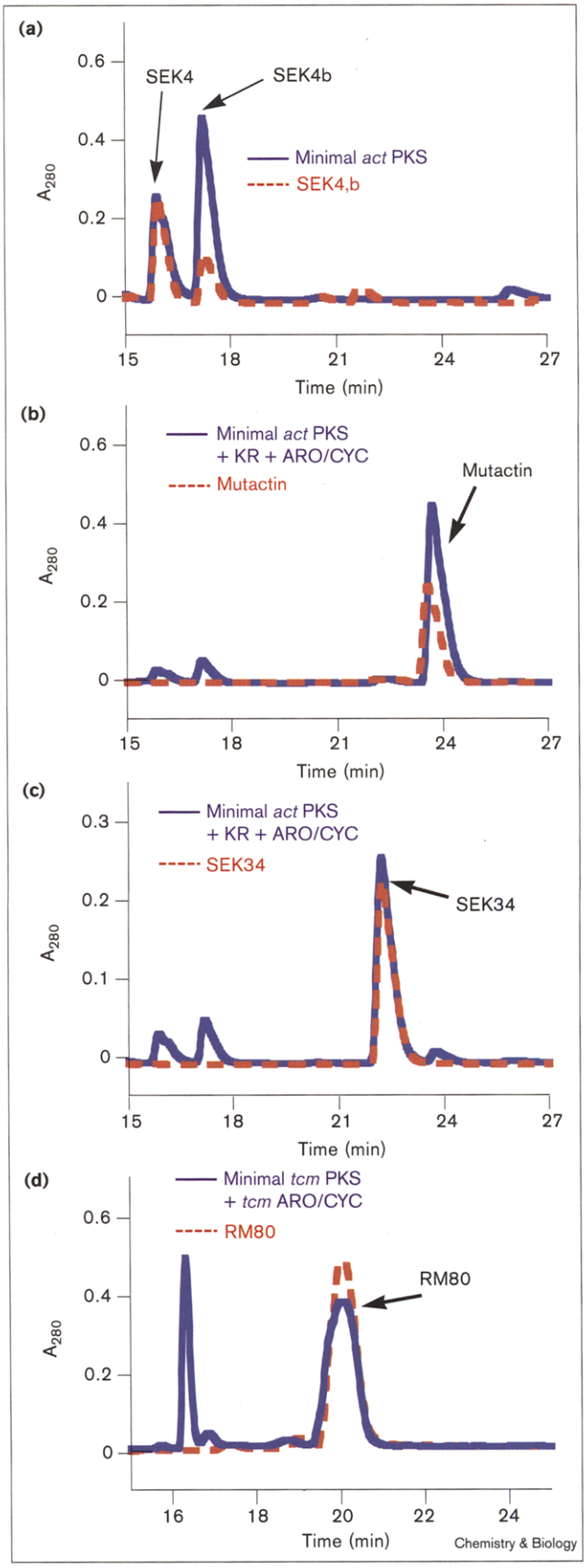


Figure 4

HPLC analysis with UV absorbance for *in vitro* assays of PKS systems. **(a)** Extract from assay of the *act* minimal PKS and SEK4,b standards. **(b)** Extract from assay of the *act* minimal PKS plus *act* KR and mutactin standard. **(c)** Extract from assay of the *act* minimal PKS plus *act* KR and *gris* ARO/CYC along with SEK34 standard. **(d)** Extract from assay of *tcm* minimal PKS plus *tcm* ARO/CYC domain and RM80 standard.

Verification of the *act* KR, *gris* ARO/CYC and *tcm* ARO/CYC activities

The activity of the *act* KR was assessed by assaying this protein in the presence of the *act* KS–CLF, *fren* ACP and MAT. The *fren* ACP was used because it was already cloned into an *E. coli* expression system that could phosphopantetheinylate the ACP. The *gris* ARO/CYC was assayed in combination with the *act* KR, *act* KS–CLF, *fren* ACP and MAT. The *tcm* ARO/CYC domain could not be assayed *in vitro* using the *act* minimal PKS because the reported biosynthetic product of the *act* minimal PKS plus *tcm*N undergoes oxidation by an unknown oxidase. The *tcm* ARO/CYC domain was therefore assayed using purified *tcm* minimal PKS, because the predicted product was expected to be generated *in vitro*.

Three reactions were set up for each of these systems. One reaction included radiolabeled malonyl CoA that was extracted and analyzed on a silica gel thin layer chromatography (TLC) plate along with purified standards. The other reactions included unlabeled malonyl CoA. One of these was analyzed using high-performance liquid chromatography (HPLC)–UV detection; the other was subjected to atmospheric pressure chemical ionization (AP–CI) mass spectroscopic analysis. Figure 4 shows the results of HPLC analysis, along with reference products for the three systems. The results obtained by mass spectroscopy agreed with the expected masses for the products.

Oligomerization studies

The *act* KR, *gris* ARO/CYC and *tcm* ARO/CYC domain were analyzed separately over a Superdex 200 column to estimate their apparent molecular weights (MWs) (Figure 5). The *act* KR eluted at 15.4 ml, which corresponds to an apparent MW of 46 kDa, the *gris* ARO/CYC eluted at 15.6 ml, which corresponds to an apparent MW of 37 kDa and the *tcm* ARO/CYC eluted at 16.9 ml, which correspond to an apparent molecular weight of 20.8 kDa. The apparent molecular weights of the *gris* ARO/CYC and *tcm* ARO/CYC domain were consistent with their monomeric molecular weights. (Note that Shen and Hutchinson [25] demonstrated by gel-filtration chromatography that the full length *tcm*N gene product exists as a dimer.) The *act* KR had an apparent MW suggestive of a dimer, however. Cross-linking experiments were therefore undertaken to investigate whether a dimer could be directly identified. Indeed, SDS–PAGE analysis revealed a cross-linked species with

an observed molecular mass consistent with that of a KR dimer when glutaric dialdehyde was used as the cross-linking agent (data not shown). Moreover, when the *act* KR was purified on Resource Q, it eluted as two distinct peaks, both of which displayed KR activity. This suggests that the *act* KR exists in equilibrium between two distinct states, possibly a monomer and dimer.

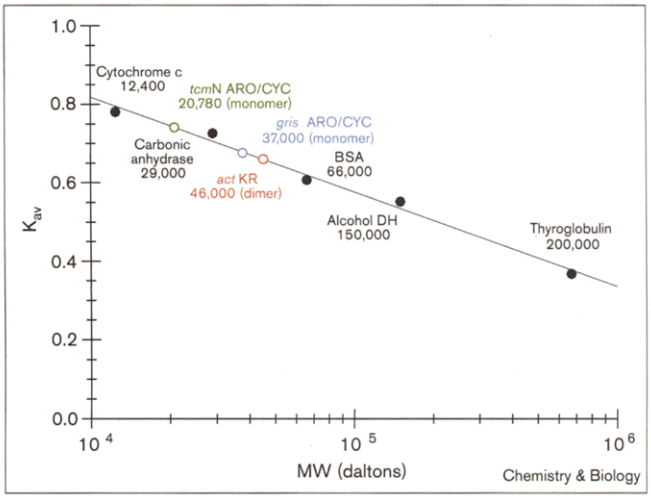
In addition to determining apparent MWs, experiments were performed on the gel-filtration column to determine if complexes between the various PKS subunits could be observed directly. The *act* KR was combined with the *gris* ARO/CYC with and without the *fren* ACP. In addition, the *act* KS/CLF and the *fren* ACP were applied in conjunction with either the *act* KR, *tcm* ARO/CYC domain, or *act* KR plus *gris* ARO/CYC. None of these combinations led to new higher MW peaks, suggesting the absence of any stable complexes in these mixed incubations.

Kinetic studies of PKSs containing the *act* KR and *gris* ARO/CYC

The rates of polyketide production for the *act* minimal PKS were found to be linear from 0–30 min (5–15 turnovers); all rates were therefore computed from samples harvested in this time range. The k_{cat} for mutactin formation was found to be 0.11 ± 0.02 min^{-1}, whereas the k_{cat} for SEK34 formation was found to be 0.44 ± 0.04 min^{-1}. These numbers compare favorably with the k_{cat} for SEK4 and SEK4b production by the minimal PKS (0.33 ± 0.06 min^{-1}). In order to determine whether the rate enhancement observed with the addition of ARO/CYC was due solely to the presence of additional nonspecific protein, 10 μM BSA was added to a reaction mixture of the *act* minimal PKS. Addition of bovine serum albumin (BSA) had no effect on the overall rate of the minimal PKS. In order to determine whether the decrease in overall rate observed when the *act* KR is included with the *act* minimal PKS was because of product inhibition, we carried out side-by-side assays of the *act* minimal PKS plus *act* KR in the presence and absence of 60 μM mutactin (the product of the *act* minimal PKS plus *act* KR). Again, addition of mutactin had no effect on the overall rate of polyketide synthesis.

In order to gain insight into the stoichiometry of the various components in these extended PKSs, the *act* KR and *gris* ARO/CYC were titrated into an assay mixture containing 0.5 μM *act* KS–CLF, 0.5 μM MAT and 20 μM *fren* ACP. This corresponds to the highest experimentally achievable concentration of the KS–CLF; moreover, under these conditions the activity of the minimal PKS is saturated with respect to MAT and ACP [24]. The results of these titrations are shown in Figures 6 and 7. From these results a clear difference can be seen between the stoichiometry of the KR and the ARO/CYC required for optimal PKS activity. The concentration of the *act* KR at which mutactin was synthesized at 50% of the maximum

Figure 5



Estimation of the molecular weights of the *act* KR, *gris* ARO/CYC and *tcm* ARO/CYC domains using gel filtration chromatography. $K_{av} = (V_e - V_0)/(V_t - V_0)$. V_0 and V_t were determined using blue dextran and tyrosine, respectively.

rate was 3.12 ± 0.62 μM. In contrast, the concentration of the *gris* ARO/CYC at which SEK34 was synthesized at 50% of the maximum rate was only 50 ± 10 nM.

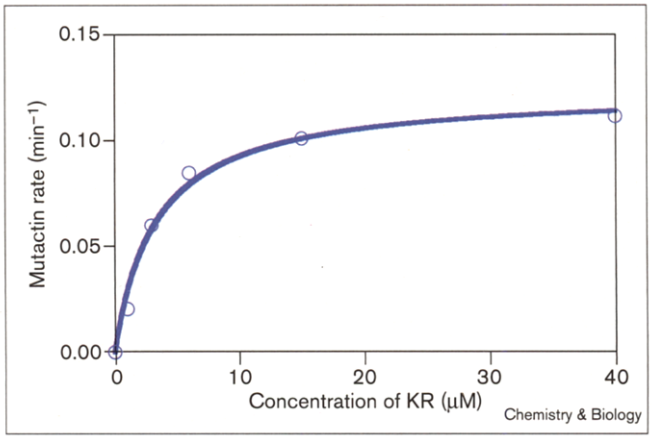
Kinetic studies of the *tcm* minimal PKS and *tcm* minimal PKS plus *tcm* ARO/CYC

The rate of polyketide production for the *tcm* minimal PKS was found to be linear from 0–30 min (10–50 turnovers); all rates were therefore computed from samples collected in this time range. Interestingly, the k_{cat} for the *tcm* minimal PKS (1.40 ± 0.25 min^{-1}) was found to be fourfold greater than that of the *act* minimal PKS (0.33 ± 0.06 min^{-1}) despite the fact that the *tcm* PKS must perform two additional condensations.

In contrast to findings *in vivo* in which the primary products of the minimal *tcm* PKS were SEK15 and SEK15b, [8,12,14] *in vitro* assays of the *tcm* minimal PKS resulted in production of SEK15 and SEK15b (~67% total polyketide) as well as significant amounts of RM80 (~33% total polyketide). The k_{cat} for SEK15 and SEK15b formation by the *tcm* minimal PKS was found to be 0.95 ± 0.11 min^{-1}, whereas the k_{cat} for RM80 formation by the minimal PKS was found to be 0.46 ± 0.15 min^{-1}. Addition of *tcm* ARO/CYC (5 μM) resulted in an increase in the overall rate of polyketide synthesis to 2.55 ± 0.31 min^{-1}, with the rate of RM80 synthesis increasing to 1.79 ± 0.17 min^{-1} and the rate of SEK15 and SEK15b synthesis decreasing to 0.75 ± 0.15 min^{-1}.

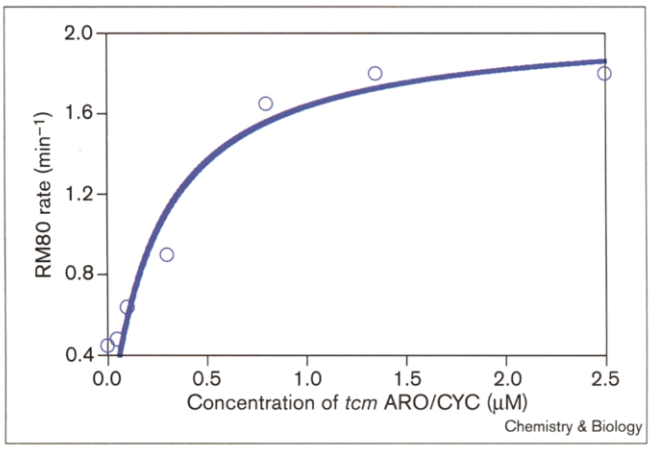
The *tcm* ARO/CYC was titrated into an assay mixture containing the *tcm* minimal PKS at concentrations sufficient for its maximal rate (0.25 μM *tcm* KS–CLF, 0.1 μM MAT, and 15 μM *fren* ACP). The results of this titration are

Figure 6



Titration of the *act* KR. The *act* KR was titrated into 0.5 μM *act* KS/CLF heterodimer and 20 μM *fren* ACP. The rates were determined from extractions harvested at 10, 20 and 30 min and divided by the concentration of the KS/CLF heterodimer.

Figure 8



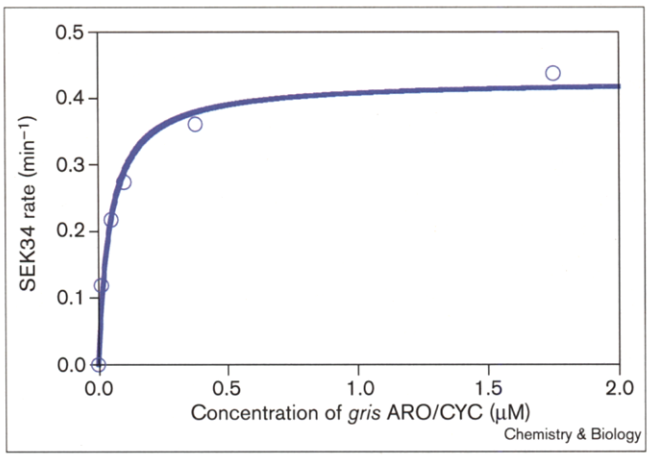
Titration of the *tcm* ARO/CYC. The *tcm* ARO/CYC was titrated into 0.25 μM *tcm* KS/CLF, 20 μM *fren* ACP and 0.1 μM MAT. The rates were computed from extractions collected at 10, 20 and 30 min and divided by the concentration of the KS/CLF heterodimer.

shown in Figure 8. The concentration of the *tcm* ARO/CYC at which RM80 was synthesized at 50% of the maximum rate was 320 ± 80 nM.

Discussion

Until recently the principal strategy for dissecting the function and specificity of PKS subunits was through heterologous expression and product analysis of recombinant PKS gene sets in *Streptomyces*. An implicit assumption in such studies was that the product isolated from whole-cell systems was indeed the dominant product of the recombinant PKS *in vivo*. The results reported here and elsewhere corroborate this assumption. For example, the primary products of the *act* minimal PKS, the *act* minimal PKS + *act* KR, and the *act* minimal PKS + *act* KR + *gris* ARO/CYC *in vivo* were reported to be SEK4 and SEK4b [11], mutactin [8,28] and SEK34 [13]. As described above and elsewhere [21,23], these are the very same products observed in reconstituted cell-free systems. Similarly, the primary product of the *tcm* minimal PKS + *tcm* ARO/CYC, RM80 [14], has also now been confirmed *in vitro*. Biosynthetic inferences drawn from structural and isotope labeling studies of recombinant polyketides observed *in vivo* can therefore be accepted as a reasonable starting point for more detailed mechanistic studies in the future.

Figure 7



Titration of the *gris* ARO/CYC. The *gris* ARO/CYC was titrated into 0.5 μM *act* KS/CLF heterodimer, 20 μM *fren* ACP and 40 μM *act* KR. The rates were computed from extractions harvested at 10, 20 and 30 min and divided by the concentration of the KS/CLF heterodimer.

In this study we succeeded in expressing a variety of auxiliary PKS subunits in *E. coli*, and reconstituting them with minimal PKSs. The availability of purified protein also yielded insights into the assembly of aromatic PKSs into higher order complexes. Specifically, size exclusion chromatography revealed that auxiliary PKS components do not form stable complexes with minimal PKS subunits. It must be kept in mind, however, that these experiments were carried out in the absence of substrates. It might be that the components only associate in the presence of a growing nascent polyketide. Indeed, our kinetic measurements suggest that interactions at least between the KR and/or minimal PKS and the ARO/CYC subunits occur. The k_{cat} for formation of the fully aromatized product formed upon the addition of both the KR and the *gris* ARO/CYC was fourfold higher than the k_{cat} for formation of mutactin (the product of the minimal PKS plus *act* KR) and 33% higher than the k_{cat} for formation of SEK4 and SEK4b (the products of the minimal PKS alone). Because the ARO/CYC acts downstream of the KR and the minimal PKS in the biosynthetic pathway, the observed

rate enhancement probably reflects the channeling of the reduced intermediate between the KR and ARO/CYC.

In addition to the extended *act* PKS system for which the minimal PKS has already been characterized [24], kinetic analysis was also performed on the *tcm* PKS. Initial characterization of *tcm* minimal PKS systems revealed that its k_{cat} (1.4 ± 0.25 min^{-1}) was significantly higher than the k_{cat} for the *act* minimal PKS systems (0.33 ± 0.06 min^{-1}). Addition of a downstream subunit, specifically the *tcm* ARO/CYC, also enhanced the rate of polyketide synthesis 1.8-fold over the rate for the minimal *tcm* PKS alone (2.55 min^{-1} versus 1.4 min^{-1}). These results are in agreement with the findings of Shen and Hutchinson [25], in which they reported an increase in total polyketide product when the *tcm* ARO/CYC was included with the *tcm* minimal PKS. These results, therefore, represent another example of a downstream component enhancing the overall biosynthetic rate, and further supports the existence of channeling of intermediates between components of extended type II PKSs.

Titration experiments revealed that the *gris* ARO/CYC saturates the system at significantly lower concentrations than the *act* KR. It is unclear whether this reflects differences in protein–protein interaction affinities, or whether it is a measure of variable enzyme–substrate affinities. It is likely that both properties are captured in these measurements, because the substrate for the auxiliary subunits is probably bound to the minimal PKS [25]. The remarkable differences in saturating concentration levels are reminiscent of the large differences between the corresponding parameters for the ACP and the MAT in the minimal PKS complex [24].

Significance

Until recently the function and specificity of polyketide synthase (PKS) subunits could only be probed through heterologous expression and product analysis of recombinant PKS gene sets in *Streptomyces*. The work presented here demonstrates the feasibility of expressing and reconstituting the activity of an extended aromatic PKS system *in vitro*. This capability has enabled us to analyze steady-state kinetic properties, the results of which in turn suggests that substrates are channeled between the subunits of type II PKSs. Further investigations should continue to uncover exciting new insights into the mechanism and structure of the type II PKSs. Moreover, such reconstituted systems may also expand our ability to synthesize novel polyketides of practical utility *in vitro*.

Materials and methods

Reagents and chemicals

[^{14}C]-Malonyl CoA was obtained from Moravek Biochemicals. Malonyl CoA was obtained from Sigma Chemical Company. The cross-linking reagent glutaric dialdehyde was obtained from Aldrich. Hydroxyapatite CHT5-I was procured from Bio-Rad, Butyl Sepharose, Phenyl Sepharose 6 FF, Phenyl Sepharose HP, HiTrap Q, HiTrap SP, Resource Q and Superdex 200 were obtained from Pharmacia Biotech.

Strains and culture conditions

S. coelicolor CH999/pSEK38 [21] and CH999/pSEK23 [12] were used to obtain *act* KS and CLF and *tcm* minimal PKS respectively. Mycelia from 2 l stationary phase cultures were harvested yielding a 15 g mycelial pellet. *E. coli* strain BL21(DE3) was used for expression of the *fren* ACP, *act* KR, *gris* ARO/CYC and *tcm* ARO/CYC genes. *E. coli* was grown in 1 l of LB (200 μg/ml carbenicillin). Protein production was induced with 1 mM IPTG at OD_{600} ~0.6, following which the cells were then grown for ~8 h before being centrifuged and resuspended in disruption buffer.

Gene expression in E. coli

The first eight codons of the *act* KR, *gris* ARO/CYC and *tcm* ARO/CYC were optimized for expression in *E. coli* using the polymerase chain reaction (PCR) and then cloned into the *Nde*I/*Eco*RI sites of pET21c (pRZ153, pRZ112 and pRZ106 respectively). The sequence for the *act* KR (pRZ153) primers were as follows (restriction enzyme sites are italicized and modified bases are shown in bold): 5′-*CATATG*-GC**G**AC**C**CAGGACTCCGAAGTCGCACTG-3′ and 3′-*CTTAAG*AGT-CATCAAGGGGTCGGGCGGCGT-5′. The primers for the *gris* ARO/CYC (pRZ112) were as follows: 5′-*CATATG*TCTCAGCC**G**GGCCTG-CGCGAGGTGGAGCAC-3′ and 3′-*CTTAAG*AGTCGGGGCGGCC-GGGCGCCGT-5′. The primers for the *tcm* ARO/CYC (pRZ106) were: 5′-*CATATG*GCAAGCGCGCACGGACAA-3′ and 3′-*CTTAAG*-AGTGTTGTCAACGACCCGCA-5′.

Purification of the PKS proteins

The *act* KS–CLF [21] and *tcm* KS–CLF [22] complexes were purified as previously described. The *fren* ACP, which was coexpressed with Sfp, a phosphopantetheinyl transferase (PPTase) from *Bacillus subtilis* [29] for *in vivo* phosphopantetheinylation, was prepared by freeze/thaw lysis [30] and Resource Q chromatography as previously reported [31]. Cells expressing *act* KR, *gris* ARO/CYC or *tcm* ARO/CYC genes were disrupted using a French press at 1300 psi. The crude extracts were then precipitated with 70% saturated $(NH_4)_2SO_4$.

The *act* KR extract was desalted into 10 mM NaH_2PO_4, pH 7/NaOH, 2 mM DTT, 2 mM EDTA, 1 M $(NH_4)_2SO_4$ (buffer A) and bound to a Butyl Sepharose FF column (HR 10/30, Pharmacia Biotech). The column was washed with 20 ml buffer A followed by a 80 ml gradient at 1 ml/min to buffer A lacking $(NH_4)_2SO_4$ that went 100–25% buffer A over 40 ml then 25–0% over 40 ml. Peak fractions (*act* KR 70% of protein) eluting at 50–0 mM $(NH_4)_2SO_4$ were pooled and applied to a hydroxyapatite column. The flow through, which contains KR (~90% of protein), was desalted into 20 mM Tris, pH 8/HCl, 2 mM dithiothreitol (DTT), 2 mM EDTA, 20% (v/v) glycerol (buffer B) and applied to an anion exchange column (Resource Q; 6 ml column). A gradient from 0–0.25 M NaCl in buffer B was run at 1 ml/min for 5 min, followed by a gradient from 0.25 to 0.4 M NaCl at 1 ml/min for 20 min. The KR elutes in a form that is greater than 95% pure between 0.30 M and 0.38 M NaCl.

The *gris* ARO/CYC was desalted into 50 mM NaH_2PO_4, pH 7/NaOH, 2 mM DTT, 2 mM EDTA, 0.75 M $(NH_4)_2SO_4$ (buffer C) and applied to a Phenyl Sepharose HP column (HR 10/30, Pharmacia Biotech). A linear gradient at 1 ml/min to buffer C lacking $(NH_4)_2SO_4$ and containing 20% (v/v) glycerol was run over 90 min. Fractions containing the *gris* ARO/CYC (5% of total protein) which eluted at the end of the gradient were desalted into buffer B and applied to a 6 ml Resource Q column. A gradient was developed at 1 ml/min to 0.15 M NaCl in buffer B over 5 min followed by a gradient to 0.25 M NaCl over 30 min. The *gris* ARO/CYC (85% of protein) eluted as a peak around 0.17 M NaCl. The purest fractions were pooled and concentrated on Centriprep 10 membranes (Amicon) to a protein concentration of 3–4 mg/ml (200 μl total volume). The *gris* ARO/CYC was then chromatographed on a Superdex 200 size exclusion column in 100 mM NaH_2PO_4, pH 7.3/NaOH, 2 mM DTT, 2 mM EDTA (buffer D) where it eluted as a 37 kDa protein that was greater than 95% pure.

The *tcm* ARO/CYC was desalted into buffer B and loaded onto an anion exchange column (HiTrap Q; 2 × 5 ml columns). A gradient at 1 ml/min was run from 0–0.15 M NaCl in buffer B over 5 min followed by a 30 min gradient at 1 ml/min from 0.15–0.30 m NaCl. Fractions at 0.18 M NaCl, which contained *tcm* ARO/CYC (75% of the total protein), were pooled and desalted into 50 mM malonic acid, pH 5.5/NaOH, 2 mM DTT, 2 mM EDTA, 20% (v/v) glycerol (buffer E) and applied to a cationic exchange column (HiTrap SP; 2 × 5 ml columns). A linear gradient to 0.20 M NaCl in buffer E was run at 1 ml/min over 30 min. The *tcm* ARO/CYC eluted as a fraction that was greater than 95% pure at 0.10 M NaCl.

Assay of PKS activity

The activity of the *act* minimal PKS was determined as described previously [20,21]. Activities of the *act* KR and *gris* ARO/CYC were determined using a similar method with purified *act* minimal PKS. Unless specified otherwise, reaction mixtures (100–200 µl) contained 0.5 µM KS/CLF, 20 µM holo-ACP, 0.1 µM MAT, 20 µM *act* KR, 5 µM *gris* ARO/CYC (not included when solely assaying KR), 1 mM [^{14}C]malonyl CoA (0.35 Ci/mol), 2 mM NADPH, 100 mM NaH_2PO_4, pH 7.3, 2 mM DTT, 2 mM EDTA, 25% glycerol (v/v). Reactions were incubated at room temperature for 90 min prior to quenching with 0.1 g of NaH_2PO_4 and extracting with 2 × 0.5 ml of ethyl acetate. Ethyl acetate extracts were dried *in vacuo*, resuspended in 10–15 µl ethyl acetate:methanol 50:50 and run on TLC on silica gel (Baker Si250F, methanol:acetic acid:ethyl acetate 9:1:90) and visualized using electronic autoradiography (Instant Imager, Packard). The activity of the *tcm* ARO/CYC was determined in conjunction with purified *tcm* minimal PKS. Reaction mixtures (100 µl) consisted of 0.2 µM *tcm* KS/CLF, 20 µM holo-ACP, 0.1 µM MAT, 15 µM *tcm* ARO/CYC, 1 mM [^{14}C]malonyl CoA (0.35 Ci/mol), 100 mM NaH_2PO_4, pH 7.3, 2 mM DTT, 2 mM EDTA and 25% glycerol (v/v). The products of assays of the *act* KR, *gris* ARO/CYC and *tcm* ARO/CYC were verified by HPLC and AP-CI mass spectrometry of extracts obtained from 500 µl reactions. The extracts were compared to standards of mutactin for the *act* KR, SEK34 for the *gris* ARO/CYC and RM80 for the *tcm* ARO/CYC. (Note the product for the *tcm* minimal PKS plus *tcm* ARO/CYC reported here, RM80, is consistent with the major product found in previous *in vivo* investigations on this system done in this laboratory [14]. However, *in vivo* and *in vitro* analysis of the *tcm* minimal PKS plus *tcm* ARO/CYC by Hutchinson and coworkers [6,25,32] resulted in a related but different product, TCMF2. (The reasons for this discrepancy remain unknown.)

Kinetic assays for act *minimal PKS,* act *minimal PKS plus* act *KR, and* act *minimal PKS plus* act *KR plus* gris *ARO/CYC*

Assays for SEK4 and SEK4b, mutactin, and SEK34 (the products of the three multienzyme systems, respectively) production were performed in 400–500 µl reactions with 100 µl time points taken in the range of 5–15 enzymatic turnovers. Each time point was extracted 3 × 400 µl ethyl acetate. The extracts were dried *in vacuo*, resuspended with 10–15 µl ethyl acetate:methanol 50:50, and loaded onto a TLC plate. The amount of product was determined using standards of known specific activity. The concentrations of the proteins were determined using the Lowry method and via densitometric scanning. Apparent k_{cat} values were calculated under the assumption that the KS and CLF formed a heterodimer.

Kinetic assays for tcm *minimal PKS and* tcm *minimal PKS plus* tcm *ARO/CYC*

Assays for SEK15 and SEK15b [8,12] and RM80 [14] (the products of the two multienzyme systems, respectively) production were performed in 400–500 µl reactions. Reactions for the *tcm* PKS plus *tcm* ARO/CYC consisted of 0.2 µM KS–CLF, 15 µM *fren* ACP, 0.1 µM MAT and varying amounts of *tcm* ARO/CYC. It was determined that the rates of polyketide formation for the *tcm* PKS were linear in the range of 10–50 enzymatic turnovers: therefore, 100 µl aliquots were extracted in this range. Each aliquot was extracted with 3 × 400 µl ethyl acetate. The extracts were dried *in vacuo*, resuspended with 10–15 µl ethyl acetate:methanol 50:50, and loaded onto a TLC plate. The TLC plate was developed using a running buffer of ethyl acetate:methanol:acetic acid 92:7:1. The amount of product was determined using standards of known specific activity. The concentrations of the proteins were determined using the Lowry method and via densitometric scanning. Apparent k_{cat} values were calculated under the assumption that the KS and CLF formed a heterodimer with one active site.

Gel-filtration chromatography

Molecular masses were estimated by applying the *act* KR (66 µM), *gris* ARO/CYC (22.6 µM) and *tcm* ARO/CYC (39 µM) onto Superdex 200 pg (AK 26/60, Pharmacia) with 100 mM NaH_2PO_4, pH 7.3, 2 mM DTT, 2 mM EDTA 20% glycerol (v/v) at 0.25 ml/min. These proteins were also loaded onto the column in combination with the *act* KS/CLF (3 µM) and ACP (350 µM) to investigate the possibility of complex formation. Elution volumes were determined by absorbance at 280 nm and verified using SDS–PAGE.

Chemical cross-linking

Purified *act* KR (46 µM) was equilibrated into buffer B without addition of DTT. The cross-linking agent used was glutaric dialdehyde at 1.9 M in the enzyme solutions (12 µl). The reactions were incubated at 4°C and quenched with sodium borohydrate at 2, 3 and 5 min. The protein samples were denatured and electrophoretically separated using 12% SDS–PAGE. The molecular masses of the cross-linked species were estimated with a protein marker ladder (New England Biolabs).

Identification of mutactin, SEK34 and RM80 by HPLC

The polyketide products mutactin, SEK34 and RM80, generated *in vitro* and extracted with ethyl acetate, were chromatographed on a C-18 reverse-phase HPLC column. A gradient from 0–20% acetonitrile in water over 5 min followed by 20–40% over 45 min at 1 ml/min was used for mutactin and SEK34. RM80 was subjected to a gradient from 0–100% acetonitrile in water over 30 min at 1 ml/min. Authentic samples were used as references. UV peaks at 280 nm were monitored using an on-line multi-wavelength detector. The peaks corresponding to mutactin, SEK34 and RM80 appeared at 29.4%, 28.6% and 67% acetonitrile, respectively. AP–CI mass spectrometry was also performed to confirm the molecular weights of the products.

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