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Complexity Generation during Natural Product Biosynthesis using Redox Enzymes

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Abstract

Redox enzymes such as FAD-dependent and cytochrome P450 oxygenases play indispensable roles in generating structural complexity during natural product biosynthesis. In the pre-assembly steps, redox enzymes can convert garden variety primary metabolites into unique starter and extender building blocks. In the post-assembly tailoring steps, redox cascades can transform nascent scaffolds into structurally complex final products. In this review, we will discuss several recently characterized redox enzymes in the biosynthesis of polyketides and nonribosomal peptides.

Introduction

The structural complexity observed among natural products is a testament to nature's amazing ability to perform chemistry with biological catalysts. Combinatorial biosynthesis is the approach to reengineer natural product pathways towards generation of new compounds in either the native or heterologous hosts through the manipulation of biosynthetic enzymes [1]. While increasing microbial genome sequences and synthetic biological tools in recent years have pushed combinatorial biosynthesis to a new frontier, it remains crucial to understand the enzymatic basis of complexity generation in order to (come close to) match the synthetic prowess of natural pathways. For major natural product families, such as polyketides and nonribosomal peptides, nature injects three levels of structural complexity when converting precursors (input) into the final product (output) (Figure 1). In the pre-modification step, simple primary metabolites such as acyl-CoAs and amino acids are modified into more elaborate precursors by dedicated enzymes [2, 3]. These precursors are incorporated into the nascent natural product scaffold by highly programmed machineries (second level), such as the assembly-line like polyketide synthase (PKS) and nonribosomal peptide synthetase (NRPS) [4]. The third level of complexity generation is performed by post-modification enzymes to complete the bioactive products [5]. While many different types of enzymes are recruited in the pre- and post-modification steps, redox enzymes such as FAD-dependent oxygenases, oxidoreductases, cytochrome P450 oxygenases, and NADPH-dependent reductases are ubiquitous and arguably catalyze the most diverse and sophisticated transformations. Especially in the post-modification steps,

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efficient redox cascades can regioselectively and stereoselectively morph the relatively simple structures into highly complex final products. In this review, we will discuss selected examples of recently discovered redox enzymes in the pre- and post-modification steps. Complexity generation in the assembly-line step is not discussed and can be found in numerous recent reviews [4, 6].

Pre-assembly modification in polyketide biosynthesis

Polyketides are a group of natural products synthesized by PKSs using successive Claisen-like condensation reactions [4]. Prior to entering the polyketide assembly line, recruitment of starter and extender acyl thioesters is required. A variety of starter units are incorporated for polyketides produced by different families of PKSs [7]. Extender units for polyketide biosynthesis are selected by acyltransferases in PKSs in the form of CoA thioesters of dicarboxylic acids. The most common malonyl-CoA and methylmalonyl-CoA are siphoned from primary metabolism pool of the cell. In recent years, a growing numbers of polyketide natural products were discovered to contain acyl-malonyl building blocks synthesized by crotonyl-CoA carboxylase/reductase (CCR) homologs (Scheme 1a) [8]. Through reductive carboxylation of the β carbons of a variety of α - β unsaturated fatty acyl-CoAs, CCRs can synthesize a number of dicarboxylic acids, including ethyl-[9], chloroethyl-[10^{**}], butyryl-, pentyl-, isopentyl-, hexylmalonyl-CoA [11^{**}].

The first dedicated CCR homolog in natural product biosynthetic pathways was SalG found in the salinosporamide A gene cluster from the marine bacterium *Salinispora tropica* [10^{**}]. SalG synthesizes chloroethylmalonyl-CoA, which is selected as a building block by the PKS SalA. The function of SalG was confirmed by gene inactivation and in vitro assay using 4-chlorocrotonyl-CoA as the substrate [10^{**}]. SalG was subsequently shown to also generate 4-bromoethylmalonyl-CoA and 4-fluoroethylmalonyl-CoA from the corresponding 4-halocrotonyl-CoAs, which was utilized by downstream enzymes to produce bromosalinosporamide [12] and fluorosalinosporamide [13], respectively. Since then a growing number of CCR homologs have been discovered in PKS pathways, including those of FK506 [14], sanglifehrins [15], divergolides [16], and ansalactams [17] etc.

The detailed mechanism of CCR-catalyzed carboxylative reduction of (*E*)-crotonyl-CoA to (2*S*)-ethylmalonyl-CoA was revealed by Alber and coworkers [18]. The reaction is initiated by a hydride transfer from NADPH onto the β carbon followed by the *anti* carboxylation with CO₂. The structure of hexylmalonyl-CoA synthase CinF involved in cinnabaramides biosynthesis was recently reported [19^{*}]. The structure of CinF in complex with 2-octenoyl-CoA provided insights into the mechanism and substrate specificity of the CCR homolog. Two smaller amino acid residues Gly362 and Ala163 in the CinF binding pocket are important for accommodating the larger 2-octenoyl chain. The corresponding residues in CCR from *S. coelicolor* are Phe370 and Ile171, which sterically prevent the binding of substrates larger than crotonyl-CoA. CinF small to large point-mutants Gly362Phe and Ala163Ile each lost activities toward 2-octenoyl-CoA, but remained active towards crotonyl-CoA [19^{*}]. The accumulation of CCR homologs and the newly gained understanding of CCR substrate specificity will enable dramatic expansion of polyketide extender unit pools. With concerted engineering of acyltransferase specificity and downstream PKS flexibility, these new extender units may lead to significantly more structural complexity in the final products.

Post-assembly modification in polyketide biosynthesis

Following the assembly-line like synthesis of the polyketide backbone, post-assembly modifications by different tailoring enzymes are required to convert the compound into its bioactive form. Among them, redox enzymes play crucial roles in rearranging the scaffolds

into highly complex, sometimes unrecognizable final products. The biochemical mechanisms of two elusive oxidative cascades were recently established and are highlighted below.

Polyketides containing spiroacetal cores are widely found in nature, including reveromycin A [11^{**}], griseorhodin A [20] *etc.* Compounds in this family exhibit a broad spectrum of biological activities, such as the potent induction of apoptosis in osteoclasts by reveromycin A. While the stereospecific spiroacetal cores are crucial for the observed activities, the enzymatic basis of the transformation from linear precursors has been unresolved until recently for the cyclization step in reveromycin A [11^{**}]. Two enzymes, a dihydroxy ketone synthase (RevG) and a spiroacetal synthase (RevJ), are involved in stereoselective spiroacetal formation (Scheme 1b). Knockout of RevG led to accumulation of the acyclic precursor RM-A1a **1** that contains the 11, 15, 19-triol. In vitro assay with RevG and **1** led to formation of an unstable intermediate that contains the dihydroxy ketone moiety, which can spontaneous form the spiroacetal without stereocontrol. Addition of the previously unknown enzyme RevJ resulted in dehydrative cyclization of the dihydroxy ketone into exclusively the *15S* product **2** that is found in the final reveromycin A structure [11^{**}]. Interestingly, homologs of RevG and RevJ are not found in the known gene clusters of other spiroacetal-containing polyketides, suggesting different mechanisms of stereocontrol may be required to generate different cyclic scaffolds.

The gilvocarcins are a group of antitumor natural products bearing a unique benzo[*d*]naphtho[1,2-*b*]pyran-6-one chromophore linked with a variety of *C*-linked deoxysugar moieties [21]. The unique chromophore is rearranged from an angucycline polyketide via multiple oxidative modifications. Rohr and coworkers performed one-pot total enzymatic synthesis of the aglycon defucogilvocarcin M **7** using purified enzymes and various redox partners/cofactors (Scheme 1c) [22^{**}]. In the “reaction pot”, the ensemble of PKS enzymes assembled acetyl-CoA and malonyl-CoA into the angucycline scaffold **3**, which was tailored into **7** by a collection of four redox enzymes (GilOI, GilOII, JadF, GilR) and two methyltransferases (GilM, GilMT) in the presence of cofactors FAD, NADPH, *S*-adenosyl methionine (SAM), and cofactor regeneration enzymes. The precise control of reaction components through removal of individual tailoring enzymes in the pot, as well as the elimination of nonspecific reactions from endogenous cellular enzymes under in vivo conditions, enabled recovery of true intermediates in the reaction cascade. Interestingly, the inability to process the angucycline UWM6 under any combinations of the tailoring enzymes indicated that UWM6 is in fact a shunt product, instead of the long-standing assignment as an intermediate. This finding also led to the assignment of the oxygenase JadF (the soluble replacement of GilOIV) in the release, decarboxylation and dehydration of the ACP-tethered polyketide product **3** to yield **4**. In addition, GilOI was identified as a bifunctional enzyme catalyzing both C12 oxidation and 4a,12b-dehydration of **4** to yield **5**, which undergoes oxidative cleavage catalyzed by GilOII to afford the dibenzochromen-6-one core. The last step in the synthesis of **7** from the hemiacetal **6** is catalyzed by the oxidoreductase GilR, which can also catalyze the conversion of pregilvocarcin V to the gilvocarcins V [23]. Crystal structure of GilR in complex with pregilvocarcin V was recently solved, which showed the cofactor FAD is covalently attached to the enzyme through His65 and Cys125 [24]. Two amino acids Tyr445 and Tyr448 located in the catalytic pocket were proposed and confirmed by mutagenesis to play crucial roles in catalysis. With the roles of the redox enzymes assigned in this cascade, one gains more appreciation of the nature's remarkable ability to control reactive intermediates en route to highly complex structures.

Pre-assembly modification in nonribosomal peptide biosynthesis

Nonribosomal peptides (NRPs) are a group of structurally and functionally diverse natural products which are synthesized by NRPSs [4]. A typical module of NRPS consists of an adenylation (A) domain, thiolation (T) domain and condensation (C) domain. Each A domain activates an amino acid as an aminoacyl adenylate, which is transferred to the phosphopantetheinyl arm of the T domain and incorporated into the NRPs [25]. Much of the structural complexity seen in NRPs is generated through the incorporation of nonproteinogenic amino acids, many of which are hydroxylated versions of the natural amino acids. Hydroxylation of amino acids can either place on the free amino acid, or on the activated aminoacyl moiety that is attached to the T domain (Scheme 2). For example, *N*^δ-hydroxyornithine (**8**) is found in siderophores coelichelin and erythrocelin, and is the product of *L*-ornithine *N*-hydroxylation by FAD-dependent monooxygenases CchB and EtcB, respectively [26, 27]. In contrast, epoxidation of the succinamoyl building block by the α -ketoglutarate dependent DdaC to afford epoxysuccinamoyl (**9**) takes place after *N*_β-fumaramoyl-*L*-2,3-diaminopropionate is transferred to the NRPS DdaD [28]. T-domain dependent oxidation is also observed in the formation of 3, 5-dihydroxy-4-methylantranilate (**10**) found in sibiromycin. Following NRPS-independent modification of 3-hydroxykynurenine to yield 3-hydroxy-4-methylantranilic acid (3H4MAA), 3H4MAA is transferred to the NRPS SibE and is hydroxylated by the FAD-dependent hydroxylase SibG to give **10** [29].

Recently, oxygenases using different mechanisms were identified to catalyze the aromatic hydroxylation of tyrosine (Tyr) and phenylalanine (Phe). SfmD was characterized as a heme containing peroxidase which regioselectively hydroxylates 3-methyltyrosine to 3-hydroxy-5-methyltyrosine (**11**) in the biosynthesis of saframycin A, using H₂O₂ as oxidant [30]. SgcC is a monooxygenase involved in the biosynthesis of the antitumor enediyne C-1207 and was shown to catalyze the hydroxylation of β -Tyr in a T-domain dependent fashion [31]. SgcC is able to hydroxylate (*S*)-3-chloro- β -tyrosine tethered to SgcC2 at the C-5 position to afford (*S*)-3-chloro-5-hydroxy- β -tyrosyl-SgcC2 (**12**) in presence of O₂ and FADH₂. In addition to **12**, SgcC can also hydroxylate *meta*-substituted bromo-, iodo-, fluoro-, and methyl- β -tyrosyl-SgcC2. 2-hydroxylphenylalanine was found as one of the building blocks in the pentapeptide antibiotics pacidamycins [32]. A phenylalanine hydroxylase (PecX) was demonstrated to be an Fe(II)-dependent enzyme that regioselectively synthesize **13** from *L*-Phe using tetrahydrobiopterin as a cofactor [32, 33]. Comparing with another group of Phe 4-hydroxylases (Phe4Hs) which catalyzes the formation of *L*-Tyr, Cys187 and Thr202 in the binding pocket of PacX are replaced by Phe and Gly, respectively. A PacX C187F/T202G mutant was shown to have dramatically altered hydroxylation regiospecificity to afford >90% *L*-Tyr instead.

SyrP is an Fe(II) and α -ketoglutarate dependent hydroxylase that acts on an aspartic residue tethered to the eighth module of SyrE to generate *L*-*threo*-3-OH-Asp (**14**) in the biosynthesis of syringomycin E [34]. In the biosynthesis of the antifungal hexadepsipeptide kutznerides, both *L*-*threo*-hydroxyl and *L*-*erythro*-hydroxyl glutamate derivatives can be incorporated [35]. After *L*-glutamic acid is transferred to the T domain of KtzH, the oxygenases KtzO and KtzP can catalyze β -hydroxylation to form **15** and **16**, respectively. Unlike the above examples, AsnO was demonstrated to catalyze the hydroxylation of free *L*-asparagine to yield *L*-3-hydroxyasparagine **17**, which is activated and incorporated by the NRPS to form the calcium dependent antibiotic (CDA) peptidyl chain [36]. Based on the AsnO crystal structure, Marahiel and coworkers engineered a D241N AsnO mutant that can hydroxylate Asp instead of Asn [37].

Post-assembly modification in nonribosomal peptide biosynthesis

Fungal indole alkaloids derived from tryptophans are NRPs consisting of relatively few amino acids. While the NRPSs construct the starting structural frameworks, which can be pyrazinoquinazolines and diketopiperazines, the seemingly exponential increase in structural complexity of the final products can be attributed to the oxidative modifications catalyzed by a few redox enzymes. In particular, epoxidation of the 2,3-double bond in the indole ring is a highly effective strategy to activate the C2 carbon and build multicyclic ring systems. For example, fumiquinazoline F **18** is the nascent NRP product of the tryptoqualanine (TQA) and fumiquinazoline A **20** biosynthetic pathways (Scheme 3a) [38*, 39]. Epoxidation of **18** by a FAD-dependent monooxygenase (Af12060 in pathway of **20**) initiates annulation of the indole ring by an amino acid residue (e.g. alanine) tethered to a monomodule NRPS **21**. Biochemical analysis suggests the stereoselective attack of the iminium **19** by the nucleophilic amine of **21** is catalyzed by a C-like domain in the monomodule NRPS [40]. Subsequent attack of the indole nitrogen on the alanyl-thioester forms the 6-5-5 imidazolindolone present in **20**. A monocovalent flavoprotein (Af12070) can then dehydrogenate the C-N bond in the pyrazinone ring in **20**, which can trigger different events including hydrolytic cleavage of the ring towards the formation of TQA, or spontaneous intramolecular cyclization to yield the spirohemiaminal fumiquinazoline C, which can be converted to the more stable aminor fumiquinazoline D [41*]. Similarly, epoxidation of the indole ring in the synthesis of notoamides, a family of prenylated fungal indole alkaloids containing the bicyclo [2.2.2] diazaoctane core, is catalyzed by a FAD-dependent monooxygenase NotB (Scheme 3b) [42]. Formation of the 2,3-epoxide initiates either a Pinacol-like rearrangement to afford notoamide C or annulation of C-2 by the tryptophan amide to yield the hexacyclic notoamide D.

Whereas the above examples illustrate how post-NRPS oxidation can lead to dramatic change in alkaloid structure, reductive modifications have also been noted in generation of unusually complex alkaloid-like natural products, as exemplified in the formation of the pentacyclic tetrahydroisoquinoline saframycin A [43*]. A single R domain in the NRPS SfmC reductively released three different peptidyl thioesters in a NADPH-dependent fashion to generate the corresponding aldehyde intermediates. Through Pictet-Spengler reactions catalyzed by the C domain, the aldehydes are cyclized with peptidyl thioesters reloaded on SfmC T-domain to afford the unique saframycin ring systems.

Newly discovered Redox enzymes in other systems

In addition to their importance in polyketide and nonribosomal peptide biosynthesis, redox enzymes with novel functions are indispensable in the modification of other classes of natural products. For example, the mononuclear iron enzyme hydroxypropylphosphonic acid epoxidase (HppE) catalyzes the final step of fosfomyin biosynthesis, in which HppE generates a C1 radical intermediate of (*S*)-2-hydroxypropylphosphonic acid (*S*-HPP) that can cyclize into the epoxide (Scheme 3c). Interestingly, HppE instead generates a C2 radical intermediate in the presence of the *R*-enantiomer, which can form the ketone 2-oxo-propylphosphonic acid [44]. The structures of HppE in complex with either enantiomer revealed variations in the positioning of the two compounds with respect to the reactive Fe (III)-superoxo species [45**]. The different orientations of the C1 and C2 carbons in the active site therefore dictate the regioselectivity of hydrogen abstraction step.

Tirandamycin A and B are dienoyl tetramic acids that contain heavily modified bicyclic ketal cores [46**]. Following the biosynthesis of tirandamycin C by a hybrid PKS-NRPS pathway, the bicyclic core is oxidized by a P450 monooxygenase (TamI) and a FAD-dependent oxidase TamL to yield tirandamycin A and B (Scheme 3d). In vitro reconstitution

by Sherman and coworkers elegantly confirmed TamI and TamL are sufficient to catalyze the dazzling multi-step oxidative cascade. In particular, TamI alone can catalyze the hydroxylation at C10 and C18, as well as epoxidation of C11-C12. TamI was also shown to be able to complete the entire oxidative cascade single-handedly by performing double hydroxylation of C10, which can spontaneously form the ketone intermediate tirandamycin D, albeit the second hydroxylation at C10 is much less efficient. TamL is hence recruited to oxidize the monohydroxylated tirandamycin E into tirandamycin D. The remarkable versatility of TamI, along with recently confirmed activities a P450 monooxygenase that converts amorpho-4,11-diene to artemisinic acid [47]; and LovA from the lovastatin biosynthetic [48], fully demonstrates how nature can use a single P450 enzyme to catalyze multiple oxidative modifications in a biosynthetic pathway and generate structural complexity in natural products [47].

Conclusions

Redox enzymes play indispensable roles in generating structural complexity during natural product biosynthesis. Continued characterization of these enzymes will undoubtedly expand our molecular toolbox for combinatorial biosynthesis. However, it remains challenging to recombine redox enzymes from different pathways towards the rational enzymatic synthesis of new compounds, largely attributed to the limited substrate specificities of the enzymes. Another difficulty in using these enzymes as synthetic biology parts is our inability to predict substrates and products of uncharacterized redox enzymes uncovered from microbial genome sequencing. Protein engineering and biochemical characterization are required to overcome these limitations and allow us to truly realize the impressive catalytic power of these enzymes.

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Highlights (for review)

Redox enzymes play important roles in pre- and post-assembly modifications.

Both PKS and NRPS pathways use redox enzymes to introduce structural complexity

Recent discoveries of novel redox enzymes are discussed

Figure 1.
Complexity generation during natural product biosynthesis can take place at three different stages.

Scheme 1.
Representative pathways to generate natural diversity in polyketide biosynthesis.

Scheme 2.

Representative chemical structures generated by pre-assembly modifications in nonribosomal peptide biosynthesis.

Scheme 3.

Representative pathways to generate natural complexity in nonribosomal peptide biosynthesis and other systems.