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

Ashish Garg,[†,∥] Xinqiang Xie,[†,∥] Adrian Keatinge-Clay,[‡] Chaitan Khosla,[§] and David E. Cane*[,†]

[†]Department of Chemistry, Box H, Brown University, Providence, Rhode Island 02912-9108, United States

[‡]Department of Chemistry and Biochemistry, the University of Texas at Austin, 1 University Station A5300, Austin, Texas 78712-0165, United States

[§]Departments of Chemical Engineering, Chemistry, and Biochemistry, Stanford University, Stanford, California 94305, United States

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 "KR^0" 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-^{2}H]-(2*R*,3*S*)-2-methyl-3-hydroxypentanoyl-SACP ([2-^{2}H]-**3b**) with the EryKR3^0 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 EryKR3^0-catalyzed epimerization of transiently generated [2-^{2}H]-2-methyl-3-ketopentanoyl-ACP (**4**). Similar results were obtained with redox-inactive PicKR3^0 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 EryKR1^0 mutants retained the intrinsic epimerase activity of the parent EryKR1 domain. These results establish the intrinsic epimerase activity of redox-inactive KR^0 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 KR^0 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.

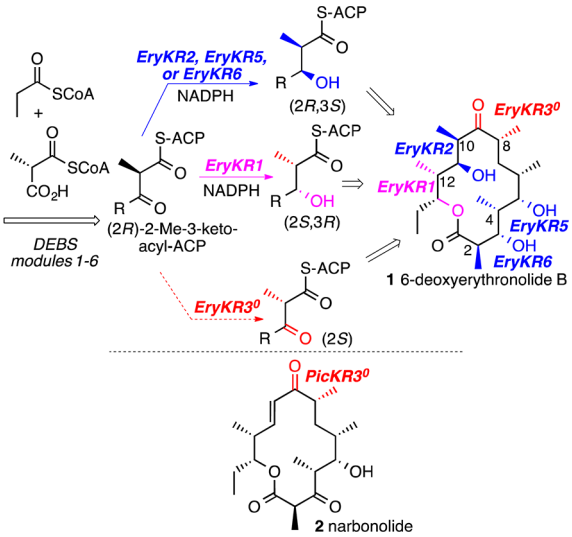

Figure 1. Roles of redox active (EryKR1 and EryKR6) and redox-inactive (EryKR3° and PicKR3°) KR domains in biosynthesis of typical macrolide aglycones **1** and **2**.

EryKR3^0 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 PicKR3^0 domain from module 3 of the closely related picromycin synthase[7] 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 KR^0 domains are strongly correlated with the presence of a

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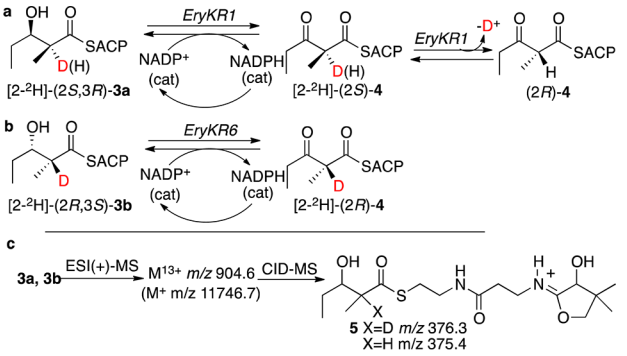
presumptively epimerized (*S*)-α-methyl ketone substructure in the final macrolide or polyether (Figure S1).[2b,d] We now report experiments demonstrating that both EryKR3^0 and PicKR3^0 harbor the heretofore cryptic but nonetheless essential catalytic epimerase function. Our results also rule out any 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-^{2}H]-2-methyl-3-hydroxypentanoyl-ACP thioesters (**3**) (Scheme 1).

Scheme 1. Equilibrium Isotope Exchange (EIX) Assay

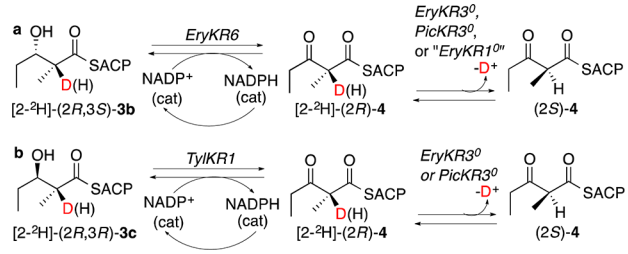


For example, incubation of [2-^{2}H]-(2*S*,3*R*)-**3a** with the epimerizing KR domain EryKR1 in the presence of a catalytic amount of NADP$^+$ results in first-order time- and cofactor-dependent washout of deuterium from **3a**, as a consequence of reversible EryKR1-catalyzed epimerization of the derived [2-^{2}H]-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-^{2}H]-(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 KR^0 domains due to the essential requirement that the reduced, epimerization-inert 2-methyl-3-hydroxyacyl-ACP substrate **3** first undergo reversible oxidization 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 KR^0 domain (Scheme 2).[10] Thus, incubation of equimolar concentrations (75 μM, 11.25 nmol) of EryKR3^0 and EryKR6 with a 4-fold excess of [2-^{2}H]-(2*R*,3*S*)-**3b** (300 μM, 45 nmol), in which the

Scheme 2. Tandem EIX Assay of Redox-Inactive KR^0 Domains



labeled diketide is tethered to EryACP6, in the presence of catalytic NADP$^+$ (15 μM, 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 ∼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 KR^0 domains

	time (min)							
	0	10	15	20	30	40	50	60
KR^0	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
EryKR3^0 [b,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

[a]Average of two or more measurements (±2%.). [b]0.05 equiv of NADP$^+$ [c]0.1 equiv of NADP$^+$. [d]0.2 equiv of EryKR3^0. [e]TylKR1 and [2-^{2}H]-**3c** in place of EryKR6 and **3b**.

control incubation, less than 5% isotope exchange was observed over 1 h in the absence of EryKR3^0, 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 EryKR3^0 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 EryKR3^0-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 EryKR3^0 has intrinsic epimerase activity. Application of the tandem EIX

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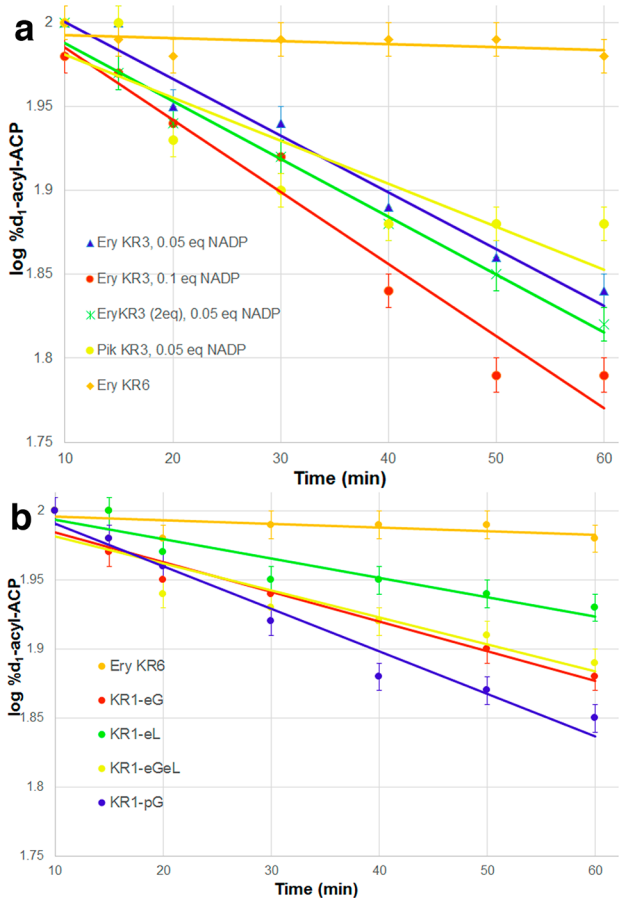

Figure 2. Time-dependent tandem EIX washout of deuterium from [2-^{2}H]-**3b**. (a) Redox-inactive EryKR3^0 or PicKR3^0. (b) Redox-inactive EryKR1^0 mutants.

assay to PicKR3^0 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* KR^0 domain in the tandem EIX assay does not alter the stereochemistry of the recovered reduced diketide substrate.[12] Separate control incubations of [2-^{2}H]-(2*R*,3*S*)-**3b** with either EryKR3^0 or PicKR3^0 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-inactive[8] TylKR1 and its cognate substrate,[6] (2*R*,3*R*)-[2-^{2}H]-**3c**, in place of EryKR6 and (2*R*,3*S*)-[2-^{2}H]-**3b** (Scheme 2b). The results with both EryKR3^0 and PicKR3^0 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 KR^0 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 EryKR3^0 and PicKR3^0, 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 KR^0 domains (Figure S2). These differences are also supported by direct superposition of the structures of EryKR1 and PicKR3^0, while reinforcing previous structural analysis of the defect in NADPH binding in redox inactive KR domains.[2d]

The method of overlap extension PCR[13] was used to generate a candidate set of four redox inactive EryKR1° mutants: (1) EryKR1^0-eG, in which the conserved **GTGGVG** motif of EryKR1 has been replaced by the corresponding AASPV**G** sequence from EryKR3^0; (2) EryKR1^0-pG, in which this same motif has been replaced by the corresponding AEEPAA sequence from PicKR3^0; (3) EryKR1^0-eL in which the conserved **VSRSGPDADGAGE** linker from EryKR1 is replaced by AGACPGDD from EryKR3^0; and (4) a double mutant, EryKR1^0-eGeL, containing both the eG and eL swaps. None of the four EryKR1^0 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 EryKR1^0 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-^{2}H]-**3b** and 0.05 equiv NADP$^+$ resulted in deuterium exchange that was essentially identical to that measured for EryKR3^0 or PicKR3^0 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 EryKR3^0.

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^0 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.

■ AUTHOR INFORMATION

Corresponding Author

David_Cane@brown.edu

Author Contributions

‖These authors contributed equally.

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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(10) Unlike the EIX assay, in which the initially generated [2-^{2}H]-**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-^{2}H]-**3b**, must dissociate from the active site of EryKR6·NADPH, then be captured, epimerized, and released by the paired redox-inactive, epimerase-active KR^0 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 EryKR3^0-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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