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Glutamate-41 of *Vibrio harveyi* acyl carrier protein is essential for fatty acid synthase but not acyl-ACP synthetase activity☆

Huansheng Gong and David M. Byers*

Departments of Pediatrics and Biochemistry and Molecular Biology, Atlantic Research Centre, Dalhousie University, Rm C-305 Clinical Research Centre, 5849 University Avenue, Halifax, Nova Scotia, Canada B3H 4H7

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Abstract

Bacterial acyl carrier protein (ACP) is a small, acidic, and highly conserved protein that supplies acyl groups for biosynthesis of a variety of lipid products. Recent modelling studies predict that residues primarily in helix II of *Escherichia coli* ACP (Glu-41, Ala-45) are involved in its interaction with the condensing enzyme FabH of fatty acid synthase. Using recombinant *Vibrio harveyi* ACP as a template for site-directed mutagenesis, we have shown that an acidic residue at position 41 is essential for *V. harveyi* fatty acid synthase (but not acyl-ACP synthetase) activity. In contrast, various replacements of Ala-45 were tolerated by both enzymes. None of the mutations introduced dramatic structural changes based on circular dichroism and native gel electrophoresis. These results confirm that Glu-41 of ACP is a critical residue for fatty acid synthase, but not for all enzymes that utilize ACP as a substrate.

Keywords: Acyl carrier protein; Fatty acid synthase; Acyl-ACP synthetase; Gel electrophoresis; Circular dichroism; Site-directed mutagenesis

Acyl carrier protein (ACP) plays a central role in the synthesis and transfer of fatty acyl groups [1]. ACP typically consists of 70–100 residues and is either an integrated domain in a larger multifunctional polypeptide of the type I fatty acid synthase (FAS) systems found in mammals, fungi, and certain mycobacteria, or is a discrete protein in a dissociated multienzyme system (type II FAS) as utilized by plants and most bacteria. In addition to fatty acid biosynthesis, bacterial ACP also supplies acyl groups for the synthesis of phospholipids [1], lipid A [2], lipoic acid [3], hemolysin [4], acyl homoserine lactones involved in quorum sensing [5], and the aldehyde substrate of luciferase in bioluminescent bacteria such as *Vibrio harveyi* [6]. Structural analysis of ACPs from a variety of bacteria [7–10] reveals a common folding pattern of three parallel α-helices. Activated fatty acids are covalently attached as thioesters to a 4′-phosphopantetheine prosthetic group at the beginning of helix II, providing a "swinging arm" to deliver acyl groups into the active sites of enzymes. As ACP is essential for microbial growth and has a distinct architecture in mammals and bacteria, enzymes that interact with ACP are potential targets for the development of new antimicrobial agents [11].

How ACP interacts with its multiple enzyme partners is largely unknown, and sequence analysis of these enzymes has not revealed any obvious ACP-binding motifs. A recent molecular docking study indicated that amino acids primarily in helix II of ACP interact directly with *Escherichia coli* FabH, a rate-limiting enzyme in FAS [12]. Site-directed mutagenesis of FabH predicted an important electrostatic interaction between Arg-249 of FabH and Glu-41 of ACP, and between Ala-253 of FabH and Ala-45 of ACP to permit close approach of the interacting helices [12]. A role for helix II of ACP in binding to other enzymes has also been indicated from the X-ray structure of *Bacillus subtilis* ACP co-crystallized with holo-ACP synthase [8]. On the other hand,

☆ *Abbreviations:* AAS, acyl-ACP synthetase; ACP, acyl carrier protein; CD, circular dichroism; DTT, dithiothreitol; FAS, fatty acid synthase; GST, glutathione *S*-transferase; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; IPTG, isopropyl β-D-thiogalactopyranoside; PAGE, polyacrylamide gel electrophoresis; rACP, recombinant *Vibrio harveyi* ACP.

* Corresponding author. Fax: +902-494-1394.
E-mail address: david.byers@dal.ca (D.M. Byers).

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different regions of *E. coli* ACP have been implicated in interactions with the glycosyltransferase involved in membrane-derived oligosaccharide biosynthesis [13], suggesting that some enzymes may bind to different regions of ACP.

In the present study, we have used the *V. harveyi* ACP gene as a template for site-directed mutagenesis to assess the importance of specific ACP helix II residues for the activities of the FAS complex and those of acyl-ACP synthetase (AAS), a *V. harveyi* enzyme that attaches long chain fatty acids directly to ACP in an ATP-dependent manner. The results indicate that an acidic residue at position 41 is critical for FAS but not AAS activity and that both activities tolerate replacements at position 45.

Materials and methods

Site-directed mutagenesis and purification of mutant V. harveyi ACPs. Plasmids encoding mutant ACPs E41D, E41K, V43I, A45C, A45G, and A45W were constructed from a pGEX-5X-3 vector containing the *V. harveyi acpP* gene [14] using the GeneEditor kit (Promega). All mutations were verified by DNA sequencing. GST–ACP fusion proteins were purified from IPTG-induced *E. coli* BL21 cells (Stratagene) using glutathione–Sepharose 4B and anion-exchange chromatography as described [14]. Wild type recombinant (rACP) and mutant *V. harveyi* ACPs produced by Factor Xa cleavage contain four extra N-terminal residues (GIPM).

Enzyme assays. Partially purified *V. harveyi* fatty acid synthase was prepared from 25 ml of cells harvested in mid-log phase by sonication (six bursts of 30 s each) in 0.6 ml of 50 mM Na^+/K^+-phosphate (pH 7.0), 5 mM DTT, and separated from endogenous ACP by ammonium sulfate fractionation (between 40% and 75% saturation) in the same buffer. ACPs (10 μM) were incubated at 37 °C for 1 h with FAS fraction (3 μg of total protein), 0.5 mM NADPH, 10 μM acetyl-CoA, 23 μM [2-^{14}C]malonyl-CoA (47,600 dpm/pmol) in 100 mM Na^+/K^+-phosphate (pH 7.0) and 5 mM DTT in a total volume of 40 μl. The reaction was stopped with 40 μl of 1 M KOH and incubation at 60 °C for 1 h. After acidification with 40 μl of 2 M HCl, fatty acids were extracted into 1 ml of petroleum ether and quantified by liquid scintillation counting.

Vibrio harveyi acyl-ACP synthetase was partially purified by DEAE–Sepharose and Sephacryl S-300 chromatography [15]. Assays were performed at 37 °C in a final volume of 15 μl containing 80 μM [9,10-^{3}H]myristic acid (890 dpm/pmol), 10 mM $MgCl_2$, 10 mM ATP, and 50 μM ACP in 100 mM Tris–HCl (pH 7.8) and 5 mM DTT. Acyl-ACP synthetase (0.3 μg of total protein corresponding to 2 milliunits [16]) was added to start the reaction and 10 μl samples were removed at 10 min. Acyl-ACP formation was measured after spotting on filter paper and washing with methanol:chloroform:acetic acid (6:3:1, v/v) to remove unbound fatty acid [16]. AAS was also used to prepare acyl-ACPs for native PAGE analysis except that 2 μg ACPs were incubated with 1.6 μg enzyme and 80 μM unlabelled myristic acid for up to 3 h at room temperature to achieve quantitative conversion to acyl-ACP.

Circular dichroism. Spectra from a Jasco J-810 spectropolarimeter were recorded at 25 °C using a 0.1 cm water-jacketed cell. ACP concentration was determined by Micro BCA protein assay (Pierce). Samples were diluted to 30 μg/ml (~3 μM) in 10 mM sodium phosphate buffer at pH 7.0 and spectra were obtained from 260 to 190 nm in continuous mode at a scanning speed of 10 nm/min directly or immediately after addition of $MgCl_2$.

Gel electrophoresis. ACPs or acyl-ACPs were mixed with 0.33 volumes of 4× native sample buffer (0.25 M Tris–HCl, pH 6.8, 25% glycerol, and bromphenol blue) and resolved by conformationally sensitive native gel electrophoresis (native PAGE) [17] under 150 V at 37 °C on a 20% polyacrylamide gel. Native PAGE at neutral pH was performed under 150 V at 4 °C on a 20% gel using a continuous buffer system of 43 mM imidazole and 35 mM Hepes at pH 7.4 after prerunning the gel for 2 h [18]. Gels were stained with GelCode Blue Stain Reagent (Pierce).

Results

Effect of ACP mutations on FAS and AAS activity

The negative charge of Glu-41 and the small size of Ala-45 have been predicted by computational docking analysis to be important for the interaction of helix II of *E. coli* ACP with the fatty acid synthase condensing enzyme FabH [12]. On this basis, we substituted both acidic (E41D) and basic (E41K) residues for Glu-41 of *V. harveyi* ACP by site-directed mutagenesis and replaced Ala-45 with either smaller (A45G), similar sized

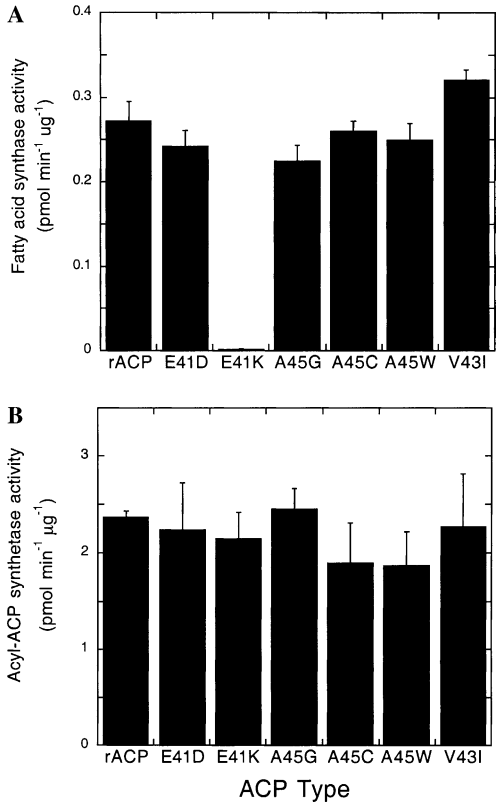

Fig. 1. Activities of *V. harveyi* fatty acid synthase and acyl-ACP synthetase with mutant ACPs. (A) FAS activity was measured as picomole incorporation of [2-^{14}C]malonyl-CoA into fatty acids in the presence of partially purified FAS fraction, ACPs (10 μM), acetyl-CoA, and NADPH as described in the text. Blank values from reactions conducted in the absence of ACP were subtracted, and data are expressed relative to total protein (FAS fraction). (B) Acyl-ACP synthetase activity was measured as ATP-dependent attachment of [^{3}H]myristic acid to ACP (50 μM) as described in the text. The mean and SD from four independent samples are shown in each case.

(A45C), or bulkier residues (A45W). The primary structure of *V. harveyi* ACP is identical to that of *E. coli* over this region (i.e., 100% between residues 31 and 71 and 86% identity over the entire protein [19]), and the modelled *V. harveyi* ACP backbone is essentially superimposable on that of *E. coli* ACP [20].

To measure the ability of recombinant ACP mutants to support FAS activity, crude extracts of *V. harveyi* were fractionated using ammonium sulfate (45–70% cut); a similar fraction has previously been shown to retain the *E. coli* FAS component enzymes while endogenous ACP remains soluble [21]. FAS activity with this enzyme fraction was completely dependent on added ACP, NADPH, and acetyl-CoA (with background values <3% of the complete mixture in each case) and was linear with both time and enzyme concentration under assay conditions (less than 15% conversion of limiting malonyl-CoA substrate). As illustrated in Fig. 1A, FAS activity was not affected when Glu-41 of ACP was replaced by another acidic residue (E41D), but was completely abolished with the E41K ACP mutant. In contrast, replacement of Ala-45 with either Gly, Cys, or Trp had no apparent effect on FAS activity. Moreover, mutation of Val-43 in this region to Ile, which has been shown to stabilize a more compact conformation of *E. coli* ACP [22], also had no effect relative to rACP.

For comparison, the effect of amino acid replacements in ACP helix II on activity of *V. harveyi* AAS was also investigated. AAS is a soluble 62 kDa enzyme that directly activates a broad range of free fatty acids to ACP in an ATP-dependent manner [15]. All ACPs tested were equally effective substrates for this enzyme (Fig. 1B). Thus, FAS (but not AAS) is sensitive to ACP mutations that reverse the charge at position 41, while both activities are insensitive to replacements of Ala-45.

Conformational analysis of ACP mutants

Circular dichroism was used to examine the effects of helix II mutations on the secondary structure of ACP. As we have reported recently [14,20], *V. harveyi* ACP (unlike *E. coli* ACP) is largely unfolded at neutral pH based on its ellipticity at 220 nm, but can adopt a native-like conformation containing ~50% α-helix in the presence of Mg^{2+} ions (Fig. 2). Similar behavior was observed for ACP mutants E41D, A45G, and A45W, while mutants E41K and V43I exhibited substantial α-helical content even in the absence of Mg^{2+}, with little further change following its addition. In contrast, the A45C mutant contained little secondary structure either in the presence or absence of Mg^{2+}, but a

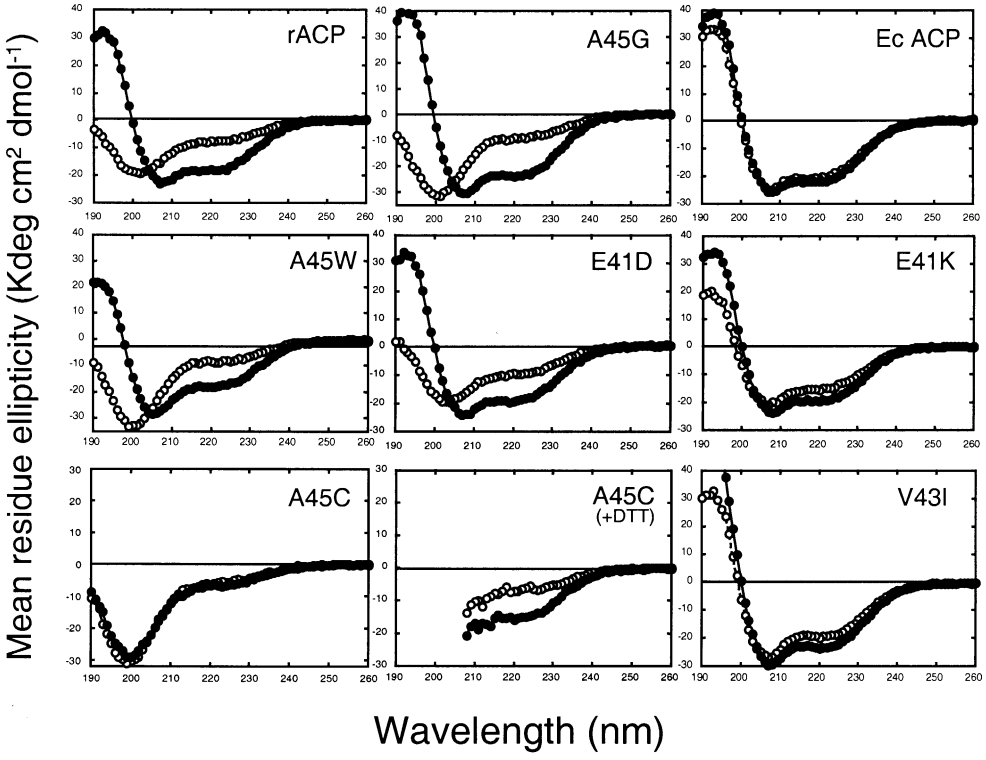

Fig. 2. Effects of Mg^{2+} on the secondary structure of ACPs. CD spectra of ACPs (3 μM) in 10 mM sodium-phosphate (pH 7.0) were recorded before (○) and after (●) addition of 2 mM $MgCl_2$. CD spectra of A45C were also recorded in the presence of 10 mM DTT. Results are representative from at least two separate preparations for each ACP mutant.

Mg^{2+}-dependent conformational change of this mutant was restored by addition of the sulfhydryl reducing agent DTT (Fig. 2). This suggests that oxidation of Cys-45 to form either intermolecular or intramolecular (i.e., with the phosphopantetheine sulfhydryl) disulfide bonds impairs proper folding of ACP. Overall, these results reveal that, although native-like structure is attainable in the presence of Mg^{2+} and/or DTT in all cases, ACP conformation is sensitive to mutations in the highly conserved helix II region.

The hydrodynamic properties of ACPs as monitored by native PAGE at pH 7.4 were consistent with the CD data (Fig. 3). In the presence of Mg^{2+} and DTT, all mutants exhibited similar electrophoretic mobility to rACP except for V43I, which migrated faster suggesting a smaller hydrodynamic radius [22]. It is noteworthy that E41K should have a decreased negative charge relative to the other ACPs at this pH, so that its similar mobility might also be indicative of a more compact shape under these conditions.

ACPs exhibit hydrodynamic expansion due to electrostatic repulsion at elevated pH and native PAGE at pH 9 is a sensitive method to monitor the conformational stability of ACP [17]. As shown in Fig. 4, mutants A45C, A45G, and A45W all exhibited decreased mobility relative to rACP, indicating that these mutations further destabilize the native conformation of ACP at this pH. Interestingly, mutants V43I and E41K migrated faster than rACP, suggesting that these mutations counteract the high pH-induced expansion of ACP. Enzymatic attachment of fatty acid, which is known to interact with residues lining a hydrophobic "fatty acid binding pocket" and stabilize a more compact conformation of ACP [17], resulted in increased electrophoretic mobility which was similar for all ACPs (Fig. 4). Nevertheless, our combined CD and electrophoresis data indicate that all helix II mutants can adopt a native-like conformation upon either acylation or Mg^{2+} binding.

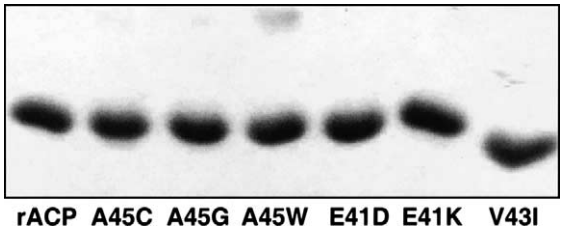

Fig. 3. Hydrodynamic analysis of ACPs using native PAGE at neutral pH. ACPs (4 μg each) were resolved on a 20% polyacrylamide gel at pH 7.4 in the presence of 10 mM $MgCl_2$ and 100 mM DTT. Gels were stained with GelCode Blue Stain Reagent.

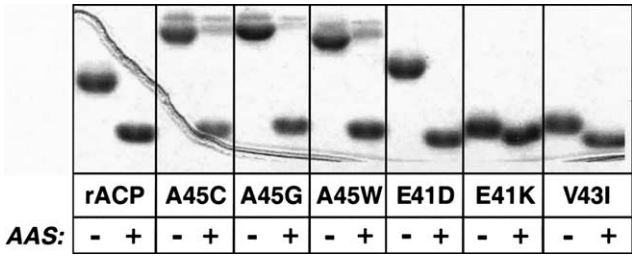

Fig. 4. Conformationally sensitive native PAGE analysis of holo- and acyl-ACPs. ACPs (2 μg each) were separated on a 20% polyacrylamide gel at pH 9.2 before (−) and after (+) ATP-dependent quantitative conversion into acyl-ACPs by AAS. Protein bands were visualized by staining with GelCode Blue Stain Reagent.

Discussion

Although early studies demonstrated that *E. coli* FAS activity requires native ACP conformation [21,23], more detailed structural information implicating specific amino acids in interactions between ACP and FAS enzymes has only recently begun to emerge. Our observation that an acidic residue at position 41 of ACP is essential for FAS activity supports evidence by Zhang et al. [12] that this residue forms a key electrostatic interaction with Arg-249 of the FAS condensing enzyme FabH. Glutamate is found at this position in almost all ACPs, and a similar interaction has been observed between Glu-41 of ACP and Arg-221 of ACP synthase in the crystal structure of the *B. subtilis* complex [8]. Indeed, all ACP-dependent enzymes of known structure possess a basic/hydrophobic patch, adjacent to the active site, that is predicted to dock the ACP substrate [12].

Somewhat surprisingly, we found no evidence that Ala-45 of ACP is essential for FAS activity. This residue is thought to interact with Ala-253 of FabH, allowing close contact between helix II of ACP and helix Cα2 of the enzyme, and mutation of the FabH Ala-253 to a bulkier residue blocked its activity [12]. One obvious explanation for our result would be differences between the FabH enzymes of *E. coli* and *V. harveyi*. The amino acid sequence of *V. harveyi* FabH has not been determined, although other FAS components from this species (i.e., FabG, FabF) are >75% identical to the corresponding *E. coli* enzymes [19]. Another possibility is that FabH may not be rate-limiting in the overall FAS reaction measured with the *V. harveyi* complex; in this case effects of Ala-45 substitutions on FabH activity might go undetected. In any case, Ala-45 is clearly not universally required for FAS activity.

The effects of ACP helix II mutations on FAS activity must be interpreted in light of their influence on ACP conformation. While all the mutants examined in this study were capable of attaining a compact helical structure under optimal conditions, native PAGE ex-

periments indicated that helix II mutations can either destabilize (e.g., A45G, A45W, and A45C) or stabilize (E41K and V43I) a more compact ACP conformation. The lack of FAS activity with E41K ACP could result from impaired electrostatic interaction with a basic residue on a FAS component enzyme(s), as predicted for FabH [12]. Alternatively, this mutation could decrease FAS activity by restricting the conformational flexibility of ACP, as *E. coli* ACP is known to exist in at least two conformers that are in dynamic equilibrium [24]. This flexibility is thought to be important for ACP's ability to reversibly interact with multiple enzyme partners, undergoing conformational changes as the attached fatty acid moiety alternately switches between interaction with an enzyme active site and the fatty acid binding pocket of ACP [12]. However, the V43I mutant also exhibited Mg^{2+}-independent helical content and increased conformational stability without any loss of FAS activity, suggesting that impaired electrostatic interaction is the correct explanation for the effect of the E41K mutation.

One caveat is that E41K and V43I mutations probably stabilize ACP conformation by different mechanisms. The E41K mutation introduces a positive charge into the highly acidic helix II and, like binding of divalent cations which also occurs in this region [25], neutralizes electrostatic repulsion. We have recently shown that a similar mechanism is likely responsible for the increased conformational stability of *E. coli* versus *V. harveyi* ACP, due to the presence of a basic residue (His-75) near the C-terminus of *E. coli* ACP [20]. The V43I mutation was previously shown to decrease the hydrodynamic radius of *E. coli* ACP [22], and (like fatty acid binding) likely stabilizes by increasing the hydrophobic character of the protein core.

In contrast to the important role of Glu-41 in FAS activity, replacement of this residue with lysine had no effect on the *V. harveyi* acyl-ACP synthetase reaction. Previous work has demonstrated that native ACP conformation is essential for AAS activity, and residues near the ACP fatty acid binding pocket (e.g., Ile-54) have been implicated in enzyme binding [14]. While we cannot definitively rule out participation of helix II in interaction with AAS on the basis of the present results, an acidic residue at position 41 is clearly not required for this activity. Another enzyme shown to interact with a different region of ACP is the *E. coli* glucosyltransferase involved in membrane-derived oligosaccharide synthesis, where helix I and the adjacent extended loop of ACP have been implicated [13]. This may reflect the unique nature of ACP's participation in the glucosyltransferase reaction, in which the phosphopantetheine moiety is not required. It is interesting to speculate that different classes of ACP-dependent enzymes, such as synthetases, synthases, acyltransferases, glucosyltransferase, etc., might recognize distinct features of ACP.

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