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High-Throughput Mutagenesis to Evaluate Models of Stereochemical Control in Ketoreductase Domains from the Erythromycin Polyketide Synthase

Helen M. O'Hare,[1,2,3] Abel Baerga-Ortiz,[1] Bojana Popovic,[1] Jonathan B. Spencer,[2] and Peter F. Leadlay[1,*]
[1] Department of Biochemistry
University of Cambridge
80 Tennis Court Road
Cambridge CB2 1GA
United Kingdom
[2] Department of Chemistry
University of Cambridge
Lensfield Road
Cambridge CB2 1EW
United Kingdom

Summary

Ketoreductase (KR) activities help determine the stereochemistry of the products of modular polyketide synthases (PKSs). For example, domains eryKR$_1$ and eryKR$_2$, contained, respectively, in the first and second extension modules of the erythromycin-producing PKS, reduce 3-ketoacyl-thioester intermediates with opposite stereospecificity. Amino acid motifs that correlate with stereochemical outcome have been identified in KRs. We have used saturation mutagenesis of these motifs in eryKR$_1$ and eryKR$_2$, and a microplate-based screen of such mutants for activity against (9*R*, *S*)-*trans*-1-decalone, to identify candidate enzymes potentially altered in stereocontrol. Active mutants were reassayed with (2*R*, *S*)-2-methyl-3-oxopentanoic acid *N*-acetylcysteamine thioester, and the alcohol products were analyzed by chiral HPLC. Variant enzymes were found with either altered substrate selectivity for the (2*R*) or (2*S*) substrate or altered stereospecificity of reduction, or both, further highlighting the importance of these motifs in stereochemical control.

Introduction

Modular type I polyketide synthases (PKSs) are multienzymes that catalyze the assembly line biosynthesis of structurally complex and clinically important polyketide natural products [1, 2], starting from simple carboxylic acid building blocks. Each modular PKS is composed of multiple enzyme domains, arranged into modules that assemble the carbon chain by a mechanism resembling that of fatty acid synthases: an acyltransferase (AT) domain loads a dicarboxylic acid extender unit onto an acylcarrier protein (ACP) domain, where it undergoes condensation with a starter acyl unit in a reaction catalyzed by a ketosynthase (KS). The product is an extended β-ketoacyl thioester intermediate that remains covalently linked to the ACP. The β-keto group may then be processed by additional domains within the module before the extended chain migrates to a KS for another cycle of extension. Such reductive activities are optionally present depending on the module and comprise: a β-ketoreductase (KR) domain, which catalyzes the reduction of the initially formed β-ketoester to a β-hydroxyester; a dehydratase (DH) domain, which dehydrates the β-hydroxyester; and an enoylreductase (ER) domain, which reduces the double bond. The diversity of polyketide structures results, in part, from combinatorial use of chemically different starter and extender units, and, also, partly from the variable degree of processing of β-keto groups. Extensive engineering of polyketide biosynthetic genes [3–6] (including other enzymes in these pathways such as glycosyltransferases [7]) has already resulted in the production of significant additional molecular diversity. Chiral centers in complex polyketides are introduced by the use of branched extender units and by the reduction of β-keto groups to alcohols. The ability to manipulate the stereochemistry of the methyl and alcohol groups would give access to many additional novel polyketides, perhaps with novel clinical properties.

The most well-studied PKS, 6-deoxyerythronolide B synthase (DEBS), catalyzes the synthesis of the macrolide precursor to the antibiotic erythromycin [8, 9]. The AT domains of DEBS load (2*S*)-methylmalonyl groups onto the ACP domains [10, 11], and condensation occurs with inversion of stereochemistry at the carbon atom derived from C-2 of the extender unit; thus, a (2*R*)-2-methyl-3-ketoacylthioester is generated [12]. In certain PKS modules, such as extension module 1 of DEBS, the initial condensation product is epimerized to give a (2*S*)-2-methyl-3-ketoacyl chain before reduction [12, 13]. In modules housing epimerizing KS domains, such as extension module 1 of DEBS, the KR also determines the methyl stereochemistry at C-2 by discriminating between (2*R*)- or (2*S*)-2-methyl-3-ketoacyl substrates [14]. The engineered exchange of KR domains between natural PKSs has shown that a KR domain in a PKS multienzyme may have an intrinsic stereospecificity and stereoselectivity (see, for example, [15, 16]). However, when confronted by "unnatural" substrates, the stereochemical outcome is not always as predicted, either in vivo or in vitro [17].

Individual KR domains of modular PKSs have recently been successfully expressed as soluble and enzymatically active proteins in recombinant *Escherichia coli*, in the absence of other PKS components [18]. The kinetics and stereochemistry of ketoreduction of model substrates on these recombinant KR domains supported the idea that alternative modes of binding to the KR active site were responsible for different stereochemical outcomes, and they revealed that the energetic differences between these binding modes are rather small. It appeared, therefore, that alteration of only a few key residues at the active site might suffice to change the stereochemistry of KR-mediated reduction.

There is as yet no high-resolution X-ray crystal structure for any KR domain from a modular type I PKS.

*Correspondence: pfl10@mole.bio.cam.ac.uk
[3] Present address: Ecole Polytechnique Fédérale de Lausanne, Institute of Chemical Sciences and Engineering, 1015 Lausanne, Switzerland.

However, homology modeling [19, 20] (see the accompanying paper in this issue of *Chemistry & Biology*) has shown that these enzymes belong to the short-chain dehydrogenase/reductase (SDR) family of NADPH-linked dehydrogenases [21], in which proton transfer is thought to occur from a conserved tyrosine to the carbonyl oxygen of the substrate, while reaction intermediates are stabilized by hydrogen bonding with a conserved serine and lysine. The active site tyrosine and serine residues are present in analogous positions in all modular PKS KRs. Experimental confirmation for this assignment to the SDR family has also been obtained by site-directed mutagenesis of catalytic residues in the KR of DEBS module 6 (eryKR$_6$) [19]. Further, the multiple alignment of natural KR domains catalyzing a known stereochemical outcome has revealed that, at two positions near the KR active site (referred to here as motif I and motif II, respectively), the presence or absence of certain amino acids is highly correlated with the stereochemistry of the alcohol center, hinting at their possible contribution to the mechanism of stereocontrol [22]. In the accompanying paper [20], we report the effects of site-specific mutagenesis of the residues in motifs I and II in eryKR$_1$ and eryKR$_2$, the KR domains derived, respectively, from the first and second extension modules of DEBS. These KR domains catalyze the production of alcohols of opposite stereochemistry, although their active sites have exactly the same architecture of catalytic residues [20]. Remarkably, substitution of only two active site residues in eryKR$_1$ led to an effective switch of alcohol stereochemistry. The results of mutation of eryKR$_2$, which normally encounters a triketide substrate and which is not fully stereoselective against diketide substrates, also showed significant changes in stereochemical outcome [20].

There is currently great interest in using the methods of molecular evolution [23–25] to achieve alterations or improvements in the enantioselectivity and stereospecificity of enzyme catalysis [26, 27]. These powerful methods, which do not require precise information about mechanism or active site structure, have not previously been applied to PKS multienzymes, but we reasoned that they might be applied to individual recombinant domains. We report here the development of a convenient microplate assay for activity of isolated KR domains, suitable for high-throughput mutagenesis. It is based on assays originally developed for fatty acid synthase [28] and PKS [29] KR domains in their multienzyme context, and it was more recently used with recombinant PKS KR domains individually expressed in *Escherichia coli* [18]. Catalytically competent enzymes were isolated by screening thousands of mutants of eryKR$_1$ and eryKR$_2$ that had been generated by saturation mutagenesis of amino acid residues in motifs I and II. Numerous active site mutants of both eryKR$_1$ and eryKR$_2$ that were active against surrogate substrates and that had altered stereocontrol were identified. In addition to shedding light on the potential role of individual active site residues, these results open the way to directed molecular evolution of KR domains via several rounds of mutation and selection, and to insertion of such mutated KR domains into PKS multienzymes as a potential new route to engineered polyketide drugs with altered chirality.

Results and Discussion

Screening Libraries of eryKR$_1$ and eryKR$_2$

The first two ketoreductase domains from 6-deoxyerythronolide B synthase (DEBS), eryKR$_1$ and eryKR$_2$, produce opposite stereochemistry in terms of both the methyl and alcohol centers in the macrolide product (see Figure 1 of the accompanying paper [20]). Using the classification system of Caffrey [22], eryKR$_1$ is B type and eryKR$_2$ is A type, corresponding to reduction from alternative faces of the keto group. The actual stereochemical course for reduction of the surrogate substrate (2*R*, *S*)-2-methyl-3-ketopentanoic acid *N*-acetylcysteamine thioester has been previously shown to be as in Figure 1. eryKR$_1$ selects the (2*S*)-enantiomer of the substrate and reduces it almost exclusively to the (2*S*, 3*R*) product, as predicted, while, for eryKR$_2$, the predicted product is only a few percent of the product mixture [18]. Broadly, mutagenesis of eryKR$_1$ was therefore expected to lead to a loss of stereocontrol, while, for eryKR$_2$, no clear prediction could be made. Figure 1 also shows the reaction that both eryKR domains catalyze on (9*R*, *S*)-*trans*-1-decalone [28, 29].

Three residues in each motif were chosen for saturation mutagenesis: residues 93–95 in motif I, and residues 141, 144, and 148 in motif II (amino acids are numbered according to [22]). Each motif was randomized in each enzyme to give four libraries (libraries 4–7, Figure 2), and the three residues making up motif I in KR$_1$ were also diversified singly in three smaller libraries (libraries 1–3, Figure 2). The change in absorbance of the cofactor NAPDH provided a convenient spectrophotometric assay for ketoreductase activity against (9*R*, *S*)-*trans*-1-decalone. The assay was optimized for use with unpurified proteins in a 96-well microplate format (Figure 3A); 96 members each of libraries 1–7 (except for libraries 4 and 5; 288 members) were screened for ketoreductase activity (Figures 3B–3H). The frequency of clones active against decalone is summarized in Figure 2. Several active mutants from libraries 1–7 were chosen for further characterization.

Screening for Compensating Mutations in Site-Directed Mutants of eryKR$_1$ and eryKR$_2$

Four recombinant KR domains have previously been constructed by mutagenesis to either motifs I and II: KR$_1$ WGG, KR$_1$ PQS, KR$_2$ LDD, and KR$_2$ LPN [20]. Of these four enzymes, only KR$_2$ LDD is sufficiently active for detection by the microplate assay. We were interested to discover whether the catalytic defects in these enzymes could be compensated by mutations at the other motif, and diversity was therefore introduced to each mutant (Figure 2) to give an additional seven libraries equivalent to libraries 1–7, but with fixed mutations at the undiversified motifs. A total of 288 members of each library were screened. Mutants with improved activity toward decalone were isolated from libraries 10 (KR$_1$ WGG 95), 12 (KR$_1$ PQS 141–148), and 14 (KR$_2$ LPN 93–95) (a single mutant from each library), and 13 (KR$_2$ LDD 141–148) (8.3% of members are active) (Figure 1). No active variants were found in libraries 8 (KR$_1$ WGG 93), 9 (KR$_1$ WGG 94), or 11 (KR$_1$ WGG motif I).

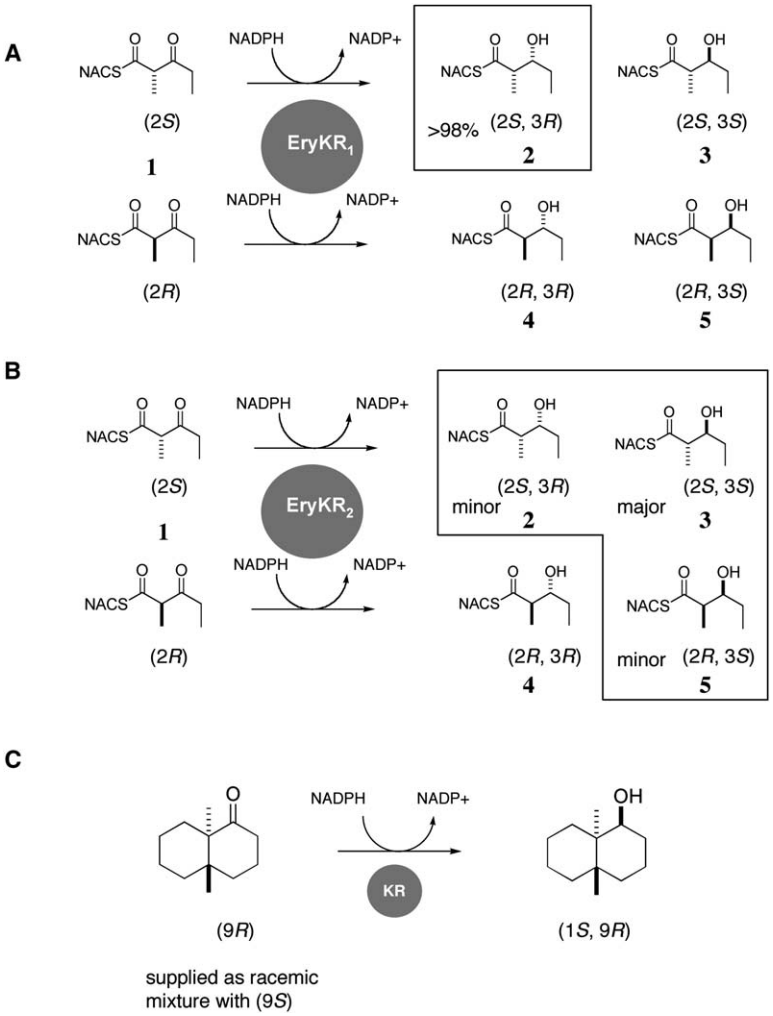


Figure 1. Surrogate Substrates Used in This Work for In Vitro Assay of Recombinant Ketoreductase Domains Derived from the Erythromycin-Producing PKS

(A–C) Surrogate substrates used in this work for in vitro assay of recombinant ketoreductase (KR) domains derived from the erythromycin-producing PKS. (A) Reduction of racemic 2-methyl-3-ketopentanoic acid *N*-acetylcysteamine thioester (**1**) by eryKR$_1$ and by eryKR$_2$. eryKR$_1$ reduces the (2*S*) stereoisomer of **1** only and produces almost exclusively the predicted diastereoisomer of the product, **2** [18, 20]. (B) eryKR$_2$ produces a mixture of three diastereomers, **2**, **3**, and **5**, in which the predicted product, **5**, is a minor component [18, 20]. (C) Reduction of (9*R*, *S*)-*trans*-1-decalone by either eryKR$_1$ or eryKR$_2$ [29].

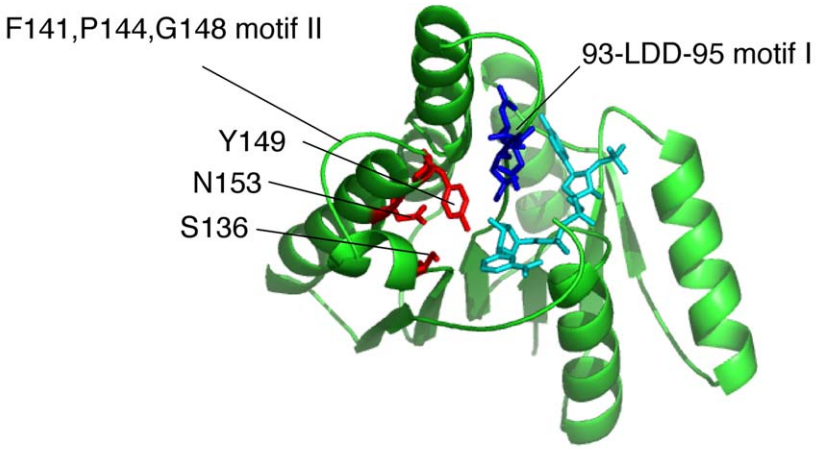
Characterization of Selected KRs

Selected genes were sequenced, and the KR domains were purified as fusion proteins with glutathione-*S*-transferase (GST) for in vitro assays, as described previously [18]. The fusion proteins were used directly in these experiments, since we have previously shown [18] that the presence of the GST domain does not significantly affect the kinetics or stereochemistry of KR-mediated reduction. Reaction rates with decalone were measured spectrophotometrically. (2*R*, *S*)-2-methyl-3-ketopentanoic acid *N*-acetylcysteamine (NAC) thioester was used as a model substrate to determine the reaction stereospecificity, by HPLC analysis of the products. The results are summarized for eryKR$_1$ in Table 1, and for eryKR$_2$ in Table 2, and the HPLC separation of the alcohol stereoisomers is shown in Figure 4. Although the mutants derived from KR$_1$ WGG (library 10) and KR$_1$ PQS (library 12) had higher activity toward decalone, they were inactive with NAC-diketide, and they are not included here. Some eryKR$_1$ mutants showed a reversal of stereospecificity (Figure 5) accompanied by a decrease in overall activity, as has been seen for certain specific mutants of this enzyme [20]. The effects of individual mutations are interpreted below with reference to recent structural models of eryKR$_1$ and eryKR$_2$ [20].

Prescreening of eryKR Mutants for Reduction of Decalone Allows for Rapid Identification of Mutants Capable of NAC-Diketide Reduction

In the present work, the theoretical total library size was 262,144 mutant genes for eryKR domains, encoding 64,000 different proteins. In practice, some of the libraries made were incomplete; for example, library 4 theoretically comprises 32,768 genes, but only 10,000 colonies were picked. Nevertheless, a representative sample of over 100,000 mutant genes was constructed, and over 2,000 of these proteins were screened by using the NADPH-linked assay. The separation of KR domains from their context in the PKS multienzyme requires the use of surrogate, untethered substrates for assays. NAC-diketide could also be used in the microtitre plate-based screen, but the use of the cheaper, more stable, decalone allowed for the rapid examination of large numbers of KRs. Despite the obvious differences between the two types of substrate, preselection of libraries for decalone reduction was shown to increase the likelihood of isolating mutants capable of NAC-diketide reduction (see the Supplemental Data available with this article online). Nevertheless, any mutants possessing activity toward NAC-diketide, but lacking decalone reductase activity, would have been discarded during

A



B

		Motif I		Motif II	Active clones in library (%)
KR_1 wt	88	HAAAT**LDD**GTVDTLTG	134	FSSFASA**F**GA**P**GLG**G**Y	-
Library 1	88	HAAAT**XDD**GTVDTLTG	134	FSSFASA**F**GA**P**GLG**G**Y	4
Library 2	88	HAAAT**LXD**GTVDTLTG	134	FSSFASA**F**GA**P**GLG**G**Y	16
Library 3	88	HAAAT**LDX**GTVDTLTG	134	FSSFASA**F**GA**P**GLG**G**Y	18
Library 4	88	HAAAT**XXX**GTVDTLTG	134	FSSFASA**F**GA**P**GLG**G**Y	14
Library 5	88	HAAAT**LDD**GTVDTLTG	134	FSSFASA**X**GA**X**GLG**X**Y	10
KR_1-WGG	88	HAAAT**LDD**GTVDTLTG	134	FSSFASA**W**GA**G**GLG**G**Y	-
Library 8	88	HAAAT**XDD**GTVDTLTG	134	FSSFASA**W**GA**G**GLG**G**Y	0
Library 9	88	HAAAT**LXD**GTVDTLTG	134	FSSFASA**W**GA**G**GLG**G**Y	0
Library 10	88	HAAAT**LDX**GTVDTLTG	134	FSSFASA**W**GA**G**GLG**G**Y	0.3
Library 11	88	HAAAT**XXX**GTVDTLTG	134	FSSFASA**W**GA**G**GLG**G**Y	0
KR_1-PQS	88	HAAAT**PQS**GTVDTLTG	134	FSSFASA**F**GA**P**GLG**G**Y	-
Library 12	88	HAAAT**PQS**GTVDTLTG	134	FSSFASA**X**GA**X**GLG**X**Y	0.3
KR_2 wt	88	HAAGL**PQQ**VAINDMDE	134	FSSGAGV**W**GS**A**RQG**A**Y	-
Library 6	88	HAAGL**PQQ**VAINDMDE	134	FSSGAGV**X**GS**X**RQG**X**Y	4
Library 7	88	HAAGL**XXX**VAINDMDE	134	FSSGAGV**W**GS**A**RQG**A**Y	5
KR_2-LDD	88	HAAGL**LDD**VAINDMDE	134	FSSGAGV**W**GS**A**RQG**A**Y	-
Library 13	88	HAAGL**LDD**VAINDMDE	134	FSSGAGV**X**GS**X**RQG**X**Y	8
KR_2-LPN	88	HAAGL**PQQ**VAINDMDE	134	FSSGAGV**L**GS**P**RQG**N**Y	-
Library 14	88	HAAGL**XXX**VAINDMDE	134	FSSGAGV**L**GS**P**RQG**N**Y	0.3

Figure 2. Homology Model of the Active Site of PKS Ketoreductase Domains

(A and B) The model of the active site of ketoreductase $eryKR_1$ [20] shows catalytic residues (in red) and amino acid motifs identified by Caffrey [22], which are putatively involved in the control of stereochemistry by KR domains from modular PKSs. (A) The strongest indicator for B-type KR domains is an LDD motif (motif I) in the region between amino acids 88 and 103, which is absent from A-type KR domains. Additional amino acids in the 134–149 region (motif II) support this assignment, specifically P144 and N148, which indicate B-type KRs and W141 in A-type domains. The active site Y149, S136, and K153 (red) position the substrate carbonyl for the transfer of the hydride by NADPH (cyan). F141, P144, and G148 are part of a loop directly adjacent to catalytic residue Y149, while LDD93–LDD95 belong in a loop adjacent to the active site (blue). (B) Sequence alignment of KR domains showing the site-directed mutations constructed in this work. The active site motifs, and the changes made, are indicated in bold (residues are numbered according to [22]). X indicates randomized residues.

the screening. This is a common drawback of surrogate substrates in directed evolution: multiple rounds of mutagenesis and selection favor enzymes that are best adapted to the surrogate, which may not necessarily be the best with the real substrate. Future work involving multiple rounds of screening and selection should preferably be based on more realistic substrate mimics.

Mutants showing activity toward decalone were found to comprise a few percent of each library (Figures 2 and 3). These proteins must be stable and well expressed, with unperturbed catalytic residues and a NADPH binding site. The sequences of mutants that meet these requirements form a consensus of amino acid combinations for active KRs. In $eryKR_1$ motif I, all three positions contained a high proportion of hydrophobic residues, particularly tryptophan (Table 1). In motif II, residue 141 was usually hydrophobic, and residue 144 was always proline or serine. A variety of mutations were seen in position 148, but mutants with alanine or glycine had the highest activity toward NAC-diketide. In $eryKR_2$ motif I, there appears to be a high proportion of hydrophilic residues, though only three mutants were examined. In motif II, residue 141 is hydrophobic, residue 144 is proline or hydrophobic, and residue 148 is alanine or glycine (Table 1).

None of the residues studied are intolerant to mutation, confirming that they do not play a direct role in NADPH binding or catalysis. Leucine 93 of $eryKR_1$ is the least tolerant to mutation, consistent with its position in the structural model—only 4 Å from the catalytic tyrosine 149. Mutations in both motifs modulate $eryKR_1$ and $eryKR_2$ activity and may increase or decrease the activity toward decalone; for example, $eryKR_1$ (D95M)

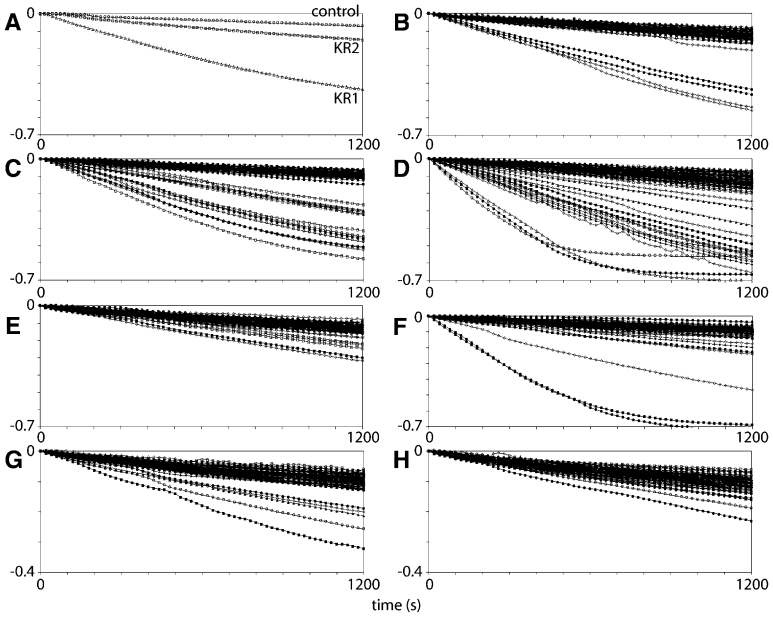


Figure 3. Screening Expression Libraries for Ketoreductase Activity

(A–H) Decalone reduction by KR was detected in cell extracts of recombinant *E. coli*. Absorption at 340 nm was measured for a period of 20 min. (A) Measurement of NADPH consumption by *E. coli* expressing eryKR$_1$ (triangles), eryKR$_2$ (squares), or no KR (circles). The other panels show measurements of NADPH consumption by cell extracts containing mutant KRs, 96 samples per panel. (B) 96 clones from library 1 KR$_1$ residue 93. (C) Library 2 KR$_1$ residue 94. (D) Library 3 KR$_1$ residue 95. (E) Library 4 KR$_1$ motif I residues 93–95. (F) Library 5 KR$_1$ motif II residues 141, 144, and 148. (G) Library 6 KR 2 motif I residues 93–95. (H) Library 7 KR 2 motif II residues 141, 144, and 148.

has 6-fold higher activity. This suggests that the residues concerned are indeed close to the active site and are involved in substrate binding or orientation. A possible alternative explanation is that they may affect a conformational transition between open (unbound) and closed (substrate bound) forms of the enzyme, analogous to

Table 1. Activity and Specificity of KR$_1$ Mutants against Decalone and (2*R*, *S*)-2-Methyl-3-Oxopentanoic Acid NAC Thioester Substrates

Library	Motifs I and II		Activity against Decalone[a]	Peak Area, (2*R*, 3*R*) (4)	Peak Area, (2*S*, 3*S*) (3)	Peak Area, (2*S*, 3*R*) (2)
KR$_1$[b]	LDD	FPG	0.260	298	198	32,469
Motif I Single Mutants						
	WDD	FPG	0.079	110	110	468
	LRD	FPG	0.057	332	513	735
	LSD	FPG	0.163	196	377	3,849
	LWD	FPG	0.154	170	351	4,124
	LDA	FPG	0.141	335	516	560
	LDM	FPG	1.614	192	373	180
	LDR	FPG	0.052	318	499	408
	LDV	FPG	0.797	nd	245	101
Motif I Triple Mutants						
	AHM	FPG	0.148	129	310	4,136
	ARM	FPG	0.172	105	294	1391
	LWR[c]	FPG	0.147	543	724	289
	MWC[c]	FPG	0.065	565	746	239
	VGR	FPG	0.333	118	299	5,250
	WEW	FPG	0.148	101	282	154
	WRP	FPG	0.080	451	632	628
	WWA[c]	FPG	0.208	677	858	478
Motif II						
	LDD	**FPS**	0.215	462	643	54,550
	LDD	**YPA**	0.787	458	639	51,503
	LDD	**ASA**	1.190	nd	193	34,113
	LDD	**TSG**	0.110	319	500	26,853
	LDD	**FSA**	0.279	nd	209	23,634
	LDD	**HPE**	0.537	300	481	12,414
	LDD	**GPI**	0.182	356	537	10,769
	LDD	**ASR**	0.269	nd	141	756

Amino acid residues within either motif I or motif II that were found to be altered in each KR mutant are shown in bold type.

[a] Units of activity are μmoles/min/mg protein, measured at 5 mM decalone, 40 μM NADPH, and 0.2 mg/ml KR.

[b] Higher column loading allowed for detection of these minor products of KR$_1$, not reported by Siskos et al. [18]. nd indicates that a peak was not detected for this isomer.

[c] The products of ketoreduction of 1 by these mutants (which show inverted stereospecificity) are shown in Figure 5. Specific activity of wild-type KR$_1$ with NAC-diketide is 0.06 μmoles/min/mg protein.

Table 2. Activity and Specificity of KR_2 Mutants against Decalone and (2*R*, *S*)-2-Methyl-3-Oxopentanoic Acid NAC Thioester Substrates

Library	Motifs I and II		Activity against Decalone[a]	Peak Area, (2*S*,3*S*) (3)	Peak Area, (2*R*,3*S*) (5)	Ratio (2*S*,3*S*): (2*R*,3*S*)	Peak Area, (2*S*,3*R*) (2)
KR_2[b]	PQQ	WAA	0.074	1,968	515	3.8:1.0	1,059
Motif I							
	LDD	WAA	0.411	6,409	2,189	2.9:1.0	nd
	KEN	WAA	0.095	931	178	5.2:1.0	nd
	LKR	WAA	0.097	1,555	1,096	1.4:1.0	nd
Motif II							
	PQQ	**LPN**	0.009	2,898	3,164	1.0:0.9	nd
	PQQ	**LPA**	0.015	30,731	13,259	2.3:1.0	nd
	PQQ	**LPG**	0.016	22,670	9,266	2.4:1.0	nd
Both Motifs	**LDD**	**LPN**	—	4,872	4,404	1.1:1.0	nd
Motif I LDD, II Selected							
	LDD	**CFG**	0.143	6,381	6,325	1.0:1.0	nd
	LDD	**FLG**	0.135	12,973	7,009	1.9:1.0	nd
	LDD	**FPG**	0.132	15,313	8,193	1.9:1.0	nd
	LDD	**IPA**	0.083	6,434	11,524	1.0:1.8	nd
	LDD	**LAG**	0.081	10,233	8,193	1.2:1.0	nd
	LDD	**WCG**	0.457	6,324	3,124	2.0:1.0	nd
II LPN, I Selected	**MKK**	**LPN**	0.056	15,398	51,366	1.0:3.3	nd

Amino acid residues within either motif I or motif II that were found to be altered in each KR mutant are shown in bold type.
[a] Activity is expressed in μmoles/min/mg protein, measured at 5 mM decalone, 40 μM NADPH, 0.2 mg/ml KR.
[b] Specific activity of wild-type KR_2 with NAC-diketide is 0.07 μmoles/min/mg protein. nd indicates that a peak was not detected for this isomer.

the transition reported for the KR involved in biosynthesis of the aromatic polyketide actinorhodin [30, 31].

Mutants of $eryKR_1$ Isolated from the High-Throughput Assay Include Variants Having Switched Stereospecificity of Ketoreduction

The most telling evidence for the direct involvement of the targeted residues in substrate binding comes from the examination of the stereospecificity of individual mutants against (2*R*, *S*)-2-methyl-3-oxopentanoic acid NAC thioester as substrate. Wild-type $eryKR_1$ yields the (2*S*, 3*R*) isomer of the alcohol as the major product (>98%) (Figure 1), as predicted from the structure of erythromycin, and as observed in the intact module. Trace amounts of the (2*S*, 3*S*) and (2*R*, 3*R*) isomers were also obtained. All mutations to motif I of $eryKR_1$ dramatically reduce the yield of the (2*S*, 3*R*) isomer, sometimes to the limits of detection (Figures 4B and 4C). Since all of the selected enzymes have high activity toward decalone, there are two possible interpretations of the dramatic loss of activity toward (2*R*, *S*)-2-methyl-3-oxopentanoic acid NAC thioester. First, the residue(s) in question might be involved in binding the substrate. Alternatively the substitution may simply block access to the active site, particularly if bulky residues, for example, L93W, are involved. For residue D95, the first explanation seems the more likely, as four different substitutions, including an alanine, all have the same effect (Table 1). The reduction in activity is dramatic, over 50-fold, which would be consistent with a direct interaction, for example, via a hydrogen bond, between D95 and the substrate. In contrast, the yield of the "wrong" alcohol stereoisomer, (2*S*, 3*S*), is unchanged by several mutations to motif I, and some mutations actually increase the yield by up to 4-fold. In mutants in which the yield of the (2*S*, 3*R*) isomer is very low, for example, in mutants containing tryptophan in motif I, the (2*S*, 3*S*) isomer is the major product (Figure 5). In other words, the stereospecificity of reduction is reversed, as reported for specific $eryKR_1$ mutants in the accompanying paper [20]. Such mutants confirm that the (2*S*, 3*R*) and (2*S*, 3*S*) isomers are likely to have arisen from two different binding modes of the NAC-diketide, involving different substrate-protein interactions, rather than alternative positioning of the catalytic tyrosine.

Motif II of $eryKR_1$ is more tolerant to mutation than is motif I, with retention of reductase activity toward NAC-diketide. In particular, the triple mutant "ASA" (Table 1) has activity and stereochemistry comparable to wild-type $eryKR_1$ (Figure 4D). In general, the amino acid requirements for decalone reduction are the same as those for diketide reduction, which may represent the requirements for enzyme folding or stability, and there is no evidence for a direct role in substrate binding. The tolerance of $eryKR_1$ to mutations at position 148 is surprising, as this residue is adjacent to the catalytic residue Y149 and it is intolerant to mutation in $eryKR_2$. Bulky residues at this position appear to decrease the activity toward NAC-diketide more severely than toward decalone, as seen with "ASR," which has 5-fold lower activity against decalone than "ASA," but 45-fold lower activity against NAC-diketide.

Mutants of $eryKR_2$ Isolated from the High-Throughput Assay Include Variants with Increased Stereospecificity and Activity of Ketoreduction against Surrogate Substrates

Wild-type $eryKR_2$ has been reported [18, 20] to reduce (2*R*, *S*)-2-methyl-3-oxopentanoic acid NAC thioester to

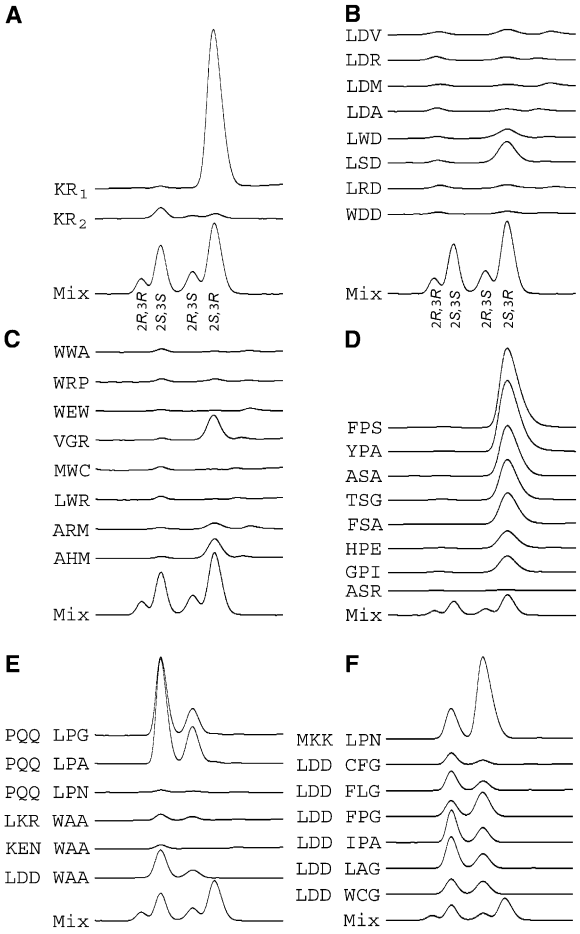


Figure 4. Stereochemical Outcome of Ketoreduction by eryKR$_1$, eryKR$_2$, and Selected Mutants

(A–F) HPLC traces showing the separation of the stereoisomers of 2-methyl-3-hydroxypentanoic acid NAC thioester. Each trace represents 20 min of elution time. Synthetic samples of all four isomers were used as standards and are shown at the bottom of each panel ("Mix"). The same standards were used in all panels, but the scale of each panel is adjusted according to the yield of product. (A) Reaction products of wild-type KR$_1$ and KR$_2$. (B) KR$_1$ mutants selected from libraries 1–3. Amino acids 93–95 of motif I are shown next to each trace. (C) KR$_1$ mutants from library 4, motif I. (D) KR$_1$ library 5, motif II amino acids 141, 144, 148. (E) KR$_2$ mutants from libraries 5 and 6 and designed mutants, motifs I and II. (F) KR$_2$ mutants from libraries 13 and 14, motifs I and II.

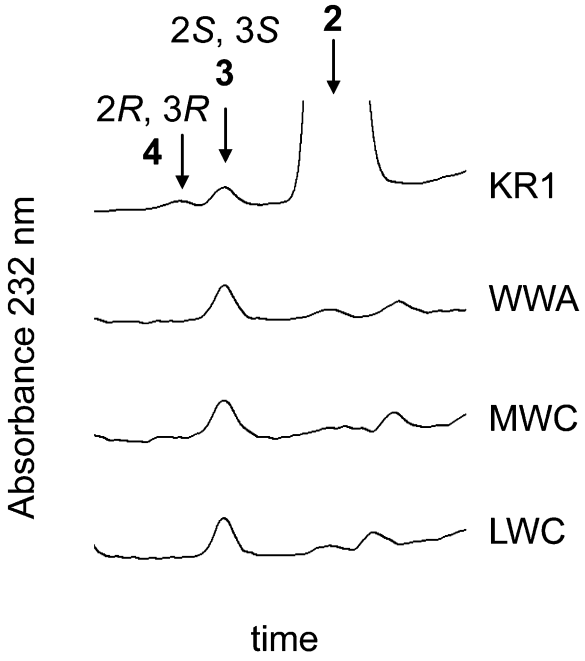


Figure 5. HPLC Separation of the Products of Reduction of a Surrogate Substrate by Recombinant PKS Ketoreductase Domains

HPLC separation of the products of reduction of (2*R*, *S*)-2-methyl-3-oxopentanoic acid *N*-acetylcysteamine thioester **1** by eryKR$_1$ mutants that show reduced catalytic activity but reversed stereospecificity compared to wild-type. The peaks corresponding to individual stereoisomers of 2-methyl-3-hydroxypentanoic acid NAC thioesters **2**, **3**, and **4** are indicated by arrows. The amino acid sequence found at positions 93–95 in each mutant is shown next to the corresponding trace.

a mixture of the (2*R*, 3*S*) isomer (predicted product), the (2*S*, 3*S*) isomer (wrong substrate selectivity), and the (2*S*, 3*R*) isomer (wrong substrate selectivity and wrong product specificity), with the (2*S*, 3*S*) isomer forming the main product (Figure 1). Surprisingly, all mutants of eryKR$_2$ yielded only 3*S* alcohols (correct specificity), with 2*R* or 2*S* methyl centers in variable proportion (Figures 4E and 4F; Table 2). Mutations at motifs I and II appear to fix the stereospecificity and manipulate the substrate selectivity. This result must be treated with caution, however, as (2*R*, *S*)-2-methyl-3-oxopentanoic acid NAC thioester is a poorer substrate mimic for eryKR$_2$. It is also shorter than the triketide chain normally acted on by eryKR$_2$ in the context of the PKS, and it is a mixture of both 2*S* and 2*R* isomers, whereas eryKR$_2$ is normally preceded by a nonepimerizing KS and is, hence, presented with only 2*R* substrates. Nevertheless, it is interesting that the majority of eryKR$_2$ mutants were more active than the wild-type, with either decalone or (2*R*, *S*)-2-methyl-3-oxopentanoic acid NAC thioester **1** as substrate. This is difficult to rationalize, apart from the observation that the mutations remove bulky residues, either proline 93 or tryptophan 141, or both. It is possible that the mutants have a more open active site or are more flexible, but further interpretation will require structural data. No single residue was absolutely essential for (2*R*, *S*)-2-methyl-3-oxopentanoic acid NAC thioester reduction by eryKR$_2$. However, the observation that mutations in motifs I or II may alter, or even reverse, the substrate selectivity clearly implicates both motifs in substrate binding.

Conclusions

Directed evolution has proved a potent tool for the selection of enzyme variants with desirable properties, including increased activity against alternative substrates, higher thermostability, and reduced product inhibition [25]. A number of complementary techniques have been used to generate enzyme variants, including introduction of single changes by (for example) error-prone PCR, saturation mutagenesis of active sites, and DNA shuffling [23], and these have been fruitfully combined with rational design approaches [25]. Some of the most interesting recent advances concern the manipulation of the stereochemical outcome of catalysis.

It has been possible, for example, to dramatically enhance the enantioselectivity of a lipase acting on a chiral ester [32] and also to invert the enantioselectivity of the enzyme, via a total of only 11 amino acid substitutions [33]. Subsequent analysis has shown, importantly, that these changes affect not only active site residues, but also more remote residues [26]. However, the residues at the active site still provide a promising starting point for attempts to alter stereoselectivity. Of particular relevance to the stereochemistry of reduced or complex polyketides is the role of ketoreductase enzymes. Recent work on two mutually homologous plant tropinone reductases, which reduce the same alkaloid substrate, tropinone, to different stereoisomeric alcohols [34], has shown that their active site geometry is highly conserved, and that the stereospecificity may be switched by site-directed mutagenesis of only a few residues at the active site [35].

Until now, directed evolution has not been applied to polyketide synthases, although attempts have been made to speed up the construction and screening of hybrid PKS multienzymes [36, 37]. The enormous size of PKS genes and their content of multiple copies of highly repetitive DNA offer a particular technical challenge. In this work, we have taken advantage of the recent finding that individual KR domains from modular PKSs can be expressed as separate and active enzymes, making them accessible to high-throughput methodology. As for tropinone reductases, the PKS KR domains all share a common architecture, and, in the accompanying paper [20], we provide evidence that mutagenesis of amino acid residues in certain motifs at the active site of the $eryKR_1$ and $eryKR_2$ domains significantly alters the stereochemical outcome of catalysis. The results of the present paper provide strong additional evidence for the importance of these residues in stereocontrol, and demonstrate the potential for molecular evolution applied to individual domains of an assembly line multienzyme.

The combination of saturation mutagenesis and high-throughput screening has enabled scrutiny of the KR active site in far more detail than conventional techniques allow. This has given additional support to a model for catalysis in which the enantioselectivity and stereospecificity of reduction are dictated by different binding modes of each substrate to a catalytic apparatus in the KR active site whose geometry is essentially identical in both $eryKR_1$ and $eryKR_2$ [19, 20]. It also has allowed rapid confirmation of the potentially useful finding made in the accompanying paper [20] that the stereochemistry of ketoreduction can be effectively switched by exchange of only a few active site residues. These were residues that had been previously highlighted as being correlated with a given stereochemical outcome in natural PKS KR domains [19, 22]. It remains to be seen whether such mutant KRs retain their altered properties when inserted into modular PKS multienzymes. If so, this approach may offer a promising new route to the production of novel polyketide products. The same approach could evidently be taken for the constituent domains/activities of nonribosomal peptide synthetase multienzymes, in which the directed evolution of the entire multienzyme also presents a challenging prospect.

A practical consideration is the significant loss of catalytic activity in many mutants studied, compared to the wild-type enzyme. Encouragingly, typical PKS KR domains have measured catalytic activities in vitro 1–2 orders of magnitude higher than those reported for the operation of an intact modular PKS multienzyme [16, 18, 20, 29]; thus, even mutant KR domains with compromised activity may not decrease the rate of overall polyketide synthesis. The present work also opens the way for directed evolution of KRs, via multiple rounds of mutagenesis and selection. This could also reveal further mutations, remote from the active site [26], which contribute to stereocontrol, and it can provide improved variants for installation in PKS assembly lines. Since several other PKS-derived domains, including AT-ACP [38, 39], KS-AT [40], and TE [41, 42] domains, have also been shown to be active in vitro, these, too, are potential targets for such directed evolution.

Significance

The engineering of modular polyketide synthases (PKSs) by the creation of hybrid multienzymes, in which domains and modules from two or more PKSs are spliced together, has proved a versatile technology for the rational production of novel, potentially bioactive polyketides, but many such hybrids are inefficient. Altering the configuration of the polyketide products has proved particularly difficult because we lack detailed information about the mechanisms of stereocontrol during polyketide chain extension. For conventional single enzymes, in vitro-directed evolution has provided a uniquely powerful approach to obtain altered enzyme catalysts, particularly when combined with rational design. Such methods are not easy to apply to giant assembly line multienzymes such as PKSs and nonribosomal peptide synthetases (NRPSs). We have developed a convenient method for high-throughput saturation mutagenesis and assay of individual ketoreductase (KR) domains from a modular PKS, and we have used it to identify active mutants, out of some 2000 mutants assayed, with the desired altered stereocontrol. The results support our current model for stereochemical control of KR-catalyzed ketoreduction, and they encourage the view that directed evolution of individual domains is a fruitful approach to understanding and altering the catalytic properties of engineered polyketide synthases.

Experimental Procedures

Library Construction

Libraries were constructed by PCR with oligonucleotides to encode randomized residues with the codon NNS, where N = A, G, C, or T and S = G or C (sequences of oligonucleotides are provided in Supplemental Data). The templates were KR_1 and KR_2 cloned in pGEX-4T-3, or mutants of these enzymes. Libraries were prepared in *E. coli* strain DH10B [43, 44], and the library size was determined by counting the number of colonies. Colonies were scraped up for preparation of plasmid DNA.

Library Screening

Plasmids were transformed in *E. coli* BL21-CodonPlus-RP (Stratagene), and individual colonies wer used to inoculate 1 ml LB medium supplemented with ampicillin (100 mg/l) and chloramphenicol (30 mg/l) in a Deepwell 96-well plate (Fisher). Plates were sealed with adhesive film, which was pierced with a fine needle (1 mm bore,

two holes per well). Master plates were grown at 30ºC with shaking at 625 rpm for 22 hr, after which a small portion (10 μl) of each culture was used to inoculate a duplicate plate for expression. After 4.5 hr of growth at 30ºC, IPTG was added to a final concentration of 0.1 mM, and, after growth for an additional 3 hr, cells were harvested by centrifugation, and the pellets were stored at −20ºC. Cells were resuspended in 200 μl lysis buffer (50 mM sodium phosphate [pH 8.0], containing 150 mM sodium chloride, 1 mg/ml lysozyme, and 3 U/ml Benzonase [Novagen]). Lysates were incubated at 25ºC for 5 min and transferred to 96-well Optical Reaction Plate (Applied Biosystems), and the insoluble fragments were removed by centrifugation.

Cell-free extracts were used directly for activity assays by the addition of 50 μl cell-free extract to 50 μl reaction mixture (100 mM potassium phosphate [pH 7.5], containing 1.6 mM NADPH and 10 mM decalone) in a UV-transparent 96-well plate (Grenier). Reactions were incubated at 30ºC in the chamber of a Spectramax Plus microplate reader equipped with Softmax Pro (Molecular Devices, Philadelphia, PA) software for data acquisition and analysis, and absorbance at 340 nm was measured at 30 s intervals for 20 min. A total of 35 active clones were chosen for further analysis.

To determine whether screening for decalone activity is an efficient means of isolating mutants with (2*R*, *S*)-2-methyl-3-oxopentanoic acid NAC thioester reductase activity, four clones were chosen from both libraries 10 (KR_1 93–95) and 12 (KR_1 141–148) without preselection. These eight enzymes were purified and assayed in parallel with the selected mutants (Supplemental Data). All eight were well expressed, although only one shows significant activity with decalone. Four of the mutants gave no detectable reduction of (2*R*, *S*)-2-methyl-3-oxopentanoic acid NAC thioester, and the remaining four yielded the (2*S*, 3*R*) isomer of the alcohol product in extremely low yield (Supplemental Data).

Purification of KR Domains and Kinetic and Stereochemical Analysis

Plasmid DNA was prepared from master plates, sequenced, and used to transform *E. coli* BL21-CodonPlus-RP for expression. Expression and purification was as described in the accompanying paper [20]. Activity was determined by both the addition of 50 μl protein (0.2 mg/ml) to 50 μl reaction mixture and the measurement of the rate of NADPH consumption, as described above. For product analysis, reactions contained 0.1 mg/ml protein, 50 mM potassium phosphate (pH 7.5), 8 mM NADPH, and 10 mM (2*R*, *S*)-2-methyl-3-oxopentanoic acid NAC thioester. Reactions were started by the addition of protein and were incubated at 30ºC for 16 hr. Products were extracted with ethyl acetate, dried, and dissolved in 50 μl isopropanol. Products were separated by HPLC with a Chiracel OC column (Chiral Technologies Europe) in 93% isohexane, 7% ethanol as described. Synthetic samples of (2*R*, 3*R*)-, (2*S*, 3*S*)-, (2*R*, 3*S*)-, and (2*S*, 3*R*)-3-hydroxy-2-methylpentanoic acid were used as standards. The substrate contained a minor impurity (0.2%, peak area 181) with the same retention time as (2*S*, 3*S*)-2-methyl-3-oxopentanoic acid NAC thioester, which was subtracted from all samples.

Supplemental Data

Supplemental Data including the synthetic oligonucleotides used in the library construction as well as data on the sequence, activity, and stereospecificity of randomly selected KR library members are available at http://www.chembiol.com/cgi/content/full/13/3/287/DC1/.

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References

1. Staunton, J., and Weissman, K.J. (2001). Polyketide biosynthesis: a millennium review. Nat. Prod. Rep. *18*, 380–416.
2. Walsh, C.T. (2004). Polyketide and nonribosomal peptide antibiotics: modularity and versatility. Science *303*, 1805–1810.
3. Weissman, K.J. (2004). Polyketide biosynthesis: understanding and exploiting modularity. Philos. Trans. R. Soc. Lond. A *362*, 2671–2690.
4. Walsh, C.T. (2002). Combinatorial biosynthesis of antibiotics: challenges and opportunities. ChemBioChem *3*, 125–134.
5. Khosla, C., and Keasling, J.D. (2003). Metabolic engineering for drug discovery and development. Nat. Rev. Drug Discov. *2*, 1019–1025.
6. Weissman, K.J., and Leadlay, P.F. (2005). Combinatorial biosynthesis of reduced polyketides. Nat. Rev. Microbiol. *3*, 925–936.
7. Mendez, C., and Salas, J.A. (2001). Altering the glycosylation pattern of bioactive compounds. Trends Biotechnol. *19*, 449–456.
8. Cortés, J., Haydock, S.F., Roberts, G.A., Bevitt, D.J., and Leadlay, P.F. (1990). An unusually large multifunctional polypeptide in the erythromycin-producing polyketide synthase of *Saccharopolyspora erythraea*. Nature *348*, 176–178.
9. Donadio, S., Staver, M.J., McAlpine, J.B., Swanson, S.J., and Katz, L. (1991). Modular organization of genes required for complex polyketide biosynthesis. Science *252*, 675–679.
10. Marsden, A.F.A., Caffrey, P., Aparicio, J.F., Loughran, M.S., Staunton, J., and Leadlay, P.F. (1994). Stereospecific acyl transfers on the erythromycin-producing polyketide synthase. Science *263*, 378–380.
11. Wiesmann, K.E.H., Cortés, J., Brown, M.J.B., Cutter, A.L., Staunton, J., and Leadlay, P.F. (1995). Polyketide synthesis *in vitro* on a modular polyketide synthase. Chem. Biol. *2*, 583–589.
12. Weissman, K.J., Timoney, M., Bycroft, M., Grice, P., Hanefeld, U., Staunton, J., and Leadlay, P.F. (1997). The molecular basis of Celmer's rules: the stereochemistry of the condensation step in chain extension on the erythromycin polyketide synthase. Biochemistry *36*, 13849–13855.
13. Böhm, I., Holzbaur, I.E., Hanefeld, U., Cortés, J., Staunton, J., and Leadlay, P.F. (1998). Engineering of a minimal modular polyketide synthase, and targeted alteration of the stereospecificity of polyketide chain extension. Chem. Biol. *5*, 407–412.
14. Holzbaur, I.E., Harris, R.C., Bycroft, J., Cortés, J., Bisang, C., Staunton, J., Rudd, B.A.M., and Leadlay, P.F. (1999). Molecular basis of Celmer's rules: the role of two ketoreductase domains in the control of chirality by the erythromycin modular polyketide synthase. Chem. Biol. *6*, 189–195.
15. Kao, C.M., McPherson, M., McDaniel, R.N., Fu, H., Cane, D.E., and Khosla, C. (1998). Alcohol stereochemistry in polyketide backbones is controlled by the β-ketoreductase domains of modular polyketide synthases. J. Am. Chem. Soc. *120*, 2478–2479.
16. Khosla, C., Gokhale, R.S., Jacobsen, J.R., and Cane, D.E. (1999). Tolerance and specificity of polyketide synthases. Annu. Rev. Biochem. *68*, 219–253.
17. Holzbaur, I.E., Ranganathan, A., Thomas, I.P., Kearney, D.J.A., Reather, J., Rudd, B.A.M., Staunton, J., and Leadlay, P.F. (2001). Molecular basis of Celmer's rules: role of the ketosynthase domain in epimerization and demonstration that ketoreductase domains can have altered product specificity with unnatural substrates. Chem. Biol. *8*, 329–340.
18. Siskos, A.P., Baerga-Ortiz, A., Bali, S., Stein, V., Mamdani, H., Spiteller, D., Popovic, B., Spencer, J.B., Staunton, J., Weissman, K.J., et al. (2005). Molecular basis of Celmer's rules: stereochemistry of catalysis by isolated ketoreductase domains from modular polyketide synthases. Chem. Biol. *12*, 1145–1153.
19. Reid, R., Piagentini, M., Rodriguez, E., Ashley, G., Viswanathan, N., Carney, J., Santi, D.V., Hutchinson, C.R., and McDaniel, R.

(2003). A model of structure and catalysis for ketoreductase domains in modular polyketide synthases. Biochemistry *42*, 72–79.

20. Baerga-Ortiz, A.J., Popovic, B., Siskos, A.P., O'Hare, H.M., Spilteller, D., Williams, M.G., Campillo, N., Spencer, J.B., and Leadlay, P. (2005). Site-directed mutagenesis alters the stereochemistry of catalysis by isolated ketoreductase domains from the erythromycin-producing polyketide synthase. Chem. Biol. *13*, this issue, 277–285.
21. Kallberg, Y., Oppermann, U., Jornvall, H., and Persson, B. (2002). Short-chain dehydrogenases/reductases (SDRs). Eur. J. Biochem. *269*, 4409–4417.
22. Caffrey, P. (2003). Conserved amino acid residues correlating with ketoreductase stereospecificity in modular polyketide synthases. ChemBioChem *4*, 649–662.
23. Stemmer, W.P. (1994). Rapid evolution of a protein in vitro by DNA shuffling. Nature *370*, 389–391.
24. Black, M.E., Newcomb, T.G., Wilson, H.M., and Loeb, L.A. (1996). Creation of drug-specific herpes simplex virus type 1 thymidine kinase mutants for gene therapy. Proc. Natl. Acad. Sci. USA *93*, 3525–3529.
25. Arnold, F.H. (1998). Design by directed evolution. Acc. Chem. Res. *31*, 125–131.
26. Reetz, M.T. (2004). Controlling the enantioselectivity of enzymes by directed evolution: practical and theoretical ramifications. Proc. Natl. Acad. Sci. USA *101*, 5716–5722.
27. Reetz, M.T., Bocola, M., Carballiera, J.D., Zha, D., and Vogel, A. (2005). Expanding the range of substrate acceptance of enzymes: combinatorial active-site saturation test. Angew. Chem. Int. Ed. Engl. *44*, 4192–4196.
28. Rangan, V.S., Joshi, A.K., and Smith, S. (1998). Mapping the functional topology of the animal fatty acid synthase by mutant complementation *in vitro*. J. Biol. Chem. *273*, 34949–34953.
29. Østergaard, L.H., Kellenberger, L., Cortés, J., Roddis, M.P., Deacon, M., Staunton, J., and Leadlay, P.F. (2002). Stereochemistry of catalysis by the ketoreductase activity in the first extension module of the erythromycin polyketide synthase. Biochemistry *41*, 2719–2726.
30. Hadfield, A.T., Limpkin, C., Teartasin, W., Simpson, T.J., Crosby, J., and Crump, M.P. (2004). The crystal structure of the actIII actinorhodin polyketide reductase: proposed mechanism for ACP and polyketide binding. Structure *12*, 1865–1875.
31. Korman, T.P., Hill, J.A., Vu, T.N., and Tsai, S.C. (2004). Structural analysis of actinorhodin polyketide ketoreductase: cofactor binding and substrate specificity. Biochemistry *43*, 14529–14538.
32. Reetz, M.T., Wilensek, S., Zha, D.X., and Jaeger, K.-E. (2001). Directed evolution of an enantioselective enzyme through combinatorial multiple-cassette mutageneis. Angew. Chem. Int. Ed. Engl. *40*, 3589–3591.
33. Zha, D., Wilensek, S., Hermes, M., Jaeger, K.-E., and Reetz, M.T. (2001). Complete reversal of enantioselectivity of an enzyme-catalyzed reaction by directed evolution. J. Chem. Soc. Chem. Commun. 2664–2665.
34. Nakajima, K., Yamashita, A., Akama, H., Nakatsu, T., Kato, H., Hashimoto, T., Oda, J., and Yamada, Y. (1998). Crystal structures of two tropinone reductases: different reaction stereospecificities in the same protein fold. Proc. Natl. Acad. Sci. USA *95*, 4876–4881.
35. Nakajima, K., Kato, H., Oda, J., Yamada, Y., and Hashimoto, T. (1999). Site-directed mutagenesis of putative substrate-binding residues reveals a mechanism controlling the different stereospecificities of two tropinone reductases. J. Biol. Chem. *274*, 16563–16568.
36. Kim, B.S., Sherman, D.H., and Reynolds, K.A. (2004). An efficient method for creation and functional analysis of libraries of hybrid type I polyketide synthases. Protein Eng. Des. Sel. *17*, 277–284.
37. Kodumal, S.J., Patel, K.G., Reid, R., Menzella, H.G., Welch, M., and Santi, D.V. (2004). Total synthesis of long DNA sequences: synthesis of a contiguous 32-kb polyketide synthase gene cluster. Proc. Natl. Acad. Sci. USA *101*, 15573–15578.
38. Aparicio, J.F., Caffrey, P., Marsden, A.F., Staunton, J., and Leadlay, P.F. (1994). Limited proteolysis and active-site studies of the multienzyme component of the erythromycin-producing polyketide synthase. J. Biol. Chem. *269*, 8524–8528.
39. Liou, F.G., Lau, J., Cane, D.E., and Khosla, C. (2003). Quantitative analysis of loading an extender acyltransferases of modular polyketide synthases. Biochemistry *42*, 200–207.
40. Kim, C.Y., Alekseyev, V.Y., Chen, A.Y., Tang, Y., Cane, D.E., and Khosla, C. (2004). Reconstituting modular activity from separated domains of 6-deoxyerythronolide B synthase. Biochemistry *43*, 13892–13898.
41. Caffrey, P., Green, B., Packman, L.C., Rawlings, B.J., Staunton, J., and Leadlay, P.F. (1991). An acyl-carrier protein-thioesterase domain from the 6-deoxyerythronolide B synthase of *Saccharopolyspora erythraea*. High-level production, purification and characterisation in *Escherichia coli*. Eur. J. Biochem. *195*, 823–830.
42. Lu, H., Tsai, S.C., Khosla, C., and Cane, D.E. (2002). Expression, site-directed-mutagenesis, and steady state kinetic analysis of the terminal thioesterase domain of methymycin/picromycin polyketide synthase. Biochemistry *41*, 12590–12597.
43. Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989). Molecular Cloning: A Laboratory Manual (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press).
44. Dower, W.J., Miller, J.F., and Ragsdale, C.W. (1998). High efficiency transformation of *E. coli* by high voltage electroporation. Nucleic Acids Res. *16*, 6127–6145.