Tracking carrier protein motions with Raman spectroscopy

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Engineering microbial biosynthetic pathways represents a compelling route to gain access to expanded chemical diversity. Carrier proteins (CPs) play a central role in biosynthesis, but the fast motions of CPs make their conformational dynamics difficult to capture using traditional spectroscopic approaches. Here we present a low-resource method to directly reveal carrier protein-substrate interactions. Chemoenzymatic loading of commercially available, alkyne-containing substrates onto CPs enables rapid visualization of the molecular cargo’s local environment using Raman spectroscopy. This method could clarify the foundations of the chain sequestration mechanism, facilitate the rapid characterization of CPs, and enable visualization of the vectoral processing of natural products both in vitro and in vivo.
Microbial combinatorial biosynthesis represents a potentially powerful route to gain access to expanded chemical diversity from renewable resources. Its success hinges on understanding how proteins within a synthase communicate. Current mix-and-match approaches often fail due to incompatibility between carrier proteins (CPs) and other enzymes, although the reason why is not entirely clear. CPs are dynamic proteins that play the most central role during the biosynthesis of pharmacologically important classes of molecules, such as fatty acids, polyketides, and non-ribosomal peptides (Supplementary Fig. 1). These small proteins interact with virtually all other proteins within the synthase, and they tether a variety of molecular building blocks and intermediates during natural product biosynthesis.

While visualizing CP conformational changes and interactions with other species is essential for creating functional hybrid synthases, directly capturing transient interactions and the full ensemble of CP conformations remains a challenge for at least two reasons. First, catalytically relevant CP movements are thought to occur on the micro- to pico-second ($\mu$s to ps) timescale, and thus neither nuclear magnetic resonance spectroscopy (NMR) nor X-ray crystallography can characterize the full ensemble of CP-substrate interactions via Raman spectroscopy (Fig. 1). In brief, molecular substrate-mimics with terminal alkyne probes are installed onto the CP via the ligase-catalyzed addition of commercially available carboxylic acids onto the Ppant arm. The alkyne C≡C stretching band is then used to report on changes in the probe environment, which can differentiate with picosecond time resolution between the non-sequestered aqueous state (lower frequency) and the sequestered hydrophobic environment (higher frequency). The modification of a native substrate to include a terminal alkyne is expected to only minimally perturb the natural system because it does not alter the overall length, volume, or hydrophobicity of the molecular cargo. The alkyne C≡C stretching band is a strong, narrow, and unique signal in the transparent region of the Raman spectrum (close to 2100 cm$^{-1}$) that does not overlap with other solvent or biomolecular signals from the untagged CP or other proteins.

**Results**

**Characterization of chain sequestration behavior.** For proof-of-concept experiments, we collected data from three acyl carrier proteins (ACP) for which chain sequestration information was previously reported via NMR and molecular dynamics (MD) simulations: the E. coli type II fatty acid synthase (FAS; EcACP), Streptomyces coelicolor type II acyltransferase polyketide synthase (PKS, Act ACP), and the mammalian rat type I FAS (Rat ACP). For EcACP, MD simulations suggested that an octanoyl acyl chain is the ideal length for complete sequestration of the molecular cargo inside the ACP hydrophobic core. Shorter acyl chains were proposed to be highly mobile and less sequestered since the ACP cavity is too large to stabilize the short substrates; and larger acyl chains are sequestered only at the terminal end of the chain. Previous NMR analysis of Rat ACP in various acylated states suggested that Rat ACP does not sequester any hydrophobic acyl-intermediates due to bulky hydrophobic...
residues that line the interior pocket\textsuperscript{11}. Rat ACP should be viewed as an analog of the EcACP with an inhibited sequestration capability because the EcACP has been shown to at least partially substitute for the EcACP in vitro, functionally interacting with the acyltransferase, ketosynthase, and reductase domains from the \textit{E. coli} FAS\textsuperscript{13}. Taken together, the EcACP and Rat ACP systems present an ideal juxtaposition for preliminary experiments. NMR studies of Act ACP reveal interesting and distinct behavior: butyryl-, hexanoyl-, and octanoyl- acyl chains bind within the hydrophobic cavity, but the substrates are situated perpendicular to their traditional orientation, possibly due to the large size of the cavity (Supplementary Data 1)\textsuperscript{12}. His-tagged ACPs were expressed and purified from \textit{E. coli} BAP1 competent cells\textsuperscript{16}. If necessary, ACPs were completely phosphopantethienylated using the R4-4 Sfp transferase from \textit{B. subtilis}\textsuperscript{17}. The \textit{V. harveyi} acyl-ACP synthetase (AasS) was used to acylate the alkane-containing carboxylic acid to the terminal thiol of the Ppant arm\textsuperscript{18}. 7-octynoic acid (a mimic for an eight-carbon C\textsubscript{8} substrate) was loaded onto both EcACP and Rat ACP, producing distinct Raman spectra in the region of the alkyne probe signal (Fig. 2a). EcACP, expected to sequester the C\textsubscript{8} cargo, produced Raman spectra in the region of the alkyl probe signal, consistent with a non-sequestered state (Supplementary Fig. 2 for more detail); any conformational change would be detected in the spectral lineshape. Conversely, the Rat ACP probe frequency and lineshape were nearly identical to that of the solvated probe, indicating that the same chain attached to Rat ACP was not sequestered. The probe Raman spectrum of Act ACP loaded with 7-octynoic acid is broader and covers frequencies associated with both hydrophobic and aqueous environments, with NMR data for octanoyl Act ACP that suggested the C\textsubscript{8} substrate was only partially sequestered (Supplementary Data 1)\textsuperscript{19}. Taken together, these results from already-characterized ACPs validate our Raman probe-based approach to visualize CP chain sequestration. The spectra in Fig. 2a highlight how the C\textsubscript{8}Raman frequency reports sequestration: the line shape directly reports the complete distribution of environments experienced by the probe, thus providing direct information about the heterogeneous nature of substrate sequestration with sufficient temporal resolution. The intrinsic timescale of Raman spectroscopy for this vibrational probe is about 10 ps (see Supplementary Fig. 2 for more detail); any configurations that interconvert more slowly can be distinguished in the spectral lineshape.

Next, we used this technique to directly evaluate the role of the acyl chain length in chain sequestration. EcACP was acylated with 4-pentoxy acid (a C\textsubscript{5} substrate) and 12-tridecenoic acid (a C\textsubscript{13} substrate). Previous crystallography and NMR studies of acyl EcACPs suggested that hexanoyl, heptanoyl, and decanoyl chains were fully sequestered, while the precise range of butyryl-bound substrate environments remained unclear\textsuperscript{7,20}. The Raman spectrum of the C\textsubscript{5} probe on EcACP indicates that this probe is not sequestered, whereas the C\textsubscript{13} probe displays a similar spectrum to that of the sequestered C\textsubscript{8} probe (Fig. 2b). These data suggest that the two longer probe-labeled chains are sequestered; this provides insight into how EcACP interacts with longer chain lengths, such as C\textsubscript{13}, which has not been the subject of previous work. These results, from different-length substrates on the same CP, point towards future application of our Raman-based technique to explore how substrate sequestration changes throughout the entire substrate elongation process.

This method provides a quick, low-cost, and effective means to analyze CP-substrate interactions that does not depend on structural rendering of the entire protein through more labor-intensive methods. All steps in the process are amenable to high-throughput approaches, which will facilitate rapid characterization of CPs for which conventional structural data are not available. To examine chain sequestration in these CPs, we ligated the C\textsubscript{8}-alkyne probe onto the Ppant arm of two previously uncharacterized type II PKS ACPs: arimetamycin (Arm ACP) and benastatin (Ben ACP), as well as the ACP from the spore pigment biosynthetic gene cluster, WhiE ACP (Fig. 2c). The Arm ACP spectrum exhibits a higher C\textsubscript{8}Raman frequency than the C\textsubscript{8} probe in buffered solution. Like Rat ACP, the Ben ACP and WhiE ACP spectra produced nearly identical signals to those of the aqueous C\textsubscript{8} probe. The spectrum of the WhiE ACP loaded with the C\textsubscript{5} probe also indicated a non-sequestered state (Supplementary Fig. 3). Alongside data from the C\textsubscript{8} probe on Act ACP, these results support the hypothesis that a general feature of type II PKS ACPs is a hydrophobic cavity too large to fully stabilize shorter and/or less polar acyl chain probes relative to the native substrate\textsuperscript{12}. Coupling the Raman probe technique described here with the synthesis of more sophisticated substrate-intermediates, and/or site-directed mutagenesis of tagged ACPs, will reveal further details of the exact molecular nature of substrate sequestration with suf...
interactions that govern chain sequestration in both ACPs and CPs from other biosynthetic pathways.

**Discussion**

The ubiquitous yet heterogeneous (across substrate lengths and proteins from different pathways) role of CP-substrate interactions is central in natural product biosynthesis, yet conventional structural methods cannot directly capture these events. The site-specific vibrational approach implemented here represents a relatively simple and broadly applicable method that will enable the rapid elucidation of dynamic structures across diverse CP-substrate interactions. The optical equipment used here (see Methods) is an ordinary continuous-wave, dispersive Raman spectrometer that does not supply any optical enhancement (i.e. UV-resonance or stimulated scattering) of the Raman signal, so signals like those we report are quite easy to access using relatively unspecialized equipment. While the interpretations that we present of the alkene frequencies and lineshapes are based on empirical comparisons, recent work has demonstrated that MD simulations coupled with effective fragment potential-based calculations can be used to quantitatively simulate vibrational probe lineshapes21,22. Our current computational work focuses on the extension of such calculations to alkane probes and the simulation of the spectra observed here; these efforts should enable a more directly quantitative and physical interpretation of the Raman data in Fig. 2 and from other CPs of future interest.

The alkene probes introduced onto the CPs in this study could also serve as biorthogonally reactive substrates capable of being processed through chain elongation, chain transfer, and tailoring events while simultaneously reporting on changes in the local substrate environment. With strong and growing evidence that CP chain sequestration and flipping (the movement of a CP-bound substrate from inside the hydrophobic core of the CP into the active site of a partner enzyme) is centrally linked to functional channeling of biosynthetic intermediates8, this approach can be applied broadly to fill a central and unmet need in understanding the molecular details of those biosynthetic pathways across many species and syntheses.

It is also anticipated that this technique will be used to elucidate the unconventional behaviors of CPs and can be applied in cases where, for example, the substrate is tucked against a non-polar patch on the surface of the CP domain23. Probe-labeled substrates, including those containing alkynes further up the chain and those with more complicated oxidation and substitution patterns, can also be utilized to provide more in-depth insight into the nature of CP-substrate interactions. Additionally, the alkynyl probes in this work could also bridge the gap between in vitro and in vivo studies of biosynthetic events, as alkynyl-labeled species can be imaged in vivo by stimulated Raman microscopy (sometimes simultaneously with other fluorescently labeled species, which could enable novel co-localization studies of direct relevance to biosynthesis)24-26. In a more general sense, data from the technique reported here could help to design hybrid natural product syntheses capable of accessing novel chemical diversity.

**Methods**

**Protein expression and purification.** BAP1 competent cells16, which feature a T7 RNA polymerase, were used to transform the respective plasmids for expression of EcACP, ActACP, RatACP, ArmACP, BenACP, and WhiEACP (all featuring 6xHis-tag) digested with NdeI/EcoRI and treated with calf-intestine alkaline phosphatase (CIP), T4 ligase, and 1X Dilution buffer. For ligation at room temperature (30 min), 10 µL of the ligation product mixture was transformed into chemically competent DH5α cells and plated on LB agar plates (50 µg/mL kanamycin). The plasmids for ArmACP, BenACP, and WhiEACP were prepared by these means for a previous study28. The plasmid for RatACP was prepared for this study (see Supplementary Fig. 4 for sequence of DNA insert). All plasmids are available from the authors upon request.

A single colony was selected for the growth of seed cultures overnight at 37 °C in 10 mL of LB media (50 µg/mL of kanamycin or 100 µg/mL of carbenicillin). Seed cultures were then added to 0.75 L production cultures (50 µg/mL of kanamycin or 100 µg/mL of carbenicillin) and were grown at 37 °C until the OD600 was 0.6. After sufficient culture growth, cells were induced with 188 µL of 1 M IPTG. The induced cultures were incubated at 18 °C overnight, while shaking. Following the induction period, cells were harvested by centrifugation (5,000 RPM, 20 min), and the cell pellets were stored at –80 °C.

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**Complete 4'-phosphopantetheinylation of ACPs.** Liquid chromatography mass spectrometry (LC-MS) analysis of ACPs (see below) revealed that some ACPs were produced as a mixture of the apo and holo forms and thus a 4'-phosphopantetheinyl transferase was used to push these ACP samples completely to the holo form. Coenzyme A (1.5 mM) was added to a solution of ACP (0.1-1.0 µM), with Sfp R4-4 (2 µM)17, magnesium chloride (10 mM) and DTT (2.5 mM), in 50 mM sodium phosphate pH 7.6 (0.8-1 mL total volume). The solution was incubated for 24 h at room temperature. All ACPs were purified into 50 mM phosphate buffer pH 7.6 using an AKTA FPLC equipped with a HiPrep 26/10 desalting column. EcACP was concentrated to 1 mM using a 3.5-kDa MWCO Centricor centrifugal filter. LC-MS was used to confirm all ACPs were completely in the holo form before substrate loading.

**Chemoenzymatic attachment of probe-containing substrates.** 4-pentylenoic acid (C5), 7-octynoic acid (C8), and 12-tridecynoic acid (C13) were all commercially acquired (C5:Enamine, C8:Enamine, C13:Mcule). NMR data were acquired to verify the purity of each probe (Supplementary Figs. 5–7). Carboxylic acids were loaded onto the ACPs using the *Vibrio harveyi* Acyl-acyl carrier protein synthetase (AasS). AasS has been shown to be a promiscuous ligase, capable of loading various fatty acids onto the terminal thiol of the ACP’s Pant arm19. The reaction was completed on a 1-mL scale, consisting of the ACP (275 µM, stock in 50 mM sodium phosphate buffer pH 7.6), dithiothreitol (2.5 mM), magnesium chloride (23 mM), ATP (18 mM, stock adjusted to pH 7.6), AasS (0.8 µM, stock in Tris buffer pH 7.6), and the desired carboxylic acid (4.6 mM, stock in isopropanol). The reaction was prepared in a glass vial, as plastic tubes have previously been shown to contain competitive carboxylic acids that could be loaded onto the ACP in place of the desired substrates. The reaction mixture was left shaking at 100 RPM for 16 h at 37 °C. Samples were spun in a centrifuge at 13,000 RPM for 5 min to pellet precipitation. Supernatant was loaded onto a Sephadex G-25 in PD-10 Desalting Column to separate the protein from salts and remaining unloaded substrate. A ThermoScientific Nanodrop 2000c spectrophotometer was used to determine protein concentration and purity. Fractions featuring characteristic protein peaks and lacking a 260-nm peak (characteristic of the unloaded carboxylic acid) were selected. Chosen fractions were pooled and concentrated using a 3.5-kDa MWCO Centricor centrifugal filter. The concentration used for visualization via Raman spectroscopy ranged from 1 to 3 mM.

**Verification of substrate loading onto ACPs.** ACPs (20 µL of a 0.1-mg/mL solution in 50 mM sodium phosphate buffer, pH 7.6) were analyzed by LC-MS.
Characterizing ACP structure after probe loading. Circular dichroism (CD) spectroscopy was performed using an Aviv 410 spectrophotometer (Aviv Biomedical, NJ). The secondary structures of hole and modified ACPs were determined by obtaining CD spectra at far-UV (260–180 nm) in a 0.1 mm path length cuvette (Hellma Analytics). All data were collected at 25 °C using a 1-nm bandwidth, a step resolution of 0.5 nm, and a 3-s averaging time. The baseline was corrected against the storage buffer, and ACP concentrations were −0.5 mg/mL. The corrected spectra were smoothed using a manual smoothing function implemented in the instrument software, using a window width of 11 data points, degree 2. Smoothed data were plotted in Origin (v.8.6.0). See Supplementary Figs. 29–30.

Raman spectroscopy. All Raman spectra were collected using a home-built CW-Raman spectrometer. A 532-nm DPSS CW laser (Coiboll, Inc.) was attenuated to 80 mW incident power was focused vertically through a 1-mm diameter glass capillary filled with 1–5 μL of sample. Scattered light was collected at 90° to the incident beam at a Nikon f/1.2 camera lens and then focused onto the slit of a P-Acceptor SpectraPro 500 nm single monochromator (with a 600 grooves/mm grating blazed at 500 nm) and collected on a P-Accept Spec10/100 liquid-nitrogen-cooled CCD camera. Rayleigh scattering was rejected using a > 532 nm long-pass filter (Edmund Optics). Spectra were collected in exposures of 1 min for up to 2 h total accumulation time. All ACP samples were 1–3 mM concentration.

Data analysis was completed in Origin 8. The raw data was imported and analyzed within a large region around the desired peak to establish a baseline (2000 cm⁻¹ to 2300 cm⁻¹). A smaller region containing the peak (2100 cm⁻¹ to 2130 cm⁻¹) was cut out to introduce a hole where the peak of interest was. The hole-containing baseline region was fit to a seventh-degree polynomial, and this polynomial was subtracted from the peak-containing baseline region data. The alkyn probe signal was then scaled to set the maximum point of the peak of interest at 1.0 and all other points a fraction relative to the maximum. The mode (by inspection), mean (calculated between 2100–2130 cm⁻¹), and FWHM (by inspection) are reported for each spectrum (Supplementary Table 1).

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

The data underlying the findings of this study are available from Open Science Framework (https://doi.org/10.17605/OSF.IO/RKK4E) and from the authors upon reasonable request. The raw data underlying Fig. 2 as well as Supplementary Figs. 3 and 27–30 are provided in a Source Data file.

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L.K.C. and C.H.L. designed the study; S.C.E., E.S.W., and A.R.H. performed research; S.C.E., E.S.W., A.R.H., C.H.L., and L.K.C. analyzed data; S.C.E., C.H.L., and L.K.C. wrote the manuscript.

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