Dynamics in an unusual acyl carrier protein from a ladderane lipid-synthesizing organism

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Abstract
Anaerobic ammonium-oxidizing (anammox) bacteria express a distinct acyl carrier protein implicated in the biosynthesis of the highly unusual “ladderane” lipids these organisms produce. This “anammox-specific” ACP, or amxACP, shows several unique features such as a conserved FF motif and an unusual sequence in the functionally important helix III. Investigation of the protein’s structure and dynamics, both in the crystal by ensemble refinement and by MD simulations, reveals that helix III adopts a rare six-residue-long $\beta$-helical conformation that confers a large degree of conformational and positional variability on this part of the protein. This way of introducing structural flexibility by using the inherent properties of $\beta$-helices appears unique among ACPs. Moreover, the structure suggests a role for the FF motif in shielding the thioester linkage between the protein’s prosthetic group and its acyl cargo from hydrolysis.

KEYWORDS
ACP, acyl carrier protein, ensemble refinement, lipid biosynthesis, molecular dynamics, protein dynamics

1 | INTRODUCTION

Bacteria and archaea use a wide array of acyl chains in their lipid membranes, ranging from saturated- and partially unsaturated linear chains to highly branched and even cyclopropyl group-containing moieties. Arguably, the most surprising and interesting acyl chains encountered in microbial lipids, however, are the ladderanes, which consist of linearly fused cyclobutane rings forming a ladder-like structure (Figure 1A). Ladderanes are exclusively found in the lipids of anaerobic ammonium oxidizing (anammox) bacteria, which use the energy released by the oxidation of ammonium with nitrite to generate a proton gradient across an internal membrane, which in turn drives ATP synthesis. As recent measurements have confirmed that membranes containing ladderane-based lipids are less permeable to protons and hydroxide ions, it is currently believed that anammox bacteria employ ladderanes in their membranes to help maintain their metabolic proton gradient. How ladderane lipids are produced, however, remains enigmatic; some mechanistic proposals have been put forward, but no direct information on ladderane biosynthesis is available to date. However, bioinformatics has identified a gene cluster unique to anammox bacteria that is both conserved and expressed at high levels, and which encodes homologs of canonical fatty acid biosynthesis enzymes as well as other proteins, such as a putative radical SAM enzyme. Currently, the proteins encoded by this gene cluster are the most likely candidates for enzymes involved in ladderane biosynthesis.

One of the unusual proteins on this gene cluster is amxACP, a unique version of an acyl carrier protein (ACP), specific to anammox bacteria. ACPs are small, four-helical-bundle proteins that shuttle nascent fatty acid molecules from enzyme to enzyme during fatty acid biosynthesis, or nascent polyketide chains during polyketide synthesis. ACPs either occur as a domain of a large, multidomain synthetase consisting of a single polypeptide (Type I ACPs), or as discrete proteins (Type II ACPs), as is the case for amxACP. ACPs bind the nascent

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FIGURE 1  Legend on next page.
acyl chain via a thioester linkage to a 4'-phosphopantetheine moiety, which in turn is bound to a conserved serine on the surface of the protein (Figure 1B). How the acyl chain interacts further with the ACP varies, but typically Type II ACPs sequester their acyl cargo in an internal cavity, which expands as needed to accommodate nascent acyl chains at various stages of maturation. This expansion is achieved without any major changes to the fold of the protein but does appear to involve a repositioning of helix III. A displacement of helix III was also implicated in opening the side of the protein to allow the acyl chain to move into and out of its binding cavity in a switchblade-like manner, to be presented to various enzymes for modification.

Biosynthetic pathways typically use a dedicated ACP that is tailor-made for its particular cargo and for the required interactions with the pathway’s enzymes. Interestingly, the amino acid sequence of amxACP shows unique features conserved in anammox organisms. A dual phenylalanine (FF) motif, located between helices I and II, was found to be typical for amxACPs. Moreover, the sequence of helix III is distinct from that in other ACPs but highly conserved among amxACPs. To investigate how these unique features might influence the properties of the protein, we determined the structure of the amxACP from the anammox model organism Kuenenia stuttgartiensis.

This protein can be loaded with various acyl chains, including ladderane-containing acyl chains (Dietl et al., in preparation). We find that in K. stuttgartiensis amxACP, the functionally important helix III forms a rare, six residue-long 3_10-helix, and we study the protein’s conformational flexibility in crystals by ensemble refinement and in silico by molecular dynamics. The results point to a hitherto unknown, distinct manner in which amxACP achieves the remarkable flexibility that all Type II ACPs require to carry out their functions.

2 | MATERIALS AND METHODS

2.1 | Protein expression and purification

The gene coding for the anammox acyl carrier protein from Kuenenia stuttgartiensis (amxACP, kuste3603) was amplified from genomic DNA by polymerase chain reaction (PCR), using primers KsE3603_F1 (5'-GGAGGACCTACCATGGACGAAGAG-3') and KsE3603_R1 (5'-CGAAATACCCCGAGCTTCCACCTGCTGG-3').

The PCR product was cloned into the pET-24d vector with a C-terminal hexa-histidine tag using the NcoI and Xhol restriction sites. The protein was heterologously expressed in Escherichia coli BL21 (DE3) cells. 2 x 2 L lysogeny broth (LB) medium supplemented with kanamycin (50 μg/ml) were inoculated with 1% (v/v) overnight overnight pre-culture and grown at 37°C while shaking at 100 rpm in baffled 5 L Erlenmeyer flasks. Protein expression was induced at an OD_600 of 0.6–0.8 by the addition of 500 μM isopropyl-β-D-thiogalactopyranoside (IPTG), after which incubation was continued at 20°C, 90 rpm for 16–18 hr. The bacterial cells were harvested by centrifugation at 8000 x g, 15 min, 4°C. The cell pellet was resuspended in wash buffer (WB, 50 mM Tris-HCl pH 8.0, 300 mM NaCl and 10 mM imidazole) with the addition of one spatula tip of each lysozyme and DNasel (Roche, Mannheim, Germany). The cells were disrupted using a microfluidizer (Microfluidics, Westwood) operated at a pressure of 0.7 MPa, and the lysate was clarified by centrifugation at 50 000 x g for 1 hr at 4°C. The clarified lysate was filtered through a 0.45 μm syringe filter and loaded onto a gravity-flow column containing 8 ml of Ni-NTA (nickel-nitriilotriacetic acid) agarose (Qiagen, Hilden, Germany) pre-equilibrated with buffer WB. The column was further washed with 30 ml of buffer WB. The C-terminally His-tagged amxACP was eluted using elution buffer EB [50 mM Tris-HCl pH 8.0, 300 mM NaCl and 250 mM imidazole] and collected in 2 ml fractions. ACP-containing fractions were pooled and concentrated using a 3-kDa cut-off Amicon concentrator (Millipore Bioscience, Schwalbach, Germany). The concentrated protein was further purified using gel filtration chromatography on a Superdex 75 (16/60) column (GE Healthcare, Uppsala, Sweden) in gel filtration buffer (GFB, 50 mM HEPES-NaOH pH 7.5 and 150 mM NaCl) at a flow rate of 1 ml/min at 8°C. Finally, ACP-containing fractions were pooled, concentrated, and buffer-exchanged to a buffer containing 25 mM HEPES-KOH pH 7.5 and 25 mM KCl using a 3-kDa cut-off Amicon concentrator up to a concentration of about 40 mg/ml. Protein purity was assessed by 15% SDS-PAGE and protein concentration was determined based on the absorbance at 280 nm using an extinction coefficient of ε_280 = 5500 M⁻¹ cm⁻¹ on a NanoDrop spectrophotometer (Peqlab, Erlangen, Germany).

The protein solution was flash-frozen in small aliquots in liquid nitrogen and stored at –80°C until further use.
2.2  |  Protein crystallization

C-terminally His-tagged apoACP from *Kuenenia stuttgartiensis* (amxACP) was crystallized using the vapor-diffusion hanging-drop method by mixing 1.5 μL protein solution (10 mg/mL in 25 mM HEPES-KOH pH 7.5 and 25 mM KCl) with 1.5 μL precipitant on glass cover slips that were placed above a reservoir containing 800 μL of precipitant solution in pre-greased 24-well crystallization plates (Crystalgen Inc, Commack, New York) at 20°C. Hexagonally shaped crystals with dimensions of 30 × 10 × 10 μm² were obtained after 2–3 days from a precipitant containing 28% (v/v) PEG 400, 100 mM sodium acetate, 100 mM sodium acetate pH 4.5, and 10 mM zinc chloride. The crystals were flash-cooled in liquid nitrogen after brief soaking in a cryoprotectant containing 35% (v/v) PEG 400, 150 mM calcium acetate, 100 mM sodium acetate, pH 4.5, and 10 mM zinc chloride.

2.3  |  Data collection and structure determination

A highly redundant, 2.5 Å resolution Zn-SAD dataset was recorded from an apo-amxACP crystal at 1000 Å wavelength (at which f² for Zn is 2.6 e⁻) at the PXII beamline of the Swiss Light Source (Paul Scherrer Institute, Villigen, CH). Data were processed using XDS²⁰ and showed appreciable anomalous signal up to 2.8 Å resolution, as judged by the significance of the anomalous correlation at the 0.1% confidence level. These data were phased by phenix.autosol²¹,²² using the anomalous signal from the zinc ions. Six sites were found automatically, and after density modification, a figure-of-merit of 0.388 was obtained. Automatic building resulted in a partial model that contained 67 residues (out of 91 in the final model) of which >50% had the correct sequence assigned. The structure was completed manually using Coot²³,²⁴ to obtain a high-quality dataset with minimal radiation damage at higher resolution for refinement, the first 100 of the data were reprocessed taking data to higher resolution into account. This dataset showed considerable anisotropy, extending to ~1.8 Å resolution along the k axis but only to ~2.5 Å along the l axis. After integration with XDS,²⁰ the data were therefore merged with STARANISO, while applying an ellipsoidal resolution cut-off.²⁵ The final single-structure model was obtained by refinement against this data set using phenix.refine.²⁶ Data and model statistics are reported in Tables 1 and 2.

| Table 1: Data collection and processing statistics |
|-----------------------------------------------|
| **amxACP, Zn-SAD** | **amxACP, high res. processing** |
| **Diffraction source** | SLS PX-II | SLS PX-II |
| **Wavelength (Å)** | 1.0000 | 1.0000 |
| **Temperature (K)** | 100 | 100 |
| **Detector** | Pilatus 6M | Pilatus 6M |
| **Crystal-detector distance (mm)** | 390 | 390 |
| **Rotation range per image (')** | 0.1 | 0.1 |
| **Total rotation range (')** | 315 | 100 |
| **Exposure time per image (s)** | 0.1 | 0.1 |
| **Space group** | P6₁ | P6₁ |
| **a, b, c (Å)** | 76.8, 76.8, 30.9 | 76.8, 76.8, 30.9 |
| **α, β, γ (°)** | 90.0, 90.0, 120.0 | 90.0, 90.0, 120.0 |
| **Mosaicity (°)** | 0.2 | 0.2 |
| **Resolution range (Å)** | 38.4–2.5 (2.6–2.5) | 38.4–1.8 (1.9–1.8) |
| **Total no. of reflections** | 61 600 (4700) | 39 342 (1123) |
| **No. of unique reflections** | 3741 (280) | 13 897 (935) |
| **Completeness (%)** | 99.9 (100.0) | 91.6 (57.8) |
| **Redundancy** | 16.5 (16.8) | 5.3 (2.8) |
| **R₁(merge)** | 33.3 (13.6) | 16.4 (1.5) |
| **CCₐₐₐ** | 0.072 (0.426) | 0.025 (0.652) |
| **Overall R factor from Wilson plot (Å²)** | 48.6 | 32.3 |

Note: Values for the outer shell are given in parentheses.

²²Treating Friedel mates as individual reflections.

²³An ellipsoidal cut-off was applied using STARANISO.

| Table 2: Structure solution and refinement |
|------------------------------------------|
| **amxACP apo stand-alone** | **amxACP apo ensemble (75 models)** |
| **PDB# 7AU5** | **PDB# 7AX5** |
| **Resolution range (Å)** | 38.4–1.8 (1.9–1.8) | 38.4–1.8 (1.9–1.8) |
| **Completeness (ellipsoidal, %)** | 91.6 (57.8) | 91.6 (57.8) |
| **σ cut-off** | F > 1.34σ(F) | F > 1.34σ(F) |
| **No. of reflections, working set** | 13 208 (715) | 13 208 (715) |
| **No. of reflections, test set** | 689 (36) | 689 (36) |
| **Final Rᵣᵣᵣᵣ** | 0.2301 | 0.2862 |
| **Final Rᵣᵣᵣᵣᵣ督察** | 0.1747 | 0.2343 |
| **ML-Based DPI** | 0.23 | 0.16 |
| **No. of non-H atoms** | | |
| **Protein** | 1451 | 1451 |
| **Ligands** | 7 Zn ions | 7 Zn ions |
| **Water** | 36 | 36 |
| **R.m.s. deviations** | | |
| **Bonds (Å)** | 0.012 | 0.008 |
| **Angles (°)** | 1.394 | 0.664 |
| **Average B factors (Å²)** | | |
| **Protein** | 57.7 | 45.7 |
| **Ligands** | 84.0 | 48.7 |
| **Water** | 46.6 | 52.6 |
| **Ramachandran plot** | | |
| **Most favored (%)** | 95.35 | 87.23 |
| **Allowed (%)** | 4.65 | 7.54 |
| **Outliers (%)** | 0.00 | 5.24 |
| **Rotamer outliers (%)** | 1.27 | 12.7 |

Note: Values for the outer shell are given in parentheses.

²⁴A large number of unfavorable (α, β) combinations and rotamers are expected, as ensemble refinement samples high-energy conformers.²⁷
2.4 | Ensemble refinement

Ensemble refinement was performed using phenix.ensemble_refinement\textsuperscript{27} according to the protocol described previously.\textsuperscript{28} Explicit hydrogens were added to the final, refined single-model structures using phenix.refine, after which a three-dimensional grid search was set up scanning different values for $p_{\text{TLS}}$ (0.6, 0.8, 0.9, and 1.0), for $T_{\text{bath}}$ (2.5, 5.0, and 10.0) and $t_x$ (0.3, 0.6, and 1.2). The individual refinements were carried out in parallel on 36 cores of a cluster of Intel Xeon CPU X7560 processors running at 2.3 GHz. The lowest $R_{\text{free}}$ (0.2343) was obtained at $p_{\text{TLS}} = 0.8$, $T_{\text{bath}} = 2.5$ and $t_x = 0.6$. The final ensemble was analyzed and the results displayed using custom-written python scripts employing NumpY\textsuperscript{29,30} and Matplotlib.\textsuperscript{31,32}

2.5 | Molecular dynamics simulations

MD calculations were performed with GROMACS 2020\textsuperscript{33,34} using the AMBER99SB-ILDAB force field\textsuperscript{35} and TIP3P water\textsuperscript{36} in a simulation box of the size of the molecule plus 10 Å on all sides. After energy minimization, 100 ps each of NVT and NpT equilibrations were performed with positional restraints on the protein atoms. Then, a production MD run of 1 μs with a time step of 2 fs was performed at 300 K using a modified Berendsen thermostat and Parrinello-Rahman pressure coupling, while employing periodic boundary conditions. Simulations were carried out on a cluster of 4 Intel Xeon CPU E7-4890 v2 processors with 15 cores each, running at 3.4 GHz.

2.6 | Bioinformatics

A custom-written python script using the PyPDB module\textsuperscript{37} was used to conduct a search of the Protein Data Bank\textsuperscript{38} for entries mentioning “acyl carrier protein” in any text record, revealing 1006 entries. These were retrieved individually and passed to the DSSP program (CMBI version 3.0),\textsuperscript{39,40} and the results searched automatically for stretches of 4 or more residues in a 310-helical conformation in a chain shorter than 150 residues. NMR structure ensembles were searched model by model. Entries flagged as possibly containing an ACP with a 310 helix were manually inspected in PyMol (Schrödinger, Inc.).

To investigate how often 310-helices of 6 residues (2 turns) or longer occur in general, we performed automated DSSP 3.0 analysis of 10 000 randomly sampled structures of any size in the PDB, searching for 5, 6, 7, 8, or more consecutive residues in a 310-helical conformation in any chain, and in any model of a multi-model structure, without visual inspection.

3 | RESULTS

Kuenenia stuttgartiensis amxACP was heterologously expressed, crystals were grown and diffraction data collected to a resolution of 1.8 Å. These were phased by single-wavelength anomalous diffraction, using the anomalous signal from zinc ions present during crystallization. Although phasing and initial model building were easily achieved by automatic means, refinement was far from trivial; various refinement strategies were evaluated but none resulted in satisfactory R-factors. Pathologies that could explain these difficulties, such as twinning or translational NCS, were not detected, but the presence of pronounced anisotropy in the data pointed to a degree of disorder in the structure, as did the fact that in some regions the electron density appeared smeared out. We therefore employed ensemble refinement\textsuperscript{27,28,41-43} to account for any such flexibility. This resulted in an ensemble model for amxACP containing 75 separate structures which has acceptable R-factors. Data collection statistics are given in Table 1, and statistics for both the stand-alone and ensemble models are given in Table 2.

The results show that amxACP displays the typical four-helical bundle fold of a Type II acyl carrier protein (Figure 2A), as demonstrated by the relative orientations of helices II, III, and IV.\textsuperscript{44} In addition to these helices, a short, two-turn α-helix between helices I and II is present that also occurs in several other ACPs, and which we call helix la. The conserved Ser41, to which the 4'-phosphopantetheine linker binds, is located at the N-terminus of helix II. Strikingly, helix III, which consists of residues 60–67, adopts a 310-helical conformation as determined by DSSP 3.0\textsuperscript{39,40} and STRIDE,\textsuperscript{45,46} rather than a regular α-helical conformation as the other helices do. This 310-helix stretches over an unusual length of six residues (two complete turns, residues 61–66). Importantly, ensemble refinement identifies this helix III as well as the loop consisting of residues 30–40 which contains the FF motif specific to amxAACPs (Phe33–Phe34), as highly flexible (Figure 2B). Within the structures obtained by ensemble refinement, these two structural elements displayed positional RMSDs of up to 0.6 Å for the Cα atoms. The rest of the protein, with the exception of the N- and C-termini, showed much lower RMSDs (Figure 2C). This apparently higher structural variability is reflected in the electron density maps (Figure 2D) and geometry statistics. However, the electron density for helix III is of sufficient quality to confidently assign a 310-helical conformation to this structural element.

The difference in flexibility between helix III and the other helices could be due to crystal packing; in the crystal, helix III has room to move whereas helices la, II and IV are involved in crystal contacts. We therefore performed a 1 μs molecular dynamics simulation of amxACP in water and compared the results with those of the ensemble refinement. Inspection of the trajectory shows that the overall fold of the molecule is maintained over the time course of the simulation. However, plotting the positional RMSD of Cα atoms reveals that the overall flexibility of the structure in the MD simulation is indeed higher than in the X-ray structure with helix III being considerably more flexible during the simulation than the rest of the protein.

During large parts of the MD simulation’s time course, the 310-helical structure in helix III is stable, with periods of unfolding in between: residues 61–65 adopt a 310-helical conformation more than 50% of the time with residue 66 doing so 28%, and 6% of the time three or more of these residues assume an α-helical conformation, which is in line with simulation results reported by Narwani and coworkers.\textsuperscript{47} In the ensemble refinement results, the 310-helix is present in about half of the individual models (Figure 3). We therefore investigated whether the high RMSD...
values observed are solely due to an unfolding of the helix or whether they, to a first approximation, represent motions of an intact helix III. We therefore split the ensemble refinement result and the MD trajectory into frames with an intact 310-helix in helix III (defining five consecutive residues in the correct conformation as “intact”) on the one hand and frames with a partially unfolded helix III on the other. We then recalculated the RMSD values for each set of frames (not shown), which revealed that the comparatively high RMSD values for helix III are due to both effects: a partial unfolding of the 310-helix as well as motions of the folded helix III as a whole (Figure 4).

Our crystal structure shows that in amxACP, helix III forms exclusively hydrophobic interactions with the rest of the protein (Figure 5A), over a surface area of 370 Å². Of this buried surface, more than 40% is accounted for by Ile67 alone, which is not part of the 310-helix. The sidechain of Ile67 contacts Ala45 and Ile47 on helix II, as well as Thr73 on helix IV. In crystal structures of acyl-loaded E. coli ACP, this conserved residue at the C-terminus of helix III was found to be the only residue undergoing a large change in backbone conformation upon ligand binding.14 Other interactions with helix III are made by Leu45 and Leu48 on helix II and Leu76 on helix IV.

To investigate how often 310-helices occur in helix III of ACPs, we mined the Protein Data Bank for other ACP structures with a 310-helix in helix III. Apart from a few NMR structures in which short 3₁₀⁺ helices occur in some of the models in the ensemble, the ACP Asl1650 from Nostoc sp. PCC 7120 (formerly called Anabaena sp. PCC 7120)⁴⁸ and spinach chloroplast ACP¹³ are the only ACPs we found in which a part of helix III predominantly adopts a 3₁₀⁻ helical conformation, albeit for only three or four consecutive residues, respectively. Thus, amxACP appears to be the only ACP structurally characterized to date with a helix III that mainly consists of two full turns of 3₁₀⁻ helix, that is, six consecutive residues. By contrast, investigation of 10 000 randomly sampled PDB structures of any size or description revealed that around 11% contain a stretch of six residues that adopt a 3₁₀⁻ helical conformation as determined by DSSP 3.0.

**FIGURE 2** (A) Structure of amxACP from Kuenenia stuttgartiensis, as determined using conventional (single model) refinement. Ser41 is shown as sticks, and helix III, which is a 3₁₀⁻ helix, is shown in orange. (B) Result of ensemble refinement, showing considerable flexibility in the 30–40 loop and in helix III. (C) RMSD of Cα positions plotted as a function of residue number for the ensemble refinement (blue) and MD (red) results. The various structural elements are indicated below the horizontal axis. In both cases, helix III (hIII, orange) shows the largest structural variability. (D) 2mFo-DFc electron density map from ensemble refinement (blue, 1.0 σ) overlayed on helices III and IV. The electron density is more well-defined for helix IV than it is for helix III.

**DISCUSSION**

ACPs require a considerable degree of conformational flexibility to perform their function. They need to be able to bind acyl chains of various lengths, and which are in various states of functionalization as encountered during their synthesis. Moreover, they need to signal these states to binding partners, and need sufficient flexibility to allow access of even very large acyl chains to and from their binding.
As shown by our crystallographic and simulation results, a large degree of conformational flexibility in amxACP resides in helix III, which largely consists of a two-turn (six residue) $3_{10}$-helix. This length is comparatively rare for a $3_{10}$-helix; the vast majority (96%) of $3_{10}$-helices are only four residues or less in length, in fact, their average length is just one turn. Most $3_{10}$-helices are found at the termini of $\alpha$-helices and it has been proposed that they represent an intermediate state between the $\alpha$-helical and random coil conformations. Indeed, they appear to be less conformationally stable than, for example, an $\alpha$-helix, and as such have been associated with structural transitions in both peptides and proteins. A switch between $3_{10}$- and $\alpha$-helix conformation was observed for a synthetic peptide in response to a change in solvent polarity and another peptide was found to be in equilibrium between the two states. A transition between the two states was suggested to explain the behavior of another peptide in response to an applied electric field. In aspartate aminotransferase, a switch of one helical turn from $\alpha$- to $3_{10}$-helical conformation assists in closing the enzyme’s active site and a switch between an $\alpha$- and $3_{10}$-helix is implicated in the functioning of certain voltage-gated ion channels.

Interestingly, the sequence of helix III in amxACP is strongly conserved among anammox genera yet distinct from that in other ACPs, and appears to have evolved specifically to favor the formation of a $3_{10}$-helix. For instance, the N-terminus of helix III consists of two
negatively charged residues in sequence (Glu61 and Glu62). The presence of N-terminal glutamate residues was found to have a stabilizing influence on 3_10-helices. Moreover, the presence of a glutamate at position 62 as well as an aspartate at position 66 results in an electrostatically unfavorable $i-i+4$ interaction, which would destabilize an $\alpha$-helical conformation, favoring a 3_10-helix formation.59

Apart from being inherently less stable than an $\alpha$-helix, the properties of 3_10-helices also dictate specific ways in which they pack with other structural elements. As pointed out before, their helical pitch of $\sim 3$ residues per turn results in "ridges" of side chains at $\sim 120^\circ$ intervals around the axis of the helix, rather than the more spread out, staggered arrangement of side chains found around an $\alpha$-helix. This results in two main modes of packing for 3_10-helices on the outside of proteins: with one ridge of side chains wedged into a cleft on the surface of the rest of the protein, or contacting the proteins' surface with one of the flat sides between the ridges. Helix III in amxACP displays the latter kind of interaction (Figure 5B), providing little opportunity for specific interactions, which likely contributes to the observed mobility of helix III. Thus, the 3_10-helical conformation of helix III explains both types of structural variability observed in our ensemble refinement and MD results: conformational variability within the helix as well as motions of the helix as a whole. As for other ACPs, this structural variability is likely required to allow the protein to adapt to various acyl chain lengths and functionalizations, as well as to allow access to and from the binding cavity. Moreover, helix III has been implicated in signaling the functionalization state of the nascent fatty acid chain to binding partners.49

In addition to helix III, the FF motif in the loop between helices Ia and II sets amxACP apart from other ACPs. Superimposing amxACP and the crystal structure of decanoyl-loaded E. coli ACP14 shows that this motif is located near the distal end of the 4'-phosphopantetheine part of the cargo (Figure 6). This suggests that the FF motif could assist with another important function of ACPs: protection of the labile thioester bond through which acyl chains are coupled to the 4'-phosphopantetheine linker. Structures and simulations of ACPs binding various acyl chains show that this moiety is buried to varying extents depending on the length of the acyl chain, with longer acyl chains leading to the thioester bond being positioned outside the binding cavity.61 Indeed, longer acyl chains render this thioester linkage more susceptible to hydrolysis.13,16,62 We hypothesize that the
The data that support the findings of this study are openly available in the Protein Databank at https://www.rcsb.org/pdb, under entry codes 7AUF and 7AX5.

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