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Understanding the crucial interactions between Cytochrome P450s and non-ribosomal peptide synthetases during glycopeptide antibiotic biosynthesis

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The importance of Cytochrome P450-catalyzed modifications of natural products produced by non-ribosomal peptide synthetase machineries is most apparent during glycopeptide antibiotic biosynthesis: specifically, the formation of essential amino acid side chains crosslinks in the peptide backbone of these clinically relevant antibiotics. These cyclization reactions take place whilst the peptide substrate remains bound to the non-ribosomal peptide synthetase in a process mediated by a conserved domain of previously unknown function — the X-domain. This review addresses recent advances in understanding P450 recruitment to non-ribosomal peptide synthetase-bound substrates and highlights the importance of both carrier proteins and the X-domain in different P450-catalyzed reactions.

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The role of Cytochrome P450s in glycopeptide antibiotic biosynthesis

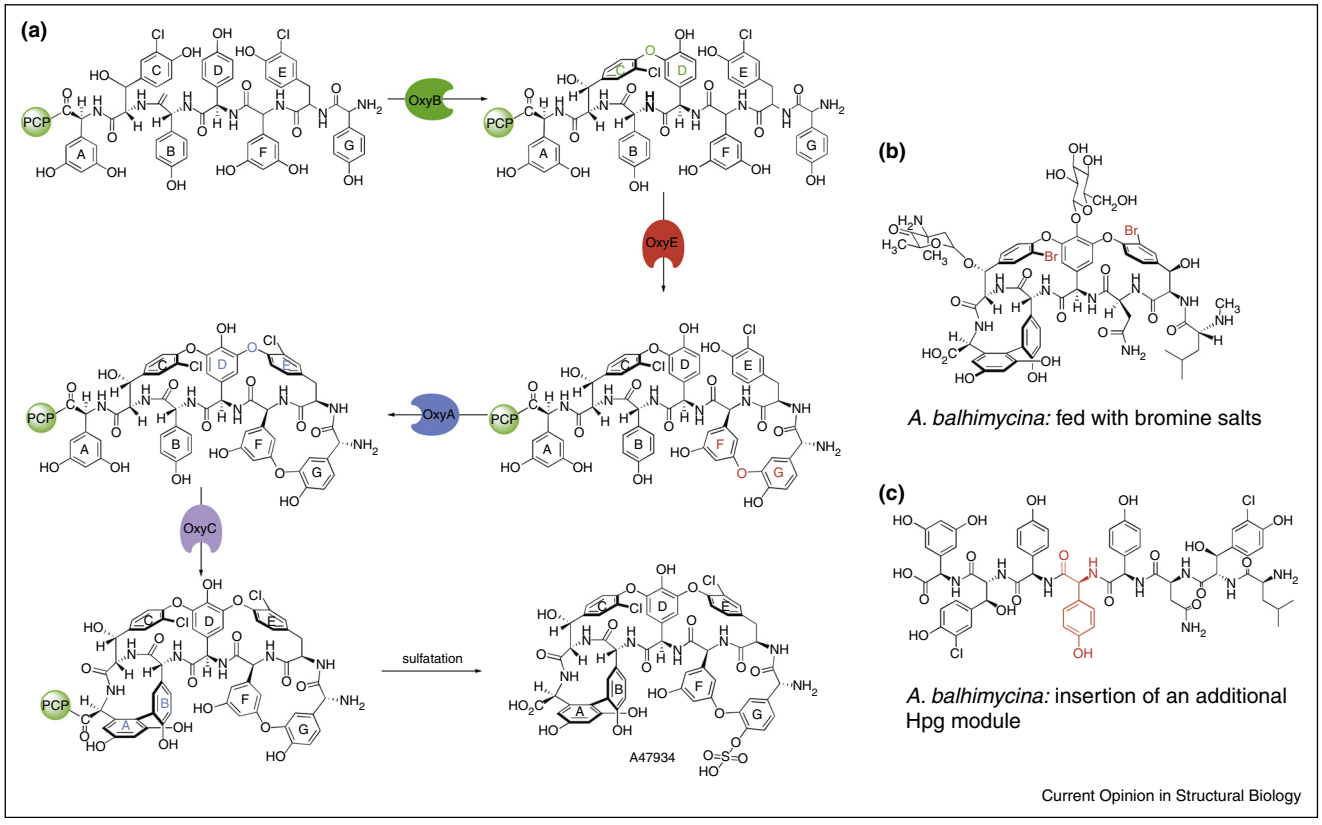
The use of glycopeptide antibiotics (GPAs) as antibiotics of last resort against methicillin-resistant *Staphylococcus aureus* (MRSA) together with their long clinical lifetime has made these highly important compounds for human health [1,2]. From a structural viewpoint GPAs have an intriguingly complex architecture, specifically their highly crosslinked state: GPAs possess three or four biaryl and biaryl-ether cross-links between the side chains of aromatic amino acids present in their heptapeptide backbone (Figure 1) [1,2]. These cross-links lead to the characteristic three-dimensional cup shape of the GPA aglycone. This structure is essential for their activity, as it confers binding towards the Lys-D-Ala-D-Ala motif present in various stages of the bacterial cell wall biosynthesis, for example, Lipid II and its precursors, via five hydrogen bonds [2,3]. Previously it has been shown that GPAs are assembled by a non-ribosomal peptide synthetase (NRPS) [4••]. Gene inactivation studies revealed that P450 monooxygenase enzymes install the crosslinks present in both balhimycin [5,6••] and in the teicoplanin-type GPA A47934 [7••] with a strict order of cyclization steps. This was determined by inactivation of each of the P450s, which resulted in the accumulation of intermediates at a distinct stage of side-chain cyclization due to stalling of the biosynthetic machinery. Hence, for the type-I GPA balhimycin it could be shown that the first ring-system to be formed is the C-*O*-D diaryl ether ring catalyzed by OxyB, followed by the OxyA-catalyzed D-*O*-E diaryl ether ring-system and finally the biaryl AB system, which is catalyzed by OxyC (Figure 1) [5,6••]. Similar experiments were performed for the teicoplanin-like GPA A47934, through which the order of cyclizations C-*O*-D → F-*O*-G (performed by the enzyme OxyE) → D-*O*-E → AB could be established (Figure 1) [7••]. Furthermore, these results indicated that P450 monooxygenases would function in close interaction with the NRPS assembly line [8••]. This knowledge set the stage for subsequent experiments on the *in vitro* reconstitution of the vancomycin P450 monooxygenase OxyB with peptide substrates [9–11,12••,13]. Here, the conversion of linear hexa-peptide peptidyl carrier protein and hepta-peptide peptidyl carrier protein (PCP) thioesters could be achieved, whilst linear heptapeptides not presented on a PCP domain were not suitable substrates [11,12••].

PCP-driven P450 recruitment

Amino acid hydroxylation

Along with the cyclization reactions performed by P450s in GPA biosynthesis, some GPAs require β-*R*-hydroxytyrosine as a precursor for NRPS-catalyzed peptide assembly [4••,14,15]. This hydroxylation is P450 catalyzed and

Figure 1



Structural overview of glycopeptide antibiotics. **(a)** Order of cyclization reactions and enzymes involved during the biosynthesis of A47934. Altered GPA structures of **(b)** bromobalhimycin, produced by supplementation of the media with bromine salts, and **(c)** an octapeptide, produced by an engineered NRPS *Amycolatopsis balhimycina* mutant.

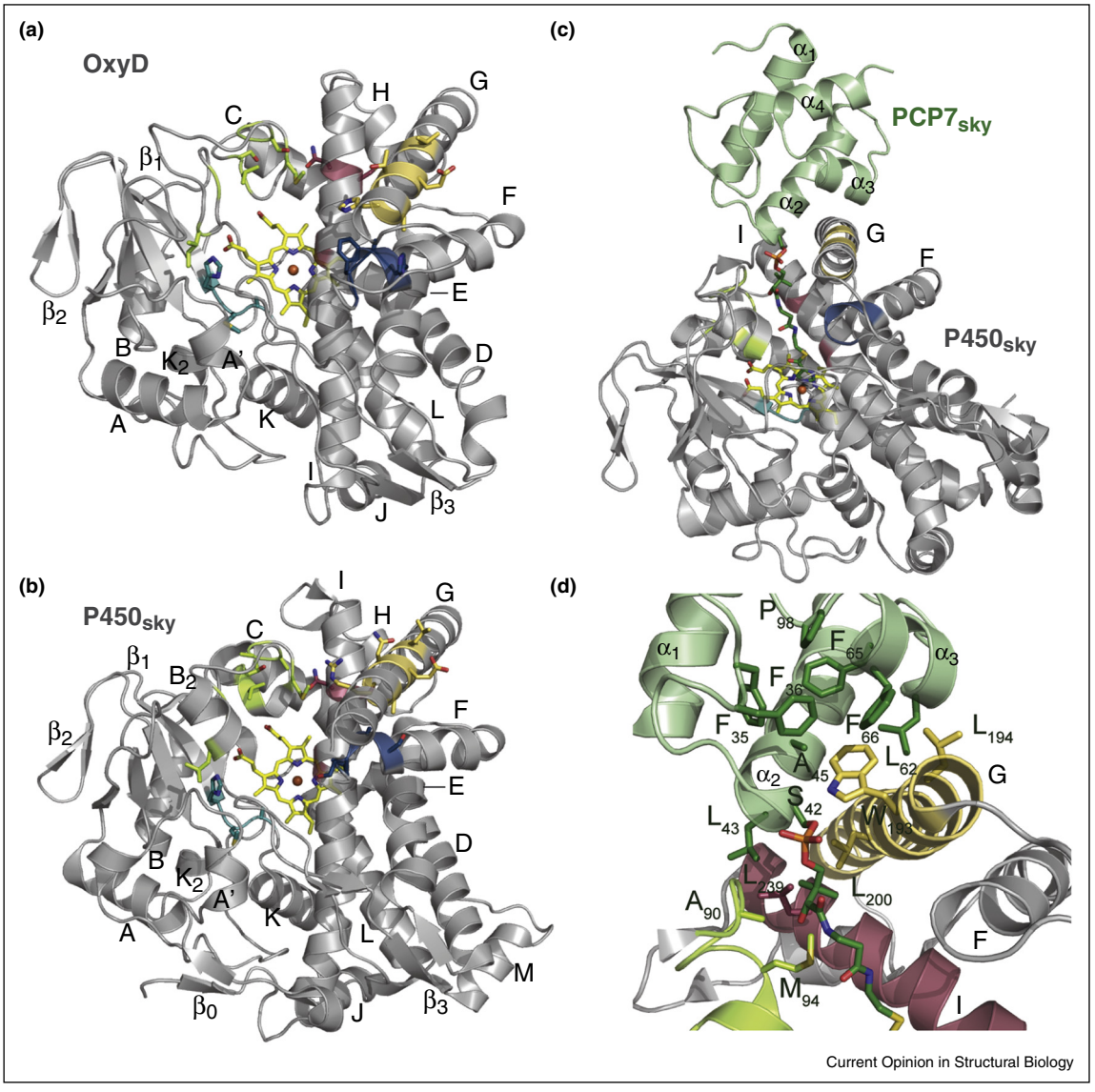
involves a separate NRPS module that generates *aminoacyl*-PCP (Tyr-PCP) substrate for this P450, OxyD [4••]. The structure of OxyD, whilst conforming to the canonical P450 fold, displays some unusual features: in particular a resolved B-C loop, which is typically unfolded in the absence of substrate [16•]. Biochemical data showing that the PCP-domain was the main source of OxyD selectivity led to the hypothesis that this structural arrangement was generating the required PCP-binding surface to allow complex formation (Figure 2) [16•]. Sequence alignments with other *aminoacyl*-PCP oxidizing P450s confirmed that many residues in this hypothesized interaction surface were highly conserved, supporting this hypothesis [16•].

Structural comparison of a related P450 responsible for *aminoacyl*-PCP oxidation from skyllamycin biosynthesis showed a highly similar tertiary structure to OxyD in spite of the low identities of the PCP binding partners (Figure 2) [17•]. In contrast to OxyD, $P450_{sky}$ selectively oxidizes specific PCP-bound AAs within the main NRPS machinery: despite this major mechanistic difference, the PCP-binding interface remains essentially the same as for OxyD [17•,18]. Structural characterization of $P450_{sky}$ in trapped complex with a skyllamycin PCP-domain was a further important step into understanding how such PCP binding is mediated (Figure 2), which revealed a different CP binding site to that of the only other example of such a complex solved to date (the $P450_{BioI}$-ACP complex) [19••,20••]. In contrast to the $P450_{BioI}$-ACP complex, the majority of interactions are hydrophobic in nature, with hydrophobic pockets from both PCP and P450 serving to bind complementary residues from the protein partner [19••,20••]. Also, as the only PCP-bound P450 complex solved to date, this structure has also proved highly valuable to model PCP binding to GPA cyclization P450s.

Peptide cyclization

$OxyB_{van}$ is the initial peptide cyclization enzyme from vancomycin biosynthesis and was the first structurally and functionally characterized GPA cyclization enzyme [12••,21]. In the structure of $OxyB_{van}$, the active site is highly solvent exposed as the F and G helices are held open above the active site; the structure is even more open than those of the *aminoacyl*-PCP oxidases [21]. Flexible loops around the active site were not resolved in the structure of $OxyB_{van}$ and thus the question of peptide orientation is difficult to assess, with peptide soaking experiments not successful to date. Following on from

Figure 2



Structures of the P450s OxyD **(a)** and $P450_{sky}$ **(b)** involved in hydroxylation of PCP-bound amino acids, with residues postulated to be involved in the PCP-binding interface shown in color; structure of the complex of an inhibitor bound PCP domain and $P450_{sky}$ showing the positioning of the PCP domain **(c)** and a close up view focusing on the major hydrophobic interaction interface between the two proteins **(d)**.

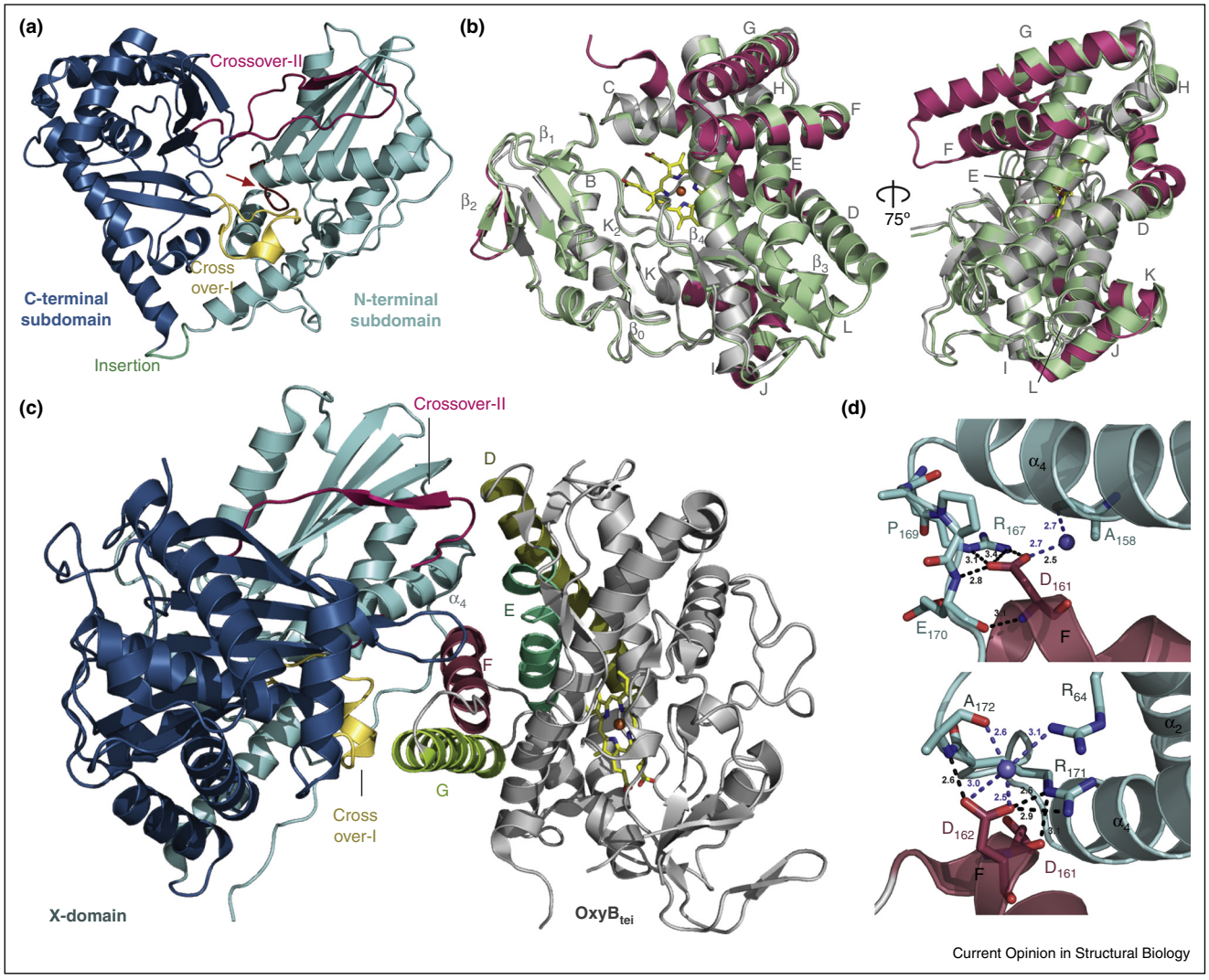
in vivo experiments, PCP substrates were tested and shown to be greatly preferred substrates for $OxyB_{van}$ over free peptides [9–11,12••]: this established the paradigm that GPA cyclization P450 catalysts accept *peptidyl*-PCP substrates, although work on other systems combined with the lack of progress in establishing subsequent steps in peptide cyclization were a clue that our understanding of peptide cyclization in GPA biosynthesis was not complete [22].

X-domain driven recruitment

The X-domain

A unique feature of the terminal module of all GPA producing systems is an additional domain between the terminal PCP and Te-domains, known as the X-domain [1,23]. Phylogenetic analysis showed that the X-domain is related to an $^{L}C_{L}$ type condensation domain, although no function in peptide synthesis could be assigned [24]. Furthermore, the conserved catalytic motif of C-domains is altered from H**H**xxxD**G** to H**R**xxxD**D**, likely rendering this domain inactive. The structure of the X-domain demonstrates the effects of these mutations [25••], whilst confirming that the X-domain indeed conforms to a C-domain type fold (Figure 3): this is described by two structurally similar subdomains that belong to the chloramphenicol-acetyltransferase superfamily fold connected at the bottom of their V-shaped arrangement. In

Figure 3



Structure of the X-domain from teicoplanin biosynthesis conforms to that of a condensation domain **(a)**; a comparison of the structures of OxyB from teicoplanin biosynthesis **(b)** both isolated (green) and in complex with the X-domain (gray with altered regions shown in magenta); structure of the complex of the X-domain and OxyB from teicoplanin biosynthesis **(c)**; and crucial interactions between the X-domain and the aspartate residues of the OxyB PRDD motif **(d)**.

comparison to other C-domain and E-domain structures [26•,27,28,29•,30,31], at least five additional residues extend this connection, whilst contact between subdomains is established by two crossover regions originating from the C-terminal subdomain: region I (known as the floor) and II (known as the latch). The altered active site of the X-domain is located in the V-shaped cleft, with residues R141 and D146 blocking the expected binding site of the PCP donor-Ppant arm during peptide bond formation. In addition, R141 forms a salt bridge to E391 in the C-terminal subdomain, which closes off the active site. Comparison to the C-domain of the AB3403 NRPS terminal module — in which the C-domain interacts with the *holo*-PCP domain — demonstrates that R23 from α1-helix of the X-domain also would block the entry of the Ppant-linker from the acceptor PCP side [26•]. Structural characterization therefore demonstrates that the X-domain is unable to play a role in peptide elongation: thus, the role of this conserved domain continued to be a mystery.

P450 recruitment mediated by the X-domain

Once binding interactions between GPA cyclizing P450s and the X-domain were revealed [25••], a potential role for the X-domain in peptide cyclization became clear. Activity assays indicated that all Oxy enzymes tested — except OxyB$_{van}$ — required the X-domain to catalyze even moderate levels of *peptidyl*-PCP cyclization [25••]:

crucially, this included the first reports of GPA peptide bicyclization catalyzed by an OxyA homologue [25••,32•,33••]. $OxyB_{van}$ is clearly an outlier in GPA cyclizing P450s: recent results have shown that although $OxyB_{van}$ can catalyze peptide crosslinking when substrates are presented solely by a PCP-domain, the binding affinity and reaction velocity is dramatically increased in the presence of the X-domain [11,13,25••,32•,33••,34]. Whilst the role of the X-domain is likely conserved *in vivo*, the properties of $OxyB_{van}$ make it an important target for use as an *in vitro* catalyst.

The nature of the P450/X-domain interaction was identified once a structure of OxyB in complex with the X-domain (both from the teicoplanin system) was resolved (Figure 3) [22,25••]. This structure demonstrated that hydrogen bonding and salt bridges dominate the interaction between the proteins, and that only minor changes occur to their structures in complex (RMSD 0.9 Å — X-domain alone, 1.1 Å — $OxyB_{tei}$ alone). $OxyB_{tei}$ binds to the X-domain via residues in the F-, G-, D- and E-helices, thus presenting its active site towards the donor side of the X-domain. This orientation leaves space for the *peptidyl*-PCP substrate, which would be located at the X-domain donor side due to its position N-terminal of the X-domain within the NRPS module.

The X-domain interaction interface involves both crossover regions between subdomains as well as the α-4 helix and subsequent loop region. To date, only X-domain mutants with substitutions in the loop region (R167A, R171A) have been shown to be essential for both P450 interaction and peptide crosslinking (Figure 3) [25••,33••]. This region interacts with the PRDD motif found at the N-terminal part of the $OxyB_{tei}$ F-helix; such a motif is also present in the same position in other structurally characterized Oxy enzymes [21,35–38], which is conserved amongst the GPA crosslinking P450s and appears to be a fingerprint for X-domain mediated recruitment.

Comparison of the structure of $OxyB_{tei}$ isolated and in complex with the X-domain shows that the main changes in the P450 are located in the F-G helices, which move slightly down towards the I-helix as a consequence of X-domain binding (Figure 3) [22,25••]. Similar to the $OxyB_{tei}$ structure, the majority of the B-C-region involved in substrate binding remains unresolved: this serves to demonstrate that the Oxy/X-domain complex is a recruitment complex and not a substrate-bound complex, which would require the presence of *peptidyl*-PCP. Although a PCP/Oxy structure has not yet been achieved, the OxyB/X-domain complex combined with the $PCP/P450_{sky}$ structure allows a model of the expected PCP-domain position for the GPA crosslinking P450s to be generated [25••]. However, alterations in this model for different GPA crosslinking P450s are to be expected due to the different positions of the crosslinks that these P450s introduce into the peptide. The recent structure of $OxyA_{tei}$ also indicated that the active site of these P450s can vary significantly between the different catalysts, with OxyA displaying far more hydrophilic active site environments to those of OxyB/C (Figure 4) [21,22,35,37].

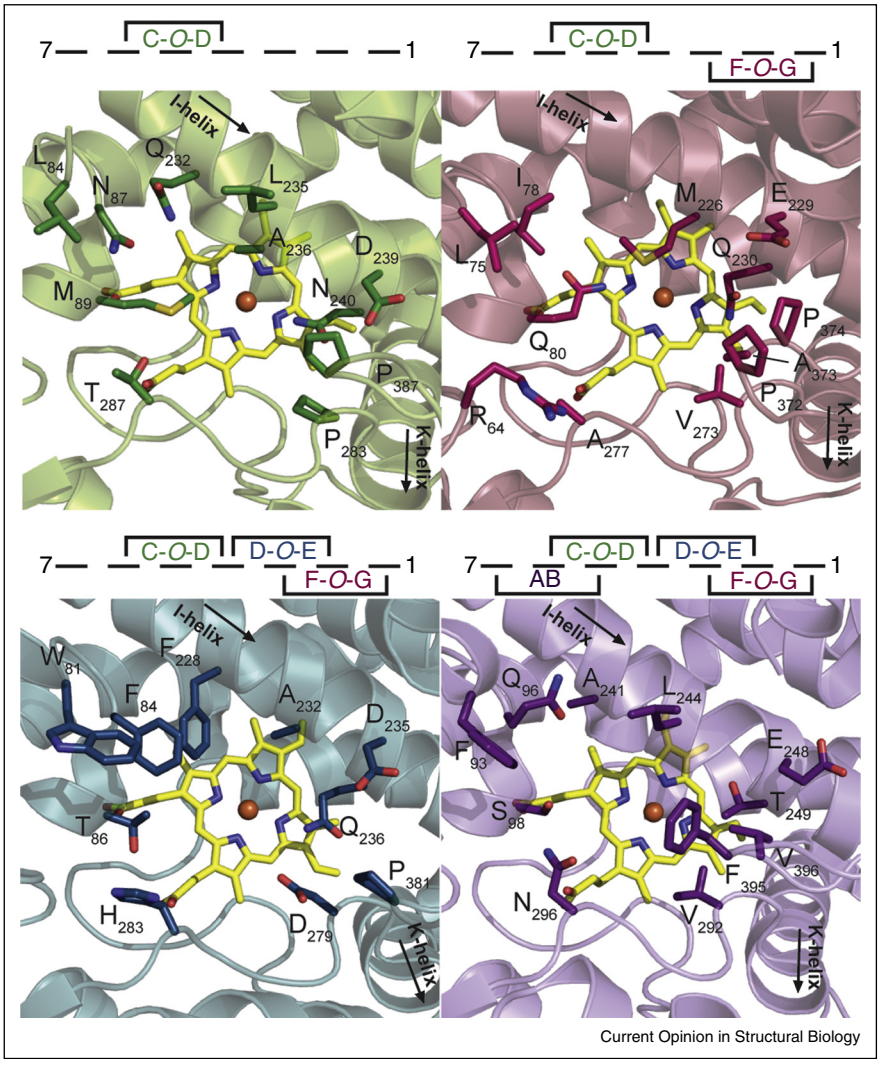
Why an X-domain?

In GPA biosynthesis, the X-domain efficiently connects NRPS-catalyzed peptide assembly with peptide modification by up to four tailoring enzymes in *trans*, all of which must occur prior to cleavage of the peptide from the NRPS machinery. In this context, the conserved 'PRDD' motif in the F-helix of all GPA crosslinking P450 enzymes but absent in other related P450s, for example, OxyD, may function as a preselection motif during X-domain recruitment that improves the efficiency of the peptide modification process. In contrast to other P450 enzymes with PCP-bound substrates, the GPA crosslinking cascade requires the efficient selection of the peptide substrate on the same PCP-domain by multiple, highly related P450s. Recent results indicate that whilst GPA crosslinking state is selected for by the P450s, the X-domain provides the platform for a constant shuffling of the GPA cyclization P450s, which appears to maintain a high enough rate of peptide cyclization so as not to impede peptide biosynthesis by the NRPS [33••]. Analysis of GPA terminal modules indicate that the X-domain was likely to have been a C-domain of the subsequent module in the ancestor NRPS to that producing GPAs [24,39], which has evolved into a P450 recruitment platform for GPAs (an intact, accessible C-domain active site could both inhibit P450 binding and cause peptide hydrolysis from the PCP-domain; this suggests the X-domain active site alterations are highly important). As no other NRPS systems have been reported to contain an X-domain, it appears to be an essential part of the unique GPA cyclization process in coordinating multiple *trans* interacting proteins towards one PCP-bound peptide substrate.

Structural implications for the role of GPA P450s *in vivo*

Previously, attempts were made to alter the structure of the GPA aglycones via biosynthesis (Figure 1): this included the replacement of chlorine substituents with bromine by precursor-directed biosynthesis [40] or the feeding of fluorinated β-hydroxytyrosines and various phenylglycines to GPA mutant strains employing mutasynthesis [41•,42]. Gene inactivation experiments also afforded GPA-like compounds with distorted aromatic side-chain bridging: such compounds lost antibacterial activity [8••]. Finally, manipulation of the balhimycin NRPS from a heptamodular to an octamodular assembly line by module insertion [43] was able to successfully generate an octapeptide: however, concomitantly the P450 monooxygenase processing was almost completely abolished. Furthermore, while the efforts to synthesize precursor compounds and to reprogram the biosynthesis were enormous, in many cases

Figure 4



Structural comparisons of the amino acids in the vicinity of the active site heme moieties of $OxyB_{tei}$ (green), $OxyA_{tei}$ (red), $OxyE_{tei}$ (blue) and $OxyC_{van}$ (purple), revealing significant differences between these P450s.

a significant drop in yields of the corresponding GPAs was observed. The structural findings on the X-domain as a platform for GPA cross-linking by P450s have shown that NRPS processing is a complex process requiring a sophisticated interplay between enzyme domains [25••]. A future goal, that is, reprogramming of the biosynthesis in order to generate new GPA backbones, will require a deeper understanding of the interaction and interplay of NRPS domains. Alternatively, restoring the NRPS-independent oxidative power of P450 monooxygenases from NRPS could bring researchers much closer to generating new glycopeptide antibiotics.

Conclusions

The structural characterization of the Cytochrome P450 enzymes responsible for GPA cyclization and related processes has greatly improved our understanding of how the protein-bound substrates of these P450s are selected and the role that these protein-protein interactions play in this process. The characterization of substrate complexes of these P450s is now a high priority, as this will not only illuminate the mechanism of these enzymes but will also reveal the origin of their substrate specificity: this information will be vital to the future success of reprogramming efforts to produce novel GPAs.

Conflict of interest statement

Nothing declared.

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