Minireview

Bacterial Long Chain Fatty Acid Transport: Gateway to a Fatty Acid-responsive Signaling System*

Exogenous long chain fatty acids (LCFAs) influence a myriad of cellular processes including intracellular signaling and patterns of gene expression. Some cell types accumulate distinct classes of LCFAs whereas others accumulate or release fatty acids following some type of external stimulus (i.e. hormonal or nutritional cues). Given the energetic cost of fatty acid synthesis on one hand and (at least in the mammalian system) the necessity for obtaining essential fatty acids from the environment, the transport of LCFAs must necessarily represent a fundamental biological process. The biochemical mechanisms underpinning fatty acid transport involve specific proteins and enzymes, which act either directly at the membrane or indirectly at the level of downstream metabolism. Distinct membrane-bound and membrane-associated fatty acid transport proteins have been identified and characterized in a number of different systems including bacteria, yeast, and mammals (1–7).

The genetic and biochemical foundations of LCFA transport were first elucidated in the Gram-negative bacterium Escherichia coli. The hallmark of this system is the coupled transport and activation of exogenous LCFAs, which in turn leads to changes in transcription patterns of the genes encoding the proteins required for fatty acid biosynthesis and degradation. The three central components of this system include FadL, an outer membrane-bound fatty acid transport protein, FadD, an inner membrane-associated long chain acyl-CoA synthetase (ACSL), and FadR, a long chain acyl-CoA-responsive transcription factor. The bacterial fatty acid transport and trafficking system leading to downstream fatty acid-responsive transcriptional regulation serves as a fundamental paradigm in biology, which provides a number of guiding principles that can be applied to more complex systems.

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†To whom correspondence should be addressed: Center for Metabolic Diseases, Ordway Research Inst., Inc., 150 New Scotland Ave., Albany, NY 12208. Tel.: 518-641-6461; Fax: 518-641-6304; E-mail: pblack@ordwayresearch.org.
‡The abbreviations used are: LCFAs, long chain fatty acids; ACSL, acyl-CoA synthetase; ACA-CoA, long chain acyl-CoA; LDAO, lauryldimethylamine N-oxide.

The transport of exogenous LCFAs across the cell envelope of E. coli requires both the transport protein FadL (encoded by the fadL gene) and the ACSL FadD (encoded by the fadD gene). The product of transport, long chain acyl-CoA, is the effector molecule that regulates the DNA binding activity of the transcription factor FadR (encoded by the fadR gene), which in turn controls the expression of nine genes primarily involved in fatty acid degradation and biosynthesis (reviewed in Refs. 8 and 9). The seminal studies in the laboratories of Peter Overath and Salih Wakil provided the framework required to establish the genetic foundations of the fatty acid transport and trafficking system in E. coli (10–13). Of particular note are studies that identified and mapped most of the genes of the fatty acid degradative regulon (fad) and distinct regulatory mutants, including fadR (10, 11). Overath et al. (11) identified the fadL gene in 1969 and at that time suggested this ACSL was a component of the fatty acid transport system. Klein et al. (14) subsequently characterized the inducible system for the uptake of exogenous fatty acids and proposed that this enzyme functioned in the process of fatty acid transport by “vectorial acylation by analogy with vectorial phosphorylation, an elegant term coined by H. R. Kaback for the phosphoenolpyruvate-dependent sugar transport system in E. coli.” FadD is associated with the plasma membrane where it is hypothesized to abstract fatty acids from the membrane concomitant with activation to CoA thioesters (14). In a related study, Hill and Angelmaier (15) identified a novel mutant that had wild type acyl-CoA synthetase activities yet was unable to incorporate exogenous fatty acids into total lipids. These investigators proposed that the affected gene product participates in the uptake of LCFAs and even suggested this was a protein, “which facilitates the diffusion of oleate through the cytoplasmic membrane” (15).

Nunn and Simons (16) identified the fadL gene in 1978 confirming the existence of a second protein involved in the transport of exogenous LCFAs. They suggested the product of the fadL gene was an inner membrane-bound fatty acid permease that facilitated the transport of exogenous LCFAs and worked in concert with FadD (16–18). However, purification and characterization of FadL by Black et al. (19) demonstrated that this protein was localized exclusively in the outer membrane and had heat-modifiable characteristics similar to those defined for OmpA. The localization of FadL in the outer membrane and its role in the transport of exogenous fatty acids are now well established facts despite the ambiguity in the earlier literature (4, 9, 19–23).

The major regulator controlling the expression of the FadL and FadD structural genes is the long chain acyl-CoA (LCA-CoA)-responsive FadR (20). As the product of fatty acid transport proceeding through FadL and FadD is LCA-CoA, this tripartite system functions to allow E. coli to quickly adapt its transcriptional output in the presence of exogenous long chain fatty acids. Several reviews by DiRusso and colleagues (8, 9, 21) provide a detailed description of the regulation of the fatty acid degradative and biosynthetic genes by FadR in E. coli. 

Outer Membrane and Long Chain Fatty Acid Transport Protein FadL

The outer membrane protein, FadL, is the only bona fide membrane-bound LCFA transport protein presently recognized. Other candidate fatty acid transport proteins have been identified in eukaryotic systems, but each of these has additional functions including roles in intermediary metabolism (fatty acid-binding protein, plasma membrane-bound (FABP/pm/aspartate aminotransferase)) (25), acting as a receptor for oxidized low density lipoproteins (fatty acid transport protein (FAT/CD36)) (1, 2) and activating very long chain fatty acids (fatty acid transport protein (FATP)) (6, 26, 27). The work on the long chain fatty acid transport protein FadL has progressed from the fundamental genetics studies detailed above to determination of two distinct crystal structures, which support the hypothesis of a ligand-induced conformational shift as part of the transport process. Within the outer membrane leaflet of the bacterial cell envelope the 421-amino acid residue FadL protein functions to bind exogenous LCFAs with a relatively high affinity. The binding of long but not medium chain fatty acids to FadL provides evidence that the length of the hydrocarbon tail provides substrate specificity (20).
Oleoyl alcohol and methyl oleate are unable to compete for FadL-specific binding of oleate arguing that the carboxylate of the fatty acid is also required (20). These studies support the notion that binding occurs through specific hydrophobic interactions between the protein and the acyl chain of the fatty acid. As the carboxylate of the LCFA is also essential for binding, several charged amino acid residues within FadL are predicted to function as contacts with this charged group.

Studies using in vitro mutagenesis of the fadL gene demonstrated the LCFA binding and transport activities associated with FadL are separable (28–30). Analysis of the linker mutation fadLS1 (insertion of E-F between residues 383 and 384) showed that transport is reduced to 30% wild type levels, whereas binding is essentially wild type arguing that residues within the C-terminal region of FadL are involved in transport. Linker mutations resulting in two amino acid insertions that are more N-terminal proximal are defective for both LCFA binding and transport. These data suggest that in general terms amino acid residues within the C-terminal region of FadL are required for transport whereas residues within the N-terminal region are required for binding (29).

Site-directed mutagenesis of fadL has refined these observations and in particular identified three amino acid residues within FadL (Phe401, Val383, and Ser370) that contribute to transport and one (His83) that contributes to binding (28, 30). Directed mutagenesis studies also identified two fadL mutations (fadLH3 substitutions of E-F between Ala511 and Gly512 and fadLP401A) that have low fatty acid binding relative to transport suggesting these two mutant forms of FadL are “locked” in an open conformation (29, 30). These data provided the first indication that FadL contains a conformationally sensitive region, which is responsive to LCFA binding. The biochemical properties of these fadL mutants are consistent with the information that has arisen from the two crystal structures of FadL.

To more fully understand the mechanism by which this protein functions in fatty acid transport, van den Berg et al. (31) solved two crystal structures (monoclinic and hexagonal) of FadL (Fig. 1). In both crystal structures the protein is a monomer consisting of a long (50 Å) β-barrel composed of 14 antiparallel β-strands. The differences in the two crystal forms are largely found within the N-terminal 42 amino acid residues of the protein, which forms a small compact domain that obstructs the barrel on the periplasmic side. This domain, referred to as the hatch, consists of three short helices connected by short loops that interact with the walls of the barrel through ~15–20 hydrogen bonds and a single salt bridge. In the monoclinic crystal form, the conformation of β-strand S3 contains a kink that points inward, thereby disrupting the interstrand β-sheet hydrogen-bonding pattern. The kink is stabilized by interactions with specific residues within the N terminus of the hatch domain; in particular Asn103 to Gly105 in β-strand S3 forms a short antiparallel β-strand with residues Phe3 through Leu4 in the N terminus.

The monoclinic crystal structure of FadL revealed two discrete regions that are likely to represent low affinity and high affinity fatty acid binding sites. The low affinity site is a solvent-exposed hydrophobic groove between the two extracellular loops L3 and L4. Within the FadL barrel, on the extracellular side of the membrane a prominent hydrophobic pocket is apparent and is proposed to represent the high affinity fatty acid binding site. A single molecule of lauryldimethylamine N-oxide (LDAO), a detergent used in protein purification, is found within this site (Fig. 2). At least 15 hydrophobic amino acid residues in β-strands S3 to S10 contribute to this proposed fatty acid binding pocket. In addition, Phe3 appears to link this region of FadL to the N-terminal hatch domain. This entrance to this fatty acid binding site on the extracellular side of the membrane is located close to one end of the hydrophobic groove between loops L3 and L4 and is accessible to solvent (31).

The hexagonal crystal structure of FadL, which also contained a bound LDAO molecule, revealed that the architecture of the high affinity binding site was altered when compared with the structure from the monoclinic crystal. In particular, the conformation of the seven N-terminal residues within the FadL were altered in such a manner that Phe3 is rotated nearly 180° (Fig. 2). In addition, the kink in S3 observed in the monoclinic crystal is absent. The bound LDAO molecule within the binding pocket is extended within the hexagonal crystal and has moved nearly 10 Å. This results in an apparent opening of the LCFA-specific channel, which allows the fatty acid to move through FadL and into the periplasmic space.

These data are consistent with the ligand-induced conformational shift initially predicted from the analyses of fadL mutants detailed above.

**Periplasmic Space and Bacterial Fatty Acid Transport**

The process of LCFA transport is partially sensitive to osmotic shock suggesting that either a protein or the integrity of the chem-
transport and proposed that this enzyme functions by vectorial esterification. In essence, this ACSL functions to abstract and activate exogenous LCFA with transport across the inner membrane. Both CoA and ATP pools are required for fatty acid transport, which reflects the requirement for these substrates by FadD for activity (32, 36). A number of the initial studies describing the E. coli ACSL noted this enzyme was found both within the inner membrane and cytosol, suggesting it moved between different cellular compartments during its catalytic cycle. On the basis of these studies it has been proposed that FadD partitions into the inner membrane in an ATP-bound form to retrieve and then activate LCFA, thus rendering the process of fatty acid transport unidirectional (36).

FadD has served as a prototype to define functional elements within the family of acyl-CoA synthetases (36, 37). Of particular note is a 25-amino acid residue signature motif (residues 431–455 of FadD), which is common to all ACSLs (37). A series of site-directed mutations within the fadD gene corresponding to this motif confirmed the importance of this region to catalytic activity. Seven of 18 substitutions that lowered the affinity for fatty acids also altered the fatty acid chain length specificity. These data showed this region of the enzyme is essential for catalytic activity, functions in part to promote fatty acid chain length specificity, and is predicted to comprise part of the fatty acid binding site (37).

Using afinity-labeling, LCFA 9-azidophenoxynonanoic acid, the fatty acid binding site within FadD was defined and found to overlap the region defined using directed mutagenesis confirming this is part of the fatty acid binding domain (38). A second region of interest in FadD corresponds to the ATP-AMP binding site (residues 205–228), a signature motif common to all adenylyl-forming enzymes (36). Using a combination of directed mutagenesis of fadD (corresponding to the ATP-AMP signature) and mechanistic enzymatic studies, the functional contribution of this region of the enzyme has been evaluated, both in terms of ACSL and fatty acid transport activities. These experiments showed amino acid substitutions within this region of the enzyme either reduce or eliminate catalytic activity and in turn reduce or eliminate fatty acid transport (36).

Although the three-dimensional structure of FadD has not been defined a partial structure has been proposed (Fig. 1) (38). More recently Hisanaga et al. (39) have determined the crystal structure of a distinctly related acyl-CoA synthetase from Thermus thermophilus and proposed a two-step enzyme mechanism. Both the proposed structure and the defined structure demonstrate the fatty acid and ATP-AMP binding sites are in close proximity to each other even though they are separated by a considerable distance in the linear amino acid sequence (38, 39). The ACSL FadD is thought to first bind ATP and then move to the membrane (36, 38). This hypothesis was derived from the observation that the enzyme becomes activated in the presence of membrane lipid (36). The model that emerges upon consideration of these data is that once FadD contacts with the membrane, this enzyme functions to abstract fatty acid concomitantly with the formation of the acyl-AMP and release of PPi. Subsequently the enzyme binds CoA with the release of AMP and the formation of acyl-CoA, which in turn is released from the enzyme (36, 39).

Bacterial Long Chain Fatty Acid Transport and Downstream Signaling

The LCA-CoA formed as a consequence of the vectorial acylation mediated by the ACSL FadD serves primarily as a substrate for β-oxidation although it can be incorporated into membrane phospholipids. One particular feature of LCA-CoA is that it functions as a signaling lipid that governs the DNA binding properties of the transcription factor FadR. In a series of studies from the DiRusso laboratory, LCA-CoA was unequivocally defined as the effector that governed the DNA binding activity of this transcription factor (24, 40–42). Three studies are of particular note. The first defined the affinity of FadR for different acyl-CoAs of varying chain lengths (24, 43); the second mapped the LCA-CoA binding domain following the selection of super-repressor mutants in fadR (44); and the third defined three crystal structures of FadR (alone, complexed with DNA, and complexed with myristoyl-CoA; Fig. 1) (40, 41).

The signaling proceeding through LCA-CoA is of particular sig-
nificance as FadR controls the expression of a significant number of genes involved in fatty acid degradation, fatty acid biosynthesis, acetate metabolism, and the stress response (9, 42). FadR is a transcriptional repressor of genes involved in fatty acid transport, activation, and β-oxidation and a transcriptional activator of at least two genes required for fatty acid biosynthesis (fadb and fabB) (9, 42). In addition FadR functions as an activator of iclR, encoding a transcriptional repressor of the glyoxylate shunt genes (acetate metabolism) and as a repressor of uspA, which functions in a number of stress response pathways (8, 45). Binding sites for FadR have been mapped within fadBA, fadL, fadD, fadF, fadH, fabA, fabB, iclR, and uspA (24, 42, 45, 46). By regulating the expression of iclR, FadR also controls acetate degradation, which is also required for bacterial growth on LCFAs. The control of the expression of uspA (encoding the universal shock protein UspA) by FadR links fatty acid degradation to the stress response. This link is further evidenced by the observation that fadF, encoding acyl-CoA dehydrogenase, is induced in starvation due to inactivation of FadR (47).

FadR responds to the intracellular levels of LCA-CoA to modulate the expression of genes involved in fatty acid and acetate metabolism and the stress response. As LCA-CoA levels rise within the cell, these compounds bind to FadR, rendering it unable to bind DNA. The net result is releasing the transcriptional repression of the fatty acid degradative genes and uspA. In addition, inactivation of FadR occurs as cells proceed through stationary phase as nutrients become depleted or during various types of environmental stress. At the same time FadR can no longer function to activate key fatty acid biosynthetic genes and iclR.

**Fatty Acid Transport, Trafficking, and Transcriptional Regulation in E. coli**

The mechanisms that govern the transport and trafficking of exogenous LCFAs are well established in E. coli. This process leads to changes in the transcriptional output of genes involved in both fatty acid degradation and fatty acid biosynthesis. Thus although this is a bacterial system, it shares many features common to higher eukaryotic systems, including mammals, and serves as a model of coordinating the transmembrane movement of exogenous fatty acids with downstream processes, most notably transcriptional control. Fig. 1 summarizes features of this system and demonstrates how the bacterial cell quickly adapts to the presence of exogenous LCFAs. Under normal growth conditions both FadL and FabD are present at low levels within the cell and the intracellular concentrations of LCA-CoA are low, which allows FadR to bind to cognate operators on the fatty acid degradative genes (acting as a repressor) and several fatty acid biosynthetic genes (acting as an activator). When the cell encounters LCFAs in the environment, these ligands bind to FadL and via a ligand-induced conformational shift within the protein are transported into the periplasmic space. The more acidic environment of the periplasmic space promotes the formation of uncharged fatty acid molecules, which partition into and flip across the membrane. The ACSL functions to activate these long chain fatty acids from the membrane in a manner that is coupled to activation. As the levels of LCA-CoA increase within the cell, these molecules bind to FadR causing a significant conformational rearrangement of the DNA binding domain, which relieves transcriptional repression of the fatty acid degradative genes (including fadL and fadD), and depressing the transcriptional activation of the fatty acid biosynthetic genes (particularly fabA and fabB). FadR is exquisitely sensitive to the changes in intracellular LCA-CoA levels, which allows the bacterial cell to quickly adapt its metabolism accordingly.