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Characterization of Molecular Interactions between ACP and Halogenase Domains in the Curacin A Polyketide Synthase

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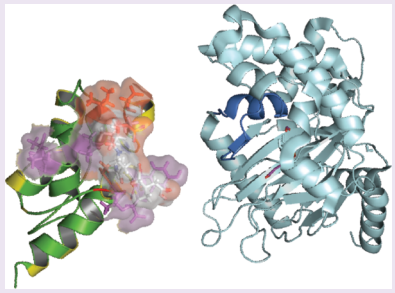
Supporting Information

ABSTRACT: Polyketide synthases (PKSs) and non-ribosomal peptide synthetases (NRPSs) are large multidomain proteins present in microorganisms that produce bioactive compounds. Curacin A is such a bioactive compound with potent anti-proliferative activity. During its biosynthesis the growing substrate is bound covalently to an acyl carrier protein (ACP) that is able to access catalytic sites of neighboring domains for chain elongation and modification. While ACP domains usually occur as monomers, the curacin A cluster codes for a triplet ACP (ACP_I-ACP_{II}-ACP_{III}) within the CurA PKS module. We have determined the structure of the isolated holo-ACP_I and show that the ACPs are independent of each other within this tridomain system. In addition, we have determined the structure of the 3-hydroxyl-3-methylglutaryl-loaded holo-ACP_I, which is the substrate for the unique halogenase (Hal) domain embedded within the CurA module. We have identified the interaction surface of both proteins using mutagenesis and MALDI-based identification of product formation. Amino acids affecting product formation are located on helices II and III of ACP_I and form a contiguous surface. Since the CurA Hal accepts substrate only when presented by one of the ACPs within the ACP_I-ACP_{II}-ACP_{III} tridomain, our data provide insight into the specificity of the chlorination reaction.



The polyketide synthases (PKSs) and the non-ribosomal peptide synthetases (NRPSs) produce natural products of a huge chemical diversity. PKS and NRPS are multidomain protein assemblies that function by sequentially elongating a growing polyketide or peptide chain by acyl units or amino acids, respectively. The growing product is attached *via* a thioester linkage to the 4′-phosphopantetheine (4′-Ppant) arm of either a holo acyl carrier protein (ACP) in PKSs or a holo peptidyl carrier protein (PCP) in NRPSs and is passed from one module to another along the chain of reaction centers. The modular arrangement makes PKS and NRPS systems an interesting target for protein engineering.[1] More than 200 novel polyketide compounds have already been created; unfortunately, however, engineered PKSs often fail to produce significant amounts of the desired products.[2−4] Structural studies may faciliate yield improvement from engineered systems by providing a more complete understanding of the interface between the different domains. One example is the 6-deoxyerythronolide B synthase (DEBS PKS) from *Saccharopolyspora erythraea*. Docking experiments of ACP domains with the X-ray structure of the ketosynthase (KS) and acyltransferase (AT) didomain of module 3 (KS3-AT3), for example, suggest that the ACP domain binds in a deep cleft between both domains with interactions mediated by helix II of the ACP.[5] Mutations in this helix II affect the specificity of the system.[6] Further studies led to the conclusion that helix II is a universal "recognition helix" for interaction with different enzymatic centers.[7−11] While some information about domain−domain interactions involving ketosynthase and acyltransferase is starting to emerge,[12] little is known about the interaction of ACP domains with other modifying enzymes such as methyltransferases, epimerases, or halogenases.

Curacin A is an anticancer natural product derived from the marine cyanobacterium *Lyngbya majuscula*. It is generated by an unusual mixed PKS/NRPS system with cyclopropane and thiazoline moieties, as well as an internal *cis* double bond, and a

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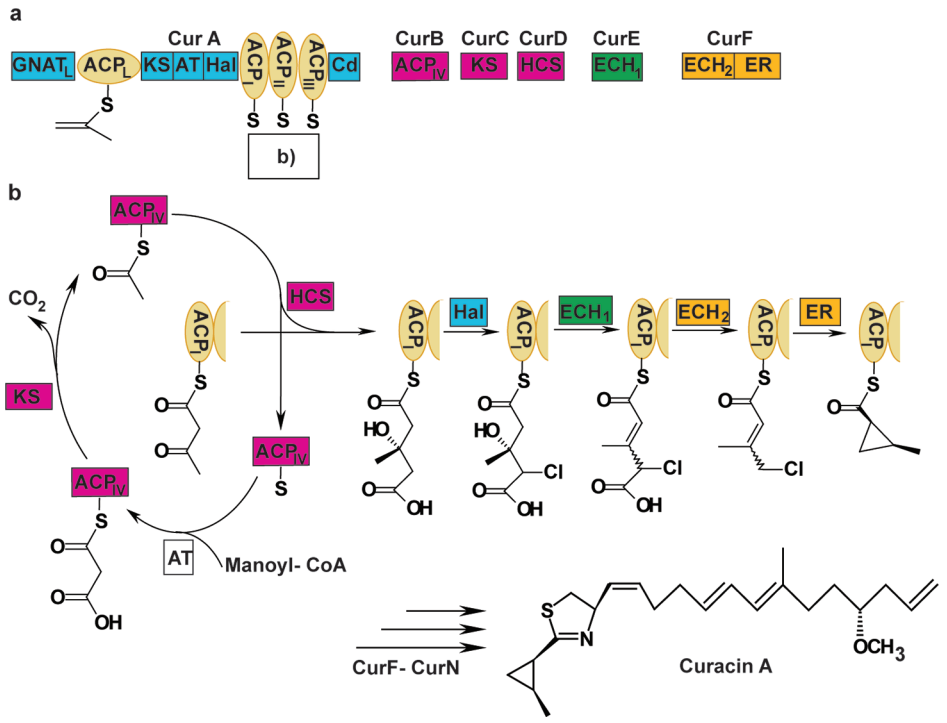

Figure 1. Ten-enzyme assembly catalyzing the cyclopropane ring formation. (a) The 10 enzymes that are involved in the cyclopropane ring formation are encoded on different proteins. (b) Representation of the biosynthesis of the cyclopropane ring formation. $GNAT_L$ = loading module, KS = ketosynthase, AT = acyltransferase, Hal = halogenase, ACP = acetyl-carrier-protein, HCS = HMG-CoA synthase-like enzyme, ECH_1 = dehydratase, ECH_2 = decarboxylase, ER = enoyl-reductase.

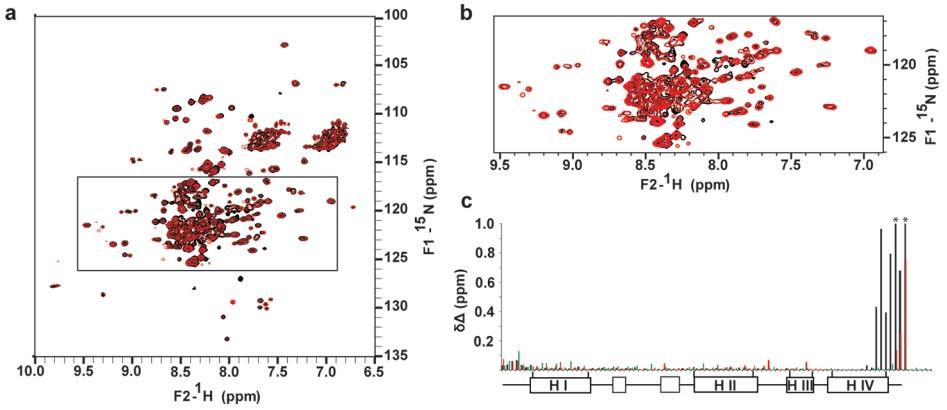

Figure 2. Dimerization does not affect the conformation. (a) Overlay of [^{15}N,^{1}H]-TROSY spectra of apo-$ACP_{I,II,III}$ (red) and apo-$ACP_{I,II,III}$-C_d (black). (b) Magnification of the central region. (c) Chemical shift differences between the isolated holo-ACP_I and holo-ACP_I as part of the triplet $ACP_{I,II,III}$ are plotted for ACP_I (black), ACP_{II} (red), and ACP_{III} (green). The CSPs for Gln2032 (1.95 ppm) and Thr2034 (1.26 ppm), indicated with an asterisk, are truncated at 1.0 ppm.

terminal alkene. The biosynthesis of curacin A is mediated by a 2.2 MDa hybrid PKS-NRPS cluster[13,14] (Figure 1). The first enzyme within the CurA PKS to act on the initial 3-hydroxyl-3-methyl-glutaryl (HMG) linked to ACP_I is the curacin halogenase (CurA Hal). This is followed by the discrete dehydratase (ECH_1) and decarboxylase (ECH_2) that comprise the β-branching steps in formation of the cyclopropane ring (Figure 1).[15] The recently solved crystal structure of CurA Hal in five individual states differing in their substrate and cofactor composition has improved our understanding of its enzymatic mechanism.[16] In addition, amino acids essential for the stereospecific substrate recognition of (*S*)-HMG-ACP by CurA Hal could be identified by mutagenesis.[16] The HMG substrate is presented by an unusual cluster of three tandem ACPs ($ACP_{I,II,III}$). Little is known about the function of these multiple ACPs, but recent studies have demonstrated that they enhance significantly the efficiency of the multienzyme reaction leading to formation of methyl-cyclopropylacetyl-ACP.[17]

In this study we elucidated the structural basis for the selectivity that drives the interaction between CurA Hal and CurA $ACP_{I,II,III}$ and show that these triplet ACP domains are structurally independent. Furthermore we report the NMR solution structures of holo-ACP_I and HMG-ACP_I. NOE experiments indicated that no sequestration of the HMG substrate occurs and that it is instead presented on the ACP surface. Using mutational analysis we have mapped the protein−protein interaction surface between CurA HMG-

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Table 1. Statistics derived from Cyana for the Structure of Holo and (*S*)-Hydroxyl-methylglutaryl (HMG)-ACP_I

NMR restraints	Holo	HMG	Structural statistics	Holo	HMG
Number of peaks			**Restraint violations**		
^{13}C NOESY-HSQC	5260	5078	Max. Dihedral angle restraint violations	/	/
^{15}N NOESY-HSQC	1557	1557	Max. distance restraint violations (A)	0.12	0.21
$^{13}C^{Arom}$ NOESY-HSQC	58	57	Cyana target function value ($Å^2$)	2.42	1.56
Hydroxy-methyl-glutaryl					
Intramolecular NOEs	-	86			
Intermolecular NOEs	-	37			
			Precision for residues 1950-2033		
Total NOE	2866	2923	**R.m.s.d to mean coordinates (Å)**		
Short range |i-j|<=1	1307	1349	Heavy atoms	0.54 ± 0.06	1.01 ± 0.29
Medium-range 1<|i-j|<5	826	819	Backbone	0.24 ± 0.04	0.11 ± 0.02
Long range |i-j|>=5	734	755	**Ramachandran plot staistics (%)**		
Hydrogen bonds	-		most favored	88.6	87.1
			additionally allowed	11.4	12.9
Dihedral angle			generously allowed	0	0
Talos+ Φ/ Ψ	162	162	disallowed	0	0

ACP_I and CurA Hal that includes not only the recognition helix II but also large parts of helix III.

RESULTS AND DISCUSSION

Impact of ACP Repetition on the Single Domain. The triplet ACP domains ($ACP_{I,II,III}$) are located at the C-terminus of the CurA module. As shown using negative-stain electron microscopy, $ACP_{I,II,III}$ has a propensity to dimerize through a C-terminal domain (C_d-domain).[17] Our initial objective was to evaluate whether the presence of the triplet $ACP_{I,II,III}$ domains or their dimer leads to domain−domain interactions that adopt a preferred conformation. [^{15}N,^{1}H]-TROSY spectra of the two proteins CurA-$ACP_{I,II,III}$ with (in black) and without C_d (in red) were recorded and compared (Figure 2). The absence of chemical shift differences indicates that no conformational changes occur. Some peaks decrease in intensity upon adding the 60 aa C_d domain. However, surprisingly we failed to observe new peaks representing the C_d domain. Due to dimerization the overall size increases from 36 to 89 kDa.[17] The increased size of the protein, in combination with some conformational exchange of the C_d domain, might broaden the resonances of the C_d domain beyond the detection limit. Parts that tumble independently, such as the individual ACP domains, are affected less by this increase in size (Figure 2).

In the absence of clear evidence that the C_d domain can affect the conformation of the entire ACP assembly, we decided to further analyze $ACP_{I,II,III}$. The [^{15}N,^{1}H]-TROSY spectrum of $ACP_{I,II,III}$ suffers from severe peak overlap due to the high sequence identity of the individual domains (93−100%). The distinction of the individual domains was achieved by segmental labeling, allowing the assignment of the full length construct.[18] Analysis of the excised ACP_I, ACP_{II}, or ACP_{III} proteins resulted in [^{15}N,^{1}H]-TROSY spectra with strong chemical shift perturbations (CSPs). Shift differences $\Delta\delta$ were calculated and plotted against the ACP sequence (Figure 2). For ACP_I and ACP_{II}, CSPs $\Delta\delta > 0.4$ ppm occurred for amino acids confined to the C-terminus, and for ACP_{III} small CSP occurred at the N-terminus reflecting differences in the environment of the linker region in the isolated domain and the $ACP_{I,II,III}$ tridomain (Figure 2). These data suggest that no detectable interaction occurs between ACP_I, ACP_{II}, and ACP_{III}. To further investigate this question, we performed titration experiments of the ^{15}N-labeled ACP_I domain (residues 1946−2043) with the $ACP_{II,III}$ didomain (2044−2248) and the $ACP_{I,II}$ didomain with ^{15}N-labeled ACP_{III}. Both experiments failed to show CSPs. Similarly, no interdomain NOEs were detected in 3D ^{13}C-edited/^{15}N-separated NOESY-HSQC experiments with a [^{15}N]ACP_I-[^{13}C]ACP_{II}-ACP_{III}, labeled version (data not shown). Taken together these data establish that no detectable interactions occur between the ACP domains of the triplet ACP cluster. To investigate the dynamics of the ACP linker regions that were found to be structured in other systems,[5,19,20] we performed {^{1}H}^{15}N heteronuclear NOE measurements. Nearly all amino acids of the structured domains ACP_I, ACP_{II}, and ACP_{III} could be identified, and therefore the additional signals can be associated with the linker region. Heteronuclear NOE values of around 0.8 represent highly structured regions, which is the case for the assigned amino acids within the ACP domains, whereas values around 0.2 were measured for the unassigned peaks, indicating that the linkers are unstructured and highly flexible (Supplementary Figure 4).

Taken together we show that the linker region is unstructured and that the ACP domains behave independently from each other in $ACP_{I,II,III}$ as well as in the dimerized form of $ACP_{I,II,III}$-C_d. This leads to the conclusion that the interaction between Cur Hal and ACP domains can be studied using one representing ACP domain of the triplet, for example, ACP_I.

Structure of Holo-ACP_I. ACP_I was expressed in its apo form and post-translationally modified *in vitro*. The purity of the apo protein and completeness of conversion to the holo form was investigated using matrix-assisted laser desorption ionization (MALDI) mass spectrometry analysis (Supplementary Figure 3). The structure was solved using standard NMR methods, and structural statistics are given in Table 1. Coordinates for holo-ACP_I have been deposited in the Protein Data Bank under the accession code 2LIU.

Holo-ACP_I consists of the expected right-hand twisted bundle formed by four major α-helices (I, II, III, VI) connected by three loops.[21−25] Figure 3 shows a ribbon diagram of the minimized mean structure. Conformational exchange has been previously reported in the ACP and PCP family;[25,26] however, for ACP_I only a single set of cross-peaks was observed with no indication of slow conformational exchange.

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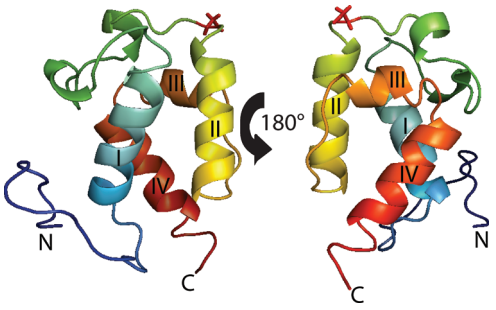

Figure 3. Ribbon diagram of the averaged and minimized NMR structure of holo-ACP_I from *Lyngbya majuscula*. Helices I–IV are colored light blue, yellow, orange, and red, respectively. The active site Ser1989 is indicated in red at the N-terminus of helix II.

The apo- to holo-ACP_I conversion is accompanied by CSPs. The amino acids surrounding the active site Ser1989 show the strongest perturbations between the apo and holo forms, including amino acids at the N-terminus of helix II and on helix III (all >0.05 ppm CSP) (Figure 4). In addition ^{15}N NOESY-HSQCs for apo- and holo-ACP_I were measured. However, comparison of the NOE pattern of amino acids undergoing strong CSPs revealed minor differences between both states (Supplementary Figure 5) excluding major conformational changes upon formation of the holo state.

Acyl-ACP. During curacin A biosynthesis ACP_I occurs in its holo, acetyl, HMG, and 4-chloro-3-hydroxy-3-methylglutaryl (Cl-HMG) forms. To investigate these different chemical states apo-ACP_I was loaded by the corresponding acyl-CoAs. For Cl-HMG loading racemic (*R*,*S*)-HMG was used and enzymatically converted to 50% γ-Cl-(*S*)-HMG-ACP_I by Cur Hal and 50% residual (*R*)-HMG-ACP_I due to the stereospecificity of the reaction.[15] [^{15}N,^{1}H]-TROSY spectra of acetyl-, HMG-, and γ-Cl-HMG- were compared to holo-ACP_I (Figure 5). The CSPs are small compared to the CSPs of the apo to holo conversion and are located near the active site Ser1989 on helices II and III. The CSPs increase from acetyl- to HMG- (~0.05 ppm) and γ-Cl-HMG-ACP_I (~0.1 ppm) (Figure 5).

In previous solution NMR investigations of ACPs, no NOEs between the protein and the prosthetic group were detected. This led to the assumption that no interactions or only transient interactions are present between the 4′-Ppant arm and the surface of the ACP domain.[21−23] If the 4′-Ppant arm is modified with larger substrates, however, association with the surface has been observed with the acyl chain bound in a cleft that is located between helices II and III. Examples are the FAS hexanoyl-ACP of *E. coli*[27] and octanoyl-ACP from *Streptomyces coelicolor*.[28] For a detailed characterization of the interaction of ACP_I with Cur Hal the localization of the substrate on the surface must first be defined. To investigate potential contacts between the 4′-Ppant arm and the protein, we expressed $^{13}C/^{15}N$-labeled ACP_I and modified it *in vitro* with unlabeled CoA and (*R*,*S*)-HMG-CoA. NOEs between ^{12}CH groups of the attached cofactor and the ^{13}CH groups of the protein were detected in a 3D F1-$^{13}C/^{15}N$-filtered, F3-^{13}C-separated NOESY (Figure 6). Surprisingly we could obtain NOEs with similar intensities for both holo- and HMG-ACP_I reflecting that in both cases the 4′-Ppant arm might be located similarly. In a next step we recorded F1/F2-$^{13}C/^{15}N$ double-filtered 2D-NOESY spectra to selectively detect the $^{12}CH-^{12}CH$ NOEs present within the 4′-Ppant arm loaded with HMG (Figure 6). Using the previous assignments of the 4′-Ppant arm loaded on other PCPs and ACPs,[25,29] we could assign the 4′-Ppant chain attached to ACP_I. The 1D spectra of CoA and HMG-CoA then gave rise to the unambiguous assignment of the protons of the HMG methyl group (66, 67, 68) (Supplementary Figure 6) and allowed us to assign the NOEs observed between the HMG and the protein (Supplementary Table 2).

To calculate the structure of HMG-ACP_I, we combined the restraints obtained for holo-ACP_I with the NOE-based distance restraints obtained for HMG, assuming that no major conformational changes occur between the different states (Supplementary Figure 5, Table 1 for structural statistics). The structure shows that the HMG-4′-Ppant arm associates with the surface of the ACP but that this interaction is transient, allowing different orientations of the HMG group to occur

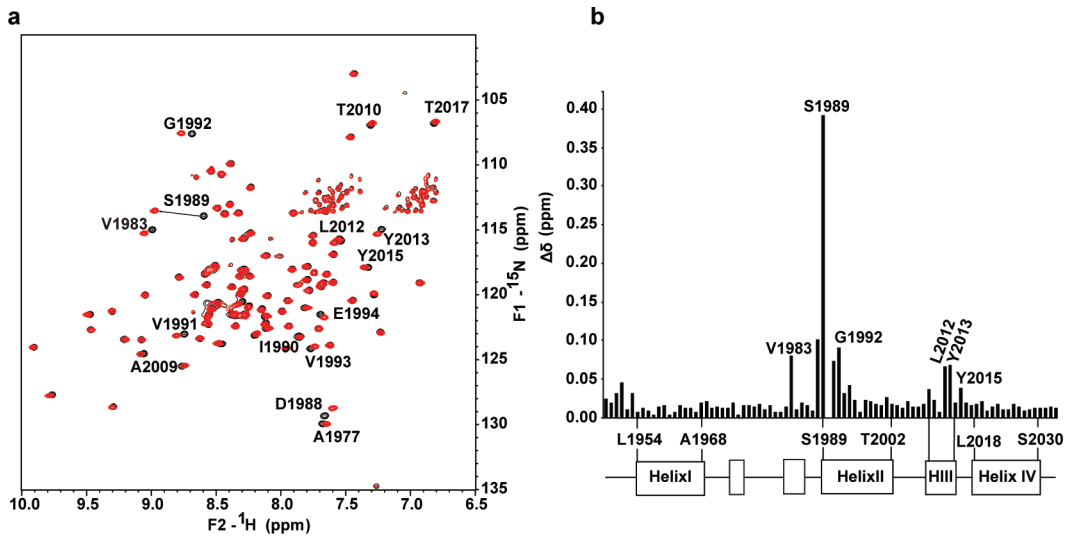

Figure 4. Comparison of chemical shifts of apo- and holo-ACP_I. (a) An overlay of [^{15}N,^{1}H]-TROSY spectra of uniformly ^{15}N-labeled apo-ACP_I (black) and ^{15}N-labeled holo-ACP_I (red). The assignments of the amino acids undergoing the strongest chemical shift perturbations are indicated. (b) Plot of chemical shift differences between holo-ACP_I and apo-ACP_I. The secondary structure elements are indicated below the sequence. No CSP is given for Ile1990, which could not be assigned in the apo form.

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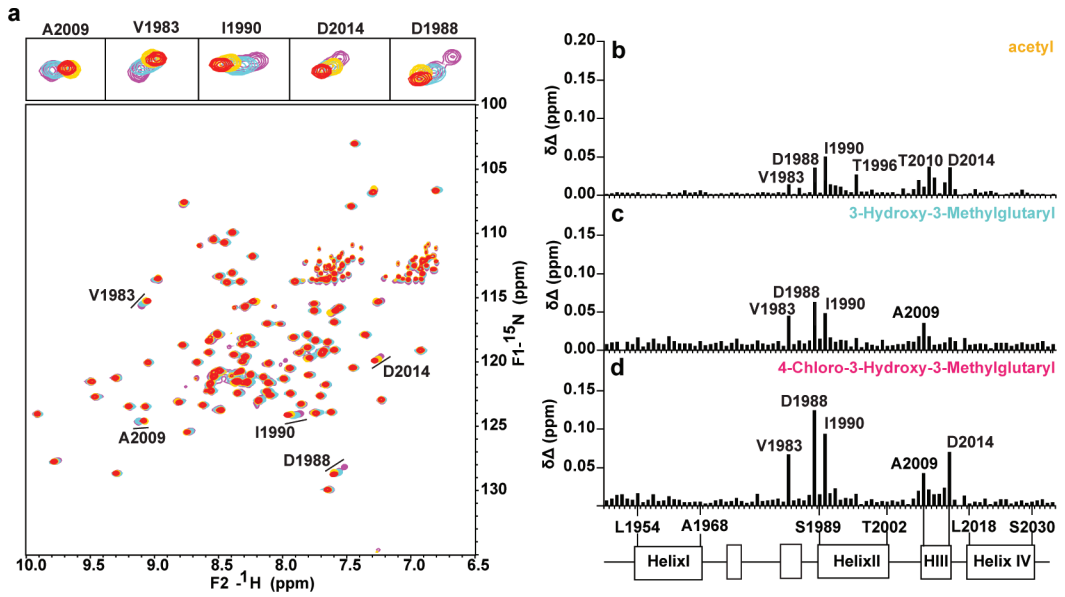

Figure 5. Comparison of the different chemical states of ACP_I. (a) An overlay of [^{15}N,^{1}H]-TROSY spectra of holo-ACP_I (red), acetyl-ACP_I (yellow), HMG-ACP_I (light blue),and Cl-HMG-ACP_I (magenta). Assignments of the amino acids undergoing the strongest chemical shift perturbations are indicated, and their magnifications are presented on the top panel. The right-hand panel presents plots of chemical shift differences between holo-ACP_I and acetyl-ACP_I (b), HMG-ACP_I (c), and Cl-HMG-ACP_I (d). The secondary structure elements are indicated below.

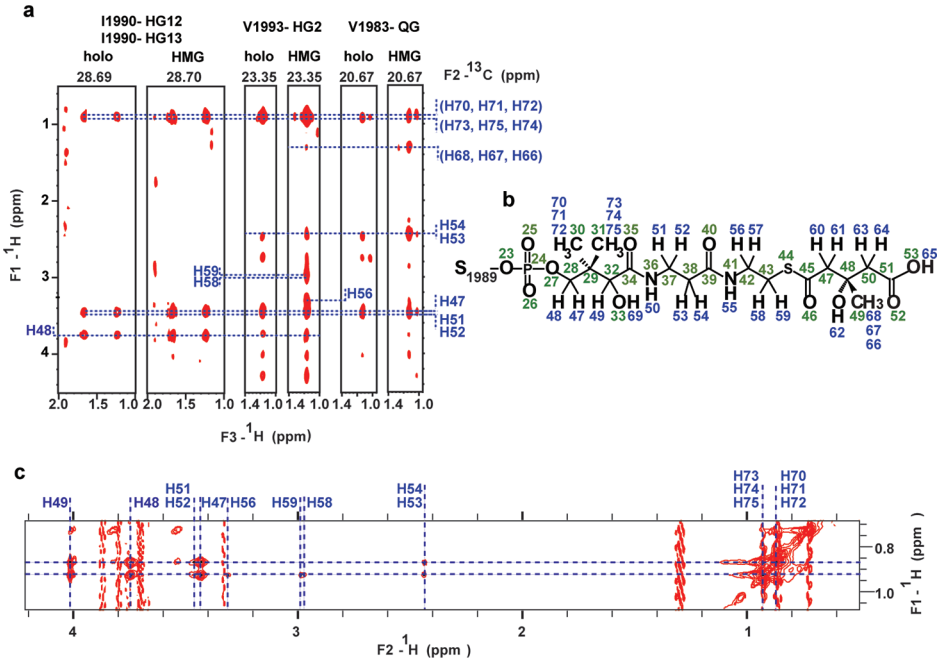

Figure 6. Isotope filtered NOESY experiments for the structure determination of HMG-ACP_I. (a) F1−F3 strips from a 3D F1-^{13}C/^{15}N-filtered, F3-^{13}C-separated NOESY-HSQC of holo- and HMG-ACP_I filtering the ^{13}CH-^{15}NH NOEs. The protein was loaded *in vitro*, and the substrate is therefore unlabeled giving strong NOEs from ^{12}CH to the ^{13}CH groups of the protein surface. (b) The cofactor S-HMG and its numeration. (c) Expansion of the two-dimensional F2-filtered NOESY spectrum of ^{13}C,^{15}N-labeled HMG-ACP_I. The 4′-Ppant chain and the HMG group are unlabeled and give rise to strong NOE connectivities within the 4′-Ppant arm.

(Figure 7). Structures of other ACP-substrate complexes had shown a more tight interaction. X-ray studies of the *E. coli* FAS II ACP demonstrated that starting from C4 chain length the substrate and the 4′-Ppant arm is sequestered into a hydrophobic pocket.[27,30] These results were similar to the NMR findings for the PKS actinorhodin system of *Streptomyces coelicor*.[28]

Interaction Studies of ACP_I with Cur Hal. The curacin A biosynthetic pathway differs from other β-branching pathways in the introduction of a γ-chlorination step on (*S*)-HMG-ACP_I mediated by Cur Hal, a non-heme Fe(II), α-ketoglutarate-dependent enzyme.[15] Recent studies showed that the chlorination step takes place before dehydration catalyzed by ECH_1- and ECH_2-mediated decarboxylation followed. Previous work has shown that ECH_1 and ECH_2 in the CurA β-branching cassette are more promiscuous enzymes. For example, ECH_1 can accommodate either (*S*)-HMG- or γ-Cl-(*S*)-HMG bound to $ACP_{I,II,III}$,[15] CurB,[31] or in the acyl-CoA form, albeit with

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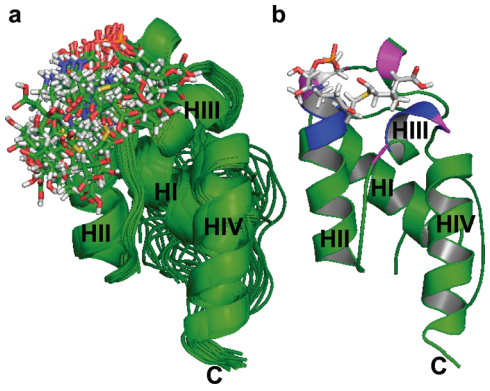

Figure 7. (a) Ribbon diagram for the 20 structures of (*S*)-HMG-ACP$_I$. (b) Mean structure of HMG-ACP$_I$. The amino acids that undergo chemical shift perturbations once the 4′-Ppant arm is added are labeled in blue (CSP > 0.04 ppm), amino acids that undergo slight chemical shift perturbations when HMG is added to the 4′-Ppant arm are indicated in magenta (CSP > 0.04 ppm). The shifts are located around the attachment site of the substrate on helix II and helix III.

lower efficiency.[32] By contrast, the halogenase CurA Hal was shown to be stereospecific as it distinguishes between (*R*)- and (*S*)-HMG[15,16] and accepts (*S*)-HMG only in an ACP-bound form. In order to assess the specificity determinants for this interaction, Cur Hal was expressed, purified, and reconstituted anaerobically as described,[15,16] and its activity was confirmed by MALDI mass spectrometry (Supplementary Figure 7). To identify the specific interaction surface on ACP$_I$, we titrated (*R*,*S*)-HMG-[^{15}N]ACP$_I$ with active, unlabeled CurA Hal, but no CSPs could be observed. The experiment was repeated under nonreactive conditions: without Cl^-, α-ketoglutarate, or O_2 or missing all components. Subsequently the inactive mutant R241A[16] or Fe^{3+}-reconstituted, inactive halogenase was tested. Finally, ACP$_I$ in apo, holo, or loaded with the substrates acetyl or malonyl were titrated with functional halogenase. In all cases, despite using a high excess of the enzyme, no CSPs or line broadening effects were observed, which would indicate formation of an HMG-ACP$_I$/Cur Hal complex. To investigate if the reaction occurs even in the absence of CSPs and to probe the specificity of the reaction, we performed activity assays using CurA ACP$_I$, CurB ACP, and TycB1 PCP as a substrate donor. Only in the case of ACP$_I$ all (*S*)-HMG-ACP$_I$ was chlorinated within 10 min (Supplementary Figure 7). Even after 1 h HMG-CurB and HMG-PCP did not convert to the chlorinated product (data not shown). This result demonstrates that Cur Hal distinguishes between these ACPs, leading to the conclusion that an interface must be present, which cannot be detected by typical NMR titrations.

To further investigate the CurA Hal/ACP$_I$ interaction surface, we mutated solvent exposed, not conserved amino acid (Supplementary Figure 8). Since we used (*R*,*S*)-HMG-ACP$_I$ as a substrate, activity was determined by assuming that chlorination of 100% (*S*)-HMG results in two peaks, one representing the unreacted (*R*)-HMG-ACP and the other one γ-Cl-(S)-HMG-ACP, separated by 34 Da and of equal intensities (see MALDI-MS in Supplementary Figure 9 and the derived activities are listed in Supplementary Table 1). The mutations D1988A and I1990A neighboring the active site Ser1989 and A2009R on helix III had the strongest impact on the chlorination efficiency (activity: 29 ± 10%; 18 ± 7%; 15 ± 12%, respectively). In these cases no product peaks were observed. However, the first and second sodium adduct peaks (∼+22 and ∼+44 Da) might overlap with a small product peak. To avoid underestimation of the product yield, we assumed the peak intensity at a distance of 34 Da to be product (Supplementary Figure 9). This leads to an overestimation rather than an underestimation of the activity, making it clear that these mutations reduce the activity significantly. The single point mutations T2010A, Y2013A, and D2014A on helix III decrease the activity to 37 ± 7%, 35% ± 2%, and 59 ± 2%, respectively. Circular dichroism (CD) curves of the less active ACP$_I$ mutants showed that the mutations did not have an impact on the global fold. Melting temperatures (T_M) derived from CD melting curves showed that the mutations D1988A and D2014A destabilize the protein (Supplementary Table 1, Supplementary Figure 10). To ensure that a strong reduction by a mutation is not simply caused by structural perturbations we compared [^{15}N,^{1}H]-TROSY spectra of mutants with the WT (Supplementary Figure 11) revealing for D1988A and I1990A CSPs for only 5−6 amino acids located near the mutation site, suggesting that these mutations do not affect the structure. The mutation A2009R leads to stronger CSPs, which might be due to structural rearrangements. We were interested whether these mutations might lead to a displacement of the cofactor, which in turn affects the Cur Hal/ACP interaction. We loaded the mutants D1988A, I1990A, and A2009R with the 4′-Ppant arm, acetyl, and HMG, mapped the CSPs against the sequence, and compared these results with the WT (Supplementary Figure 12). Interestingly in all cases the same amino acids showed CSPs, suggesting that the localization of the cofactor is not affected (Supplementary Figure 12). Mapping the mutations with the strongest effects onto the structure of HMG-ACP$_I$ shows that these amino acids surround the substrate and form a contiguous surface, suggesting its importance for Cur Hal recognition and selectivity (Figure 8).

Overall our data demonstrate that Cur Hal interacts with ACP$_I$ specifically, which is likely applicable to Cur Hal interaction also with ACP$_{II}$ and ACP$_{III}$ of the tridomain. Cur Hal recognizes an interface consisting mainly of the N-terminus of helix II, the 4′-Ppant arm with its substrate, and helix III. Substrate attached to a different carrier domain (either ACP or PCP) is not recognized by the halogenase. The importance of presenting the substrate as part of an entire substrate−ACP complex is also demonstrated by the fact that the isolated HMG-CoA does not serve as a substrate for CurA Hal. In contrast to high specificity of the Hal, it is evident that ECH$_1$ and ECH$_2$ β-branching cassette enzymes are less specific and can accommodate acyl-CoAs, albeit with significantly lower efficiency. The radical mechanism involved in the chlorination step (inherently highly reactive and less specific) is possibly the driving force for the Hal acyl-ACP specificity. The requirement of presenting the correct substrate within a specific environment might be necessary to prevent nonspecific chlorination or a competing hydroxylation reaction of substrates presented by other ACPs. For example, CurB is a discrete ACP that can freely diffuse, and should therefore be able to interact with Cur Hal.

Surprisingly, it was not possible to directly detect the interactions between ACP$_I$ and Cur Hal by NMR spectroscopy. Weak or transient interactions are difficult to detect using standard NMR experiments due to the low population and transiency of the complex. One technique for investigating these transient interactions is paramagnetic relaxation enhancement (PRE) measurements. Spin labels attached to one partner result in relaxation enhancement in the other partner even

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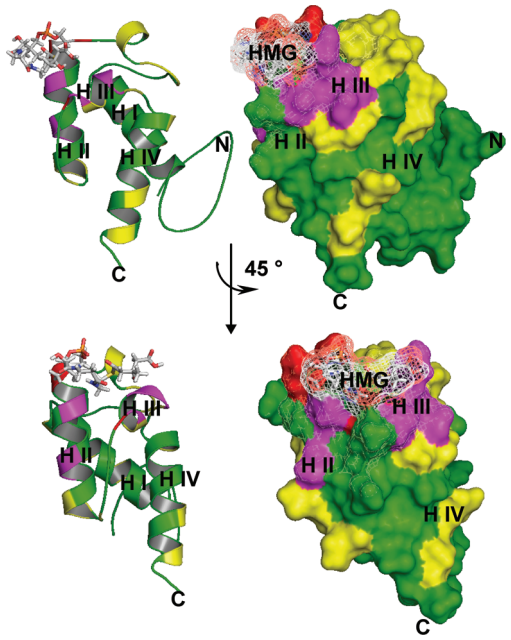

Figure 8. Effect of ACP_I mutation on its activity. Color coding has been applied according to the strength of activity decrease. Mutations that decreased the activity to 0−30% are marked in red (D1988A and I1990A amino acids neighboring the active site Ser1989 and A2009R located on helix III), mutations that decreased the activity to 30−70% are marked in magenta (*e.g.*, the multiple mutant V1993N/T1998M/T1999 M located on helix II, the single mutants T2010A, Y2013A, and D2014A all located on helix III), and mutations with minor or no effect with an activity of over 70% are marked in yellow.

when the contact time is short and the complex is unstable.[33−36] We therefore attempted to apply PRE experiments to our system, in order to visualize low populated states. Unfortunately no spin label could be attached to Cur Hal because the removal of the WT cysteine (Cys19Ala) as well as the insertion of new cysteines into Cur Hal (C19S, I61C, H109C, G111C, Y274C, H168C, and S178C) resulted in protein precipitation.

Our results demonstrate that the individual CurA ACPs of the $ACP_{I,II,III}$ tridomain do not interact with each other. Previous studies have shown that the advantage of a triplet assembly over a single ACP is the increase of the efficiency of the entire enzyme reaction.[17] It has been proposed that this clustering of ACP-linked substrates might result in binding of adjacent enzymes to corresponding substrates through nonspecific enzyme−substrate interactions.[17] Further kinetic and enzymatic investigation of Cur $ACP_{I,II,III}$ tridomain involving intact CurA module might reveal the basis for tandem ACP function and whether increasing the local concentration of potential substrate rather than distinct conformational effects affect higher efficiency during polyketide chain elongation in the curacin A pathway.

METHODS

Protein Expression and Purification. Apo-ACP (amino acids 1946−2034), the mutants, the triplet $ACP_{I,II,III}$ (1946−2248), and $ACP_{I,II,III}$-C_d (1946−2311) were expressed in the *E. coli* strain BL21(DE3) (Novagen) using the pET28a(+)plasmid[17,18] at 15 °C for 7−10 h. Media were supplemented with 0.8 mL of 0.05 M Fe(II) solution in order to prevent modifications by an endogenous PPTase.[37] All proteins were purified by Ni-NTA affinity chromatography. The constructs of Cur Hal and CurB have been described previously.[15,31] Hal was expressed, purified, and reconstituted as described previously[31] (Supporting Information and Methods). CurB was expressed in BL21 star-pRARE (Novagen) and expressed and purified like ACP_I. TycB1 PCP was cloned into pBH4 using and expressed and purified as ACP_I.

Loading of ACP. The recombinant ACPs (200 μM) were artificially loaded with either coenzyme A (CoA) or analogues (acetyl-, malonyl-, HMG-CoA) by using the promiscuous *Bacillus subtilis* phosphopantetheinyl (Ppant) transferase Sfp (3 μM). The loading reaction was incubated at RT for 1 h in buffered aqueous solution (pH 7.5, 20 mM HEPES, 50 mM NaCl, 20 mM $MgCl_2$). To ensure that complete loading was achieved the reaction mixture was analyzed directly by MALDI-MS (Supplemantary Figure 3).

Activity Assay. For the reactivity test Hal was purified and reconstituted as described. A 100 μL portion of 100 μM HMG-ACP in 20 mM Hepes, 50 mM NaCl, pH 7.2 was supplemented with 0.5 mM fresh α- KG and 75 μM Fe(II)SO_4 and 5 μM Hal. The reaction was incubated at 25 °C under shaking (800 rpm), and after 10 min the reaction was completed. The reaction products were analyzed using MALDI-MS. The experiments were performed three times.

Mass Spectrometry. Mass spectra were recorded on a MALDI-TOF mass analyzer (Voyager STR, Applied Biosystems) in linear positive ion mode. Samples were diluted with water to a concentration of 5−10 μM, and 1 μL was mixed with 1 μL of MALDI matrix (sDHB, Bruker) directly on the sample target. Spectra were accumulated over 50−100 laser shots.

NMR Spectroscopy of Holo-ACP. Solutions of ACP_I (0.6−1 mM) were prepared in 50 mM arginine/50 mM glutamate buffer at pH 6.8 containing 5% D_2O and 0.15 mM DSS (4,4-dimethyl-4-silapentane-1-sulfonate). NMR spectra were collected at a temperature of 291 K at 950, 900, 800, and 600 MHz on Bruker Avance spectrometers equipped with cryogenic 5 mm *z*-axis gradient triple resonance probes and a 500 MHz Bruker Avance spectrometer equipped with 5 mm *x,y,z*-axis gradient triple resonance RT probe. For backbone and aliphatic side chain assignments [^{15}N,^{1}H]-TROSY[38] versions of HSQC, HNCACO, HNCO, HNCACB, (H)C(CCO)NH-TOCSY, and H(CCCO)NH-TOCSY were recorded. Aromatic ring resonances were assigned with (H)CB(CGCC-TOCSY)H^{ar} experiments.[39] ^{15}N- and ^{13}C-separated 3D NOESY experiments were recorded with 70 ms mixing time. All spectra were processed with TopSpin (Bruker Biospin) and analyzed with Sparky.[40] For the acyl chain binding studies, standard sensitivity-enhanced [^{15}N,^{1}H]-TROSY spectra were acquired. Steady state {^{1}H}^{15}N hetNOE measurements were performed on a 600 MHz instrument using a TROSY-type pulse sequence.[41]

NMR of HMG-ACP and Holo-ACP. To observe NOEs between the 4′ Ppant arm and HMG loaded 4′ Ppant arm and the ACP domain, highly ^{13}C, ^{15}N-enriched ACP_I was loaded with unlabeled ^{12}C CoA or HMG-CoA. The protein was concentrated to 1 mM in 50 mM arginine/50 mM glutamate buffer at a pH of 6.8 and a 3D F1-^{13}C/^{15}N-filtered, F3-^{13}C-separated NOESY-HSQC was acquired at 800 MHz using a mixing time of 80 ms and filter delays of 93 Hz for $^{1}J_{NH}$ and to 125 and 147 Hz for $^{1}J_{CH}$. For the assignment of the unlabeled cofactor a 2D F1/F2-^{13}C/^{15}N double-filtered NOESY experiment[42] with 150 ms mixing time was acquired at 950 MHz. Fully ^{13}C/^{15}N-labeled ACP_I was loaded with unlabeled ^{12}C HMG-CoA, and the protein was concentrated to 1 mM in 25 mM NaPi and 50 mM NaCl at a pH of 6.8 and measured at 291 K.

Structure Calculation. Backbone torsion angle restraints were obtained from chemical shift data using the TALOS+ algorithm.[43] NOE-based distance restraints from 3D ^{15}N-NOESY-HSQC and ^{13}C-NOESY HSQC spectra were assigned automatically by CYANA,[44,45] which was also used for the structure calculations by torsion angle dynamics.[46] The final structure calculations included 2852 distance restraints and 162 backbone torsion angle restraints (Table 1); 100 conformers were computed using 10000 torsion angle dynamics steps. The 20 conformers with the lowest target function values were subjected to restrained energy refinement with the program OPALP[47] using the AMBER force field.[48] The quality of the structures was checked by PROCHECK[49] and WHATCHECK.[50]

Structure Calculations with HMG. A model of HMG was created using the online version of the program CORINA for 3D structure generation (http://www.molecular-networks.com/online_demos/corina_demo). HMG was attached covalently to the Ser1989 residue. For the structure calculation with CYANA,[51] a residue consisting of a serine backbone connected to the HMG cofactor was added to the standard residue library, and the data set was complemented with additional NOE information for the cofactor. The resulting structures were subjected to restrained energy minimization using OPALp.[47] The necessary partial charges for HMG-serine were calculated using the PRODRG server.[52]

■ ASSOCIATED CONTENT

Supporting Information

This material is available free of charge *via* the Internet at http://pubs.acs.org.

Accession Codes

Coordinates for holo-ACP_I have been deposited in the Protein Data Bank under the code 2LIU. The coordinates for (*S*)-hydroxy-methylglutaryl ACP_I have been deposited in the protein databank under the code 2LIW.

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■ REFERENCES

(1) Fischbach, M. A., and Walsh, C. T. (2006) Assembly-line enzymology for polyketide and nonribosomal Peptide antibiotics: logic, machinery, and mechanisms. *Chem. Rev. 106*, 3468−3496.

(2) Menzella, H. G., Carney, J. R., and Santi, D. V. (2007) Rational design and assembly of synthetic trimodular polyketide synthases. *Chem. Biol. 14*, 143−151.

(3) Menzella, H. G., and Reeves, C. D. (2007) Combinatorial biosynthesis for drug development. *Curr. Opin. Microbiol. 10*, 238−245.

(4) Kittendorf, J. D., and Sherman, D. H. (2006) Developing tools for engineering hybrid polyketide synthetic pathways. *Curr. Opin. Biotechnol. 17*, 597−605.

(5) Tang, Y., Chen, A. Y., Kim, C. Y., Cane, D. E., and Khosla, C. (2007) Structural and mechanistic analysis of protein interactions in module 3 of the 6-deoxyerythronolide B synthase. *Chem. Biol. 14*, 931−943.

(6) Alekseyev, V. Y., Liu, C. W., Cane, D. E., Puglisi, J. D., and Khosla, C. (2007) Solution structure and proposed domain domain recognition interface of an acyl carrier protein domain from a modular polyketide synthase. *Protein Sci. 16*, 2093−2107.

(7) Zhang, Y. M., Wu, B., Zheng, J., and Rock, C. O. (2003) Key residues responsible for acyl carrier protein and beta-ketoacyl-acyl carrier protein reductase (FabG) interaction. *J. Biol. Chem. 278*, 52935−52943.

(8) Zhang, Y. M., Rao, M. S., Heath, R. J., Price, A. C., Olson, A. J., Rock, C. O., and White, S. W. (2001) Identification and analysis of the acyl carrier protein (ACP) docking site on beta-ketoacyl-ACP synthase III. *J. Biol. Chem. 276*, 8231−8238.

(9) Rafi, S., Novichenok, P., Kolappan, S., Zhang, X., Stratton, C. F., Rawat, R., Kisker, C., Simmerling, C., and Tonge, P. J. (2006) Structure of acyl carrier protein bound to FabI, the FASII enoyl reductase from Escherichia coli. *J. Biol. Chem. 281*, 39285−39293.

(10) Gong, H., Murphy, A., McMaster, C. R., and Byers, D. M. (2007) Neutralization of acidic residues in helix II stabilizes the folded conformation of acyl carrier protein and variably alters its function with different enzymes. *J. Biol. Chem. 282*, 4494−4503.

(11) Arthur, C. J., Williams, C., Pottage, K., Ploskon, E., Findlow, S. C., Burston, S. G., Simpson, T. J., Crump, M. P., and Crosby, J. (2009) Structure and malonyl CoA-ACP transacylase binding of streptomyces coelicolor fatty acid synthase acyl carrier protein. *ACS Chem. Biol. 4*, 625−636.

(12) Buchholz, T. J., Geders, T. W., Bartley, F. E. 3rd, Reynolds, K. A., Smith, J. L., and Sherman, D. H. (2009) Structural basis for binding specificity between subclasses of modular polyketide synthase docking domains. *ACS Chem. Biol. 4*, 41−52.

(13) Chang, Z., Sitachitta, N., Rossi, J. V., Roberts, M. A., Flatt, P. M., Jia, J., Sherman, D. H., and Gerwick, W. H. (2004) Biosynthetic pathway and gene cluster analysis of curacin A, an antitubulin natural product from the tropical marine cyanobacterium Lyngbya majuscula. *J. Nat. Prod. 67*, 1356−1367.

(14) Verdier-Pinard, P., Lai, J. Y., Yoo, H. D., Yu, J., Marquez, B., Nagle, D. G., Nambu, M., White, J. D., Falck, J. R., Gerwick, W. H., Day, B. W., and Hamel, E. (1998) Structure-activity analysis of the interaction of curacin A, the potent colchicine site antimitotic agent, with tubulin and effects of analogs on the growth of MCF-7 breast cancer cells. *Mol. Pharmacol. 53*, 62−76.

(15) Gu, L., Wang, B., Kulkarni, A., Geders, T. W., Grindberg, R. V., Gerwick, L., Hakansson, K., Wipf, P., Smith, J. L., Gerwick, W. H., and Sherman, D. H. (2009) Metamorphic enzyme assembly in polyketide diversification. *Nature 459*, 731−735.

(16) Khare, D., Wang, B., Gu, L., Razelun, J., Sherman, D. H., Gerwick, W. H., Hakansson, K., and Smith, J. L. (2010) Conformational switch triggered by alpha-ketoglutarate in a halogenase of curacin A biosynthesis. *Proc. Natl. Acad. Sci. U.S.A. 107*, 14099−14104.

(17) Gu, L., Eisman, E. B., Dutta, S., Franzmann, T. M., Walter, S., Gerwick, W. H., Skiniotis, G., and Sherman, D. H. (2011) Tandem acyl carrier proteins in the curacin biosynthetic pathway promote consecutive multienzyme reactions with a synergistic effect. *Angew. Chem., Int. Ed. 50*, 2795−2798.

(18) Busche, A. E., Aranko, A. S., Talebzadeh-Farooji, M., Bernhard, F., Dötsch, V., and Iwai, H. (2009) Segmental isotopic labeling of a central domain in a multidomain protein by protein trans-splicing using only one robust DnaE intein. *Angew. Chem., Int. Ed. 48*, 6128−6131.

(19) Maier, T., Leibundgut, M., Boehringer, D., and Ban, N. (2010) Structure and function of eukaryotic fatty acid synthases. *Q. Rev. Biophys. 43*, 373−422.

(20) Tang, Y., Kim, C. Y., Mathews., Cane, D. E., and Khosla, C. (2006) The 2.7-Angstrom crystal structure of a 194-kDa homodimeric fragment of the 6-deoxyerythronolide B synthase. *Proc. Natl. Acad. Sci. U.S.A. 103*, 11124−11129.

(21) Kim, Y., Kovrigin, E. L., and Eletr, Z. (2006) NMR studies of *Escherichia coli* acyl carrier protein: dynamic and structural differences of the apo- and holo-forms. *Biochem. Biophys. Res. Commun. 341*, 776−783.

(22) Xu, G. Y., Tam, A., Lin, L., Hixon, J., Fritz, C. C., and Powers, R. (2001) Solution structure of B. subtilis acyl carrier protein. *Structure 9*, 277−287.

(23) Wong, H. C., Liu, G., Zhang, Y. M., Rock, C. O., and Zheng, J. (2002) The solution structure of acyl carrier protein from *Mycobacterium tuberculosis*. *J. Biol. Chem. 277*, 15874−15880.

(24) Li, Q., Khosla, C., Puglisi, J. D., and Liu, C. W. (2003) Solution structure and backbone dynamics of the holo form of the frenolicin acyl carrier protein. *Biochemistry 42*, 4648−4657.

(25) Koglin, A., Mofid, M. R., Löhr, F., Schäfer, B., Rogov, V. V., Blum, M. M., Mittag, T., Marahiel, M. A., Bernhard, F., and Dötsch, V. (2006) Conformational switches modulate protein interactions in peptide antibiotic synthetases. *Science 312*, 273−276.
(26) Zornetzer, G. A., Fox, B. G., and Markley, J. L. (2006) Solution structures of spinach acyl carrier protein with decanoate and stearate. *Biochemistry 45*, 5217−5227.
(27) Roujeinikova, A., Simon, W. J., Gilroy, J., Rice, D. W., Rafferty, J. B., and Slabas, A. R. (2007) Structural studies of fatty acyl-(acyl carrier protein) thioesters reveal a hydrophobic binding cavity that can expand to fit longer substrates. *J. Mol. Biol. 365*, 135−145.
(28) Evans, S. E., Williams, C., Arthur, C. J., Ploskon, E., Wattana-amorn, P., Cox, R. J., Crosby, J., Willis, C. L., Simpson, T. J., and Crump, M. P. (2009) Probing the Interactions of early polyketide intermediates with the Actinorhodin ACP from *S. coelicolor* A3(2). *J. Mol. Biol. 389*, 511−528.
(29) Ploskon, E., Arthur, C. J., Evans, S. E., Williams, C., Crosby, J., Simpson, T. J., and Crump, M. P. (2008) A mammalian type I fatty acid synthase acyl carrier protein domain does not sequester acyl chains. *J. Biol. Chem. 283*, 518−528.
(30) Roujeinikova, A., Baldock, C., Simon, W. J., Gilroy, J., Baker, P. J., Stuitje, A. R., Rice, D. W., Slabas, A. R., and Rafferty, J. B. (2002) X-ray crystallographic studies on butyryl-ACP reveal flexibility of the structure around a putative acyl chain binding site. *Structure 10*, 825−835.
(31) Geders, T. W., Gu, L., Mowers, J. C., Liu, H., Gerwick, W. H., Hakansson, K., Sherman, D. H., and Smith, J. L. (2007) Crystal structure of the ECH2 catalytic domain of CurF from *Lyngbya majuscula*. Insights into a decarboxylase involved in polyketide chain beta-branching. *J. Biol. Chem. 282*, 35954−35963.
(32) Gu, L., Jia, J., Liu, H., Hakansson, K., Gerwick, W. H., and Sherman, D. H. (2006) Metabolic coupling of dehydration and decarboxylation in the curacin A pathway: functional identification of a mechanistically diverse enzyme pair. *J. Am. Chem. Soc. 128*, 9014−9015.
(33) Iwahara, J., Tang, C., and Clore, G. M. (2007) Practical aspects of (1)H transverse paramagnetic relaxation enhancement measurements on macromolecules. *J. Magn. Reson. 184*, 185−195.
(34) Iwahara, J., and Clore, G. M. (2006) Detecting transient intermediates in macromolecular binding by paramagnetic NMR. *Nature 440*, 1227−1230.
(35) Clore, G. M., and Iwahara, J. (2009) Theory, practice, and applications of paramagnetic relaxation enhancement for the characterization of transient low-population states of biological macromolecules and their complexes. *Chem. Rev. 109*, 4108−4139.
(36) Tang, C., Iwahara, J., and Clore, G. M. (2006) Visualization of transient encounter complexes in protein-protein association. *Nature 444*, 383−386.
(37) Lambalot, R. H., Gehring, A. M., Flugel, R. S., Zuber, P., LaCelle, M., Marahiel, M. A., Reid, R., Khosla, C., and Walsh, C. T. (1996) A new enzyme superfamily−the phosphopantetheinyl transferases. *Chem. Biol. 3*, 923−936.
(38) Pervushin, K., Riek, R., Wider, G., and Wüthrich, K. (1997) Attenuated T2 relaxation by mutual cancellation of dipole-dipole coupling and chemical shift anisotropy indicates an avenue to NMR structures of very large biological macromolecules in solution. *Proc. Natl. Acad. Sci. U.S.A. 94*, 12366−12371.
(39) Löhr, F., Hänsel, R., Rogov, V. V., and Dötsch, V. (2007) Improved pulse sequences for sequence specific assignment of aromatic proton resonances in proteins. *J. Biomol. NMR 37*, 205−224.
(40) Goddard, T. D., and Kneller, D. G. *SPARKY 3*, University Of California, San Francisco
(41) Ferrage, F., Piserchio, A., Cowburn, D., and Ghose, R. (2008) On the measurement of 15N-{1H} nuclear Overhauser effects. *J. Magn. Reson. 192*, 302−313.
(42) Ogura, K., Terasawa, H., and Inagaki, F. (1996) An improved double-tuned and isotope-filtered pulse scheme based on a pulsed field gradient and a wide-band inversion shaped pulse. *J. Biomol. NMR 8*, 492−498.
(43) Shen, Y., Delaglio, F., Cornilescu, G., and Bax, A. (2009) TALOS+: a hybrid method for predicting protein backbone torsion angles from NMR chemical shifts. *J. Biomol. NMR 44*, 213−223.
(44) Güntert, P. (2009) Automated structure determination from NMR spectra. *Eur. Biophys. J. 38*, 129−143.
(45) Herrmann, T., Güntert, P., and Wüthrich, K. (2002) Protein NMR structure determination with automated NOE assignment using the new software CANDID and the torsion angle dynamics algorithm DYANA. *J. Mol. Biol. 319*, 209−227.
(46) Güntert, P., Mumenthaler, C., and Wüthrich, K. (1997) Torsion angle dynamics for NMR structure calculation with the new program DYANA. *J. Mol. Biol. 273*, 283−298.
(47) Koradi, R., Billeter, M., and Güntert, P. (2000) Point-centered domain decomposition for parallel molecular dynamics simulation. *Comput. Phys. Commun. 124*, 139−147.
(48) Ponder, J. W., and Case, D. A. (2003) Force fields for protein simulations. *Adv. Protein Chem. 66*, 27−85.
(49) Laskowski, R. A., Rullmannn, J. A., MacArthur, M. W., Kaptein, R., and Thornton, J. M. (1996) AQUA and PROCHECK-NMR: programs for checking the quality of protein structures solved by NMR. *J. Biomol. NMR 8*, 477−486.
(50) Hooft, R. W., Vriend, G., Sander, C., and Abola, E. E. (1996) Errors in protein structures. *Nature 381*, 272.
(51) Güntert, P. (1997) Calculating protein structures from NMR data. *Methods Mol. Biol. 60*, 157−194.
(52) Schüttelkopf, A. W., and van Aalten, D. M. (2004) PRODRG: a tool for high-throughput crystallography of protein-ligand complexes. *Acta Crystallogr, 60*, 1355−1363.