

Elucidation of the Cryptic Epimerase Activity of Redox-Inactive Ketoreductase Domains from Modular Polyketide Synthases by Tandem Equilibrium Isotope Exchange

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S Supporting Information

ABSTRACT: Many modular polyketide synthases harbor one or more redox-inactive domains of unknown function that are highly homologous to ketoreductase (KR) domains. A newly developed tandem equilibrium isotope exchange (EIX) assay has now established that such “KR⁰” domains catalyze the biosynthetically essential epimerization of transient (2*R*)-2-methyl-3-ketoacyl-ACP intermediates to the corresponding (2*S*)-2-methyl-3-ketoacyl-ACP diastereomers. Incubation of [²-²H]-2-*R*,3-*S*-2-methyl-3-hydroxypentanoyl-S-ACP ([²-²H]-**3b**) with the EryKR3⁰ domain from module 3 of the 6-deoxyerythronolide B synthase, and the redox-active, nonepimerizing EryKR6 domain and NADP⁺ resulted in time- and cofactor-dependent washout of deuterium from **3b**, as a result of EryKR3⁰-catalyzed epimerization of transiently generated [²-²H]-2-methyl-3-ketopentanoyl-ACP (**4**). Similar results were obtained with redox-inactive PicKR3⁰ from module 3 of the picromycin synthase. Four redox-inactive mutants of epimerase-active EryKR1 were engineered by mutagenesis of the NADPH binding site of this enzyme. Tandem EIX established that these EryKR1⁰ mutants retained the intrinsic epimerase activity of the parent EryKR1 domain. These results establish the intrinsic epimerase activity of redox-inactive KR⁰ domains, rule out any role for the NADPH cofactor in epimerization, and provide a general experimental basis for decoupling the epimerase and reductase activities of a large class of PKS domains.

Individual ketoreductase (KR) domains control the ultimate stereochemistry of the vast majority of both hydroxyl- and methyl-bearing stereocenters in the wide array of chemically complex and medicinally important polyketides produced by multimodular polyketide synthases (PKSs) (Figure 1).^{1–3} Intriguingly, many PKSs also contain one or more modules that harbor redox-inactive homologues of KR domains (Figures 1 and S1) whose actual biochemical function has long been an enigma.^{2d,3,4} While in theory such redox-inactive KR⁰ domains might represent mere artifacts resulting from gene duplication of a primordial precursor module with subsequent loss of ketoreductase function,⁵ we sought to test the hypothesis that they have a specific catalytic function.

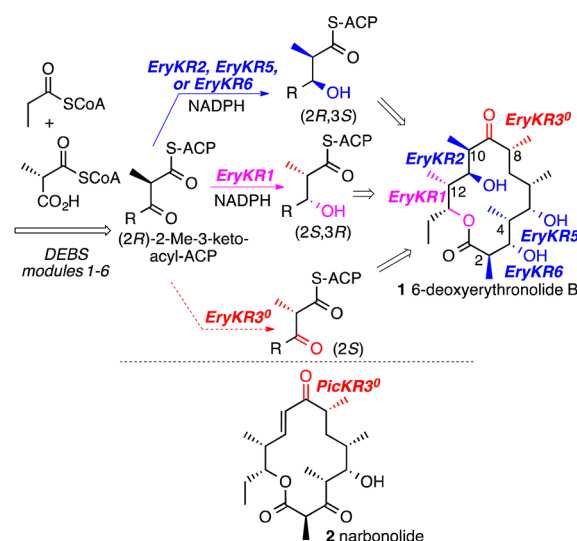


Figure 1. Roles of redox active (EryKR1 and EryKR6) and redox-inactive (EryKR3⁰ and PicKR3⁰) KR domains in biosynthesis of typical macrolide aglycones **1** and **2**.

EryKR3⁰ is derived from module 3 of the 6-deoxyerythronolide B (**1**) synthase (DEBS), which generates an unreduced 2-methyl-3-ketoacyl-ACP tetraketide intermediate that is the source of the ultimately derived (8*S*)-8-methyl-9-keto substructure of the eventually formed macrolide aglycone **1** (Figure 1).³ Since the EryKS3 domain of DEBS module 3 initially generates an unepimerized (2*R*)-2-methyl-3-ketoacyl-ACP intermediate,^{6a} formation of the actual (2*S*)-2-methyl-3-ketoacyl-ACP product by DEBS module 3 requires epimerization of the 2-methyl group at some as yet undefined stage. Similarly, the orthologous PicKR3⁰ domain from module 3 of the closely related picromycin synthase⁷ is responsible for the analogous (8*S*)-8-methyl-9-keto substructure of the narbonolide aglycone (**2**) (Figure 1), suggesting the need for an epimerase activity in this module as well.^{2d} Indeed, a survey of PKS sequences reveals that modules harboring redox-inactive KR⁰ domains are strongly correlated with the presence of a

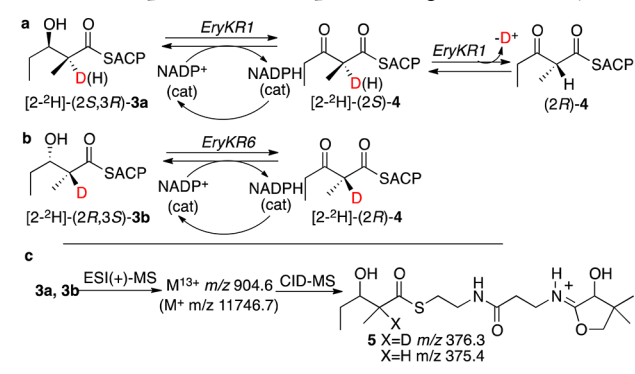
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presumptively epimerized (*S*)- α -methyl ketone substructure in the final macrolide or polyether (Figure S1).^{2b,d} We now report experiments demonstrating that both EryKR3⁰ and PicKR3⁰ harbor the heretofore cryptic but nonetheless essential catalytic epimerase function. Our results also rule out any role for the NADPH cofactor in KR-catalyzed epimerization.

We recently described an equilibrium isotope exchange (EIX) assay that provided the first unambiguous proof of the intrinsic epimerase activity of specific PKS ketoreductase domains.⁸ The assay exploits the reversibility of the KR-catalyzed reductase-dehydrogenase reaction for the controlled transient oxidative generation of configurationally labile (2*R*)- or (2*S*)-2-methyl-3-ketoacyl-ACP derivatives (**4**) from the corresponding reduced, configurationally stable, [2-²H]-2-methyl-3-hydroxypentanoyl-ACP thioesters (**3**) (Scheme 1).

Scheme 1. Equilibrium Isotope Exchange (EIX) Assay

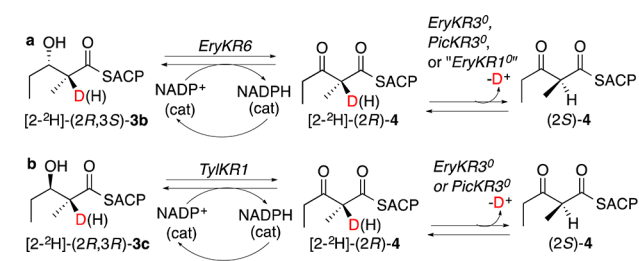


For example, incubation of [2-²H]-(2*S*,3*R*)-**3a** with the epimerizing KR domain EryKR1 in the presence of a catalytic amount of NADP⁺ results in first-order time- and cofactor-dependent washout of deuterium from **3a**, as a consequence of reversible EryKR1-catalyzed epimerization of the derived [2-²H]-2-methyl-3-ketopentanoyl-ACP (**4**). The exchange of deuterium is conveniently monitored by LC-ESI(+)-MS/MS observation of the pantetheinate ejection fragment **5** derived from recovered **3a** (Scheme 1c).⁹ By contrast, incubation of [2-²H]-(2*R*,3*S*)-**3b** with EryKR6 and catalytic NADP⁺ does not result in any significant washout of deuterium over a period of more than 1 h, as expected for a nonepimerizing KR domain. The EIX assay has been used to verify the presence or absence of intrinsic epimerase activity in a variety of redox active KR domains.⁸

The original EIX assay is not suitable for analysis of redox-inactive KR⁰ domains due to the essential requirement that the reduced, epimerization-inert 2-methyl-3-hydroxyacyl-ACP substrate **3** first undergo reversible oxidation so as to generate transiently the epimerizable 2-methyl-3-ketoacyl-ACP intermediate **4**. (The intermediate **4** cannot simply be prepared and utilized as a substrate due to its propensity to undergo rapid buffer-catalyzed exchange.) We have therefore modified and extended the original EIX assay in order to allow detection and monitoring of the proposed epimerase activity of redox-inactive KR domains.

Tandem EIX utilizes a coupled enzyme assay in which the redox-active, epimerase-inactive EryKR6 domain is used to generate *in situ* the substrate for a redox-silent KR⁰ domain (Scheme 2).¹⁰ Thus, incubation of equimolar concentrations (75 μ M, 11.25 nmol) of EryKR3⁰ and EryKR6 with a 4-fold excess of [2-²H]-(2*R*,3*S*)-**3b** (300 μ M, 45 nmol), in which the

Scheme 2. Tandem EIX Assay of Redox-Inactive KR⁰ Domains



labeled diketide is tethered to EryACP6, in the presence of catalytic NADP⁺ (15 μ M, 2.25 nmol, 0.05 equiv) at rt in pH 7.2 phosphate buffer resulted in the loss of up to 30% of the original deuterium label from **3b** after 1 h, as established by LC-ESI(+)-MS/MS analysis of the derived pantetheinate ejection fragments at *m/z* 376 (*d*₁) and 375 (*d*₀). Withdrawal of periodic aliquots from the incubation reaction and LC-MS/MS analysis revealed a small but reproducible initial lag time of \sim 15 min followed by time-dependent loss of the deuterium label, as evidenced by the plots of the log(% residual deuterium) vs time for the linear phase of the exchange (Table 1, Figure 2a). In a

Table 1. Tandem EIX Assay of Redox-Inactive KR⁰ domains

KR ⁰	time (min)							
	0	10	15	20	30	40	50	60
	Deuterium exchange of [2- ² H]- 3b (%) ^a							
EryKR3 ^{0 b}	0	0	0	11	13	21	25	30
EryKR3 ^{0 c}	0	5	7	13	16	30	36	38
EryKR3 ^{0b,d}	0	0	7	13	16	23	29	33
PicKR3 ^{0 b}	0	0	0	14	20	24	27	27
EryKR1-pG ^b	0	0	4	9	17	24	25	29
EryKR1-eG ^b	0	0	7	11	13	16	19	22
EryKR1-eGeL ^b	0	0	5	12	14	16	18	21
EryKR1-eL ^b	0	0	0	5	9	9	11	13
EryKR3 ^{0 b,e}	0	0	5	12	16	22	29	34
PicKR3 ^{0 b,e}	0	0	4	11	19	24	27	31

^aAverage of two or more measurements (\pm 2%). ^b0.05 equiv of NADP⁺. ^c0.1 equiv of NADP⁺. ^d0.2 equiv of EryKR3⁰. ^eTyIKR1 and [2-²H]-**3c** in place of EryKR6 and **3b**.

control incubation, less than 5% isotope exchange was observed over 1 h in the absence of EryKR3⁰, as previously reported for EryKR6 alone.⁸ Doubling the proportion of NADP⁺ cofactor resulted in a ca. 25% increase in both the first-order rate of deuterium exchange and the total amount of isotope loss (38%) after 1 h, while shortening the apparent lag time to less than 10 min. Doubling the concentration of EryKR3⁰ protein, on the other hand, resulted in a more modest 10% increase in total deuterium exchange. These observations are all consistent with rate-determining EryKR6-catalyzed oxidation of deuterated ACP-bound diketide **3b**, followed by more rapid EryKR3⁰-catalyzed epimerization and consequent exchange of deuterium from the transiently released 2-methyl-3-ketoacyl-ACP intermediate **4**. The observed lag time most likely reflects the time required for the concentration of this EryKR6-bound oxidation product to reach steady state.¹¹ The combined results unambiguously establish that the redox-inactive EryKR3⁰ has intrinsic epimerase activity. Application of the tandem EIX

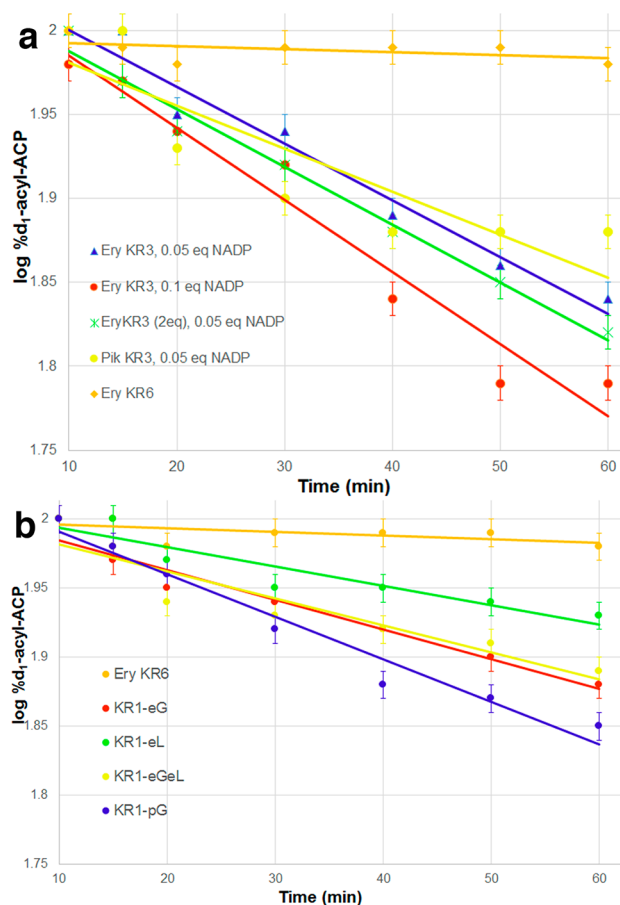


Figure 2. Time-dependent tandem EIX washout of deuterium from [2-²H]-3b. (a) Redox-inactive EryKR3⁰ or PicKR3⁰. (b) Redox-inactive EryKR1⁰ mutants.

assay to PicKR3⁰ established that this redox-inactive domain also possesses cryptic epimerase activity (Scheme 2, Table 1, Figure 2a).

As expected, the presence of a *redox-inactive* but *epimerase-active* KR⁰ domain in the tandem EIX assay does not alter the stereochemistry of the recovered reduced diketide substrate.¹² Separate control incubations of [2-²H]-(2*R*,3*S*)-3b with either EryKR3⁰ or PicKR3⁰ plus EryKR6 in the presence of catalytic NADP⁺ (0.05 equiv) were performed for 20 min in pH 7.2 phosphate. After quenching with aq. base and 20 min of hydrolysis at 65 °C followed by treatment with trimethylsilyldiazomethane, the resultant methyl ester was shown by chiral GC-MS to be diastereomerically pure methyl (2*R*,3*S*)-2-methyl-3-hydroxypentanoate (Figure S6).

To further test the generality of the tandem EIX assay, we carried out analogous incubations using the redox-active, epimerase-inactive⁸ TyIKR1 and its cognate substrate,⁶ (2*R*,3*R*)-[2-²H]-3c, in place of EryKR6 and (2*R*,3*S*)-[2-²H]-3b (Scheme 2b). The results with both EryKR3⁰ and PicKR3⁰ showed comparable rates and end points of deuterium exchange compared to those obtained with EryKR6 (Table 1). No exchange was observed in the absence of the requisite KR⁰ domain. The tandem EIX assay is therefore not restricted to the use of a single redox-active KR domain.

In order to probe further the epimerization mechanism, we also disrupted the NADPH binding site of EryKR1 so as to produce redox-inactive forms. We wished to test whether these mutants still retained the intrinsic epimerase activity of the

wild-type enzyme. Multiple sequence alignments of 5 redox-inactive KR domains, including EryKR3⁰ and PicKR3⁰, and comparison with the NADPH-binding regions of 16 redox active domains identified 3 conserved motifs within each of the 4 stereochemical classes of redox-active KR domains that are absent in the corresponding regions of redox-inactive KR⁰ domains (Figure S2). These differences are also supported by direct superposition of the structures of EryKR1 and PicKR3⁰, while reinforcing previous structural analysis of the defect in NADPH binding in redox inactive KR domains.^{2d}

The method of overlap extension PCR¹³ was used to generate a candidate set of four redox inactive EryKR1⁰ mutants: (1) EryKR1⁰-eG, in which the conserved GTGGVG motif of EryKR1 has been replaced by the corresponding AASPVG sequence from EryKR3⁰; (2) EryKR1⁰-pG, in which this same motif has been replaced by the corresponding AEEPAA sequence from PicKR3⁰; (3) EryKR1⁰-eL in which the conserved VSRSGPDADGAGE linker from EryKR1 is replaced by AGACPGDD from EryKR3⁰; and (4) a double mutant, EryKR1⁰-eGeL, containing both the eG and eL swaps. None of the four EryKR1⁰ mutants bound NADPH, as determined by fluorescence enhancement assay (Figure S4),¹⁴ and all four were devoid of residual ketoreductase activity when incubated with the standard KR assay substrate 1-decalone (Figure S5).¹⁴

Analysis by tandem EIX assay of the four redox-inactive EryKR1⁰ mutants that could no longer bind NADPH confirmed that all retained the intrinsic epimerase activity of wild-type EryKR1 (Table 1, Figure 2b). Thus, incubation of EryKR1-pG plus EryKR6 with [2-²H]-3b and 0.05 equiv NADP⁺ resulted in deuterium exchange that was essentially identical to that measured for EryKR3⁰ or PicKR3⁰ under the same conditions, but with a small reduction in the apparent lag time. EryKR1-eG and the double mutant EryKR1-eGeL each showed only slightly lower levels of deuterium washout, while the EryKR1-eL loop-swap mutant showed 2–2.5-fold reduced but nonetheless significant rates of equilibrium isotope exchange compared to EryKR3⁰.

As demonstrated for EryKR1, it should now be possible to suppress the intrinsic redox activity of any epimerase-active PKS KR domain by rational abolition of the natural NADPH binding site while retaining the native epimerase activity. The demonstration that epimerase activity in both redox-active and redox-inactive KR domains does not require NADPH firmly excludes a recent suggestion that KR-catalyzed epimerization occurs by conjugate addition of NADPH to the enol-ester tautomer of the 2-methyl-3-ketoacyl-ACP substrate.¹⁵ The path is also now open to further systematic modifications of native or engineered redox-inactive, epimerase-active KR domains in order to establish the protein structural basis for catalysis of the biosynthetically critical but mechanistically still mysterious KR-catalyzed epimerization reaction itself.

■ ASSOCIATED CONTENT

📄 Supporting Information

Experimental methods, including mutant design, construction, and characterization, and details of tandem EIX assay. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Notes

The authors declare no competing financial interest.

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(3) KR domains that generate (2*R*,3*S*)-2-methyl-3-hydroxyacyl thioester products have been classified as Type A1, those that generate the (2*S*,3*R*) diastereomer as Type B2, and those that generate the (2*R*,3*R*) diastereomer as Type B1. Redox-inactive domains that yield epimerized (2*S*)-2-methyl-3-ketoacyl-ACP products are denoted Type C2. Compare ref 2.

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(5) The superscript “0” designates a redox-inactive KR domain.

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(10) Unlike the EIX assay, in which the initially generated [2-²H]-4 undergoes reversible epimerization followed by reduction back to 3a in the same active site in which it was originally generated, the requirements for successful tandem EIX are considerably more stringent. Thus the transiently generated 4, derived from EryKR6-catalyzed oxidation of [2-²H]-3b, must dissociate from the active site of EryKR6-NADPH, then be captured, epimerized, and released by the paired redox-inactive, epimerase-active KR⁰ domain, before being rebound by EryKR6-NADPH and reduced back to unlabeled 3b.

(11) Since the concentration of EryKR6 exceeds that of the catalytic NADP⁺ by a factor of 10–20 while the concentration of the cofactor is ~1% of its *K_m*, the bulk of the EryKR6 protein will be present as the free enzyme. Increasing the concentration of EryKR6 should therefore have little effect on the observed rate of coupled EryKR3⁰-catalyzed equilibrium isotope exchange.

(12) Only the redox-active EryKR1 (or TylKR1) can carry out the stereospecific reduction of the appropriate diastereomer of the transiently generated 2-methyl-3-ketoacyl-ACP intermediate. The standard EIX assay also does not result in any loss of stereochemical purity in the recovered substrate. Compare ref 8.

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