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STRUCTURAL ENZYMOLOGY OF POLYKETIDE SYNTHASES

Shiou-Chuan (Sheryl) Tsai^{*} and Brian Douglas Ames[†]

^{*}Department of Molecular Biology and Biochemistry, Department of Chemistry, Department of Pharmaceutical Sciences, University of California, Irvine, California, USA

[†]Department of Molecular Biology and Biochemistry, Department of Chemistry, University of California, Irvine, California, USA

Abstract

This chapter describes structural and associated enzymological studies of polyketide synthases, including isolated single domains and multidomain fragments. The sequence–structure–function relationship of polyketide biosynthesis, compared with homologous fatty acid synthesis, is discussed in detail. Structural enzymology sheds light on sequence and structural motifs that are important for the precise timing, substrate recognition, enzyme catalysis, and protein–protein interactions leading to the extraordinary structural diversity of naturally occurring polyketides.

1. INTRODUCTION

The past decade has witnessed significant advances in PKS structural biology for different types of PKSs that help visualize polyketide biosynthesis at all stages, including chain initiation, elongation, reduction, cyclization, and chain termination. Using x-ray crystallography or NMR, these studies help correlate PKS three-dimensional structures with substrate specificity and also help in elucidating the sequence–function–structure relationships that predict product outcome. With the recent publication of the striking porcine FAS crystal structure, we summarize advances in PKS structural biology during the past decade, and compare different types of FASs and PKSs, as well as their individual enzyme domains.

The major methods used to determine PKS protein structures are NMR and x-ray crystallography. With the current advances in NMR instrumentation, software, and pulse sequence development, the upper limit of protein molecular weight for a given target can be as high as 80 kDa (Redfield, 2004; Zeeb and Balbach, 2004). Various pulse sequences offer a further powerful probe for protein conformational change, as well as residues that are important for protein–protein interaction and protein–ligand binding (Redfield, 2004; Zeeb and Balbach, 2004). The application of these NMR methodologies to PKS enzymology is presented in section 4 on the structure of the acyl-carrier protein (ACP). The general methodology for x-ray crystallography includes protein expression/purification, crystallization, crystal harvesting, x-ray diffraction, data processing, structure solution, model refinement and model validation. While mammalian fatty acid synthase (FAS) architecture can be visualized by electron microscopy (EM) (Asturias *et al.*, 2005), PKS flexibility has resulted in highly variable conformations as detected by electron microscopy (Grant Jensen, personal communication), and such flexibility may also contribute to the relative difficulty in obtaining PKS megasynthase crystals (as compared to crystallizing the mammalian FASs). Nevertheless, the majority of single and didomain PKS fragments can be cloned into standard expression vectors (such as pET vectors), expressed in large quantity (up to 200 mg/l culture), and purified to more than 95% purity using Ni-affinity chromatography. The pure single and didomain PKS fragments can then be crystallized using standard vapor diffusion methods (Derewenda, 2004; McPherson, 2004; Rupp and Wang, 2004), followed by crystal harvest in cryoprotectant and cooling in liquid nitrogen (Pflugrath, 2004). Robotic automation of crystallization has

greatly accelerated the discovery of initial crystal leads (Bard *et al.*, 2004). Due to the high structural homology to FAS domains, many PKS domains have been solved by molecular replacement, using programs such as CNS (Brunger *et al.*, 1998) or the CCP4 suite (Xx, 1994), although heavy-atom methods are also applied using programs such as SOLVE (Terwilliger, 2004). Protein models are then built and refined by programs such as Coot (Emsley and Cowtan, 2004), further refined by programs such as CNS (Brunger *et al.*, 1998) or the CCP4 suite (Xx, 1994), and verified by programs such as PROCHECK (Laskowski *et al.*, 1993). As discussed in detail in Section 4, the conformational flexibility of many PKS domains renders the binding of enzyme inhibitors or substrates very useful to stabilize protein conformation for crystallization and improve diffraction data quality. However, the reactivity of these PKS enzyme domains sometimes also results in difficulty in the detection of substrate electron density maps. The above issues in the methodology of PKS structural enzymology, as well as the application and subsequent outcome of these methodologies, are discussed in details in Sections 3 and 4.

2. FATTY ACID SYNTHASE

The fatty acid synthase (FAS) is a multidomain protein complex (Maier *et al.*, 2006) consisting of seven conserved protein domains (MAT, KS, KR, DH, ER, ACP and TE) that catalyze more than 50 reactions *en route* to the final fatty acid product. FASs can be classified as type I or type II (Fig. 2.1A). The crystal structures of all type II FAS domains have been solved, including the type II KS, MAT, KR, ER, DH, ACP, and TE domains (Leesong *et al.*, 1996; Olsen *et al.*, 1999; Price *et al.*, 2001; Serre *et al.*, 1995), and White *et al.* have provided an excellent review of type II FAS structural biology (White *et al.*, 2005). The crystal structure of a type I FAS TE domain, as well as the NMR structure of a type I FAS ACP domain, have also been reported (Chakravarty *et al.*, 2004) and reviewed (Smith and Tsai, 2007). The recently published full-length mammalian type I FAS, solved to 3.2 Å, has greatly expanded our knowledge about the complicated domain–domain interactions in the megasynthase (Maier *et al.*, 2008) (Fig. 2.1B, C), showing a homodimer that confirms the head-to-head model based on electron microscopy (Asturias *et al.*, 2005) and biochemical results (Joshi *et al.*, 1997, 1998). The porcine FAS is separated into two portions: the lower condensing portion which contains the KS and MAT domains, and the upper chain-modifying portion which contains the DH, ER, and KR domains (Fig. 2.1B, C). Two additional nonenzymatic domains, termed ψ ME (an inactive methyltransferase) and ψ KR (a truncated KR), lie at the periphery of the upper portion (Fig. 2.1B, C). The central core of the X-shape architecture of type I FAS consists of KS, DH, and ER domains from both monomers, with an extensive dimer interface between KS, DH, and ER domains. The dimer is held together by KS–KS, DH–DH and KS–DH interactions. The head-to-head arrangement also implies that the ACP in either monomer (A or B) can interact with active sites of both monomers, consistent with previous biochemical studies (Joshi *et al.*, 1997; Witkowski *et al.*, 2004). The DH–KS interaction connects the top and bottom of the X-shaped architecture, and strongly suggests that the DH fold is important in maintaining FAS architecture. The AT and KR domains extend from the bottom and top of the protein, respectively, to form two asymmetric reaction chambers, one on either side of the central KS–DH–ER core. The flexible linker regions between KS–AT, AT–DH, DH–ER and ER–KR (Fig. 2.1C) facilitate the opening and closing of each reaction chamber, thus allowing the ACP-bound fatty acyl intermediate access to the active-site of each domain. Although there is no solid proof, the type II FAS is proposed to adopt a similar X-shaped architecture (Smith and Tsai, 2007) due to the high degree of conservation between type I and II FAS domains.

3. DIFFERENT TYPES OF PKS AND A SUMMARY OF STRUCTURAL WORK

Based on domain architecture, there are at least four distinct PKS types: modular type I, iterative type I, type II, and type III PKSs (Shen, 2003). Extensive progress has been made on the

structural biology of DEBS (the erythromycin PKS) (Khosla *et al.*, 2007), including crystal structures of KS3–AT3 (Tang *et al.*, 2007), KS5–AT5 (Tang *et al.*, 2006), KR1 (Keatinge-Clay and Stroud, 2006), DH4 (Keatinge-Clay, 2008), and TE (Tsai *et al.*, 2001), and an NMR structure of ACP2 (Alekseyev *et al.*, 2007) (the number following the domain name abbreviation indicates the module number), as well as KR1 of the tylosin PKS (Keatinge-Clay, 2007) and the TE domain from the pikromycin PKS (PIKS) (Akey *et al.*, 2006; Giraldez *et al.*, 2006; Pan *et al.*, 2002; Tsai *et al.*, 2002). An NMR structure of the intermodular linker region in DEBS has also been reported (Weissman, 2006). For the type II PKS, extensive progress has been made on the structural biology of *Streptomyces coelicolor* MAT (Keatinge-Clay *et al.*, 2003), actinorhodin (*act*) KS/CLF (Keatinge-Clay *et al.*, 2004), *act* KR (Hadfield *et al.*, 2004; Korman *et al.*, 2004, 2008), the R1128 priming ketosynthase ZhuH (Pan, *et al.*, 2002; Witkowski *et al.*, 2004), and the NMR structure of holo and apo *act* ACP (Crump *et al.*, 1997; Evans *et al.*, 2008), frenolicin (*fren*) ACP (Li *et al.*, 2003), and oxytetracycline (*otc*) ACP (Findlow *et al.*, 2003). The crystal structures of the tetracenomycin (*tcm*) ARO/CYC, the *whiE* ARO/CYC, and the ZhuI ARO/CYC have been solved (Ames *et al.*, 2008). Three fourth-ring cyclase structures have also been reported that are not included in this chapter (Kallio *et al.*, 2006; Sultana *et al.*, 2004; Thompson *et al.*, 2004). For iterative type I PKSs, the unpublished crystal structures of the PksA PT and TE domains are discussed briefly. The linker regions in type I PKSs have been reviewed elsewhere (Smith and Tsai, 2007; Weissman, 2006).

4. STRUCTURAL ENZYMOLOGY OF INDIVIDUAL DOMAINS

4.1. The acyltransferase

The crystal structures of DEBS AT3 and AT5, *S. coelicolor* MAT, *E. coli* MAT (FabD) and the porcine FAS AT domain have been reported (Keatinge-Clay *et al.*, 2003; Serre *et al.*, 1995; Tang *et al.*, 2006, 2007). All five structures are highly similar, with an RMSD of 1.3 to 1.9 Å. The AT structure has two domains: the larger core subdomain is similar to an α/β -hydrolase fold, with a parallel β -sheet flanked on two sides by α -helices (Fig. 2.2A in grey); the smaller subdomain, an insertion typically between residues 130 to 200, has a ferredoxin fold that consists of a four-stranded antiparallel β -sheet capped by two helices (Fig. 2.2A in black). The active site lies in a cleft formed between the two subdomains. Protein–protein docking simulations were conducted using the DEBS AT3 and ACP3 homology models (Tang *et al.*, 2007), although no apparent solution was obtained. The most important structural difference between the type I modular PKS AT domains and other MAT domains is that DEBS AT3 and AT5 have a much longer C-terminal helix (residues 857 to 867), presumably important for protein–protein interactions between AT and the KS-AT linker (Tang *et al.*, 2006, 2007). Based on enzymological studies of the type II FAS MAT domain, a ping-pong bi bi mechanism is proposed for both type I and type II ATs of FAS and PKS that involves the active site Ser and His (Fig. 2.2B) (Ruch and Vagelos, 1973), while the 3-carboxylate of malonyl- or methylmalonyl-CoA can form charge–charge interactions with the side-chain of a highly conserved Arg (Keatinge-Clay *et al.*, 2003; Rangan and Smith, 1997; Serre *et al.*, 1995). In the *S. coelicolor* MAT structure, the backbone amides of Gln9 and Val98 were identified as the oxyanion hole (Keatinge-Clay *et al.*, 2003). Remarkably, for both type I and type II AT domains, the acyl-enzyme complex is stable enough for detection (Dreier *et al.*, 2001; Lau *et al.*, 2000; Liou *et al.*, 2003; Szafranska *et al.*, 2002). The deacylation of acyl-enzyme occurs only in the presence of specific thiol nucleophiles such as phosphopantetheinylated ACP. This is a unique behavior among structurally related enzymes with an α/β hydrolase fold (Serre *et al.*, 1995). In addition, based on the crystal structures of all five AT domains, the hydrophobic nature of the substrate-binding pocket should presumably discourage water binding in the cleft.

For type I modular PKSs, the loading AT domain is promiscuous and can accept acetyl-, propionyl-, isopropionyl-, isobutyryl-, crotonyl-, phenylacetyl-, hydroxybutyryl-, and

isopentyl-CoA both *in vivo* and *in vitro* (Del Vecchio *et al.*, 2003; Hong *et al.*, 2005; Lau *et al.*, 1999, 2000; Liou and Khosla, 2003; Liou *et al.*, 2003). On the other hand, the AT domains in the extender modules (such as DEBS modules 1 to 6) are highly specific toward (2S)-methylmalonyl-CoA and the (2S)-methylmalonyl thioester of N-acetylcysteamine (NAC) (Lau *et al.*, 1999; Liou and Khosla, 2003; Marsden *et al.*, 1994). Therefore, the extending AT in DEBS serves as an important gatekeeper in macrolide biosynthesis (Khosla *et al.*, 1999). Past biochemical and structural work identified four motifs to explain the observed AT specificity (Fig. 2.2A): (1) the “RVDVVQ” motif lies 30 residues upstream of the active-site Ser (Haydock *et al.*, 1995; Yadav *et al.*, 2003); (2) the GHSXG motif around the catalytic Ser (Haydock *et al.*, 1995; Yadav *et al.*, 2003); (3) the YASH motif 100 residues downstream of the active-site Ser (Haydock *et al.*, 1995; Reeves *et al.*, 2001), which based on a systematic mutational analysis of DEBS AT domains is the dominant substrate specificity motif in type I modular PKSs among motifs 1, 2, and 3 (Reeves *et al.*, 2001); and (4) the C-terminal region shown to be important for substrate specificity from domain swapping experiments (Lau *et al.*, 1999). A detailed review of the above four motifs can be found in reference (Smith and Tsai, 2007). The recent 3.2-Å porcine FAS structure further demonstrates that F682 (part of Motif 1) and F553 (part of Motif 3) form a hydrophobic cavity, which may allow M499 to flip in and out to accommodate both methylmalonyl and malonyl moieties. In conclusion, the specificity between malonyl- versus methylmalonyl-CoA (or methylmalonyl- vs. propionyl-CoA) is likely to be a combinatorial result of different structural elements that interact throughout the entire protein fold, rather than an influence of a limited number of residues.

4.2. The ketosynthase

The crystal structures of DEBS ketosynthase (KS) 3 (Tang *et al.*, 2007) and KS5 (Tang *et al.*, 2006), the actinorhodin KS/CLF (*act* KS/CLF) (Keatinge-Clay *et al.*, 2004) and the R1128 priming KS (ZhuH) (Pan *et al.*, 2002) will be compared with those of the type III PKS (Austin and Noel, 2003), type I (the porcine FAS) (Maier *et al.*, 2008), and type II (the *E.coli* FabH, FabB, and FabF) KS domains (Olsen *et al.*, 1999; Price *et al.*, 2003; Qiu *et al.*, 2005). All KS crystal structures from FASs and PKSs reveal a highly similar thiolase fold (Austin and Noel, 2003), consisting of two copies of α - β - α - β - α folds that form a five-layered core (2α - 5β - 2α - 5β - 2α): three layers of α -helices interspersed by two layers of β -sheet, with extensive connecting loops. A pseudo-twofold axis lies between N α 3 and C α 3 parallel to the dimer axis. The KSs can be divided into three subfamilies: (1) KAS I and II, including FabB, FabF, DEBS KS3 and KS5, the porcine FAS KS domain, and the *act* KS/CLF (Keatinge-Clay *et al.*, 2004; Maier *et al.*, 2008; Olsen *et al.*, 1999; Price *et al.*, 2003; Tang *et al.*, 2006, 2007); (2) KAS III and the CHS-like type III PKS enzymes, including the priming KS ZhuH (Pan *et al.*, 2002) from the R1128 PKS; (3) the biosynthetic and degradative thiolases. All three subfamilies conserve the core structural features, the extensive dimer interface, and the location of the active-site residues. There is also absolute conservation of the active-site Cys for covalent attachment of substrates and intermediates. However, there are major structural differences among the three subfamilies, mainly concerning the extent and structure of the loops on the opposite side of the substrate-binding pocket. These loops affect the position and identity of key catalytic residues (except the universally conserved Cys) as well as the different substrate chain-length specificities for CoA-linked or ACP-linked thioesters (Austin and Noel, 2003). The KSs are dimers with an extensive dimer interface stabilized by a pair of hydrogen-bonded, antiparallel β -strands, which creates a 14-stranded β -sheet spanning both monomers. In the DEBS KS5 structure, there is a long helix at the N-terminal that may facilitate KS dimerization and serve as a docking point for the upstream C-terminal docking domain of the previous module 4 (Tang *et al.*, 2006). In the type I FAS and PKS, KS deletion experiments show that the dimeric nature of the KS domains is key in facilitating dimerization of the entire megasynthase (Witkowski *et al.*, 2004).

Similarly to the well-studied FabB and FabF KSs of *E. coli*, the extending KSs, including DEBS KS3 and KS5 and *act* KS/CLF, employ a Cys-His-His triad at the active center. The active-site triad and oxyanion hole of DEBS KS3 and KS5, *act* KS/CLF and porcine FAS KS domains can be overlapped perfectly, suggesting a similar catalytic mechanism (Witkowski *et al.*, 2002; Zhang *et al.*, 2006), initiated by the docking of acyl-ACP to an electropositive patch of KS. The proposed mechanism is similar between the priming (ZhuH) and extending (DEBS KS3 and KS5 and *act* KS/CLF) KSs, except that the catalytic triad of a priming KS consists of His-Asn-Cys. Furthermore, acyl-CoA, rather than acyl-ACP, first binds to ZhuH. The Asn versus His difference between the priming and elongation KSs helps explain why inhibitors such as cerulenin and thiolactomycin preferentially bind the elongation KSs (White *et al.*, 2005).

Despite a similar thiolase fold and enzyme mechanism, the specificity of type I and II FAS and PKS KSs varies significantly. The type I FAS KS domains are highly specific toward saturated acyl chains (Witkowski *et al.*, 2002). In contrast, type I modular KS domains, such as the six KS domains in DEBS, have a wide range of substrate specificities that vary in length from diketide to decaketide, although some PKS KS domains appear to possess some specificity with regard to different β -carbon status (Khosla *et al.*, 1999). The type II systems possess highly specific chain-length control that is pathway-specific for the priming and extending KSs. Examples include the C2–C4 priming preference of ZhuH (Pan *et al.*, 2002; Qiu *et al.*, 2005), or the *act*, *tcm*, and *whiE* KS/CLF which extend the polyketide chain to 16, 20, and 24 carbons, respectively (Tang *et al.*, 2003). The observed substrate specificity of each KS domain can be explained by the size and shape of the KS substrate-binding channel, which can be divided into two halves, corresponding to the substrate-binding and PPT-binding regions (Fig. 2.3). The PPT-binding region stretches from the enzyme surface to the active-site Cys and this region is relatively well conserved, reflecting its universal role in binding the PPT moiety. In contrast, the acyl-binding region varies significantly. While the binding pockets of FAS KS domains are hydrophobic and promote fatty acyl binding (Maier *et al.*, 2008; Olsen *et al.*, 1999; Price *et al.*, 2003; Qiu *et al.*, 2005), the acyl-binding pockets of PKS KS domains (such as DEBS KS3 and KS5, *act* KS/CLF and ZhuH) are amphipathic and allow hydrogen-bonding interactions with the carbonyl groups of the growing polyketide chain (Keatinge-Clay *et al.*, 2004; Pan *et al.*, 2002; Tang *et al.*, 2006, 2007). The FAS KS domains have a hydrophobic, narrow pocket of a suitable size to specifically accommodate their corresponding fatty acyl substrates (Fig. 2.3B) (Maier *et al.*, 2008; Olsen *et al.*, 1999; Price *et al.*, 2003; Qiu *et al.*, 2005), whereas the substrate pockets in DEBS KS3 and KS5 are much wider (Fig. 2.3F) (Tang *et al.*, 2006, 2007), consistent with the substrate tolerance reported for type I modular PKS KS domains. In contrast, both priming and extending KSs of the type II PKS are more substrate-specific, reflected by the narrower acyl-binding pocket, similar to those in FAS KS domains. Significantly, mutations of four residues that define the bottom of the acyl pocket in *act* and *tcm* KS/CLF confirmed that the pocket size and shape indeed control polyketide-product chain length (Tang *et al.*, 2003), with mutations in CLF being sufficient to alter chain length.

4.3. The ketoreductase

Three ketoreductase (KR) crystal structures have been reported: for type I modular PKSs, crystal structures of the DEBS KR1 (EryKR1) (Keatinge-Clay and Stroud, 2006) and KR1 of tylosin PKS (TyIKR1) (Keatinge-Clay, 2007) have been solved (Fig. 2.4A–B) and for type II PKS, the actinorhodin KR (*act* KR) crystal structure has been reported (Hadfield *et al.*, 2004; Korman *et al.*, 2004, 2008) (Fig. 2.4D). EryKR1 and TyIKR1 reduce a diketide substrate C=O to C-OH with “2R, 3R” and “2R, 3S” stereochemistry, respectively, so these two KRs choose opposite diketide epimers (at the 2 position) for the reduction reaction. In contrast, the actinorhodin KR (*act* KR) specifically reduces the C9 carbonyl group of a 16-carbon (octaketide) preassembled polyketide chain, which folds into the C7–C12 first-ring cyclized

shunt-product mutactin when expressed without downstream enzymes. The ketoreduction catalyzed by *act* KR, as well as by other type II PKS KR domains, is chemically identical to the corresponding fatty acid ketoreduction, yet with very different regio-specificities (O'Hagan, 1993).

In both FASs and PKSs, the type I KR has two domains with the same protein fold: the catalytic subdomain and a truncated, noncatalytic structural subdomain. Both EryKR1 and TylKR1 are monomeric in solution and in crystal structure (Keatinge-Clay, 2007; Keatinge-Clay and Stroud, 2006). In contrast, the type II KR exists as a tetramer (Fig. 2.4) (Hadfield *et al.*, 2004; Korman *et al.*, 2004), and each monomer contains a single domain. Each domain (or each subdomain in type I KR) contains a short-chain dehydrogenase/reductase (SDR) fold consisting of a highly conserved Rossmann fold with two right-handed α - β - α - β - α motifs connected by α 3, and the core region consists of a seven-stranded β -sheet flanked by α -helices (Fig. 2.4). The cofactor NADPH is bound at the junction of two α - β - α - β - α motifs in a highly conserved groove characteristic of the Rossmann fold (Persson *et al.*, 2003). The polyketide substrate-binding pocket consists of a large cleft formed by helices α 6- α 7 and the loops between α 4 and α 5 (Fig. 2.4). The catalytic subdomains of EryKR1 and TylKR1, as well as *act* KR, have the typical SDR motifs, such as the TGxxxGxG motif (residues 2 to 19), the D63 and NNAG motifs (residues 89 to 92), the active-site tetrad Asn-Ser-Lys-Tyr, and the PG motif (residues 187 to 188) (Persson *et al.*, 2003). The biggest difference between the type I and II PKS KRs is a long insertion between helices 6 and 7 for *act* KR, and this may account for the different substrate specificities of type I and II PKS KRs. The monomeric type I KR orients its two subdomains (Fig. 2.4A-B) very similarly to a type II KR dimer (Fig. 2.4D), with extensive, mainly hydrophilic, interactions. The structural subdomains in EryKR1 and TylKR1 lack a cofactor-binding motif and the substrate-binding portion, thus rendering the structural subdomain inactive (Keatinge-Clay, 2007; Keatinge-Clay and Stroud, 2006). The type I KRs have additional β 1- β 8 and α 4- α F interactions bridging the structural and catalytic subdomains and stabilizing the pseudodimeric KR protein fold.

The *act* KR active-site tetrad consists of N114-S144-Y157-K161 (42 to 44). In contrast, the Asn and Lys positions are switched in EryKR1 and TylKR1 (K1776-S1800-Y1813-N1817) (Keatinge-Clay, 2007; Keatinge-Clay and Stroud, 2006). The KR tetrad lies near the nicotinamide ring of NADPH, where Tyr and Lys form hydrogen bonds with the NADPH ribose and nicotinamide ring. In *act* KR, four crystalline water molecules form extensive hydrogen bonds with N114 and K161 (Fig. 2.5A). These waters form a proton-relay network that is very similar to the one observed in *E. coli* FabG-NADP⁺ (Price *et al.*, 2004), leading to the hypothesis that the water-relay mechanism for FabG may also be applicable to *act* KR (Fig. 2.5A). *In vitro* assays indicate that type II PKS KRs have a different substrate specificity from that of FAS (Dutler *et al.*, 1971; Joshi and Smith, 1993) and modular type I PKS KRs (Ostergaard *et al.*, 2002), both of which can reduce linear and monocyclic ketones. The strong preference of *act* KR toward bicyclic polyketides supports the conclusion that its natural substrate is a cyclic polyketide, and detailed kinetic analysis showed that *act* KR proceeds through an ordered bi bi mechanism (Korman *et al.*, 2008), in which the cofactor NADPH binds KR prior to the substrate *trans*-1-decalone. The above results imply that the first ring is cyclized prior to ketoreduction, thus sterically constraining the ketoreduction and leading to a highly specific C9-reduction. The inhibitor emodin-bound *act* KR structure also revealed that *act* KR can exist with at least two different conformations (Korman *et al.*, 2008): open and closed forms that differ in the 10-residue loop region (residues 199 to 209) between helices 6 and 7 (Fig. 2.5B). EryKR1 and TylKR1 also show similar conformational changes and loop movement in this region (Keatinge-Clay, 2007; Keatinge-Clay and Stroud, 2006), which may reflect different binding motifs during ketoreduction (Keatinge-Clay, 2007; Korman *et al.*, 2008).

The stereoselective signature motifs for the modular PKS KRs were previously proposed to be “LDD” and PxxxN (Caffrey, 2003; Reid *et al.*, 2003), and the presence or absence of these motifs produce the 3R or 3S stereomer, respectively. The EryKR1 crystal structure shows that the 93–95 “LDD” motif lies in a loop adjacent to the active site (Keatinge-Clay and Stroud, 2006), and KR1 mutation indeed resulted in a switch of alcohol stereochemistry (Baerga-Ortiz *et al.*, 2006; O’Hare *et al.*, 2006). The 2-position stereochemistry is also affected by KR and its upstream KS domain. Based on extensive bioinformatic analysis guided by the crystal structures of EryKR1 and TylKR1, Keatinge-Clay categorized the type I KRs into six types to explain their observed stereochemistry at the 2- and 3-positions and developed a protocol to assign substituent stereochemistry accordingly (Keatinge-Clay, 2007). Similar studies on type II KRs (Korman *et al.*, 2008) support the hypothesis that type II KR substrate specificity is defined by a combination of enzyme conformation, specific molecular interactions between the substrate and active-site residues, as well as substrate and protein flexibility due to the dynamic nature of the binding cleft.

4.4. The dehydratase of modular type I PKS

DEBS DH4 catalyzes dehydration of a 2R-methyl-3R-OH pentaketide to afford a *trans* double bond (Keatinge-Clay, 2008). The recent report of the 1.8-Å DEBS DH4 crystal structure, combined with the porcine FAS structure, reveals that the DH domain in type I PKSs and FASs consists of two subdomains with limited sequence homology, yet each subdomain consists of the “hot-dog-in-a-bun” fold (Dillon and Bateman, 2004). The double hot-dog (DHD) fold exhibited by type I FAS and PKS DHs is similar to that of the dimeric type II bacterial DHs (such as *E. coli* FabA and FabZ (Kimber *et al.*, 2004; Leesong *et al.*, 1996)), consisting of one hot-dog fold per monomer (Fig. 2.6A–B). The *E. coli* and human TE II are also reported to contain the DHD fold (Li *et al.*, 2000). In each hot-dog subdomain, the long central helix—the hot-dog—is packed against a seven-stranded antiparallel β -sheet that forms the bun. In both the porcine FAS and DEBS DH4 structures, the two hot-dog subdomains of DH interact extensively to form a 14-strand β -sheet with additional interactions between the hot-dog helices from each subdomain. In DH4, the catalytic H44 lies in the first subdomain as part of the HXXGXXXXP motif (Joshi and Smith, 1993), where the conserved Gly is necessary to make a turn that enables van der Waals interactions between H44 and P53. The catalytic D206 within the DXXX(Q/H) motif lies in the second subdomain and hydrogen-bonds with the side chain of Q210 (itself anchored to Y158). The organization and interaction of catalytic residues described for DH4 are conserved in the porcine FAS DH. Additionally, both the “GYXYGPXF” and “LPFXW” motifs are highly conserved and help define the substrate pocket.

The reaction catalyzed by type I and II FAS DHs is freely reversible with equilibrium favoring hydration (Heath and Rock, 1995; Witkowski *et al.*, 2004). In type I PKS DHs, the equilibrium may also lean toward the hydrated polyketide, and a downstream KS or TE may pull the reaction forward toward the dehydrated polyketide (Tang *et al.*, 1998; Wu *et al.*, 2005). However, there is no obvious sequence motif associated with DHs that dehydrate substrates with or without α -substituents, indicating that the α -substituents may not be recognition factors for the PKS DHs. A catalytic mechanism was proposed for DH4 (Fig. 2.6D) in which H44 serves as the active-site base to deprotonate the α -carbon, while the β -hydroxyl group may be polarized by the helix-1 dipole, facilitating water elimination. The DH4 structure shows that the stereochemistry of the β -hydroxyl group in an incoming polyketide substrate may be the primary factor that determines if a *cis* or *trans* double bond is produced by the DH domain. For example, when an A-type KR provides DH with the 3S-OH substrate, the 3S-OH is hydrogen-bonded to D206 and the polyketide chain must rotate 120 degrees about the C α –C β bond. Thus, an elimination results in the formation of a *cis* double bond, as in the phoslactomycin DH1 and DH2 (Palaniappan *et al.*, 2008) or rifamycin DH10 (Tang *et al.*,

1998). Sequence comparison indicates that if DH does catalyze epimerization, the Leu and Pro in the “LPFXW” motif may be important (Keatinge-Clay, 2008). Further work is necessary to distinguish the above hypotheses.

4.5. The aromatase/cyclase (ARO/CYC) of type II PKS

Based on previous studies of first-ring cyclization, there are at least three different classes of ARO/CYC (Fig. 2.7A): (1) C9–C14 cyclization associated with monodomain ARO/CYC, such as *tcm* ARO/CYC, *WhiE*-ORFVI (*whiE* ARO/CYC), and *RemI* (Alvarez *et al.*, 1996; Fritzsche *et al.*, 2008; Moore and Piel, 2000; Motamedi and Hutchinson, 1987; Zawada and Khosla, 1997); (2) C7–C12 cyclization in the absence of KR by monodomain ARO/CYC, such as *ZhuI* from the R1128 biosynthetic pathway (Tang *et al.*, 2004), or didomain ARO/CYC, such as *MtmQ* from the mithramycin pathway (Lombó *et al.*, 1996; Zhang *et al.*, 2008) (3) the didomain ARO/CYC associated with KR-containing type II PKSs that aromatize C7–C12 first-ring cyclized polyketides, such as the actinorhodin (McDaniel *et al.*, 1995) and griseusin (Zawada and Khosla, 1997) ARO/CYC. The best-studied monodomain ARO/CYC is *tcm* ARO/CYC, which consists of the N-terminal 160 residues of the bifunctional protein, *TcmN* (McDaniel *et al.*, 1995; Zawada and Khosla, 1999). Following the production of the linear decaketide intermediate, *tcm* ARO/CYC is proposed to fold, cyclize and aromatize the ACP-tethered polyketide *via* aldol condensation and dehydration reactions (Shen and Hutchinson, 1996). Based on genetic analyses, it had been argued that the first cyclization of a linear polyketide chain may occur either in the active site of KS–CLF (Keatinge-Clay *et al.*, 2004), in solution (without enzyme catalysis) (Hertweck *et al.*, 2004), or in the binding pocket of KR or ARO/CYC (Zawada and Khosla, 1999). Similar observations on more than ten aromatic PKSs (except in enterocin, discussed below (Hertweck *et al.*, 2004)) have led to the general conclusion that KR promotes C7–C12 cyclization (Crump *et al.*, 1997; McDaniel *et al.*, 1994), whereas the monodomain ARO/CYC in nonreducing type II systems promote C9–C14 cyclization. The crystal structure and mutagenesis of *act* KR and *tcm* ARO/CYC support the hypothesis that cyclization in the KR or ARO/CYC substrate pocket may be a likely event. However, further validation is necessary.

Three ARO/CYC structures have been solved: the *tcm*, *whiE*, and *ZhuI* ARO/CYC, all three of which have a helix-grip fold (Gajhede *et al.*, 1996; Iyer *et al.*, 2001) consisting of a seven-stranded antiparallel β -sheet that partially surrounds (“grips”) a long C-terminal α -helix (Fig. 2.8). Two small helices between β 1 and β 2 form a helix-loop-helix motif that acts to seal one end of the β -sandwich. The topology of ARO/CYC is highly similar to that of members of the Bet v1-like superfamily (Iyer *et al.*, 2001; Radauer *et al.*, 2008), which commonly includes a large solvent-accessible pocket that binds small molecules such as phytosteroid hormones, lipids, enediyne, and cholesterol (Markovic-Housley *et al.*, 2003; Mogensen *et al.*, 2002; Pasternak *et al.*, 2005; Radauer *et al.*, 2008; Tsujishita and Hurley, 2000). However, unlike many Bet v1-like proteins with hydrophobic pockets, the ARO/CYC pocket is amphipathic with an approximately equal distribution of hydrophobic and hydrophilic residues. The ARO/CYC pocket dimensions and residue composition are appropriate for binding cyclized polyketide intermediates (Fig. 2.8E). Whereas the type II PKS ARO/CYC contains a helix-grip fold, all FAS DH (such as *E. coli* FabA) contain a hot-dog fold (Dillon and Bateman, 2004; Leesong *et al.*, 1996) (Fig. 2.8A–B) that is topologically different but similar in shape. In the hot-dog fold, the β -sheets have strand-order 1–2–3–5–6–7–4, whereas the strand-order is 1–7–6–5–4–3–2 for the helix-grip fold. Also, the central helix is tucked against the β -sheets in FabA, thus precluding formation of an interior pocket between the β -sheet and central helix α C. As multiple dehydration events are necessary in order to aromatize the rings formed during polyketide biosynthesis it has been suggested that PKS ARO/CYC may act as DHs to catalyze aromatic ring formation (Hopwood, 1997). That both ARO/CYC and DH catalyze dehydration

reactions in evolutionarily related complexes suggests that they may play similar biological roles implied by their similar topologies.

Based on site-directed mutagenesis, the *tcm* ARO/CYC crystal structure, and computer-simulated docking, a catalytic mechanism was proposed for *tcm* ARO/CYC (Ames *et al.*, 2008) in which the polyketide carbonyl oxygens are anchored in close proximity to S67, R69, Y35, and R82, and subsequent aldocyclization is promoted by electrostatic stabilization of pocket residues (with special attention to the possible involvement of the strictly conserved pocket residue, R69). The above proposal is further supported by the crystal structures of *whiE* and ZhuI ARO/CYC (Fig. 2.8C, D). The *whiE* ARO/CYC promotes C9-C14 first-ring and C7-C16 second-ring cyclizations of a 24-carbon polyketide, and ZhuI is the only reported monodomain C7-C12 first-ring-only cyclase (Marti *et al.*, 2000; Yu *et al.*, 1998). The overall structure and residue composition are similar between *whiE* and *tcm* ARO/CYCs, but subtle conformational and residue changes in the *whiE* ARO/CYC pocket increases the pocket space (compared to *tcm* ARO/CYC) to accommodate the binding of a C24 polyketide. In contrast, ZhuI has a much smaller pocket that accommodates a monocyclic C7-C12 cyclized intermediate (Fig. 2.8C, D).

There are two observed folding patterns of aromatic polyketides which generally lead to unique cyclization regio-specificities (Thomas, 2001). S-type folding, promoted by type II PKSs, leads to C7-12 or C9-C14 first-ring cyclization depending on whether KR is present. In contrast, F-type folding patterns, promoted by the produce template (PT) domain of fungal nonreducing type I PKSs (Crawford *et al.*, 2006, 2008; Udvary *et al.*, 2002), include C2-C7, C4-C9, and C6-C11 first-ring cyclization. The 1.8-Å crystal structure and mutational analyses of the PksA PT domain (manuscript in preparation) revealed that PT also has a DHD fold (Fig. 2.6C) and nearly all secondary structure elements are aligned with both DEBS DH4 and porcine FAS DH domain with only 3 Å of RMSD. Significantly, similarly to ARO/CYC, PT has an interior pocket (Fig. 2.6C), and the reported F-fold patterns may be directly related to their corresponding PKS PT domain, in which the cyclization pattern is determined by pocket shape, while chain length is correlated with pocket size. In conclusion, PT may bind a fully extended linear polyketide that is “kinked” in the cyclization chamber to promote the F-folded cyclization pattern, while the ARO/CYC likely bends the ACP-bound polyketide into a hairpin, thus promoting the S-folded cyclization pattern.

4.6. Acyl carrier protein

Recently, the first solved structure of an acyl carrier protein (ACP) from a type I modular PKS was reported for DEBS ACP2 (Alekseyev *et al.*, 2007). Similar to the type I FAS ACP structure, the 10-kD DEBS ACP2 contains a three-helical bundle, and an additional short helix in the second loop also contributes to core helical packing. The conserved Ser in the universal “DSL” motif (where PPT is covalently attached) lies at the N-terminal end of helix-2, which is regarded as a universal “recognition helix” involved in interactions with other proteins (Crump *et al.*, 1997; Findlow *et al.*, 2003; Li *et al.*, 2003; Reed *et al.*, 2003; Zhang *et al.*, 2003). Homology models of DEBS ACP domains (ACP1-6) (Alekseyev *et al.*, 2007) suggest that protein-protein recognition of ACP domains is highly specific for their corresponding KS domains (Chen *et al.*, 2006). Similar results were reported for type II PKS ACP domains, such as the act *apo*-ACP (Crump *et al.*, 1997), the *fren* holo-ACP and *otc* ACP (Findlow *et al.*, 2003; Li *et al.*, 2003). These results are consistent with the “switch blade” theory based on the yeast FAS crystal structure (Leibundgut *et al.*, 2008), in which the growing acyl chain (the blade) switches its nestling cavity from ACP to the KS (or other PKS enzymes) binding pocket, and the timing of blade switching is closely related to the degree of exposure between the polyketide intermediate and the solvent, which depends not only on the ACP “recognition helix” property, but also on the chemical structure of a given polyketide intermediate.

4.7. Thioesterase

Three PKS thioesterase (TE) structures have been reported: the DEBS TE (Tsai *et al.*, 2001), and the homologous pikromycin (PIKS) TE (Akey *et al.*, 2006; Giraldes *et al.*, 2006; Tsai *et al.*, 2002), and the PksA TE in a nonreducing iterative type I PKS from aflatoxin biosynthesis (manuscript in preparation). All three structures exhibit the classic features of the α/β hydrolase fold (Fig. 2.9A–B), which consists of a central seven-stranded β -sheet with the second strand (β_2) antiparallel to the remaining strands (Chakravarty *et al.*, 2004; Giraldes *et al.*, 2006; Tsai *et al.*, 2001, 2002). While the active-site triad (Ser-His-Asp) and nearly all important secondary structure components are highly conserved, substrate specificity and regio-specificity vary significantly among different TEs. The PKS TE domains lack the characteristic α D helix of the α/β -hydrolase family, instead having an inserted “lid” region that is consistently observed in the DEBS (Tsai *et al.*, 2001), PIKS (Akey *et al.*, 2006; Giraldes *et al.*, 2006; Tsai *et al.*, 2002), surfactin synthetase (*SrfA-C*) (Bruner *et al.*, 2002), fengycin synthetase (*FenB*) (Samel *et al.*, 2006), and enterobactin (*EntF*) (Frueh *et al.*, 2008) TE structures. The lid region is also the most variable region among the TE domains (Fig. 2.9C). Because the substrate-binding region of the megasynthase TE is formed between the α/β -hydrolase core and the lid region inserted between β_6 and β_7 , variability of the lid region is reflected in the highly variable substrate channel shape among different TEs. In the modular DEBS and PIKS TEs (Akey *et al.*, 2006; Giraldes *et al.*, 2006; Tsai *et al.*, 2001, 2002), an unusual, 20-Å long amphipathic substrate channel passes through the entire protein, implying passage of the substrate through the protein (Fig. 2.9C) with the catalytic triad that carries out macrolactonization of a hydrophilic polyketide substrate located at the center. In contrast, the PksA TE substrate pocket adopts a closed conformation, sealed from both ends of the channel, to turn the pocket into a sealed hydrophobic “cyclization chamber” and protect the polyketide substrate from hydrolysis. The flexibility of the lid region is evident when a series of PIKS TE structures solved from crystals at different pH values were compared (Tsai *et al.*, 2002), showing that the size of the substrate channel increases with increasing pH. The variable channel geometry is proposed to influence the regio-selectivity between the hydrolysis and macrocyclization/Claisen cyclization activities of type I PKS TEs. The crystal structures of DEBS and PIKS TE help rationalize the observed substrate specificity of modular PKS TEs (Giraldes *et al.*, 2006; Tsai *et al.*, 2001, 2002), and the chemical structures of the polyketide substrates are as important as the TE substrate residues in determining cyclization versus hydrolysis activity (Gokhale *et al.*, 1999; Lu *et al.*, 2002). Further work is necessary to fully determine residues important for PKS TE substrate specificity.

5. SUMMARY AND FUTURE PROSPECTS

Recent revelations from crystallographic analysis of type I and type II PKSs have raised awareness of the extraordinary architecture of these megasynthases and offered a new perspective in visualizing some of the unsolved questions concerning polyketide biosynthesis. The 3.2-Å porcine FAS crystal structure has provided a framework for megasynthase architecture that may also apply to type I PKSs (Maier *et al.*, 2008). Further, the DEBS KS-AT structures clearly show that the KS-AT and post-AT linkers are highly structured and closely interacting with both KS and AT domains, so that the linker regions contribute extensively to stabilization of the overall KS-AT structure (Tang *et al.*, 2006, 2007). Clearly, we need to reconsider the original notion that the linkers merely serve as semi-flexible tethers that hold adjacent domains in proximity (Gokhale *et al.*, 1999). In the future, studies of interdomain, intermodule, and interpolypeptide linker regions in type I PKSs should further determine their importance to dimer formation, polyketide chain transfer, and reaction timing. In the arena of type II PKSs, the detection of multienzyme complex formation should help shed light on how the ACPs gain access to each of the PKS enzyme domains. Because of the dynamic nature of this complex, x-ray crystallography, if successful at all, may only trap one snapshot

of such a transient complex, and other techniques such as electron microscopy or NMR may be necessary to capture a series of protein motions during different stages of polyketide biosynthesis. The early successful application of electron microscopy to capture the FAS dynamic structures (Asturias *et al.*, 2005) can serve as an excellent example for a similar study with type I and type II PKSs.

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Figure 2.1.

(A) Domain organization of type I and type II FAS. (B) Cartoon ribbon representation and (C) domain illustration of the 3.2-Å porcine type I FAS structure.

Figure 2.3.

The substrate binding channel of (A) the priming KS ZhuH, (B) the *E. coli* KAS I, (C) actinorhodin KS/CLF, (D) chalcone synthase, (E) porcine FAS KS domain, (F) DEBS KS5. The active site Cys is shown in *spheres*, and (G) The cyclization chamber of chalcone synthase (in green) compared to the linear polyketide-extending chamber in the act KS/CLF (in purple).

Figure 2.4. Crystal structures of (A) EryKR1, (B) TylKR1, (C) porcine FAS KR, (D) *act* KR dimer, (E) the *act* KR tetramer, colored as in (C), NADP in *spheres*. A to D are in the same orientation.

Figure 2.5.

(A) Proposed proton-relay mechanism for *act* KR. (B) The asymmetric unit contains monomers A (middle panel) and B (left panel) in open and closed conformations, respectively. Surface potentials are colored from negative (red) to positive (blue). When the open and closed conformations are overlapped (right panel, open in yellow, closed in purple), the major conformational change is in the flexible $\alpha 6$ – $\alpha 7$ region.

Figure 2.6.

Comparison of the double hot dog-fold and interior pocket of (A) DEBS DH4, (B) porcine FAS DH domain, and (C) PKSA PT domain. The active-site His and Asp are shown in *sphere* and *sticks* in upper and lower panels, respectively. The N- and C-terminal subdomains in each model are shown in darker and lighter shades, respectively. (D) Proposed mechanism for DH4.

Figure 2.7.

The three types of ARO/CYCs. Class 1 proteins possess monodomain architecture and promote C9-C14 first-ring and C7-C16 second-ring cyclization of an un-reduced polyketide intermediate. Class 2 ARO/CYCs are monodomain or didomain proteins that promote C7-C12 first-ring cyclization of an un-reduced intermediate. Class 3 enzymes are didomain proteins that aromatize the first ring of an intermediate that has undergone C9-specific ketoreduction and C7-C12 first-ring cyclization.

Figure 2.8.
Crystal structures of (A) *tcm* ARO/CYC, (B) FabA, (C) *whiE* ARO/CYC, (D) Zhul ARO/CYC, and (E) The *tcm* ARO/CYC interior pocket.

Figure 2.9. (A, B) A typical TE structure with the PksA TE as an example. (C) Comparison of the lid region (yellow) of different megasynthase TE domains. The active site is red.