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COMMUNICATION

Insights into the stereospecificity of ketoreduction in a modular polyketide synthase†

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Ketoreductase enzymes are responsible for the generation of hydroxyl stereocentres during the biosynthesis of complex polyketide natural products. Previous studies of isolated polyketide ketoreductases have shown that the stereospecificity of ketoreduction can be switched by mutagenesis of selected active site amino acids. We show here that in the context of the intact polyketide synthase multienzyme the same changes do not alter the stereochemical outcome in the same way. These findings point towards additional factors that govern ketoreductase stereospecificity on intact multienzymes *in vivo*.

Modular polyketide synthases (PKSs) are multienzyme assembly lines responsible for the biosynthesis of diverse complex polyketide natural products including the macrolide antibiotic erythromycin A, the anticancer epothilones, and the immunosuppressant rapamycin.[1–4] Amongst these enzymes, the 6-deoxyerythronolide B synthase (DEBS), which produces the aglycone of erythromycin A, has undoubtedly been the most intensively studied.

Type I modular PKSs such as DEBS consist of multiple catalytic domains, distributed over several multienzyme polypeptides, that assemble a polyketide chain by successive head-to-tail condensation of acyl-CoA esters, with the intermediate species remaining covalently bound to the enzyme.[1,3] In contrast to the type I multienzyme fatty acid synthases (FASs), where multiple cycles of chain elongation proceed by iterative use of a single set of enzymes, type I modular PKSs use distinct sets of domains, or 'modules', to catalyze one round of chain extension, the modules being arrayed in the order in which they are used. Each module consists of a minimum of three core domains: a ketosynthase (KS), an acyltransferase (AT), and an acyl-carrier protein (ACP). Whereas for FASs the initially formed β-ketoacyl intermediates (anchored to the ACP *via* thioester linkage to a 4′-phosphopantetheine (PPant) cofactor) are fully reduced to a fatty acyl chain by the successive action of ketoreductase (KR), dehydratase (DH), and enoylreductase (ER) domains, for PKSs some or all of these reductive domains may be absent from a given module. This results in the presence of unreduced or partially reduced centres in the final polyketide product. In modules containing only KR and the core domains, the reduction of the β-keto functionality generates hydroxyl groups resulting in different stereocentres. Depending on the active site geometry of the KR domain, reduction can occur by delivery of an NADPH-derived hydride to either the *re* or the *si* face at the C-3 of the β-ketoacyl intermediate, resulting in a 3*S* or 3*R* β-hydroxyacyl intermediate, respectively.[5,6]

The KR stereospecificity is described as either A-type, resulting in a 3*S* β-hydroxyacyl product (where C-2 has higher priority than C-4), or B-type, giving the 3*R* β-hydroxyacyl product. For example the KR domains from the first and second extension modules of DEBS ($eryKR_1$ and $eryKR_2$) are B-type and A-type, respectively. This specificity has been correlated to the nature of key amino acids which flank the active site and are proposed to coordinate the binding of the β-ketoacyl thioester substrate in one of the two possible orientations, leading to ketoreduction from alternative faces.[5–7] Two amino acid regions differ consistently between the A- and B-type KRs: (a) residues 93 to 95 (numbering according to Caffrey, 2003)[5] in the B-type KRs are typically conserved as Leu93, Asp94, and Asp95 (usually absent in A-type KRs);[5,6] (b) in the region spanning residues 141 to 148, A-type KRs have a highly conserved Trp141, whereas B-type KRs often have Pro144 and Asn148.[5] X-ray crystal structures of both A-type[8] and B-type[9,10] recombinant KRs have confirmed the modelling that placed these motifs at the active site, and have suggested additional residues that might contribute to stereocontrol.

Understanding the molecular basis of stereocontrol during ketoreduction of PKS-bound intermediates is crucial for success in the rational design of engineered PKSs for the generation of novel polyketides[7,11] and in recent years this has been the subject of considerable research effort.[5–7,11–18] However, we still do not have a complete picture of all the factors that collectively govern the stereochemical course of ketoreduction. Recombinant KR domains have been individually cloned and successfully expressed in *Escherichia coli*, allowing the intrinsic (stereo)specificity of the

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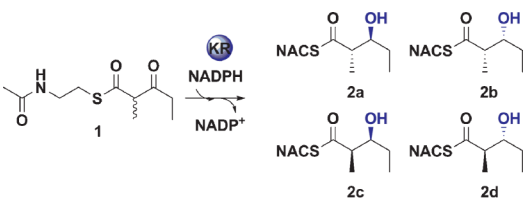
recombinant enzymes to be determined towards surrogate diketide substrates (as their *N*-acetyl cysteamine (NAC) thioesters).[7,11,13] The NAC moiety mimics the end of the PPant cofactor to which the β-ketoacyl intermediate is normally attached. When (±)-2-methyl-3-oxopentanoyl-NAC (**1**) was incubated with NADPH and purified KRs derived from different DEBS modules, analysis of the resulting products (**2a–d**) of ketoreduction (Scheme 1) showed that for eryKR$_1$, which naturally accepts a diketide substrate, the reduction was completely stereospecific and stereoselective, even in the absence of the PKS context or of ACP tethering.[13] In contrast, other eryKRs gave variable and often high proportions of the "wrong" stereoisomeric products.[13] Meanwhile, experiments with hybrid model PKSs, in which KR domains from other PKSs were substituted for eryKR$_2$, have shown that tethering to an ACP domain maintains stereocontrol even for KRs whose normal substrates are penta- or hexaketides. Similar conclusions have been reached using mix and match experiments *in vitro* combining recombinant KS, AT, ACP and KR domains from different sources.[16–18] Nevertheless, even *in vivo* natural KRs may show aberrant stereochemical control when confronted with an "unexpected" substrate delivered from the previous module.[19] The recognition of the active site motifs in A-type and B-type KRs led to the first attempts at KR active site engineering to alter the stereochemical outcome of ketoreduction. These were carried out with eryKR$_1$ and eryKR$_2$ (Fig. 1A).[7,11] Site-directed mutagenesis was used to exchange the amino acid residues conserved in A-type KRs (such as eryKR$_2$) for those typical of B-type (such as eryKR$_1$) (and *vice versa*). It was found possible to alter the stereospecificity of reduction at the C-3 hydroxyl position of the surrogate diketide substrate **1** and even to switch it completely, in certain mutants of eryKR$_1$ (Table 1).[7,11]



Scheme 1 The ketoreduction of the diketide substrate mimic (±)-2-methyl-3-oxopentanoyl-*N*-acetyl cysteamine (NAC-diketide, **1**) can generate four possible isomers (**2a–d**). The stereochemistry of the product is dictated by the KR (Table 1).

We have re-investigated these mutants using the same diketide substrate as its PPant thioester, to see whether the presence of

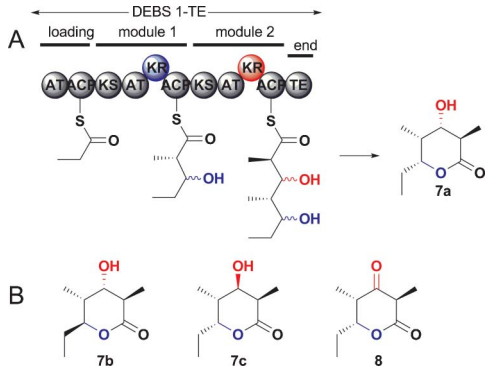
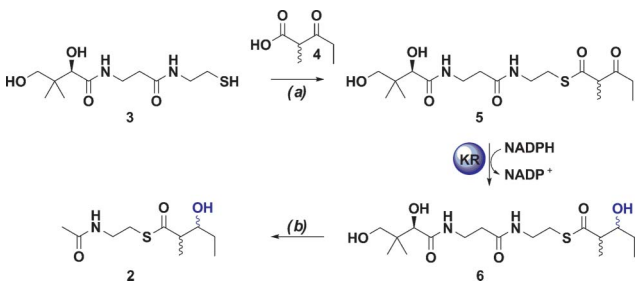


Fig. 1 (A) The modular type I PKS DEBS1-TE produces the triketide lactone **7a**.[23,25,26] (B) The triketide lactones **7b** and **7c** would arise from a 'stereochemical switch' at eryKR$_1$ and eryKR$_2$, respectively, by mutation of key amino acid residues (Table 3); the unreduced triketide lactone **8** is produced when reduction by eryKR$_2$ is inefficient or absent.[24]

the cofactor "arm" is able on its own to affect the efficiency and/or stereoselectivity of *in vitro* ketoreduction. We synthesized (±)-2-methyl-3-oxopentanoyl-pantetheine (**5**, Scheme 2(a) and ESI†),[20,21] which more closely resembles the natural ACP-bound substrate tethered *via* the PPant cofactor, and used it as a substrate of recombinant eryKR$_1$ and the mutants used in the earlier studies.[7,13] These were cloned into new constructs using the vector pGEX6P-1, allowing facile purification and then removal of the GST-tag by PreScission™ proteinase digestion (the earlier constructs suffered non-specific cleavage upon thrombin digestion and so were used as the fusion protein).[7,13]



Scheme 2 Synthesis of (±)-2-methyl-3-oxopentanoyl-pantetheine **5** and transesterification of the KR products **6** with *N*-acetyl cysteamine (NAC) to establish the KR stereospecificity. Reagents and conditions: (a) HATU, DIPEA, THF (dry), 0 °C (3 h) then rt (18 h); (b) *N*-acetyl cysteamine (3 eq), $NaHCO_3$ (aq), 22 h.

Table 1 Stereochemistry of the products resulting from the reduction of NAC-diketide **1** by recombinant eryKR1 or eryKR2 and mutants thereof[7]

			A-type		B-type	
	KR	Mutations	**2a** (2*S*,3*S*)	**2c** (2*R*,3*S*)	**2b** (2*S*,3*R*)	**2d** (2*R*,3*R*)
a	eryKR$_1$	None	—	—	100%	—
b	eryKR$_1$	L93P, D94Q, D95S	~50%	—	~50%	Minor
c	eryKR$_1$	F141W, P144G	>95%	—	—	Minor
d	eryKR$_1$	L93P, D94Q, D95S, F141W, P144G	100%	—	—	—
e	eryKR$_2$	None	74%	6%	20%	—
f	eryKR$_2$	P93L, Q94D, Q95D	~50%	—	~50%	—
g	eryKR$_2$	W141L,A144P, A148N	~50%	—	~50%	—
h	eryKR$_2$	P93L, Q94D, Q95D, W141L, A144P, A148N	~50%	—	~50%	—

Table 2 Kinetic analysis of eryKR$_1$ with model substrates **1** and **5** in different contexts

	Enzyme	Substrate	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (s^{-1} M^{-1})
a	eryKR$_1$ (DKS)[22]	**1**	0.27 ± 0.01	13 ± 2	21.7 ± 5.0
b	GST-eryKR$_1$[13]	**1**	0.26 ± 0.01	35 ± 4	7.0 ± 1.0
c	eryKR$_1$ (untagged)	**1**	0.24 ± 0.01	12 ± 1	20 ± 2.5
d	eryKR$_1$ (untagged)	**5**	0.25 ± 0.02	12 ± 2	20.8 ± 5.1

NADPH-dependent kinetic assays using **1** and **5** were carried out with the tag-free eryKR$_1$ construct (Table 2, entries c and d). These kinetic parameters agree well with those previously measured for the GST-eryKR$_1$ (entry b)[13] except that, encouragingly, the K_m for the diketide substrate is now the same, in the absence of the GST tag, as when assayed with the eryKR$_1$ domain in the context of an intact DEBS-derived diketide synthase (DKS, entry a).[22] We then investigated the stereospecificity of the untagged eryKR$_1$ and its mutants towards **5**. In the absence of pantetheinyl-diketide standards, the pantetheinyl reduction products **6** were transesterified with free *N*-acetyl cysteamine (NAC) to give the corresponding NAC-diketides (**2**, Scheme 2(b)), which were directly compared with the previously-synthesized NAC standards **2a–d** by chiral HPLC analysis. The stereospecificity of the parent eryKR$_1$ and of each of the mutants in the reduction of **5** was revealed to be identical to that towards **1** as previously determined (Table 1S in ESI†, compare to Table 1). The pantetheinyl side chain does not apparently influence the interaction of the substrate with the (mutant) KR active site.

We then wished to assess whether the observed altered stereospecificity of mutant KR domains would be retained within the context of an intact multienzyme PKS. To do this, we have used the model PKS DEBS1-TE,[23,24] which consists of the DEBS loading module (AT and ACP domains), module 1 and module 2 fused at its C-terminus to the chain-terminating thioesterase (TE). When assayed *in vitro* as a purified recombinant PKS[25] or when harboured in a genetically modified bacterial strain (*Saccharopolyspora erythraea*[23] or *Streptomyces coelicolor*[26]), DEBS1-TE catalyzes the formation of triketide lactone **7a** from propionyl-CoA, methylmalonyl-CoA and NADPH (Fig. 1A and Table 3). Mutations were introduced into either the eryKR$_1$ or the eryKR$_2$ domain of DEBS1-TE (ESI†). These mutations were identical to those previously studied for the isolated recombinant eryKR$_1$ and eryKR$_2$.[7] The parent and mutant DEBS1-TE genes were introduced into an engineered strain of *S. erythraea* (BIOT1717-JC2) from which the PKS genes of erythromycin biosynthesis had been deleted (Δ*eryAI – eryAIII*).[23,27,28] Following fermentation of the mutant strains, the triketide lactone products were extracted into ethyl acetate and analyzed by LC-MS, along with authentic standards of three of the isomers potentially generated by the mutant enzymes **7a–c** (ESI†).

In contrast to our previous experiments using isolated KR domains,[7] we found that none of the DEBS1-TE mutants carrying substitutions within their KR active sites gave rise to diastereoisomers of **7a** with an altered hydroxyl configuration (Table 3 and ESI†). There was no detectable stereochemical switch, either from an A-type to a B-type, or *vice versa*. In several cases, the original triketide lactone **7a** was still produced at significant levels (entries c and e, Table 3). For eryKR$_1$ within DEBS1-TE, mutation of residues Leu93, Asp94 and Asp95 (the LDD amino acid motif found in B-type KRs) dramatically lowered polyketide production (entries b and d, Table 3). An additional factor here in lowering the rate still further is that the nascent polyketide chain with altered configuration at the C-5 hydroxyl group is known, from previous *in vitro* studies, to be more poorly processed by the downstream domains of the PKS.[29–31] It is striking that production of the lactone **7a** by the eryKR$_1$ F141W P144G mutant shows undiminished selectivity (entry c) (showing that the β-ketoacyl substrate is still preferentially positioned for hydride delivery to the *re* face) because changes in this loop with the isolated KR domain did lead to a switch in hydroxyl group configuration.[7] For eryKR$_2$ within DEBS1-TE, the W141L, A144P, A148N substitutions led to a shift towards the production of 3-keto lactone **8** (entries f and g, Table 3), whereas the *in vitro* experiments with the KR domain had led to ketoreduction with an apparent shift towards B-type specificity.[7] It is possible that in the altered DEBS1-TE, ketoreduction is sufficiently slowed that it competes poorly with hydrolysis of the ketoacyl-ACP substrate by the adjacent thioesterase (TE) domain.[24] Once again, the native preference for A-type reduction in module 2 was retained even in the presence of the introduced LDD (B-type) motif at residues 93–95 (entry e).

These results clearly indicate that within the intact PKS system there are factors that override the effect of the mutations targeting the KR sequence motifs. This is not unexpected, first because any recalcitrance of downstream enzymes in the PKS assembly-line to accept the altered product (here, the TE domain) will tend to favour the product with the original configuration; and secondly, because there is evidence that the discrete thioesterase enzymes (TE II enzymes) that are almost invariably associated with modular PKS systems may act to hydrolyse prematurely such (more slowly-processed) intermediates.[32–34] Also, the *in vitro* results with eryKR domains have convincingly shown that the energetic differences between the two alternative pathways for reduction of the ketoacyl substrate are finely balanced and so stereocontrol

Table 3 Stereochemistry of the triketide lactone products from *S. erythraea* containing DEBS1-TE active site mutants

	eryKR$_1$	eryKR$_2$ mutations	Desired product	Major products (relative yield, %)	Minor product
a	None	None	**7a**	**7a** (100 ± 14)	**8**
b	L93P, D94Q, D95S	None	**7b**	**7a** (10.4 ± 0.3)	**8**
c	F141W, P144G	None	**7b**	**7a** (99 ± 4)	**8**
d	L93P, D94Q, D95S, F141W, P144G	None	**7b**	**7a** (2.0 ± 0.5)	**8**
e	None	P93L, Q94D, Q95D	**7c**	**7a** (56 ± 7), **8**	—
f	None	W141L, A144P, A148N	**7c**	**8**	—
g	None	P93L, Q94D, Q95D, W141L, A144P, A148N	**7c**	**8**	—

is sensitive to small changes in substrate structure and active site architecture.[7,11,13] Wholly analogous results have recently been obtained, using exactly the same *in vitro* methodology, for isolated A-type KRs from the amphotericin PKS.[8] Stereocontrol of NADPH-linked enoylreduction catalysed by PKS ER domains has also been found to be finely balanced: it was found possible, on a model triketide lactone synthase *in vivo*, to switch the configuration of the product (complete in one direction and partial in the other) by substitution of a critical active-site residue.[27,28]

The relative importance of tethering the substrate to an ACP domain, for the correct processing of a ketoacyl thioester intermediate by a KR domain, remains a matter of debate. Recent studies have demonstrated that the TE domain can interact with ACP-tethered acyl substrates in the absence of contact with the ACP domain itself,[21,35] but the same may not be true for the KR domain. The exact identity of the ACP appears though not to be critical, since isolated DEBS KRs do not display any specificity for their cognate ACPs,[36] while many whole-domain KR swaps are highly successful.[12,24] For example, a switched triketide lactone (**7c**) can be obtained when the eryKR$_2$ domain within DEBS1-TE is completely replaced with a KR of intrinsically opposite stereospecificity.[24]

Given the fine energetic balance between different stereochemical outcomes of ketoreduction, it remains an attractive idea that future engineering of alterations in polyketide natural product configuration could be accomplished by active site manipulation. More work is needed to define amino acid residues at or near KR active sites that may be involved. It has already proved possible to screen, *in vitro*, libraries of KR mutants altered in the LDD and Trp active site motifs to identify unexpected combinations of residues that support catalysis.[11] Directed evolution of polyketide ketoreductases, with screening and selection based on more realistic substrate mimics, could reveal candidate residues for stereocontrol, even ones remote from the active site, and pave the way to an effective and efficient switch of stereospecificity.

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