Unusual flavoenzyme catalysis in marine bacteria

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Ever since the discovery of the flavin cofactor more than 80 years ago, flavin-dependent enzymes have emerged as ubiquitous and versatile redox catalysts in primary metabolism. Yet, the recent advances in the discovery and characterization of secondary metabolic pathways exposed new roles for flavin-mediated catalysis in the generation of structurally complex natural products. Here, we review a selection of key biosynthetic flavoenzymes from marine bacterial secondary metabolism and illustrate how their functional and mechanistic investigation expanded our view of the cofactor's chemical repertoire and led to the discovery of a previously unknown flavin redox state.

Introduction

Various marine organisms, ranging from invertebrates and plants to microbes, produce structurally diverse secondary metabolites with a vast potential for medical application (e.g., as antibiotics or in cancer treatment) [1-3]. Marine bacteria, in particular, were recognized as prolific and underestimated producers of natural product chemicals [1,4], whose biosyntheses frequently require redox tailoring enzymes containing riboflavin (vitamin B2)-derived cofactors in the form of flavin mononucleotide (FMN) and, more frequently, flavin adenine dinucleotide (FAD) [5]. Aside from secondary metabolism, ubiquitous flavoenzymes catalyze numerous essential redox reactions in all domains of life, e.g., in central metabolism, drug metabolism, immune defense, cell signaling, protein folding, DNA repair, apoptosis, light emission, and neural development [6]. The key to the unique reactivity and versatile redox chemistry of the flavin cofactor is the reactive N5-C4a locus of the isoalloxazine ring system, which serves as entry/exit points for electrons as well as a site for covalent adduct formation (Figure 1) [5,7]. Under aerobic conditions, flavins are commonly oxidized (Fl_{ox}), while radical, single-electron reduced neutral (blue, $\text{FlH}^\bullet$) or anionic (red, $\text{Fl}^\bullet$) semiquinones (SQs) and two-electron-reduced hydroquinones (Fl_{red}) represent catalytically important redox states (Fig 1) [8-14]. Typical flavin-dependent reactions include the electron exchange between obligatory one-electron (e.g., $\text{Fe}^{\text{III}}/\text{Fe}^{\text{II}}$) and two-electron (e.g., NAD(P)H) donors/acceptors or the dehydrogenation of diverse organic substrates (Figure 1). As a rare exception among organic cofactors, protein-bound Fl_{red} furthermore enables the efficient reduction of molecular oxygen. H_2O_2 -forming flavin-dependent oxidases thereby exploit O_2 as electron acceptor, as exemplified by NADPH oxidase or monoamine oxidase [13]. Flavin-dependent monooxygenases, by contrast, employ O_2 -derived covalent flavin oxygen adducts as oxygenating species in the form of the flavin-C4a-peroxide ($\text{Fl}_{\text{C4a}}[\text{OOH}]$) [14] or the flavin-N5-

oxide ($\text{Fl}_{\text{N5[O]}}$) [8,9] (Figure 1). The electrophilic character of the well-studied transiently produced $\text{Fl}_{\text{C4a[OOH]}}$ is comparable to other organic hydroperoxides [15] and allows the monooxygenation of organic substrates, for example in the *p*-hydroxybenzoate hydroxylase that catalyzes an electrophilic aromatic substitution reaction [10,16-19]. Enzyme-assisted deprotonation of the hydroperoxide, however, gives rise to a C4a-peroxyanion that serves as a nucleophilic oxygenating agent, e.g., in Bayer-Villiger type oxidations of ketones to esters [20]. As a further variation of this chemistry, flavin-dependent halogenases exploit the $\text{Fl}_{\text{C4a[OOH]}}$ as oxidant for the formation of halonium ions, which add to organic substrates through electrophilic substitutions [21,22]. The recently discovered $\text{Fl}_{\text{N5[O]}}$ oxygenating species was shown to mediate an oxidative Favorskii-type carbon-carbon rearrangement and may conceivably constitute a versatile redox catalyst, albeit its chemistry is less understood at present (see discussion below) [8,9] (Figure 1) [5]. In this review, we highlight a small selection of recently described marine bacterial flavin-dependent redox tailoring enzymes that adopt crucial roles in the formation of structurally distinct natural products and illustrate the broad diversity of flavin-mediated catalysis. For a comprehensive overview of biosynthetic flavoenzymes, we would like to recommend the excellent work of Walsh and Wencewicz [5].

Figure 1. Overview of flavin redox states and catalysis (R = ribityl-ADP (FAD) or phosphoribityl (FMN)). Oxidases and dehydrogenases (DHase) oxidize organic substrates (S1) and utilize O_2 or alternative substrates (S2) for the reoxidation of Fl_{red} to the catalytically active Fl_{ox} , respectively. Monooxygenases employ a flavin-C4a-(hydro)peroxide or a flavin-N5-oxide for the oxygenation of organic substrates (S1). The radical formation of these oxygenating species proceeds via reduction of Fl_{ox} to Fl_{red} by NAD(P)H or the substrate, which may

enable the single electron reduction of O_2 and subsequent radical coupling of the formed superoxide and the flavin SQ at the spin density sites C4a or N5. Note that the tentative pathway for $Fl_{N5[O]}$ formation requires further investigation.

Bromination in the biosynthesis of toxic polybromophenols and polybromopyrroles

The marine environment is an exceptionally rich source of halogenated natural products [23], in particular of brominated natural products that are perhaps exclusively of marine origin. While the participation of flavoenzymes as halogenating catalysts in chlorinated natural product biosynthesis is well established (Figure 2AB) [24], it is surprising to note that physiological marine brominating flavoenzymes, those that cannot catalyze the oxidative incorporation of chlorine [25], had evaded discovery until very recently. Querying the genetic and molecular bases for the biosynthesis of the cytotoxic [26] marine natural product pentabromopseudilin revealed the ‘*bmp*’ gene locus that incorporates two intriguing flavin-dependent brominases [27]. Of these, the enzyme Bmp2 catalyzes the tribromination of L-proline derived pyrrole ring that is acylated to an acyl carrier protein (ACP) (Figure 2C). It is intriguing to note that despite the presence of a 900-fold excess of chloride in sea-water, Bmp2 demonstrates extraordinary halide specificity, in that no chloride incorporation was observed either during *in vitro* enzymological investigations, or during *in vivo* pentabromopseudilin production in growth media lacking bromide [27]. Another flavin-dependent brominase, AltN, shares the remarkable halide selectivity of Bmp2, although the sequence of events leading to the incorporation of the L-tyrosine-derived bromophenyl moiety in the suite of the bromoalterochromide marine natural product lipocyclic peptides remains to be revealed [28]. Identification of Bmp2 and AltN brominases possessing high sequence homology and functional similarity to chlorinases such as Mpy16 (Figure 2C) [29] and SgcC3 [30], respectively, provides avenues to explore the molecular basis for halide specificity in flavin-dependent halogenases. It is thus of note that while postulated halide binding sites in the crystal structures of flavin-dependent chlorinases have been identified that demonstrate resemblance to chloride binding sites in membrane halide transporters [31], their functional relevance remains in question. This is primarily due to the observations that non-halogenating flavoenzymes also bind halide anions in structurally analogous sites (reviewed by Blasiak and Drennan [25]) and that the halide in this position is neither desolvated nor correctly positioned with respect to the N5 or the C4a position of the flavin isoalloxazine ring to allow the oxidation of the halide to the halonium ion.

The extraordinary selectivity for bromide is shared by the other flavin-dependent halogenase coded within the *bmp* gene locus, the phenol brominase Bmp5 [27]. In primary sequence, Bmp5 differs from canonical two-component flavin-dependent halogenases such as Bmp2 that require an exogenous flavin-reductase enzyme to supply the necessary Fl_{red} . Rather Bmp5 uniquely resembles single-component flavoenzymes with a distinct NAD(P)H binding domain that catalyze the *cis* reduction of Fl_{ox} to Fl_{red} [27]. Bmp5 further provides the first example of a flavin-dependent halogenase-mediated electrophilic substitution reaction in which a carboxyl group is lost upon halonium addition rather than a proton, a reaction that has previously been reported for flavin-dependent hydroxylases (Figure 2D) [32]. Hence, Bmp5 likely represents an evolution of function from flavin-dependent oxygenases, an assertion that is supported by the postulated halide oxidation scheme for flavin-dependent halogenases, in which the $Fl_{C4a[OOH]}$ intermediate decomposes to a $Fl_{C4a[OX]}$ species ($X=halide$), that then transfers the halonium via the catalytic lysine side chain to the substrate [21]. The flavoenzyme Bmp5 participates in the formation of natural analogs of some of the most toxic anthropogenic molecules ever synthesized, such as polybrominated diphenyl ethers and dioxins [27,33]. While ubiquitously and abundantly present in the marine environment [34], naturally-produced polybrominated molecules have been detected in the human population as

well, likely being transferred via our trophic connection to the oceans [35]. Moreover, pentabromopseudilin (Figure 2C) is extraordinarily toxic to brine shrimp, likely representing an ecological role as a feeding deterrent [26]. Similarly, brominated pyrroles have been proposed as chemical cues for coral settlement and metamorphosis [36], thus underscoring the fact that polybrominated molecules generated by the extraordinary selective marine brominases such as Bmp2 and Bmp5 have well defined ecological roles attached to them in the marine metabolome. The mechanism via which marine brominases selectively incorporate bromide in natural product scaffolds against a selection pressure of much higher concentration of chloride in sea-water remains to be elucidated, but is unlikely to be realized by a steric differentiation between chloride and bromide ions within the halogenating enzyme active site. The fact that chlorinating flavin-dependent halogenases catalyze bromination of aromatic substrates but brominases do not catalyze chlorination reactions hints towards differential dehydration penalties for chloride and bromide ions, together with different oxidation potentials for the generation of halonium intermediates as the selectivity filter determining the halide specificity for halogenating enzyme catalysts.

Figure 2. Flavin-dependent halogenation chemistry. (A) Reaction mechanism for flavin-dependent halogenases. Flavin-dependent halogenases are O₂-dependent enzymes, and the first half of the reaction is identical to flavin-dependent oxygenases, in which the transient Fl_{C4a}[OOH] transient species is generated. This decomposes to a postulated Fl_{C4a}[Ox] transition state with the concomitant release of a water molecule, whereupon the halonium is transferred to an appropriately positioned catalytic lysine side chain. The lysine side chain halo-amine is postulated to be the active halogenating agent. Note that in canonical flavin-dependent halogenases, Fl_{red} is provided by a partner flavin-reductase enzyme. **(B) Active site representation of the canonical flavin-dependent halogenase PrnA (37)** in complex with cofactor FAD (in stick-ball representation with carbon atoms colored yellow), a chloride ion (in sphere representation colored orange), and the product 6-chloro-tryptophan (in stick ball representation with carbon atoms colored blue). Also shown

are the haloamine bearing lysine side chain, the catalytic base glutamate side chain that abstracts the proton subsequent to chloronium addition to the substrate, and the water molecules in close proximity to the chloride ion. The C4 and N5 positions of the flavin isoalloxazine ring are marked. **(C) Reactions catalyzed by Bmp2 and Mpy16. (D) Two-step reaction for Bmp5 leading to the formation of 2,4-dibromophenol from *p*-hydroxybenzoic acid.** 2,4-dibromophenol further undergoes oxidative coupling to generate bioaccumulative and persistent pollutant natural products such as the polybrominated diphenyl ethers 2OH-BDE68 and biphenyl-2,2'-diOH-BB80.

Pyrrolizine ring formation in chlorizidine A biosynthesis

The berberine bridge enzyme (BBE)-like flavin oxidase Clz9 likely contains a bicovalently attached FAD and was recently shown to mediate an unusual oxidative cyclization reaction in the biosynthesis of the tetrachlorinated alkaloid chlorizidine A by the marine *Streptomyces* sp. CNH-287 [38]. Chlorizidine A features rare pyrroloisindolone and pyrrolizine rings connected through a single C–C bond and its assembly requires an extraordinary interplay between enzymes originating from polyketide and fatty acid biosynthesis [38]. *In vitro* studies confirmed that the final pathway step, however, is catalyzed by the redox tailoring enzyme Clz9 and involves the formation of an intramolecular C–N bond between C13 and the pyrrole nitrogen of prechlorizidine A, thus giving rise to the pyrrolizine ring (Figure 3A) [38]. Flavin oxidases generally catalyze (non-oxygenating) 2-electron oxidation reactions of organic substrates and employ O₂ for the subsequent required reoxidation of Fl_{red} to the catalytically active Fl_{ox}. The stereoselective, oxidative cyclization reaction in chlorizidine biosynthesis may therefore proceed via hydride abstraction from prechlorizidine A by the electrophilic N5 of Clz-bound Fl_{ox}, before the intermediate is attacked by the nucleophilic pyrrole-nitrogen to yield chlorizidine A (Figure 3A). The bicovalent link of the flavin with Clz9 possibly increases the oxidative power of the Fl_{ox} and enables this unfavorable reaction, as has been similarly demonstrated in other cases [39–41]. The proposed mechanism is reminiscent of other BBE-family members [42], yet structural and detailed mechanistic studies are required to further illuminate this intriguing flavin-dependent cyclization reaction.

Radical indoloterpene coupling and cycloether formation

Until recently, it was assumed that flavin-dependent oxygenases employ a universal oxygen activation pathway, which leads to the formation of C4a-bound peroxides. One possible exception may be realized by XiaH, a homologue of flavin-dependent aromatic ring hydroxylases that is essential for the conversion of the indolosesquiterpene xiamycin into oxiamycin and various bixiamycins in bacterial endophytes of mangrove trees (Figure 3B) [43,44]. The dimerization of xiamycin to the bixiamycins was proposed to proceed via radical N–C and N–N aryl-aryl coupling reactions, in which XiaH-Fl_{ox}-catalyzed single-electron oxidation of xiamycin gives rise to xiamycin-N1-radicals that undergo homocoupling at the spin density sites N1, C6, or C21 (Figure 3B). Such radical aryl-aryl coupling chemistry is highly unusual for flavoenzymes and generally a domain of cytochrome P450 enzymes [45]. In addition, XiaH exhibited oxygenase activity and enabled the formation of the cycloether oxiamycin through insertion of an oxygen atom between C11 and C12 of xiamycin. The authors suggested that in this alternative route, the flavin SQ species (produced by the initial xiamycin oxidation step) may be reoxidized to Fl_{ox} through molecular oxygen, which produces a reactive superoxide that reacts with the xiamycin-radical at C11 to afford a transient xiamycin-C11-hydroperoxide. The subsequent peroxide-mediated ring expansion to tetrahydrooxepin under elimination of water would additionally require a (possibly NAD(P)H-dependent) reduction step and complete the pathway to oxiamycin [44]. XiaH may

thus represent an unusual member of the “internal monooxygenases” that use their substrate as electron donor for the activation of oxygen. Related flavin-dependent oxygenases, however, commonly use NAD(P)H as an external electron source, which XiaH may instead expend for the required reduction step *en route* to oxiamycin [44]. Direct evidence for the intriguing XiaH catalytic mechanism was not presented, yet the treatment of xiamycin with a radical forming agent allowed the biomimetic generation of several bixiamycins as well as oxiamycin and thereby significantly substantiated a radical process [44].

Figure 3. Selected flavin-catalyzed coupling reactions. (A) Proposed pyrrolizine ring formation by Clz9. Prechlorizidine A is dehydrogenated by Clz9-Fl_{ox} followed by the regio- and stereoselective pyrrolizine-forming cyclization reaction through pyrrole coupling that affords chlorizidine A. The C8 α and C6 of the flavin cofactor are likely covalently linked to His99 and Cys157 of Clz9, respectively. Oxidation of Fl_{red} by O₂ regenerates Fl_{ox} (not shown). **(B) Tentative mechanism for the XiaH-catalyzed radical xiamycin coupling and oxiamycin formation.** XiaH is proposed to catalyze the single-electron oxidation of the indoloterpenoid xiamycin, which then undergoes various radical dimerization reactions to bixiamycins or reacts with superoxide and possibly NAD(P)H to oxiamycin. The asterisks indicate a mixture of the respective atropoisomers with stable *P*- and *M*-configurations. **(C) Proposed mechanism for C-N bond formation by the Mpy10-Mpy11 enzyme pair.** Cryptic halogenation at either the C3 or the N1 positions of the monodeoxypyoluteorin monomer leads to the formation of a trihalo intermediate that undergoes nucleophilic halide displacement to yield the marinopyrrole dimer. Note that a radical mechanism is plausible too.

Another probable example of atroposelective biaryl coupling catalyzed by a flavoenzyme is provided by the postulated biosynthetic scheme for the marinopyrrole suite of halogenated marine natural products [34,46]. Using genetic experiments, it was demonstrated that two flavoenzymes coded by the genes *mpy10* and *mpy11*, along with the flavin-reductase coded by the gene *mpy1*, catalyze the coupling of monomeric monodeoxypyoluteorin to marinopyrrole A (Figure 3C) [34]. An *in vitro* reconstitution of the activity of Mpy10 and Mpy11 has not been reported as yet, consequently, physiological intermediates in the C-N bond formation event have not been elucidated. However, as Mpy10 and Mpy11 possess high sequence similarity to flavin-dependent halogenases, and because the coupling is dependent on the presence of the flavin-reductase Mpy1, two possible routes leading to the formation of the C-N bond have been proposed. In each of the scenarios, cryptic trihalo-pyrrole

intermediates have been invoked that undergo a second electrophilic substitution reaction to yield the final product. Cryptic halogenated intermediates that, in essence, act as intra- or intermolecular alkylating agents by nucleophilic displacement of the halide have been proposed in the biosynthesis of other marine natural products such as curacin [47]. The fact that these intermediates have not been observed when either or both genes *mpy10* and *mpy11* were expressed *in vivo* (in the presence of *mpyI* and fed monodeoxypyoluteorin substrate), points to the fact that the trihalo intermediates might not be released prior to the C-N bond formation by the catalytic enzyme complex. A radical coupling mechanism similar to xiamycin formation, however, cannot be ruled out. Notably, in contrast to the formation of bixiamycins, only a single atropoisomer is generated in high selectivity by the Mpy10-Mpy11 enzyme complex.

A flavin-N5-oxide-mediated oxidative carbon-carbon rearrangement

The flavoenzyme EncM is another remarkable internal monooxygenase that is involved in the biosynthesis of the unusual polyketide antibiotic enterocin in *Streptomyces maritimus* [48]. EncM is a structural homologue of flavin-dependent oxidases/dehydrogenases (rather than oxygenases) and was first shown to single-handedly catalyze an oxidative Favorskii-type carbon-carbon rearrangement that entails the formation of a tricyclic caged core, the structural hallmark of enterocin (Figure 4) [49]. Recently, detailed biochemical studies revealed EncM's unprecedented oxygen reactivity and led to the surprising discovery of the Fl_{N5[O]} cofactor [9]. ¹⁸O-labeling studies furthermore established Fl_{N5[O]} as the oxygenating species in the EncM-catalyzed reaction, which formally involves a hydroxylation-dehydrogenation dual oxidation of the linear, acyl carrier protein (EncC) - bound octaketide substrate that triggers the Favorskii rearrangement [9]. The Fl_{N5[O]} species constitutes a superoxidized flavin that is stably maintained under aerobic conditions in contrast to the transiently formed Fl_{C4a[OOH]}. Both oxygenating species may be produced, however, via Fl_{red}-mediated single electron reduction of O₂ to superoxide, which undergoes rapid radical coupling with the concomitantly formed SQ species (Figure 1). The nature of the radical SQ intermediate is controlled by the protein-based microenvironment and might be pivotal for the regiospecific coupling at the high spin-density sites C4a (producing the Fl_{C4a[OOH]}) or N5 (producing the Fl_{N5[O]}) of the neutral and anionic SQ, respectively [8]. The oxygenation pathway may involve single-electron transfer and radical coupling to produce the hydroxylated intermediate (Figure 4, step I, route a) or, alternatively, be envisaged as a nucleophilic attack of the substrate enolate on the electrophilic oxoammonium resonance form of the Fl_{N5[O]} (step I, route b) [9]. The following dehydrogenation of the introduced hydroxyl by N5 of Fl_{ox} affords Fl_{red} independent of external reductants (step II) and allows the subsequent regeneration of the Fl_{N5[O]} in the presence of O₂ (step III). Notably, the Fl_{N5[O]} likely represents a less precarious oxygenating species compared to the flavin-C4a-peroxide, which readily collapses to Fl_{ox} and toxic H₂O₂. Two strategies for preventing the formation of H₂O₂ are known from flavin-C4a-peroxide-dependent oxygenases. "Cautious" oxygenases generate the Fl_{C4a[OOH]} only in the presence of substrate, whereas "bold" oxygenases produce and stabilize this intrinsically labile species (even in the absence of substrate), resulting in half-lives of up to several minutes [14]. EncM, by this analogy, might be considered a "smart" flavin-dependent oxygenase that employs a safe form of activated oxygen adapted for reactive substrates. To date, EncM is the only reported enzyme utilizing a Fl_{N5[O]}, possibly because this newly recognized flavin redox state has been overlooked in the past [8]. Since the Fl_{N5[O]} may conceivably participate in nucleophilic, electrophilic, and radical oxidation reactions, we posit that the discovery of additional Fl_{N5[O]}-dependent enzymes may be forthcoming [8].

Figure 4. Proposed EncM- $\text{Fl}_{\text{N5[O]}}$ -catalyzed poly(β)-ketone dual oxidation. The C8 α of the flavin cofactor is covalently attached to the His78 of EncM. Note that the EncM-mediated Favorskii rearrangement and subsequent reactions required for enterocin-formation are not shown. The steps indicated by the roman numerals are discussed in the main text. For details of the $\text{Fl}_{\text{N5[O]}}$ -regeneration pathway see Figure 1.

Concluding remarks

The importance of the flavin cofactor for countless redox reactions was recognized long ago, albeit mechanistic studies were heavily focused on a moderate assortment of primary metabolic flavoenzymes. While this granted deep insight into many aspects of flavin-dependent catalysis, the more recent detailed biochemical characterization of secondary metabolic pathways has proven a true treasure trove for flavin enzymology and unearthed a broader catalytic diversity, as exemplified by the discovery of the $\text{Fl}_{\text{N5[O]}}$ redox state [8,9]. The astonishing redox versatility of flavoenzymes appears ideally suited for secondary metabolism, nature's biosynthetic tinkering ground, and significantly contributes to the vast chemical diversity of natural products via functionalization (e.g. bromination, hydroxylation) or substrate activation reactions (e.g. dehydrogenation). Notably, a large number of prokaryotic biosynthetic pathways remain untapped [50], particularly in marine bacteria that constitute an underexplored source of natural product molecules [1,4]. Already, flavoenzymes are being used as cues to mine complex metagenomes, both genetically [51], as well as computationally [27] in the search for biosynthetic gene loci for marine natural products of pharmaceutical interest. Future studies of such biosynthetic routes will therefore not cease to unveil novel flavin-dependent enzyme reactions and ultimately provide a more complete picture of the cofactor's intricate chemistry.

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