An Isoleucine to Valine Substitution in Escherichia coli Acyl Carrier Protein Results in a Functional Protein of Decreased Molecular Radius at Elevated pH*

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Escherichia coli acyl carrier protein (ACP) has been reported to exist in at least two distinct conformers in solution. A novel form of ACP having an increased electrophoretic mobility on polyacrylamide gel electrophoresis was noted previously during work on \( \beta \)-ketoacyl carrier protein synthase II (fabF) mutants of \( E. \) coli (Jackowski, S., and Rock, C. O. (1987) J. Bacteriol. 169, 1469–1473). These workers reported that the increased electrophoretic mobility of the ACP from fabF strains occurred irrespective of prosthetic group attachment or the state of acylation of the prosthetic group. Since these workers were unable to detect a difference between the amino acid sequence of the ACP from the fabF mutants and that of wild type ACP, they suggested that the increased electrophoretic mobility was due to an unknown post-translational modification of the polypeptide chain. We have reinvestigated these mutants and report that the increased electrophoretic mobility is due to a mutation within the gene (acpP) that encodes ACP. This mutation results in substitution of isoleucine for valine 43 of ACP. Site-directed mutagenesis of a synthetic ACP gene demonstrated that the amino acid substitution at residue 43 is the cause of the increased electrophoretic mobility. Gel filtration experiments indicated that the increased electrophoretic mobility results from the more compact structure of V43I ACP at high pH. The altered residue lies within the ACP region of greatest conformational liability, and thus the V43I substitution may shift the equilibrium toward the more compact conformation(s). The disulfide-linked dimer of V43I ACP was readily formed and had an electrophoretic migration greater than the dimer of wild type ACP, suggesting that formation of ACP-ACP dimers does not require structural deformation of the protein.

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¶ The abbreviations used are: ACP, acyl carrier protein; PAGE, polyacrylamide gel electrophoresis; F-ACP, fast migrating (V43I mutant) ACP; PCR, polymerase chain reaction.

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had the same amino acid sequence and thus proposed that the increased mobility of the apo form resulted from an unknown post-translational modification of the protein which occurred only in fabF strains.

The realization that E. coli ACP exists in multiple conformers together with isolation of the gene encoding ACP (13) led us to reexamine the ACP of fabF strains. Moreover, if this ACP species carried a modification other than 4-phosphopantetheine, newly available mass spectral techniques should allow detection of this modification. We report that substitution of isoleucine for valine 43 is responsible for the altered electrophoretic mobility of the ACP of fabF strains, and we show that this mutant protein has an altered solution structure.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains—**All strains used in this work were derived from E. coli K12. For marker rescue analysis, strain CY244 (fabB+ fabF1) (14) was transformed with plasmid pKM44 (15) to yield strain DK780. Strain DV79 (metB1 panD2 coaA16fr) zj::Tn (10) (16) was transformed with plasmid pDK746 to yield strain DK747, which was used as the source of V43I ACP. Strain SJ16 (metB1 relA1 spoT1) x L'gyrA216 panD2 zad-220::Tn10 (16) was transformed with plasmid pDK746 to yield strain DK746. Strain SJ16 was transformed with plasmid pDK767 (pKK223-3 containing a synthetic acpP gene) (13, 17) to yield strain DK676, which was used as the source of wild type ACP. Strain DK537 (fabB+ fabF1 panD2 zad-220::Tn10) was transformed with plasmid pDK746 to yield strain DK801, which was used as a source of prophetic group-labeled V43I ACP.

**Media—**Culture medium was either rich broth (19) or minimal salts media (18) supplemented with 0.4% glucose or as needed. Alanine (5 μM), pantothene (120 μM), ampicillin (100 μg/ml), tetracycline (25 μg/ml), kanamycin (40 μg/ml), or chloramphenicol (0.05%), Oleate-containing medium was prepared by adding Brij-58 detergent (0.5%) and oleate (0.05%) to autoclaved rich broth. Isopropyl-1-thio-D-galactopyranoside was added to 1 mM, and the culture was allowed to grow for 6 h. The cells were then collected by centrifugation and resuspended in 10 mM Tris-HCl, pH 7, followed by trichloroacetic acid precipitation as described previously (21).

**DNA Manipulations—**Sequencing of wild type and V43I mutant strain was performed by direct sequencing of PCR-amplified DNA. Briefly, a primer (5'-GCACATCTGAGAAACGGTGT-3') (Genetic Engineering Center University of Illinois, Urbana-Champaign) complementary to the amino-terminal part of acpP and a primer (5'-CAACATCTGCAGTGCGATTCC-3') complementary to the amino-terminal part of fabF were used to amplify the acpP region by PCR. The PCR-generated fragment was then fractionated by agarose gel electrophoresis and the sample eluted from the gel by extraction with Quiagen (Qiagen). The resuspended sample was then directly sequenced using a primer (5'-CCGATGTTGCAGTATGCC-3') complementary to a region internal to the amplified fragment by the chain termination method (22).

Codon 43 was converted from valine to isoleucine by site-directed mutagenesis as described previously (21). Briefly, pmR16 was digested with EcoRI and HindII, the acpP-containing fragment was fractionated on an agarose gel, and the fragment eluted and ligated into pTZ19U digested with the same enzymes. This plasmid was then used as a source of single-stranded urad-containing DNA (23). Double-stranded DNA was then synthesized in vitro using the mutagenic oligonucleotide (GTTGACGAGATAGGCCT) with the use of the Muta-Gen site-directed mutagenesis kit (BioRad) according to the manufacturer's instructions. The resulting double-stranded DNA was then transformed into a suitable host and the mutations determined by sequencing the region containing the acpP gene. Plasmids containing the desired mutation were then purified, digested with EcoRI and HindII, and ligated into expression vector pKK223-3 to yield plasmid pDK746.

**Gel Filtration Analyses of Wild Type and V43I ACP—**Gel filtration studies were performed on a FPLC unit (Pharmacia) equipped with a column of eluting Pharmacia Sepharose 5-200 (16 mm × 60 cm) or Superose 12 (10 × 300 mm). All gel filtration runs were run in 50 mM Tris-HCl buffers of various pH values. The flow rate was 0.5 ml/min, and 0.2-1 ml fractions were collected. Elution of ACP species was monitored by absorbance at 280 nm, scintillation counting, or conformationally sensitive PAGE. A mixture of standard globular proteins (ribonuclease A, 13.7 kDa; chymotrypsinogen A, 25 kDa; ovalbumin, 43 kDa; bovine serum albumin, 67 kDa; and blue dextran 2000, 2,000 kDa) was used to calibrate the columns at neutral pH. We were unable to calibrate the columns at the higher pH values because of the denaturation of some of the protein standards. ACP labeled in the prosthetic group was obtained by culturing strain SJ16 (for wild type ACP) or strain DK802 for the V43I mutant ACP in minimal medium containing β-[3-H]alanine (24). The labeled ACPs were then purified as described previously (24). V43I mutant ACP was purified from a derivative of strain DV79 which expressed V43I mutant ACP at very high levels. The fraction on medium containing 10 mM pantothene, the coaA16fr strain of strain DV79 results in increased coenzyme A pools (16), allowing a greater fraction of the overproduced ACP to be modified post-translationally (21).

**Mass Spectral Analysis of Wild Type and Mutant ACP—**Using Matrix-assisted laser desorption ionization (MALDI) matrix was prepared by mixing 1 μl of 100 nM ACP with 1 μl of matrix solution containing 25 mg/ml α-cyano-4-hydroxycinnamic acid (Merck) and 0.1% trifluoroacetic acid (TFA). The sample was allowed to dry and was then loaded onto a 1/10-inch MALDI-TOF mass spectrometer (Applied Biosystems, Foster City, CA). 7-AEEF (TFA), or 0.05% trifluoroacetic acid in acetonitrile (1:1), was used as the matrix. The protein sample was then allowed to dry before being subjected to MALDI-TOF mass spectrometry. A synthetic ACP (15) was used as a standard for calculating protein molecular mass under these instrument conditions. The average mass of wild-type ACP was 15,906 ± 10 Da. The mass of V43I ACP was 15,925 ± 10 Da. The difference in mass of V43I ACP is 20 Da, which is consistent with the expected mass of a valine to isoleucine mutation. This result was also confirmed by mass spectrometry of the synthetic ACP (15).

**RESULTS**

**The Mutation Conferred Increased Electrophoretic Mobility**

Expression of the V43I mutant gene resulted in two major protein bands that migrated faster on conformationally sensitive PAGE than wild-type ACP. The desalted ACP was diluted 1/10 with 10 mM ammonium bicarbonate, pH 8, and lyophilized. The pellet was then dissolved in ammonium acetate, pH 5, at a concentration of 30-100 pmol/μl and analyzed on a VQ Quattro electrospray mass spectrometer at the mass spectrometry facility of the University of Illinois, Urbana-Champaign.

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electrospray mass spectrometry of the purified protein (Fig. 2) indicating that no unknown modification was present. Therefore, the V43I mutation is responsible for the increased electrophoretic mobility. Note that the presence of the apo forms is due to the high rate of apo-protein production which exceeds the rate of post-translational modification (21).

The V43I Mutant ACP Is Conformationally Distinct from Wild Type ACP—Various modified and acylated species of ACP can be separated by use of PAGE run in the absence of sodium dodecyl sulfate. In these systems the gel reaches a pH of about 9.5 during the electrophoresis, a pH that induces a hydrodynamic expansion of ACP (9, 25, 26). The structure of ACP in these PAGE gels is further perturbed by a partial unfolding of the protein caused either by running the gel at elevated temperature (37°C) (26) or by including urea in the gel (20). The modified and acylated species of ACP are more stable to these conditions and thus have smaller molecular radii accounting for their faster electrophoretic migration rates relative to apo-ACP.

Based on these observations, the increased electrophoretic migration rate of the V43I ACP species strongly suggested that these molecules have smaller hydrodynamic radii than the respective wild type species. However, gel electrophoretic mobility is not a direct measure of molecular radius because mobility is charge-dependent. Although, the V43I mutation involves no change in total charge, an indirect effect on net charge due to an altered protein conformation remained possible. To avoid this ambiguity we used gel filtration to study the stability and molecular radius of the V43I mutant. Analysis by urea-PAGE of fractions collected from a Sephacyr S-200 column loaded with a mixture of purified wild type and V43I ACPs showed no resolution of the two ACP forms at pH 7 (Fig. 3A) whereas at pH 9 (Fig. 3B) the two proteins eluted much earlier with the V43I ACP eluting after the wild type protein.

Further gel filtration analyses were done with a more quantitative system in which the columns were loaded with a mixture of radiochemically pure wild type ACP (labeled in the prosthetic group) and a large molar excess of purified V43I ACP, thus allowing measurement of wild type ACP by scintillation counting and of V43I ACP by absorbance at 280 nm (the concentration of radiolabeled wild type ACP was below detection by absorbance). When the proteins were analyzed at pH 9 on a Sephacyr S-200 column (Fig. 4B) or at pH 9.3 on a Superose 12 column (Fig. 4D) the wild type protein eluted ahead of the mutant protein. We conclude that V43I ACP has an effective molecular radius at high pH which is smaller than that of wild type ACP. At pH 7 (as expected from Fig. 3A) the V43I mutant protein eluted from the Sephacyr S-200 column together with the wild type ACP protein (Fig. 4A). However, when a Superose 12 column was used the wild type protein eluted ahead of the mutant protein (Fig. 4C). In agreement
with earlier work we found that the gel filtration behavior of wild type ACP at neutral pH was that of a much larger protein (when compared with standard globular proteins); on Sephacryl S-200 ACP had an apparent molecular mass of 33.8 kDa whereas a value of 31.2 kDa was obtained from the Superose 12 column. It should be noted that gel electrophoretic analysis similar to those of Fig. 3 was also done on the fractions from the Superose column (data not shown) which were entirely consistent with the profiles of Fig. 4, C and D.

FIG. 3. Separation of purified wild type and V43I ACPs by gel filtration. Urea-PAGE analysis of elution of a mixture of wild type (W. T.) and V43I ACPs run on a Sephacryl S-200 gel filtration column at either pH 7 (panel A) or pH 9 (panel B). Equivalent amounts of wild type and V43I ACPs were mixed and injected onto a Sephacryl S-200 column as described under “Experimental Procedures.” The proteins of the column fractions were then concentrated by trichloroacetic acid precipitation, separated by urea-PAGE, and stained with Coomassie Blue.

The Prosthetic Group of V43I ACP—Little is known of the contribution the prosthetic group makes to the overall structure of ACP. Two-dimensional NMR studies indicate that the prosthetic group makes minimal contact with any amino acid residues other than the serine to which it is covalently bound (7). However, gel electrophoretic studies indicate that the presence of the ACP prosthetic group stabilizes the ACP structure as measured by the increased electrophoretic mobility of holo-ACP relative to apo-ACP (24, 25). The V43I mutant has a more tightly folded conformation than wild type protein, and thus it seemed possible that the prosthetic group might be less accessible to solvent. Thus, we compared the accessibility of the prosthetic groups of the wild type and V43I ACPs with the sulfhydryl reagent 5,5'-dithiobis(2-nitrobenzoic acid) and with disulfide cross-linking of two ACP molecules catalyzed by Cu²⁺-o-phenanthroline (29). The V43I and wild type ACPs were indistinguishable in these two assays (data not shown). Interestingly, the cross-linked V43I ACP species retained the high electrophoretic mobility characteristic of the monomeric protein; the V43I dimer migrated more rapidly than the dimer of wild type ACP (Fig. 5).

We also assayed the efficiency of post-translational modification of V43I ACP by measurement of the incorporation of
Altered ACP Conformation

**Fig. 5. PAGE analysis of β-l-alanine-labeled wild type and V43I ACPs.** Strains producing ACP of various sequences were grown to early log phase, and ACP production was induced by the addition of 1 mM isopropyl-1-thio-β-D-galactopyranoside followed by growth in the presence of β-[3-H]alanine. The trichloroacetic acid-treated cell samples were then fractionated on urea-PAGE as described under “Experimental Procedures” and detected by fluorography. Lane 1, strain DK676 (which contains a plasmid encoding wild type (WT) ACP); lane 2, strain DK685 (which contains a plasmid encoding S36T ACP, a mutant protein that cannot be modified); lane 3, strain DK746 (which contains a plasmid encoding V43I ACP); and lane 4, strain S516 (no plasmid). [Holo]2 denotes disulfide linked ACP.

**Fig. 6. Sequence comparisons of the V43 region in various ACPs.** The amino acid sequence of the regions surrounding the prokaryotic-gene attachment site in ACP or ACP-like domains in several proteins is shown. R. mel, Rhizobium meliloti; A. thal., Arabidopsis thaliana; S. ole, Spinacia oleracea; G. gal, Gallus gallus. Conservative substitutions are designated by plus sign; nonconservative changes are denoted by a space.

**DISCUSSION**

We have shown that the increased electrophoretic mobility of the ACP of fabF mutants (12) is due to a mutation within acpP rather than within fabF, and thus the increased electrophoretic mobility is not due to an unknown post-translational modification as proposed previously. The acpP mutation results in conversion of residue 43 from valine to isoleucine. It should be noted that Jackowski et al. (12) detected the V43I alteration in the ACP from fabF strains by automated Edman degradation but reported that wild type ACP had the same sequence. However, these workers were unfortunate in that the residues in question were located at the end of sequences obtained by successive degradation from the amino terminus, and thus the high background accumulated from the previous 42 sequencing cycles probably caused the mistaken amino acid identification. DNA sequencing (13) and NMR analysis of the protein (4–7) clearly demonstrate that residue 43 of wild type ACP is valine.

In the electrophoretic systems used in ACP analysis, the pH values of the separating gels vary from an initial pH of 9 to a final pH of about 9.5 (10), and thus the smaller effective molecular radius of V43I ACP detected at similar pH values by gel filtration can fully explain the observed increase in electrophoretic mobility. The relative stability to pH-induced expansion of V43I ACP and persistence of a more compact form during gel electrophoresis in the presence of urea or at elevated temperatures strongly suggests that V43I ACP is more stable than the wild type molecule. Studies of the thermodynamics and NMR structure of the V43I mutant are in progress. It should be noted that the larger molecular radius of wild type ACP at high pH values was observed previously by gel filtration (9) and is consistent with the pH-induced expansion of ACP observed by circular dichroism (27, 28).

NMR studies of ACP indicate that the protein is atypical in that it is a protein of unusually mobile structure which exists in dynamic equilibrium between two or more conformers (3–7). The major difference between the two proposed structures is the presence or absence of a short helix (helix II). Moreover, recent amide exchange experiments indicate that helix II is considerably less stable than the other ACP helices (3). Given this conformational freedom it is surprising that the V43I mutation (which adds only a single methylene group) changes the structure of ACP. Valine 43 lies within helix II, and thus a plausible interpretation of our data is that the V43I substitution can shift the equilibria toward the more compact conformers, perhaps through stabilization of helix II. Helix II forms a surface of the hydrophobic cavity which interacts with the fatty acid acyl chain. Since acylation of the prosthetic group increases the electrophoretic mobilities of both wild type and V43I ACPs (12), it seems that the amino acid substitution does not greatly affect interactions with the acyl chain. Consistent with this conclusion NMR data suggest that the valine 43 region of wild type ACP does not undergo a major structural change upon acylation (11). The valine corresponding to position 43 is widely conserved among different ACPs (Fig. 6). Only two ACPs were found which contain isoleucine at the position corresponding to position 43 of the E. coli protein, both of which are chloroplast proteins.

At high pH values the effective molecular radii observed for the wild type and V43I ACPs were similar on two different gel filtration matrices: Superose 12, an agarose-based matrix, and Sephacryl S-200, a dextran-acrylamide matrix (Fig. 4). However, at neutral pH the Superose 12 column resolved the two forms of wild type and V43I ACPs coeluted from the Sephacryl S-200 column (Fig. 4). Since the elution positions compared with globular marker proteins of known radii were essentially identical on the two matrices, the differing behavior may be due to differential hydrophobic interactions of the two ACP species with the Superose matrix. According to the manufacturer Superose is capable of hydrophobic interactions with proteins whereas Sephacryl was designed to minimize such interactions. Thus, the resolution of the wild type and V43I ACPs on Superose 12 suggests that V43I ACP might have a greater exposed hydrophobic surface area at neutral pH than does the wild type protein. Moreover, the lack of an increased retention of V43I ACP on Sephacryl (relative to Sephacryl) at higher pH values might indicate that the V43I ACP conformers present at high pH values lack the putative exposed hydrophobic surfaces present at neutral pH. However, it seems much more likely that these results reflect the fact that the markedly less compact forms of the protein present at alkaline pH values are largely excluded from the matrix and thus would have fewer opportunities to interact with either matrix. Unfortunately, these considerations are based on largely anecdotal evidence from the manufacturer of the columns, and we cannot say with certainty which of the two columns gives more valid
data. Other evidence suggests that V43I ACP has a smaller effective radius than wild type ACP at neutral pH consistent with the Superose results. V43I ACP was not retained efficiently by dialysis membranes made to retain molecules >12–14 kDa whereas wild type ACP was retained quantitatively by these membranes (data not shown). In any event it is clear that ACP is a protein with little static structure and lacks a clearly defined and characteristic molecular radius. (For this reason we have chosen not to report our results as Stokes radii.)

Surprisingly, the cross-linked V43I mutant retained its altered electrophoretic migration relative to the dimer of wild ACP (Fig. 5), suggesting that formation of ACP-ACP dimers does not require structural deformation of the protein. This finding argues that the prosthetic group can swing out from the hydrophobic pocket. This argument is supported by the inability to observe the prosthetic group in the NMR studies in the absence of acylation. It is, presumably because of rapid mobility on the NMR time scale (7). Moreover, in the absence of acylation the prosthetic group has previously been suggested to extend away from protein into the bulk solution (7). Upon acylation however, contacts between the hydrophobic fatty acyl chain and the nonpolar amino acid side chains hold the prosthetic group within the fatty acid binding pocket.

Despite the altered nature of the protein observed in vitro, the V43I protein functions efficiently in vivo; strains that carry the mutation are fully viable. Our results are also consistent with the results which showed that the V43I mutation is an efficient substrate for in vitro fatty acid biosynthesis (12). The V43I mutant form of ACP has only been found associated with mutants in fabF. The fabF mutant was originally isolated in a strain that also contained a mutation in the fabA gene encoding the dehydrase/isomerase necessary for unsaturated fatty acid biosynthesis (30). The rationale for the association between the fabA and fabF mutations was that the fabF mutation prevented elongation of palmitoleoyl-ACP, the preferred substrate for 1-acyl-glycerol-3-phosphate acyltransferase and thereby increased the efficiency of unsaturated fatty acid utilization. Since the V43I mutation is present in both independently isolated fabF mutants of E. coli, it seems possible that the V43I mutation also increases the efficiency of unsaturated fatty acid utilization. Studies are currently in progress to assess the role of this mutation in E. coli physiology.

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