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Structure of a specialized acyl carrier protein essential for lipid A biosynthesis with very long chain fatty acids in open and closed conformations

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Abstract

The solution NMR structures and backbone ¹⁵N dynamics of the specialized acyl carrier protein (ACP), RpAcpXL, from *Rhodopseudomonas palustris*, in both the apo-form and holo-form modified by covalent attachment of 4'-phosphopantetheine at S37, are virtually identical, monomeric, and correspond to the closed conformation. The structures have an extra α -helix compared to the archetypical ACP from *Escherichia coli*, which has four helices, resulting in a larger opening to the hydrophobic cavity. Chemical shift differences between apo- and holo-

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SUPPORTING INFORMATION AVAILABLE

Figures, tables, and text containing supplementary methods, rotational correlation time estimation, mass spectrometry data, overlaid NMR ¹H-¹⁵N spectra, changes in ¹H and ¹⁵N chemical shifts upon removal of Ca²⁺, an titration with MgCl₂, and upon conversion to holo-RpAcpXL, ¹⁵N dynamics, and rpa2022 genomic context, are included. This material is available free of charge via the Internet at <http://pubs.acs.org>.

RpAcpXL indicated some differences in the hinge region between helices $\alpha 2$ and $\alpha 3$ and in the hydrophobic cavity environment, but corresponding changes in NOE crosspeak patterns were not detected. In contrast to the NMR structures, apo-RpAcpXL was observed in an open conformation in crystals that diffracted to 2.0 Å resolution, which resulted from movement of helix $\alpha 3$. Based on the crystal structure, the predicted biological assembly is a homodimer. Although the possible biological significance of dimerization is unknown, there is potential that the resulting large shared hydrophobic cavity could accommodate the very long-chain fatty acid (28 to 30 carbon chain length) that this specialized ACP is known to synthesize and transfer to lipid A. These structures are the first representatives of the AcpXL family and the first to indicate that dimerization may be important for the function of these specialized ACPs.

Keywords

Structural genomics; Northeast Structural Genomics Consortium (NESG); Protein Structure Initiative; Solution NMR; X-ray crystal structure; ACP-XL

INTRODUCTION

The specialized acyl carrier proteins (ACPs) known as AcpXLs are small acidic proteins that are involved in the biosynthesis of lipid A, involving incorporation of a very long-chain fatty acid (VLCFA), typically with a 28 to 30 carbon chain length. The designation AcpXL was first given to the specialized *Rhizobium leguminosarum* ACP that has an attached VLCFA, 27-hydroxyoctacosanoic acid (27-OH-C₂₈) (1). AcpXL proteins are conserved among bacteria from the order *Rhizobiales*, including species from the genera *Rhodopseudomonas*, *Rhizobium*, *Sinorhizobium*, *Brucella*, and *Bartonella*. The *acpXL* gene is typically found in a six-gene cluster that is responsible for the synthesis of the VLCFA-modified lipid A (2–4). Lipid A is the membrane-located component of lipopolysaccharide (LPS), the major surface component of Gram-negative bacteria. VLCFA-modified LPS has been identified in many species of nitrogen-fixing bacteria, as well as some intracellular pathogens, like *Brucella* and *Bartonella* species, which can form chronic intracellular infections (5).

AcpXL is essential for the biosynthesis of VLCFA-modified lipid A in both the free-living and symbiotic states in the well-studied *Rhizobiales* species, *Sinorhizobium meliloti* and *R. leguminosarum* (3, 4, 6–9). Although not indispensable for their growth or colonization of legumes, the absence of VLCFA-LPS results in increased sensitivity to environmental stresses, such as changes in salt, detergents, or increased acidity, and abnormal bacteroid development or reduced competitiveness for legume symbiosis (3, 4, 6–9). It has also been hypothesized that VLCFA-LPS is needed for infection of medically important pathogens like *Brucella* species that are incorporated into membrane-bound acidic compartments in the mammalian host (reviewed in (10)). With mutations in the *acpXL* gene, neither *S. meliloti* or *R. leguminosarum* can produce VLCFAs (4, 6, 7, 11).

In the well-studied synthesis of lipid A in *Escherichia coli*, the ACP shuttles the growing fatty acid chain between component enzymes during chain elongation and modification. To enable this, the active form, holo-ACP, has a long 4'-phosphopantetheine (4'-PP) arm of ~18 Å that carries the growing acyl intermediates as thioesters attached to the terminal thiol of

the 4'-PP. Conversion to holo-ACP is carried out by holo-ACP synthase (AcpS), which covalently attaches a 4'-PP moiety of coenzyme-A to the hydroxyl group of a serine residue located within a highly conserved DSL sequence via a phosphodiester bond. Movement of the flexible 4'-PP alternately shelters the intermediate within the hydrophobic core of the ACP or exposes it to enzymes for further modification. In the "prototypical" lipid A synthesis, 3-hydroxymyristoyl- (3-OH-C₁₄)-ACP is involved in the first two primary acylation reactions, and lauroyl- and myristoyl-ACPs provide the FA for secondary acylation reactions. However, for synthesis of the specialized VLCFA-lipid, a long OH-FA, such as the saturated 27-OH-C₂₈ or 29-OH-C₃₀, is typically incorporated at the secondary or "piggy back" position, resulting in a pentacylated lipid A.

Here we report the solution structure of the small (10.2 KDa), acidic (pI 4.4) AcpXL from *Rhodopseudomonas palustris*, RpAcpXL, (gene *rpa2022*; GenBank, NP_947367.1; UniProtKB ID, Q6N882; KEGG ID, rpa:RPA2022; NESG ID, RpR324), in both the apo- and 4'-PP-modified forms. *R. palustris* are Gram-negative purple non-sulfur bacteria that can switch between four different modes of metabolism. Notably, *R. palustris* can fix nitrogen, like many other rhizobia, although it is not in the same *Rhizobiaceae* family as *S. meliloti* and *R. leguminosarum*, but rather belongs to the *Bradyrhizobiaceae* family. Since RpAcpXL shares > 70% sequence identity with AcpXL proteins found in other *Rhizobiales* bacteria (over 60 sequences found in blast search of Kegg orthologs), it is a structural representative for this family of proteins. High-resolution structures of both apo- and holo-RpAcpXL were determined by solution NMR spectroscopy and the apo-RpAcpXL structure was also solved by X-ray crystallography at 2.0 Å resolution. Given that ACPs must interact with dozens of proteins in order to synthesize and transfer acyl chains to lipid A (12), competition and regulation may be important. Since it has been suggested that structural adjustments of the ACP resulting from phosphopantetheinylation and subsequent acylation might result in allosteric regulation of interactions with other enzymes, we have examined the structure and backbone ¹⁵N dynamics of RpAcpXL both with and without the 4'-PP modification. This is a first step towards understanding the structure and function of proteins in the AcpXL family.

EXPERIMENTAL PROCEDURES

Expression, Purification, and Characterization of >90% apo-RpAcpXL

The *RpAcpXL* gene was cloned into a pET21-based expression vector (NESG Clone ID RpR324-21.4), which has been deposited in the PSI:Biology Materials Repository (13) (<http://psimr.asu.edu/>) with Clone ID RpCD00339419. The RpAcpXL protein, which included a C-terminal His⁶ tag (LEHHHHHH), was expressed, and purified following standard protocols of the Northeast Structural Genomics Consortium (NESG) in order to prepare [*U*-¹³C, ¹⁵N] and *U*-¹⁵N, 5% biosynthetically-directed ¹³C (NC5) samples (14); see Supporting Information for a detailed description of the sample preparation, NMR data acquisition, and structure determination methods. Se-Met-RpAcpXL for crystallization trials was primarily monomer based on analytical static light scattering in-line with gel filtration chromatography. NMR samples were purified from the monomer fraction during gel filtration chromatography and were characterized as monomeric under the conditions used in

the NMR experiments (1.0 mM protein, 10% v/v D₂O, 20 mM MES, 200 mM NaCl, 10 mM DTT, 5 mM CaCl₂, pH 6.5, 25°C) based on correlation time estimates from one-dimensional ¹⁵N *T*₁ and *T*₂ relaxation data (estimated τ_c of 8 ns, Figure S1, Supporting Information). Biosynthetic incorporation of 4'-PP was determined to be < 10% by mass spectrometry. Interestingly, the N-terminal methionine was about 50% cleaved in both the NC5 and [*U*-¹³C, ¹⁵N] RpAcpXL samples.

Overexpression and Purification of *E. coli* AcpS

The p15Rv-L expression vector containing the gene encoding AcpS from *E. coli* K12 was transformed and expressed as previously described (15), however a modified purification procedure is reported here. Soluble AcpS was obtained by denaturation of the pellet at 6 M urea, followed by refolding, rather than the previous method of purification from the soluble fraction after lysis and centrifugation (15). Pelleted cells were resuspended in 25 mL of lysis buffer (50 mM Tris, 10 mM MgCl₂, 5% glycerol, pH 8.0) and lysed by three passes through a French pressure cell (SLM Instruments). The resulting lysate was spun at 24,000 x g for 60 min. The insoluble pellet was resuspended in 25 mL of lysis buffer containing 6 M urea by stirring overnight at room temperature. After centrifugation at 12,000 x g for 5 minutes, the soluble portion was loaded onto a 10 mL Ni-NTA affinity column (Qiagen) and washed with 50 mL of lysis buffer containing 30 mM imidazole and 6 M urea. The His₆-tagged AcpS was eluted from the column with lysis buffer containing 300 mM imidazole and 6 M urea. The purified protein (~ 10 mL) was diluted to 1.5 M urea by a 4-fold dilution with buffer and centrifuged at 12,000 x g for 5 minutes to remove precipitation. Concentration of this protein by centrifugation, resulted in a final protein concentration of ~400 µg/mL.

Enzymatic conversion of <10% holo-RpAcpXL to >95% holo-RpAcpXL

To prepare samples of >95% [*U*-¹³C, ¹⁵N] and NC5 holo-RpAcpXL, the <10% holo-RpAcpXL was incubated with *E. coli* AcpS for Coenzyme A (CoA) and Mg⁺²-dependent conversion. The reactions contained 10 mM MgCl₂, 200 mM NaCl, 5% glycerol, 300 µM CoA, 100 µM RpAcpXL, 6.0 µM AcpS, 300 mM urea, and 30 mM Tris, pH 8.0, in a total volume of 10 mL. Reactions were incubated overnight at room temperature and conversion to holo-RpAcpXL was confirmed by mass spectrometry (Bruker Autoflex III MALDI-TOF, Figure S2, Supporting Information). Samples were then buffer-exchanged to NMR buffer and concentrated by centrifugation to 1 mM holo-RpAcpXL. The >95% [*U*-¹³C, ¹⁵N] holo-RpAcpXL contained ~10% ¹³C- and ¹⁵N-labeled 4'-PP and ~85–90% unlabeled 4'-PP since isotopic enrichment was dependent on the amount of ¹³C and ¹⁵N incorporated during expression and modification during biosynthesis within *E. coli*. Further incorporation of the ~85–90% unlabeled 4'-PP was added by in vitro enzymatic conversion by AcpS using unlabeled CoA.

NMR spectroscopy and structure determination of apo- and holo-RpAcpXL

NMR data for apo-RpAcpXL was collected at 25°C on [*U*-¹³C, ¹⁵N] and NC5 samples of about 30 µL in 1.7 mm microprobe NMR tubes on a Bruker Avance 600 MHz spectrometer equipped with a 1.7 mm TCI Micro CryoProbe. NMR data collection, chemical shift assignments, and structure calculations were conducted as previously described (16) and as

described briefly in the Supporting Information. The final coordinates for the ensemble of 20 structures and NMR-derived constraints for apo-RpAcpXL were deposited to the Protein Data Bank (PDB ID, 2KW2) and chemical shifts and NOESY peaks lists were deposited to BioMagResDB (BMRB accession number, 16805).

Additional NMR data for apo-RpAcpXL and data for holo-RpAcpXL was collected at 25°C on [U-¹³C, ¹⁵N] and NC5 samples of about 250 μl in 5 mm Shigemi NMR tubes on 600 MHz Varian Inova and 850 MHz Bruker Avance III NMR spectrometers. D₂O-exchanged samples were prepared by freezing [U-¹³C, ¹⁵N] samples followed by overnight lyophilization and resuspension in 99.9% D₂O (Acros Organics). Assignments of holo-RpAcpXL ¹H, ¹³C and ¹⁵N resonances were based on assignments for apo-RpAcpXL and confirmed by analysis of conventional triple-resonance spectra, including three 3D NOESY experiments (τ_m = 70 ms). The NOESY spectra were collected on both apo- and holo-RpAcpXL with identical parameters and the same NMR instrument (850 MHz Bruker Avance III NMR) in order to facilitate the comparison. Additional NOEs for holo-RpAcpXL were assigned from a 4D ¹³C-¹³C-HMQC-NOESY-HMQC (τ_m = 70 ms) in D₂O solution. Amide backbone one bond ¹H-¹⁵N residual dipolar coupling (RDC) values of apo- and holo-RpAcpXL were measured by comparing data for isotropic and two partially aligned NC5 samples in Pf1 phage (12.5 mg/mL) and polyethylene glycol bicelles (4.2%) as previously described (17–19). Spectra were processed with NMRPipe (20) and analyzed with Sparky 3.110 (Goddard, T. D. and Kneller, D. G.) Chemical shifts observed in an overlay of the ¹H-¹⁵N HSQC spectra of apo- versus holo-RpAcpXL (Figure S3, Supporting Information) indicated amide crosspeaks that were shifted by > 0.05 ppm in the ¹H dimension. Chemical shift peak doubling was observed for residues S3 and T4 as a result of the incomplete processing of the N-terminal methionine after expression in *E. coli*.

The solution NMR structures of apo- and holo-RpAcpXL were calculated using NOESY data collected under identical conditions and parameters and refined with an identical protocol, with the exclusion of 4'-PP in the apo-RpAcpXL structure calculation. Initial structures were calculated with CYANA 3.0 (21) using resonance assignments, NOESY peak lists from three or four NOESY spectra, dihedral restraints derived from TALOS+ (22), and two sets of ¹H-¹⁵N RDCs. Chemical shifts for the 4'-PP prosthetic group were assigned manually. CYANA structures were refined with CNS in explicit water (23, 24) using molecular parameter and topology files created for the 4'-PP prosthetic group with fmcGui (25). Structural statistics for apo- and holo-RpAcpXL are presented in Table 1. The final coordinates for the ensemble of 20 structures and NMR-derived constraints for both apo- and holo-RpAcpXL were deposited to the Protein Data Bank (PDB ID, 2LPK and 2LL8) and chemical shifts and NOESY FIDs and peak lists were deposited to BioMagResDB (BMRB accession number, 18263 and 18032), respectively. The 4'-PP chemical shifts were deposited to the BMRB along with holo-RpAcpXL shifts and are reported in Table 2.

Backbone ¹⁵N dynamics data were collected on apo- and holo-RpAcpXL samples at a ¹⁵N Larmor frequency of 60.8 MHz at 25 °C on a Bruker Avance III 600 MHz spectrometer running Topspin 3.0. Residue specific ¹⁵N longitudinal and transverse times (*T*₁ and *T*₂) and ¹⁵N{¹H} heteronuclear NOE values were calculated from cross-peak intensities in the respective 2D experiments. *T*₁ and *T*₂ experiments were collected as pseudo-3D

experiments. T_1 spectra were acquired with the relaxation delays, 0.05, 0.1, 0.2, 0.3, 0.4, 0.6, 0.8, 1.0, 1.2, 1.5, 2.0 s and a recycle delay of 3 s. T_2 spectra were acquired with CPMG delays, 16, 32, 48, 64, 80, 96, 128, 160, 192, and 240, 304, 384 ms and a recycle delay of 1.5 s. Data for overlapped resonances was omitted from all analyses. T_1 and T_2 were computed by Protein Dynamics Center (Bruker v.1.2.3) by fitting peak intensity as a function of delay times according to standard equations for exponential decay. Error bars were estimated by Protein Dynamics Center based on the fit to the data. T_1 and T_2 data are plotted vs. residue in Figure 2. The $^{15}\text{N}\{^1\text{H}\}$ heteronuclear NOE, was collected as two 2D interleaved spectra with and without the default proton saturation delay of 3 s and a recycle delay of 5 s. $^{15}\text{N}\{^1\text{H}\}$ heteronuclear NOE values were determined from the ratio of the peak intensities obtained after processing with NMRPipe (20), peak picking in Sparky, and using Microsoft Excel to obtain and plot the ratio versus the residue (Figure 2).

Crystallization and X-ray structure determination of apo-RpAcpXL

Crystals of Se-Met labeled apo-RpAcpXL were grown using the microbatch method at 4°C; 2 μL of protein solution in buffer containing 100 mM NaCl, 5 mM DTT, 0.02% NaN_3 , and 10 mM Tris-HCl (pH 7.5) were mixed with 2 μL of crystallization cocktail composed of 0.1 M sodium citrate (pH 4.2), 12% PEG 20k, and 0.1 M lithium sulfate. Crystals appeared after a few days and were subsequently flash-frozen using the above crystallization cocktail that contained 15% glycerol as cryoprotectant. A 2.0 Å resolution single-wavelength anomalous diffraction (SAD) data set was collected at the peak absorption wavelength of selenium at the X4C beamline of the National Synchrotron Light Source. The diffraction images were processed with the HKL package (26). Initial attempts at structure determination by the SAD method were unsuccessful as the initiating Se-Met, which was the only Se-Met in the protein, was disordered. Subsequently, the structure was solved by molecular replacement using Molrep (27). The solution state NMR structure of apo-RpAcpXL was initially used as the search model for structure determination (residues 3-91 with hydrogen atoms removed), which gave a solution with $R_{\text{work}} / R_{\text{free}}$ values 45.8 / 53.3 after refinement using CNS (23). However, inspection of the electron density map revealed that residues 57-73 comprising helix α_3 and the loop that connects it to helix α_4 had very poor electron density. After removing the biased region (residues 57-73) from the structure, a second round of refinement was performed, which significantly improved the map and $R_{\text{work}} / R_{\text{free}}$ values to 39.1 / 43.3. Consequently, model building was performed using XtalView (28) and refined by CNS. Data collection and refinement quality statistics are summarized in Table 3 and the structure and structure factors are deposited in the PDB (PDB ID, 3LMO). Crystallization attempts of holo-RpAcpXL under the same conditions were unsuccessful.

RESULTS

Apo- and holo-RpAcpXL NMR structures

The solution NMR structures of apo- and holo-RpAcpXL (PDB IDs 2LPK and 2LL8) were essentially the same (Figure 1A and B). The average pairwise backbone and heavy atom RMSD between the apo- and holo-structures was $0.6 \text{ Å} \pm 0.1$ and $1.0 \text{ Å} \pm 0.1$, respectively. Structure and quality statistics are given in Table 1. There are five helices, with four of them (α_1 , α_2 , α_3 , and α_5) forming a righttwisted four-helix bundle with an up-down-up-down

topology. The shortest helix $\alpha 4$ is perpendicular to $\alpha 3$ and $\alpha 5$ and is across from S37 at the N-terminus of $\alpha 2$. Table 2 shows a schematic view of 4'-PP and its linkage to the O γ side chain atom of S37, which is located in a conserved DSL motif. S37 is located at the entrance to a hydrophobic cavity that extends down the central core of the protein and is surrounded by all five helices. The solvent-accessible volume of the hydrophobic pocket was $\sim 70 \text{ \AA}^3$ calculated using CASTp (24). There is a large, 21-residue loop between $\alpha 1$ and $\alpha 2$ prior to S37 that is well-defined in the NMR ensemble and stabilized by hydrophobic residues that interact with the protein core (I18, I23, and I30) and by backbone hydrogen bonds (Figure 1C). Loop 2, between $\alpha 2$ and $\alpha 3$, is stabilized by a hydrogen bond between L55 H $_N$ and the side chain COO $^-$ of D48 in helix $\alpha 2$ (Figure 1C). This hydrogen bond is observed in most ACP structures and is believed to act as a hinge to allow for expansion of the cavity via an outward movement of helix $\alpha 3$. In holo-RpAcpXL, the only NOEs observed between the 4'-PP and the protein were to residues S37 and L38 (6 NOEs). In addition, 4'-PP resonances were strong with narrow linewidths indicating a dynamic structure. The 4'-PP chemical shifts were also typical of those expected for unstructured 4'-PP (Table 2). This is similar to results previously reported for other ACPs (30–32).

The structure of RpAcpXL differs from other previously solved ACP structures because it has an extra helix, $\alpha 4$, in the position following $\alpha 3$, making a total of five helices whereas in the *E. coli* ACP, and most other ACPs, the structure consists of a four-helix bundle. Structural alignment of holo-RpAcpXL and *E. coli* ACP is shown in Figure 1D. The resulting effect is that the hydrophobic cavity in RpAcpXL is slightly deeper because the lip on the cup ($\alpha 3$ and $\alpha 4$) extends higher.

For the *E. coli* ACP, cation binding stabilized the structure and stimulated FA synthesis (33). Binding to divalent cations, such as Ca $^{+2}$, Mg $^{+2}$, and Mn $^{+2}$, resulted in stabilization of the structure (33, 34). Two Mn $^{+2}$ -binding sites were characterized by paramagnetic relaxation perturbation measurements: near the modified S36, and near the hinge region carboxylate, E47 (*E. coli* numbering) (35). Small H $_N$ chemical shift changes were observed for L33, D36, S37, D39, D48, F51, and I53 in ^1H - ^{15}N HSQC spectra of upon removal of Ca $^{+2}$ and by titration with Mg $^{+2}$ (Figure S3A, Supporting Information). Although only the first site is conserved in RpAcpXL, shifts for both D36 and D48 indicate weak binding at two sites with K_d of 10 – 20 mM (Figure S3B, C, Supporting Information). Weak Mg $^{+2}$ binding can likely be attributed to the high concentration of monovalent cations in the NMR buffer, containing 200 mM NaCl. This was the case for *E. coli* ACP, where high concentrations of KCl or NaCl reduced the divalent cation binding affinities (33, 34). It is likely that both types of cations can result in stabilization of the structure by neutralization of the acidic character of the protein. The weak Mg $^{+2}$ binding and small spectral changes suggest that the structure of RpAcpXL was not significantly perturbed by binding.

An overlay of apo- and holo-RpAcpXL ^1H - ^{15}N HSQC spectra showed shifted resonances, with only S37 and L55 shifted by more than 0.1 ppm in the ^1H dimension (Figure S4 and S5, Supporting Information). The chemical shift differences between H $_N$ resonances for apo- and holo-protein samples are plotted onto the structure in Figure 1E. As expected, the largest chemical shift differences were for conserved resonances, D36, S37, and L38, a result previously reported for *E. coli* ACP (36). These chemical shift changes are the result

of covalent and transient interactions with the 4'-PP. Interestingly, the H_N resonances in loop 2, including L55, also had chemical shift changes. This indicated that there were differences in this hinge region between the apo- and holo-conformations. This was further supported by side-chain 1H and ^{13}C chemical shifts changes ($^1H > 0.05$, $^{13}C > 0.3$) for residues within the hydrophobic cavity and $\alpha 4$, in addition to those for I35 and S37 (Figure 1E). The chemical shift changes upon modification by 4'-PP may reflect small differences in the hydrophobic cavity environment that were not detected by the NOE-based structure determination.

Apo- and holo-RpAcpXL backbone ^{15}N dynamics and hydrogen/deuterium (H/D) exchange experiments

Despite the observed chemical shift differences between apo- and holo-RpAcpXL, no differences in backbone dynamics on the ps to ns timescale were detected based on analysis of two-dimensional ^{15}N T_1 , T_2 , and $^{15}N\{^1H\}$ heteronuclear NOE data (Figure 2). The average T_2 values were 85 ms, which is shorter than the 100 ms T_2 expected for a monomer of this molecular weight. This could indicate slower overall backbone motions due to global conformational flexibility. However, the contribution of a small amount of oligomer could also cause this shorter T_2 and cannot be ruled out. The T_1/T_2 ratios for both apo- and holo-RpAcpXL (Figure 2) were also very similar with the exception of S37 with the largest ratio (12.2 for apo and 16.4 for holo), indicating that μ s to ms timescale motion contributed to relaxation for this residue. It is interesting that S37 showed slower timescale motion than the rest of the protein even in apo-RpAcpXL, a feature that could influence binding to AcpS. After modification by 4'-PP, the HN of S37 exhibited even slower timescale motion with the largest change in T_1/T_2 ratio between apo- and holo-RpAcpXL. This change in dynamics resulting from the 4'-PP modification could be an allosteric control mechanism for regulating interactions with other proteins.

Although the backbone ^{15}N dynamics data did not indicate that any of the helices had more flexibility on the ps to ns timescale compared to the others, H_N H/D exchange data revealed that helices $\alpha 1$ and $\alpha 5$ had significantly slower amide proton exchange rates, on the order of days to weeks, compared to $\alpha 3$ and $\alpha 4$. In both apo- and holo-RpAcpXL, several residues in $\alpha 1$, $\alpha 2$, and $\alpha 5$ were still not exchanged by D_2O two months after lyophilization and resuspension into D_2O (Figure 1C and Figure S6, Supporting Information). These regions of slow H/D exchange indicated a stable core on the "backside" of this protein that experiences less local conformational dynamics associated with breaking of hydrogen bonds that allow amide proton exchange. The loops, helices $\alpha 3$ and $\alpha 4$, and the N-terminal half of $\alpha 2$ did not have any amides with slow H/D exchange, suggesting greater conformational flexibility. These results are similar to those reported for *E. coli* ACP where residues in loop 1 and $\alpha 3$ exchanged the fastest (37). All of the *E. coli* ACP amides exchanged in less than one day under the conditions studied (37). The slower exchange rates for residues in RpAcpXL indicated that it was more stable to conformational unfolding than the *E. coli* ACP. In RpAcpXL, $\alpha 3$ and $\alpha 4$ and the "front" of the protein contain more conserved surface residues, based on ConSurf analysis (38) indicating that these residues are important for the function of proteins in this AcpXL family (Figure 1E). There were no differences in amide proton D_2O exchange rates between apo- and holo-RpAcpXL, indicating that the 4'-PP

modification of S37 did not stabilize protein structure or alter the conformational dynamics in the vicinity of the modification as probed by H/D exchange.

Apo-RpAcpXL X-ray structure

The X-ray structure of apo-RpAcpXL has an open conformation of the hydrophobic pocket compared to either the apo- or holo-NMR structures because of movement of helix $\alpha 3$ (Figure 3A, B, C). Structure and quality statistics for this crystal structure are given in Table 3. Although RpAcpXL was primarily a monomer in solution at the high concentrations required for structure determination by NMR, with up to 13% dimer measured in solution by analytical static light scattering in-line with gel-filtration chromatography (data not shown), analysis of the molecular interface properties in the crystal structure by PDBePISA (39) indicated that the most likely biological assembly of RpAcpXL is a homodimer with $\sim 880 \text{ \AA}^2$ of buried protein surface (Figure 3D). In the predicted homodimer structure, helices $\alpha 2$ and $\alpha 3$ from each subunit pack together in a reciprocal manner, so the proteins are “front to front” and the S37 residues from each chain are close together (Figure 3D, E). According to CASTp calculations (24), each subunit has a hydrophobic cavity in the center of the helical bundle with S37 at the entrance to the cavity with a volume of $120 \text{ \AA}^3$ (Figure 3D). These hydrophobic cavities are close together and several hydrophobic residues in the cavity are less than $5 \text{ \AA}$ away from residues in the other subunit (Figure 3E). The conserved surface residues are located at the dimer interface as visualized by ConSurf (38), giving further evidence that this interface represents the biological assembly (Figure 3F). Biological interfaces are typically conserved and the largest contact in the crystal (40). Using information about conserved residues was able to improve predictions of homodimer biological vs non-biological interfaces to high accuracy (40). It is possible that the biological significance of the homodimer, if it is the biologically active assembly, is to create a large shared hydrophobic cavity between the subunits that could accommodate a very long-chain fatty acid during synthesis and transfer to lipid A.

Discussion

The structure of RpAcpXL is the first for the family of AcpXL proteins. According to the DOOR web resource (Database for prokaryotic Operons) (41), the genomic context of RpAcpXL is the same as for *S. meliloti* and *R. leguminosarum* (Figure S7, Supporting Information). There are six genes with a two-operon transcriptional organization with the first operon encoding the AcpXL along with three other proteins involved in VLCFA synthesis, and a second operon encoding two proteins, a putative alcohol dehydrogenase, AdhA2XL, and VLCFA acyl transferase, LpxXL. The LpxXL is believed to be responsible for transferring VLCFA from the AcpXL to lipid A (6, 11, 42). In fact, mutation of any of the genes in the *acpXL-lpxXL* gene cluster in *S. meliloti* resulted in loss of VLCFA-LPS (3).

In order to synthesize holo-RpAcpXL, *E. coli* AcpS was used via enzymatic conversion. The rapid conversion to completion was surprising since RpAcpXL shares only 32% sequence identity with the ACP from *E. coli*. However, proteins with even less sequence similarity were able to undergo the 4'-PP modification via enzymatic conversion by AcpS (15).

RpAcpXL contains the conserved DSL sequence motif as well as acidic residues in $\alpha 2$, D42 and D48, that are important for recognition by the AcpS (43).

The structure of RpAcpXL has an extra helix compared to most other previously solved ACP structures. As a result, the opening to the hydrophobic cavity is larger. Upon modification by 4'-PP there were no observable changes in structure or backbone dynamics on the ps to ns timescale, although there were chemical shift perturbations. It is possible that subtle differences in chemical structure may be necessary to modulate interactions with enzymes involved in the synthesis and transfer of the VLCFA to lipid A, although it may be that addition of the 4'-PP is all that is needed to provide functionality to the AcpXL.

In addition, the apo-RpAcpXL crystal structure indicated that dimerization may be important for the biological activity of AcpXL. This hypothesis is based on the fact that (i) dimerization occurs in solution to some extent under various buffer conditions, (ii) the predicted dimerization interface has $> 800 \text{ \AA}^2$ of buried protein surface and contains conserved surface residues, (iii) dimerization results in structural changes that create a large shared hydrophobic cavity, and (iv) RpAcpXL synthesizes a VLCFA that is twice as long as the well-studied *E. coli* ACP, and therefore may require a hydrophobic cavity that is twice as large. However, since the biological relevance of dimerization has not been experimentally confirmed it is possible that (i) it is an artifact of the high protein concentrations in solution, (ii) the predicted dimerization interface is a crystal packing artifact, (iii) the structural rearrangement to the open conformation upon dimerization may reflect intrinsic changes in RpAcpXL that may occur in response to binding to other enzymes in the VLCFA pathway and may not be an allusion to biological significance of dimerization. NMR-based estimations of τ_c and GF/LS screening of RpAcpXL with the Tris-HCl, pH 7.5 buffer used to set up the crystal trials, indicate that the protein is monomeric under these conditions (data not shown). Interestingly, lowering the pH below 5, resulted in some precipitation (data not shown). This suggests that oligomerization may occur at lower pH values, such as those determined for crystallization, mixing the protein in the high pH buffer with the 0.1 M sodium citrate, pH 4.2 buffer.

The binding partners for AcpXL have not yet been characterized, with the exception of LpxXL, the long chain acyltransferase that transfers the VLCFA from AcpXL to lipid A. It is likely that AcpXL interacts with all the proteins produced by genes in the six-gene cluster responsible for the synthesis of the VLCFA-modified lipid A (2–4). It also remains unknown whether AcpXL interacts with FA synthesis or other canonical LPS synthesis enzymes. The fatty acid chain could be transferred from the “standard” FA synthesis ACP to the AcpXL and then subsequently elongated with other enzymes found in the *acpXL-lpxXL* gene cluster in order to synthesize acyl-AcpXL with a VLCFA.

Structures of AcpXL in complex with the VLCFA-enzymes and the possible biological relevance of AcpXL dimerization remains to be determined. However, if dimerization is biologically relevant, it might preclude certain enzymes from binding to helix $\alpha 2$ in AcpXL. Based on the predicted dimer structure of RpAcpXL, binding proteins could still interact with both the N- and C-terminus of helix 2. However, helix 3 in the other subunit is perpendicular to helix 2 and crosses in the middle of helix 2 (near F45). In *E. coli* ACP,

helix $\alpha 2$ is of crucial importance in binding with AcpS as well as enzymes involved in FA and LPS synthesis (reviewed in (44–46). AcpS and other ACP-binding proteins typically have a conserved positively charged Arg residue in a hydrophobic patch adjacent to their active site, which can form contacts with the hydrophobic and acidic residues in helix 2 of ACP (reviewed in (44, 46). For FA synthesis ACPs, it has been determined that residues at the N-terminus of helix 2 (near S37) were important for binding to all partners, and residues near the C-terminus of helix 2 were also important for binding to some enzymes (44, 47, 48). For example, binding to LpxA, the acyltransferase that catalyzes the first step of lipid A biosynthesis, is affected by residues along the length of the *Vibrio harveyi* ACP helix 2 (48, 49). It remains to be determined whether dimerization has an effect on binding of VLCFA synthesis proteins to AcpXL and if it is important for the function of the AcpXL family proteins.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

ACP	acyl carrier protein
NMR	nuclear magnetic resonance
RDC	residual dipolar coupling
NOE	nuclear Overhauser effect
HSQC	heteronuclear single-quantum coherence spectroscopy
RMSD	root mean squared deviation
FAS	fatty acid synthesis
LPS	lipopolysaccharide
4'-PP	4'-phosphopantetheine
CoA	Coenzyme A
LPS OH-FA	lipopolysaccharide hydroxy-fatty acids
VLCFA	very long-chain fatty acid

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Figure 1.

(A) Backbone diagram of solution NMR structure of apo- (residues 2-93, PDB ID 2LPK) and (B) holo-RpAcpXL (residues 2-93, PDB ID 2LL8) with one selected 4'-PP indicated. (C) Left, back cartoon view of representative holo-RpAcpXL structure. Hydrogen bonds stabilizing loop 1 are shown (A29 HN - F75 CO, L77 HN - S27 CO, K78 HN - P25 CO). Right, front cartoon view with the hydrogen bond stabilizing loop 2 shown (L55 H^N - side chain COO⁻ of D48). In both views, the backbone representation of residues with amide protons that have not exchanged with D₂O after two months are shown in blue (α1: V8, A9, I11, I12, A13; α2: I47 α5: L80, A81, R83, I84, D85, L87). (D) Structural comparison of holo-RpAcpXL, yellow, and with *E. coli* heptanoyl-ACP, purple (PDB ID 2FAD-A). (E) Worm representation of holo-RpAcpXL scaled by the weighted average chemical shift difference between ¹H_N and ¹⁵N resonances (54) in apo- and holo-RpAcpXL, δ_{av} ; where $\delta_{av} = \{1/2[(\delta H^N)^2 + (\delta N/5)^2]\}^{1/2}$, $\delta H^N = \delta H^N_{apo} - \delta H^N_{holo}$ and, $\delta N = \delta N_{apo} - \delta N_{holo}$. Side chains with chemical shifts changes (¹H 0.05, ¹³C 0.3) are shown: I35, S37, L55 in loop 2, and Q73 and Y74 in α4. Smaller chemical shift differences (¹H 0.04, ¹³C 0.2) were observed for L57 and W60 in α4 and F40 in α2 across from L57. (F) Back and front view of ConSurf (38) image (same orientation as C). 60 Kegg orthologs were used in the alignment. Magenta is highly conserved and cyan is variable. All structure figures in this paper were created with PyMol.

Figure 2.

Summary of ^{15}N dynamics for apo- and holo-RpAcpXL. T_1 , T_2 , $^{15}\text{N}\{^1\text{H}\}$ heteronuclear NOE values, and T_1/T_2 ratios are displayed as a function of amino acid residue. Grey is apo- and black is holo-RpAcpXL

Figure 3.

(A) Ribbon drawing with transparent surface representation of holo-RpAcpXL NMR structure (PDB ID 2LL8) with closed conformation. (B) The same representation for apo-RpAcpXL crystal structure (PDB ID 3LMO) in open conformation with the entrance to hydrophobic cavity indicated by an arrow (C) Structural comparison of holo-RpAcpXL NMR (yellow) and apo-RpAcpXL crystal (purple) structures indicating the difference conformation of helix $\alpha 3$. (D) Homodimer representation of apo-RpAcpXL crystal structure with hydrophobic pocket predictions from CASTp (29) (residues A29, F40, I43, A44, I47, L55, L57, W60, Y74, F75, L80; pockets 4 and 17, using a 1.4 Å probe radius to determine the solvent accessible pocket volumes, vol_ms) indicated with yellow spheres. (E) Homodimer with hydrophobic side chains that are < 5 Å from residues in the other subunit shown as sticks (I30, I35, L38, F40, L41, L57, W60, F75). (F) ConSurf (38) image for apo-RpAcpXL homodimer using the same sequences and colors as Fig 1F.

Table 1Summary of NMR Structural Statistics for apo- and holo-RpAcpXL ensembles. ^a

Completeness of resonance assignments^b	apo	holo
Backbone (%)	98.9	98.3
Side chain (%)	99.2	99.2
Stereospecific methyl (%)	100	100
Conformationally-restricting constraints^c		
NOE constraints		
Total	1621	1735
Intra-residue ($i = j$)	328	344
Sequential ($ i - j = 1$)	404	433
Medium range ($1 < i - j < 5$)	481	515
Long range ($ i - j \geq 5$)	408	443
NOE constraints / residue	17.4	18.5
Constraints for 4'-PP (Intra / Inter)	-	4 / 6
Dihedral angle constraints	144	144
Hydrogen bond constraints	94	94
NH RDC constraints (PEG/Phage)	58 / 73	57 / 70
Number of constraints / residue (total / long range)	20.0 / 4.5	21.0 / 4.8
Residual constraint violations^c		
Average distance restraint violations / structure		
0.1 – 0.2 Å	5.3	5.5
0.2 – 0.5 Å	0.6	1.4
> 0.5 Å	0	0
Average RMS of distance violation / restraint (Å)	0.01	0.01
Maximum distance violation (Å)	0.4	0.4
Average RMS dihedral angle violations / structure		
> 1–10°	1.2	1.4
> 10°	0	0
Average RMS dihedral angle violation / restraint	0.2°	0.3°
Maximum dihedral angle violation	5.0°	5.3°
RDC Q_{RMSD} (PEG/Phage) ^d	0.1 / 0.2	0.2 / 0.3
Model Quality^c		
RMSD from average coordinates (Å)		
Backbone atoms (N, C $^{\alpha}$, C')	0.4	0.5
Heavy atoms	0.8	0.9
RMSD Bond lengths (Å)	0.02	0.02
RMSD Bond angles (°)	1.2	1.2
MolProbity Ramachandran summary ^e		
Most favored regions (%)	98.4	98.1

Completeness of resonance assignments^b	apo	holo
Allowed regions (%)	1.4	1.9
Disallowed regions (%)	0.2	0.1
Global quality scores (Raw / Z-score)		
Procheck G-factor (ϕ , ψ) ^e	0.2 / 1.2	0.2 / 1.0
Procheck G-factor (all dihedrals) ^e	0.1 / 0.7	0.1 / 0.7
Verify3D	0.4 / -0.8	0.4 / -1.0
ProsaII	0.9 / 1.0	0.9 / 0.9
MolProbity clashscore	14.2 / -0.9	13.1 / -0.7
RPF Scores^f		
Recall / Precision	99 / 92	99 / 92
F measure / DP score	95 / 85	95 / 86

^aStructural statistics were computed for the ensembles of 20 deposited structures (PDB IDs, 2LPK and 2LL8)

^bComputed for residues 1-93, using AVS software (50). Resonances that were not included were exchangeable protons (N-terminal NH_3^+ , Lys NH_3^+ , Arg NH_2 , Cys SH, Ser/Thr/Tyr OH) and Pro N, C-terminal carbonyl, side chain carbonyl and non-protonated aromatic carbons.

^cCalculated using PSVS 1.4 program (51). Average distance constraints were calculated using the sum of r^{-6} .

^dRDC goodness-of-fit quality factor, Q_{RMSD} determined using PALES (52).

^eOrdered residue ranges [$S(\phi) + S(\psi) > 1.8$] : 4-92.

^fRPF scores (53) reflecting the goodness-of-fit of the final ensemble of structures (residues 1-101, including His₆ tag) to the NOESY data and resonance assignments (residues 1-96).

Table 2

Chemical shifts of NMR assignable atoms in 4'-phosphopantetheine (4'-PP) in holo-RpAcpXL.

Position	C/N atom	Protons	¹³ C/ ¹⁵ N [¹ H] (ppm)
1	C43	H43*	26.2 [2.54, 2.54]
2	C42	H42*	45.2 [3.29, 3.29]
3	N41	H41	123.7 [8.15]
4	C39		176.3
5	C38	H38*	38.2 [2.49, 2.49]
6	C37	H37*	38.2 [3.51, 3.51]
7	N36	H36	119.6 [8.02]
8	C34		177.3
9	C32	H32	77.1 [4.00]
10	C31	H31*	26.0 [0.95]
11	C30	H30*	26.0 [0.95]
12	C28	H28*	73.8 [3.45, 3.74]

Table 3

X-ray data collection and refinement statistics for apo-RpAcpXL, 3LMO.

Crystal Parameters	Se-Met
Space group	P3 ₂ 21
Unit-cell dimensions	
<i>a</i> , <i>b</i> , <i>c</i> (Å)	62.73, 62.73, 60.12
α, β, γ (°)	90, 90, 120
Matthews coefficient (Å ³ /Da)	3.0
Data Quality	
X-ray wavelength (Å)	0.97852
Resolution range (Å)	30-2.0 (2.07-2.00)
<i>R</i> _{merge} (%) ^a	3.3 (8.6)
Number of observations	109,780
Number of reflections	16,061
Number of reflections in <i>R</i> _{free} set	784
Mean redundancy	6.9 (4.8)
Completeness (%)	89.9 (67.1)
Mean <i>I</i> / σ _{<i>I</i>}	51.2 (14.6)
Refinement Residuals (<i>I</i> - 2σ_{<i>I</i>})	
<i>R</i> _{free} (%) ^b	25.4 (25.7)
<i>R</i> _{work} (%) ^b	21.6 (21.1)
Model Quality ^c	
RMSD bond lengths (Å)	0.006
RMSD bond angles (°)	1.0
MolProbity Ramachandran summary	
Most favored regions (%)	100.0
Allowed regions (%)	0.0
Disallowed regions (%)	0.0
Global quality scores (Raw / Z-score)	
Procheck G-factor (φ, ψ)	0.3 / 1.5
Procheck G-factor (all dihedrals)	0.3 / 1.7
Verify 3D	0.5 / -0.2
ProsaII	1.0 / 1.5
MolProbity clashscore	3.5 / 0.9
Model Contents	
Protomers in asymmetric unit	1
Protein residues	2-95
Number of atoms in protein	728
Number of water molecules	192

Crystal Parameters	Se-Met
B-factor ($\text{\AA}^2$)	
Protein	31.4
Water molecules	35.0

^a $R_{\text{merge}} = \frac{\sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle|}{\sum_{hkl} \sum_i I_i(hkl)}$, where $I(hkl)$ is the intensity of reflection hkl , $\sum_{hkl}$ is the sum over all reflections and $\sum_i$ is the sum over i measurements of reflection hkl

^b $R_{\text{free}} = \frac{\sum_{hkl} ||F_{\text{obs}}| - |F_{\text{calc}}||}{\sum_{hkl} |F_{\text{obs}}|}$; R_{free} is calculated for a randomly chosen set (5%) of reflections which were not used for structure refinement and R_{work} is calculated for the remaining reflections

^c Calculated using PSVS 1.4 program (51).