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Probing the Phosphopantetheine Arm Conformations of Acyl Carrier Proteins Using Vibrational Spectroscopy

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Supporting Information

ABSTRACT: Acyl carrier proteins (ACPs) are universal and highly conserved domains central to both fatty acid and polyketide biosynthesis. These proteins tether reactive acyl intermediates with a swinging 4′-phosphopantetheine (Ppant) arm and interact with a suite of catalytic partners during chain transport and elongation while stabilizing the growing chain throughout the biosynthetic pathway. The flexible nature of the Ppant arm and the transient nature of ACP−enzyme interactions impose a major obstacle to obtaining structural information relevant to understanding polyketide and fatty acid biosynthesis. To overcome this challenge, we installed a thiocyanate vibrational spectroscopic probe on the terminal thiol of the ACP Ppant arm. This site-specific probe successfully reported on the local environment of the Ppant arm of two ACPs previously characterized by solution NMR, and was used to determine the solution exposure of the Ppant arm of an ACP from 6-deoxyerythronolide B synthase (DEBS). Given the sensitivity of the probe's CN stretching band to conformational distributions resolved on the picosecond time scale, this work lays a foundation for observing the dynamic action-related structural changes of ACPs using vibrational spectroscopy.

Polyketide synthases (PKSs) are large enzyme assemblies that produce chemically diverse and pharmaceutically relevant compounds (Figure 1). Central to all PKSs is the presence of at least one acyl carrier protein (ACP), which shuttles extender units and reactive intermediates between catalytic domains as needed.[1] Movement of the ACP's phosphopantetheine (Ppant) arm is thought to be coordinated with protein−protein interactions and significant rearrangement of the PKS enzyme assembly.[2−5] The dynamic structures of ACPs and their Ppant arms are difficult to track due to the flexibility of ACPs and ACPs' transient interactions with catalytic partners.[6]

While nuclear magnetic resonance (NMR) spectroscopy and crystallography have been used to determine structures of bacterial, fungal, plant and mammalian ACPs,[7−18] neither approach provides a clear dynamic picture of how ACPs change conformation during catalysis. NMR can reveal dynamic structural changes, but the use of radio frequencies precludes direct measurements of any conformational changes faster than the μs time scale and therefore cannot resolve the fast movements or structural distributions of ACPs. To overcome this challenge, we sought to develop a vibrational spectroscopic probe to report on ACP structural dynamics. Due to the relatively high frequencies utilized in vibrational spectroscopy, the spectral readout from a single site includes a superposition of signals from all structures that are dynamically resolved on the fs−ps time scale. A vibrational labeling group with a unique frequency can provide the site-specificity necessary to reveal the distribution of environments around the labeled site.[19]

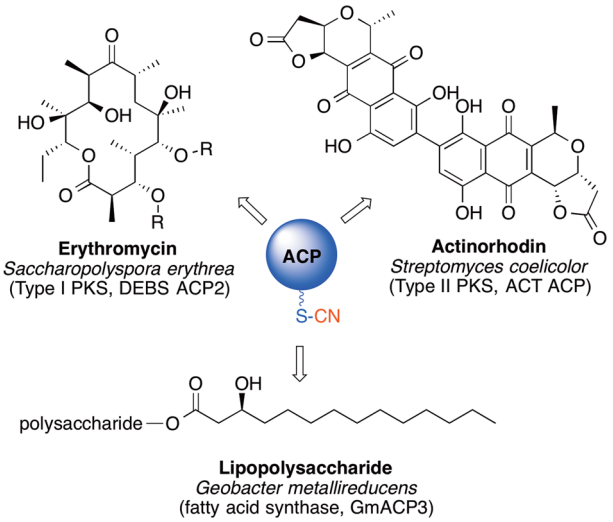

Figure 1. ACPs are central to biosynthesis of structurally diverse molecules produced by microorganisms. Polyketide natural products of the three species of interest to this study are shown.

We installed a site-specific vibrational spectroscopic probe on the terminal thiol of the ACP's 18 Å Ppant arm, which acts as a tether between the protein and reactive cargo for all ACPs. The simple conversion of the terminal thiol to a thiocyanate turns the reactive site of the ACP into a probe that has been previously shown to effectively report on hydrogen bonding and local dynamics in model[20−23] and complex[23−30] systems. The CN stretching frequency mainly reports the degree of water exposure of the probe group, while the line shape reports the heterogeneity and ps dynamics of the probe's local dipolar environment. The SCN moiety has been introduced in several systems by aqueous cyanylation of cysteine residues. The free thiol of the Ppant arm provides a novel pre-existing site that is accessible to this same chemical modification, and because this site is central to the catalytic action of the enzyme, the SCN probe in this context directly reports dynamic changes of the

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ACP conformation at the ACP's reactive site. Since the thiocyanate CN stretching peak appears between 2154 and 2164 cm^{-1}, this band is readily distinguishable from any other infrared spectral features of the ACP. Frequency and line shape variations in this band should reflect subtle changes in the distribution of probe environments related to ACP catalytic function.

To evaluate the cyanylated Ppant (Ppant*) arm as a reporter of ACP structure, we collected the CN stretching absorption bands of two ACPs previously characterized by solution NMR as having their Ppant arms in distinct environments: (i) the ACP from the *Streptomyces coelicolor* actinorhodin PKS (ACT ACP), which has been characterized as having its Ppant arm solvent exposed,[31] and (ii) the ACP from the *Geobacter metallireducens* lipopolysaccharide biosynthetic machinery (GmACP3), characterized as burying its Ppant arm within a hydrophobic cavity[11] (Figures 1 and 2). Both proteins were

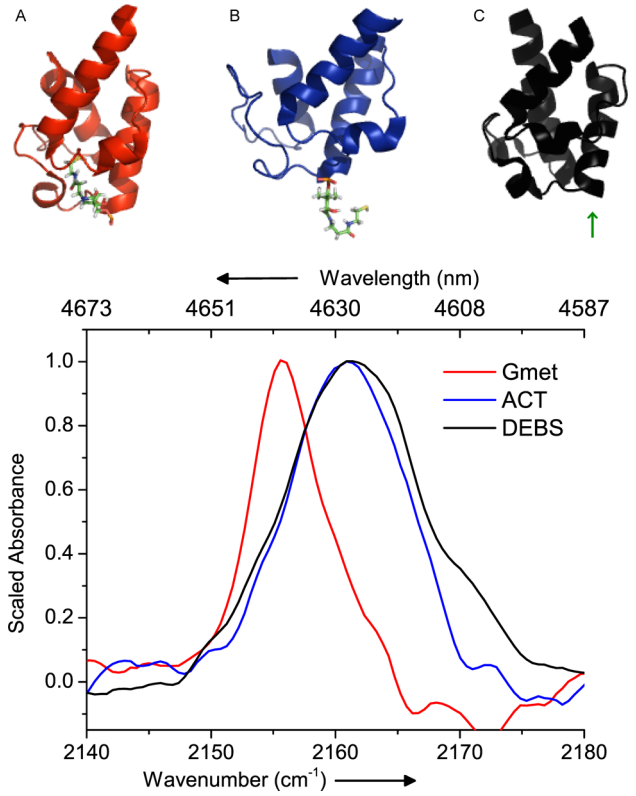

Figure 2. CN infrared absorption bands of cyanylated GmACP3 (red), ACT ACP (blue), and DEBS ACP2 (black), indicating the burial of the Ppant arm of GmACP3 (A, PDB code 2LML) and the solvent exposure of the Ppant arms of ACT ACP (B, PDB code 2K0X) and DEBS ACP2 (location of attachment noted with a green arrow on structure C, PDB code 2JU1). Actual absorbances are 100−200 μOD.

expressed in their *holo* forms and cyanylated using the standard one-pot, two-reaction methodology introduced by Degani[32] and used recently by several others (see Supporting Information for details).[24,27,33] Far-UV circular dichroism (CD) was used to verify that the cyanylation did not induce changes to the helical secondary structure of the peptide backbone (Figure S1). In the case of ACT ACP, which contains a buried cysteine at end of helix I, the *apo*-protein was exposed to cyanylating reagents to verify that only the sterically accessible Ppant arm was cyanylated and not the free cysteine residue in the protein sequence (Figure S2).

Previous studies on the solvent dependence of the thiocyanate CN stretching band indicate that the frequency is sensitive to its local solvation environment, with solvent exposed −SCN moieties exhibiting frequencies near 2163 cm^{-1} and buried probe groups displaying red shifts of varying magnitude.[20−23,30] Thus, the GmACP3 probe vibration should exhibit a pronounced redshift compared to the cyanylated ACT ACP probe according to prior reports.

The IR data were entirely consistent with this hypothesis: the CN stretching band for cyanylated GmACP3 is red-shifted by 6−7 cm^{-1} compared to the cyanylated ACT ACP (Figure 2), in agreement with previous findings that the Ppant arms of these two proteins are in markedly different environments[11,31] and confirming that the Ppant* can serve as a site-specific reporter of the Ppant arm's local environment.

Further, the shape of the IR band reflects the conformation of the Ppant* arm and its local environment. The GmACP3 band is quite narrow, which is consisted with the Ppant* arm's experiencing a relatively homogeneous environment inside the helical bundle. It is likely that this arm is tucked into one particular site inside its ACP, rather than sampling a number of different solvent-excluded "hiding places" inside the active site. The SCN location inside the ACP active site is not necessarily the same as for the unmodified Ppant arm, since the SCN group presents a sterically different profile than the free thiol and interacts differently with its environment (without strong, specific contact interactions).[20] The SCN group's specific environment might be clarified via molecular dynamics simulations.[34] In contrast to the narrow GmACP3 band, the cyanylated ACT ACP band is broad, indicating that the Ppant* arm is experiencing a heterogeneous environment by sampling various solvent dipolar structures around the exposed probe.

We next applied the SCN probe group to an uncharacterized system. The 6-deoxyerythronolide B (DEBS) polyketide synthase has emerged as the prototypical type I modular PKS.[35] The solution structure of the *apo* ACP from module 2 has been solved[14] and the effects of protein−protein interactions on the location of the Ppant arm have been probed by NMR analysis (^{19}F and $^{1}H-^{15}N$ HSQC) of a chemo-enzymatically synthesized trifluoromethylated *holo*-ACP reagent, ACP-S-CF_3, in the presence and absence of catalytic partners.[36] While the Ppant arms of type I ACPs are typically thought to be flexible and only interact transiently with the polypeptide portion of the ACP, the environment of the Ppant arm of the DEBS ACP2 has not been fully characterized. Our IR data for the cyanylated DEBS ACP2 indicates that the Ppant* arm is indeed solvent exposed (Figure 2), with a CN stretching frequency similar to that observed for the ACT ACP.

Together, the spectra in Figure 2 indicate that the cyanylation of ACPs turns the terminal thiol of the Ppant arm into a site-specific vibrational probe that reflects the local environment of the Ppant arm, the catalytically most important piece of the ACP. This is a simple and facile experiment that can be readily used to characterize the catalytic active site of any Ppant-containing enzyme. Solvent exposure of the Ppant arm could also be tracked by monitoring the Stokes shift of a fluorescently modified phosphopantetheinyl appendage of the ACP.[37] While fluorescence would allow the use of lower ACP concentrations, we favored the thiocyanate vibrational spectroscopic probe because of its small size, noninteractivity, and ease of installation. Advantages of using this site-specific methodology over more traditional NMR approaches to interrogate solution ACP structure include (i) sample preparation is

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simpler and less expensive, since the protein need not be expressed in isotopically labeled media nor analyzed in deuterated solvent, and can be evaluated as the His-tagged construct; (ii) less protein is required, as 10 μL of ∼1 mM protein is sufficient for analysis by IR while NMR analysis requires at least 10 times more protein; and most importantly, (iii) vibrational spectra directly report the conformational distribution of the Ppant arm, which is not directly accessible to NMR.

The unidirectional channeling of polyketide intermediates is at least in part facilitated by the movement of the Ppant arm and ACP conformational changes, which in turn modulate coordinated movements within and between modules.[38] Recently, the first crystallographic snapshots of substrate delivery by fatty acid synthase ACPs revealed that concerted movement of the Ppant arm is a molecular trigger that promotes specific protein−protein interactions in fatty acid biosynthesis.[2,3] In this picture, the Ppant arm of the *holo* ACP is solvent exposed until it is acylated or interacts with its catalytic partner, at which point it undergoes considerable conformational change centered about helix II and helix III which in turn drives the complex formation and disassociation steps that lead to catalysis. Our observation that the Ppant arm of DEBS ACP2, similar to the Ppant arm of the ACT ACP, is in the "ready position" with its nucleophilic thiol group solvent exposed suggests PKSs might also call upon the Ppant arm to guide the association−dissociation process during biosynthesis. Notably, this role is entirely consistent with the most recent model put forth by Khosla and co-workers that explains assembly line polyketide biosynthesis as a coupled vectorial process.[39]

A definitive view of the movements involved in polyketide biosynthesis requires approaches that capture transient interactions, fast movements, and conformational distributions.[39] ACP motions during catalysis are thought to occur on the μs time scale, and therefore, the vibrational spectra, unlike NMR spectra, reflect **all** solution structures. Since each PKS contains at least one ACP that interacts with a suite of enzymes, the SCN probe on the Ppant arm can serve as a "helmet camera" that tracks the movements of these remarkable biosynthetic machineries. It is our hope that the proof-of-concept experiments presented here will spark the use of the Ppant* probe as a tool to understand the previously inaccessible operational rules that govern PKSs. The Ppant* probe could find use in a wide range of applications ranging from identifying the rate-limiting step in a PKS assembly line to efficiently reporting on productive versus nonproductive hybrid ACP-protein interactions.

■ ASSOCIATED CONTENT

Supporting Information

Detailed experimental procedures, SDS−PAGE protein gels of all proteins, and CD spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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