Functional Role of Fatty Acyl-Coenzyme A Synthetase in the Transmembrane Movement and Activation of Exogenous Long-chain Fatty Acids

AMINO ACID RESIDUES WITHIN THE ATP/AMP SIGNATURE MOTIF OF ESCHERICHIA COLI FadD ARE REQUIRED FOR ENZYME Activity and Fatty Acid Transport

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Fatty acyl-CoA synthetase (FACS, fatty acid:CoA ligase, AMP forming; EC 6.2.1.3) plays a central role in intermediary metabolism by catalyzing the formation of fatty acyl-CoA. In Escherichia coli this enzyme, encoded by the fadD gene, is required for the coupled import and activation of exogenous long-chain fatty acids. The E. coli FACS (FadD) contains two sequence elements, which comprise the ATP/AMP signature motif (213YTG-GTTGVAKGA224 and 356GYGLTE361) placing it in the superfamily of adenylate-forming enzymes. A series of site-directed mutations were generated in the fadD gene within the ATP/AMP signature motif site to evaluate the role of this conserved region to enzyme function and to fatty acid transport. This approach revealed two major classes of fadD mutants with depressed enzyme activity: 1) those with 25–45% wild type activity (fadD621A, fadD217A, fadD221A, and fadD222A) and 2) those with 10% or less wild-type activity (fadD213A, fadD214A, and fadD381A). Using anti-FadD sera, Western blots demonstrated the different mutant forms of FadD that were present and had localization patterns equivalent to the wild type. The defect in the first class was attributed to a reduced catalytic efficiency although several mutant forms also had a reduced affinity for ATP. The mutations resulting in these biochemical phenotypes reduced or essentially eliminated the transport of exogenous long-chain fatty acids. These data support the hypothesis that the FACS FadD functions in the vectorial movement of exogenous fatty acids across the plasma membrane by acting as a metabolic trap, which results in the formation of acyl-CoA esters.

Fatty acyl-CoA synthetase (FACS, fatty acid:CoA ligase, AMP forming; EC 6.2.1.3) plays a central role in intermediary metabolism by catalyzing the formation of fatty acyl-CoA. These bioactive fatty acid metabolites are involved in protein metabolism by catalyzing the formation of fatty acyl-CoA. In Escherichia coli this enzyme, encoded by the fadD gene, is required for the coupled import and activation of exogenous long-chain fatty acids. The E. coli FACS (FadD) contains two sequence elements, which comprise the ATP/AMP signature motif (213YTG-GTTGVAKGA224 and 356GYGLTE361) placing it in the superfamily of adenylate-forming enzymes. A series of site-directed mutations were generated in the fadD gene within the ATP/AMP signature motif site to evaluate the role of this conserved region to enzyme function and to fatty acid transport. This approach revealed two major classes of fadD mutants with depressed enzyme activity: 1) those with 25–45% wild type activity (fadD621A, fadD217A, fadD221A, and fadD222A) and 2) those with 10% or less wild-type activity (fadD213A, fadD214A, and fadD381A). Using anti-FadD sera, Western blots demonstrated the different mutant forms of FadD that were present and had localization patterns equivalent to the wild type. The defect in the first class was attributed to a reduced catalytic efficiency although several mutant forms also had a reduced affinity for ATP. The mutations resulting in these biochemical phenotypes reduced or essentially eliminated the transport of exogenous long-chain fatty acids. These data support the hypothesis that the FACS FadD functions in the vectorial movement of exogenous fatty acids across the plasma membrane by acting as a metabolic trap, which results in the formation of acyl-CoA esters.

The formation of fatty acyl-CoA by a two-step process that proceeds through the hydrolysis of ATP to yield pyrophosphate. One of the key features of this catalysis is the formation of an adenylated intermediate (21). This activation step involves the linking of the carboxyl group of the fatty acid through an acyl bond to the phosphoryl group of AMP. Subsequently a transfer of the fatty acyl group to the sulphydryl group of coenzyme A occurs releasing AMP. By analogy with acetyl-CoA synthetase, it is likely this reaction precedes via a Bi-Uni, Uni-Bi Ter-molecular ping-pong mechanism with fatty acid, ATP, and CoA all serving as substrates (22, 23).

Fatty acid + ATP \(\rightarrow\) fatty acyl – AMP + Pi (Eq. 1)

Fatty acid + AMP + CoA \(\rightarrow\) fatty acyl-CoA + AMP (Eq. 2)

The formation of an enzyme-bound adenylated intermediate is a common mechanism used by a number of enzymes to activate their substrates. Sequence comparisons of adenylate-forming enzymes have identified two highly conserved sequence elements (YTGTTGXPKGV and GYGXTE) that comprise the ATP/AMP signature motif (24). The first sequence is generally 125–130 residues upstream from the second.

The focus of the present work was to identify specific resi-
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EXPERIMENTAL PROCEDURES

Bacterial Strains and Growth Conditions—The bacterial strains used in this study were: BMH 71-18 (thi supE Δlac prophAB mutS::Tn10 [F prophAB lacZ::Z15]), JM109 (endA1 relA1 gyrA96 thi hsdR17 (rK-, mK-) relA supE44 Δ(lacProAB) [F' traD36 proAB lacZ::Z15]), LS6928 (fadD fadD88 zea::Tn10), LS1547 (prototrophic wild-type), LS1548 (fadR), LS1908 (fadR fadD::Kan·), LS6952 (recBC), and BL21 (DE3/pLYsS). Strain LS1908 was constructed by replacing the coding region of fadD with the kanamycin resistance gene cartridge (Kan·; Amersham Biosciences) by a two-step process. First, the coding region of fadD in pN300 (24) was deleted and replaced by the Kan· cartridge thereby maintaining >500 bp of flanking DNA to generate pJW201. Second, this plasmid was digested with BamHI to generate a linear DNA molecule, transformed into strain LS6952 and movement of fadD::Kan· by P1 transduction into strain LS1548 to generate the fadR fadD::Kan· strain LS1908. The fadD deletion in LS1908 was confirmed by PCR of chromosomal DNA using primers specific to bacterial DNA flanking the deletion and by a loss of acyl-CoA synthetase activity. Bacterial cultures were grown at 37 °C in a gyratory shaker in Luria broth or tryptone broth (TB). When minimal media was required, medium E supplemented with vitamin B1 (EB1) was used (26). Carbon sources, sterilized separately, were added to final concentrations of 25 mM glucose, 25 mM potassium acetate, 5 mM deanoate, or 5 mM oleate. When oleate or deanoate were used as a carbon source, polyoxyethylene 20 cetyl ether (Brij 58) was added to a final concentration of 0.5% by volume. Sodium oleate was added to a final concentration of 0.1%. When required to maintain plasmids, antibiotics were added to 100 μg/ml ampicillin, 40 μg/ml kanamycin, 10 μg/ml tetracycline, and 40 μg/ml chloramphenicol. Growth of bacterial cultures was routinely monitored using a Klett-Summerson colorimeter equipped with a blue filter.

Amino Acid Sequence Comparisons—Protein sequence comparisons were performed using MultAlign version 5.3.3 (27) with sequences that were conserved among the fatty acyl-CoA synthetases or members of the adenylate forming superfamily. Sequence comparisons were directed against the SWISS-PROT data base with a 0.01 probability threshold with complexity filtering and blusum62 score table.

Site-directed Mutagenesis—Site-directed mutations within the fadD gene were generated using the Altered Sites mutagenesis system from Promega as previously described (28). Specific mutations were confirmed using dideoxysequencing and fadD-specific oligonucleotides (24). Once the mutation was confirmed, BamHI or SacI-SalI fragments containing the different fadD alleles were purified and ligated into pACYC177. The designations of the final constructions are given in Table 1. The plasmid constructs were then transformed into the host strain LS1908 (fadR fadD::Kan·) for expression analysis.

H<sup>14</sup>fadD Overexpression and Purification—Strain BL21 (DE3) (pLYsS) was transformed with plasmid pN3576 encoding the bacterial fatty acyl-CoA synthetase FadD containing a hexameric histidine tag (25). The expression and purification of H<sup>14</sup>fadD has been previously described (25). Briefly, cells were induced with isopropyl-1-thio-β-D-galactopyranoside (1 mM) for 90 min and the enzyme was purified from a clarified cell extract using Ni<sup>2+</sup>-NTA-agarose affinity chromatography (Qiagen). The His-tagged enzyme elutes as a single band between 250 and 300 mM imidazole. Antisera were prepared against purified FadD using a commercial vendor (BioWorld, Dublin, OH). Selected fadD alleles were subcloned as cassettes into pN3576 for expression and purification of mutant forms of the enzyme as detailed under “Results.”

Western Blotting—Western blotting of cell extracts from wild-type and fadD strains transformed with the plasmid constructs harboring the different fadD alleles was performed as previously described (30). Briefly total cellular protein or isolated membrane and cytosolic fractions were subjected to SDS-polyacrylamide gel electrophoresis and electrophoretically transferred onto nitrocellulose membranes (pore size, 0.45 μm) (Micron Separations Inc.). Following transfer to nitrocellulose, membranes were pretreated for 1 h at room temperature with gentle shaking in 5% powdered milk + 0.1% Tween 20 in Tris-buffered saline (TTBS; 20 mM Tris, 0.5 mM NaCl, pH 7.5). Following this blocking step, anti-FadD sera (1:25,000 dilution) was added directly and incubated an additional hour. Membranes were washed with TTBS three times and subsequently incubated with goat anti-rabbit IgG horseradish peroxidase conjugate (1:7,500) in 5% powdered milk + TTBS for 1 h at room temperature. Membranes were washed in TTBS as detailed above and developed using enhanced chemiluminescence (ECL; Amer sham Biosciences) or by the addition of the peroxidase substrate tetramethylbenzidine as specified by the vendor (Promega).

The soluble and particulate fractions of cells containing the different fadD alleles were separated and probed for FadD and mutant forms of FadD using Western blots as described above. FadD was used as a loaded control. The immunoreactive bands were quantitated as described above and lysed using a French pressure cell at 12,000 p.s.i. The cell lysates were clarified to remove unbroken cells and the supernatants were subjected to ultracentrifugation (60,000 × g for 30 min; TLA-100 rotor) to separate the membrane (particulate) fraction from the cytosolic fraction. The membranes were resuspended and centrifugation repeated. The protein concentrations of the membrane and cytosolic fractions were adjusted to 8–10 μg/10 μl and analyzed by Western blots as detailed above. The distribution between the membrane and cytosolic fractions of the mutant forms of FadD were compared with those from wild-type FadD.

Measurement of Fatty Acyl-CoA Synthetase Activity—FACS activity was monitored using a modification of a protocol defined by Kameda and Nunn (29). Cell extracts were prepared from wild-type fadD strains by sonication on ice as previously described (25). Cell extracts were added to reaction mixtures containing 200 mM Tris-HCl, pH 7.5, 1.25 mM ATP, 8 mM MgCl<sub>2</sub>, 2 mM EDTA, 100 mM NaF, 0.1% Triton X-100, 10 μM [<sup>3</sup>H]oleate, 0.5 mM coenzyme A and incubated 10 min at 37 °C. For the kinetic experiments the water content of ATP was varied between 0.05 and 2.5 mM. Reactions were initiated by the addition of CoA and terminated by the addition of isopropyl alcohol, n-heptane, 1 M H<sub>2</sub>SO<sub>4</sub> (40:10:1). The aqueous phase, containing acyl-CoA formed during the reaction, was extracted 3 times with 2.5 ml of n-heptane and subjected to scintillation counting.

Fatty Acid Transport—Oleate transport was measured as previously described (30). Following growth to mid-log phase (5 × 10<sup>6</sup> cells/ml) in TB, cells were harvested, washed once with minimal media EB<sub>1</sub>, and resuspended in EB<sub>1</sub> containing 0.5% Brij 58 and 200 μg/ml chloramphenicol. The cells were starved for an exogenous energy source with aeration for 30 min at 30 °C. Following this starvation period, 1 ml of cell extract was added to 1 ml of an assay mixture containing 200 μM [<sup>3</sup>H]oleate (potassium salt). At appropriate time points (t = 0, 2, and 4 min), duplicate aliquots (100 μl) were rapidly pipetted onto prewetted GN-6 filters (Gelman), washed twice with EB<sub>1</sub>, containing 0.5% Brij 58, air-dried, and counted. Results were expressed as picomole of oleate transported/min/mg of cell protein. For the kinetic experiments on selected fadD mutant alleles, the final concentration of oleate was varied between 10 and 100 μM. The values presented from the transport experiments represent the means ± S.E from at least three independent experiments. All transport data were subjected to analysis of variance using StatView (Abacus Concepts, Inc.).

Chicken—[<sup>3</sup>H]Oleic acid and α-[<sup>32</sup>P]ATP were purchased from PerkinElmer Life Sciences. [14C]Decanoate was obtained from Sigma. Enzymes for routine DNA manipulations were obtained from Promega, Invitrogen, or New England Biolabs. Antibiotics and other supplements for bacterial growth were obtained from Difeo and Sigma. All other chemicals were obtained from standard suppliers and were of reagent grade.
RESULTS

Identification of an ATP/AMP-binding Signature Motif within the Fatty Acyl-CoA Synthetase FadD—One of the key features of the catalytic mechanism of FACS is the formation of an adenylated intermediate (21). This activation step involves the linking of the carboxyl group of the fatty acid to the phosphoryl group of AMP and subsequent transfer of the acyl chain to the acceptor molecule coenzyme A. The formation of an enzyme-bound adenylated intermediate was a common mechanism used by a number of enzymes to activate their substrates. Sequence comparisons of enzymes, which share this catalytic property, identified two highly conserved sequence elements that comprise the ATP/AMP-binding signature motif (Fig. 1). Within the family of adenylate forming enzymes there was a third sequence element of this signature that was less well conserved and partially overlaps the FACS signature motif (25). In the E. coli FACS FadD, the two regions comprising the ATP/AMP signature have been identified on the basis of sequence similarities as

\[
\begin{align*}
&\text{fadD} & \text{Y213A} & \text{TTCTGCAAGCCACCGGCG} & \text{pNJW108} \\
&\text{fadD} & \text{T214A} & \text{TGCAATACGCCGGCGGC} & \text{pNJW109} \\
&\text{fadD} & \text{G216A} & \text{CACCGGCGCCACCACTG} & \text{pNJW111} \\
&\text{fadD} & \text{T217A} & \text{CGCGGCGGCACACTGGT} & \text{pNJW112} \\
&\text{fadD} & \text{G219A} & \text{CACCACTGCTGTGGCG} & \text{pNJW114} \\
&\text{fadD} & \text{K222A} & \text{GTGTGGCGGCAGGCGCGA} & \text{pNJW115} \\
&\text{fadD} & \text{E361A} & \text{GGCCTTACCGCGTGTGCGCCG} & \text{pN3551} \\
\end{align*}
\]

Construction of Site-directed Substitutions within the ATP/AMP-binding Signature of FACS—The homologies noted above served to guide studies using site-directed mutagenesis to define whether this region of FACS was essential for enzyme activity. Based on data from other adenylate-forming enzymes, the two highly conserved sequence elements contribute to a region of the enzyme hypothesized to be required for ATP binding and catalysis. A number of specific amino acid residues within the ATP/AMP signature motif were substituted with alanine to assess their contribution to enzymatic activity (Fig. 1, Table I). Western blots using anti-FadD sera demonstrated the protein levels of wild-type FadD and the mutant forms of FadD were equivalent (Fig. 2). These data support the notion that the mutant forms of FadD were expressed at levels approximating the wild type and there was no indication the proteins had reduced stability compared with the wild type.
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| fadD allele | Expressiona | Acyl-CoA synthetase activity, fatty acid substrate |
|-------------|-------------|--------------------------------------------------|
|             |             | C18:0                                               |
| Wild type   | +           | 17.33 (1.02) 31.70 (0.85)                           |
| ΔfadD       | −           | 0.05 (0.03) 0.12 (0.05)                              |
| fadDY2113A  | +           | 0.04 (0.03) 0.47 (0.03)                              |
| fadDT2144A  | +           | 0.08 (0.02) 1.62 (0.26)                              |
| fadDY216A   | +           | 7.55 (0.75) 8.23 (0.78)                              |
| fadDT217A   | +           | 2.17 (0.83) 10.52 (2.29)                             |
| fadDY222A   | +           | 5.91 (0.79) 9.52 (0.95)                              |
| fadDK222A   | +           | 1.98 (0.81) 5.52 (0.95)                              |
| fadDE361A   | +           | 0.01 (0.03) 0.23 (0.27)                              |

| fadD allele | Expressiona | Acyl-CoA synthetase activity, fatty acid substrate |
|-------------|-------------|--------------------------------------------------|
|             |             | C18:1                                               |
| Wild type   | +           | 17.33 (1.02) 31.70 (0.85)                           |
| ΔfadD       | −           | 0.05 (0.03) 0.12 (0.05)                              |
| fadDY2113A  | +           | 0.04 (0.03) 0.47 (0.03)                              |
| fadDT2144A  | +           | 0.08 (0.02) 1.62 (0.26)                              |
| fadDY216A   | +           | 7.55 (0.75) 8.23 (0.78)                              |
| fadDT217A   | +           | 2.17 (0.83) 10.52 (2.29)                             |
| fadDY222A   | +           | 5.91 (0.79) 9.52 (0.95)                              |
| fadDK222A   | +           | 1.98 (0.81) 5.52 (0.95)                              |
| fadDE361A   | +           | 0.01 (0.03) 0.23 (0.27)                              |

a Detection of FadD and mutant proteins using Western blots and anti-FadD sera; +, levels detected comparable to wild type in whole cell extracts and soluble and particulate fractions; −, FadD not detected (see Fig. 2).

b Protein concentrations in the reaction mixtures ranged between 36 and 50 µg/ml. S.E., standard error of the mean; n = 4.

catalytic center of the enzyme, it seems likely that subtle conformational changes will result that cannot be detected using these methods. Whereas the anti-FadD sera was prepared using highly purified His<sub>6</sub>FadD, two additional proteins were recognized, which are likely to be other adenylate-forming enzymes that share epitopes in common with FadD (Fig. 2). One of these was likely to be the undefined open reading frame designated YDID, which encodes an acyl-CoA synthetase-like protein that was 30% identical to FadD. YDID does not, however, contribute long-chain acyl-CoA synthetase activity.

Fatty Acyl-CoA Synthetase Activities of Whole Cell Extracts from fadD Mutants Containing Substitutions within the Putative ATP/AMP-binding Signature Motif—This work was driven by the hypothesis that the ATP/AMP signature sequence elements were required for ATP binding and the formation of the adenylated intermediates. FACS activities were monitored in whole cell extracts using oleate (C<sub>18</sub>:1) and decanoate (C<sub>10</sub>:0) as substrates in the ΔfadD strain LS1908 transformed with plasmids containing the different fadD alleles (Table II). The alleles fadDY213A, fadDT2144A, fadDY216A, fadDT217A, fadDY222A, and fadDK222A (within the first sequence element of the signature) and fadDE361A (within the second element) had markedly reduced enzymatic activities using oleate as the fatty acid substrate. As with our previous studies describing the FACS signature motif (25), the present studies also revealed two major classes of mutants with depressed enzyme activity: 1) those with 25–45% wild type activity (fadDY216A, fadDT217A, fadGY221A, and fadDK222A) and 2) those with 10% or less wild type activity (fadDY213A, fadDT214A, and fadDE361A) (Table II). Given that these mutant forms of FadD were expressed at wild-type levels and had the same localization patterns, these data would argue that these residues were crucial for catalytic activity.

Determination of V<sub>max</sub>, K<sub>m</sub>, and k<sub>cat</sub> Values for Fatty Acyl-CoA Synthetase Activities from fadD Mutants as a Function of ATP Concentration—The data presented above indicated that specific residues within the ATP/AMP signature were important for enzymatic activity. To investigate this further, kinetic studies were undertaken to specifically evaluate the role of ATP in catalysis (Table III, Fig. 3). We suspected that several of the fadD mutants with substitutions within the ATP/AMP signature would be defective for ATP binding and thus would have a significantly higher K<sub>m</sub> for ATP when compared with the wild-type enzyme. As shown in Fig. 3, the fadDY213A and fadDE361A alleles resulted in enzymes with no measurable activity over a range of ATP concentrations. FadDY213A may be defective in ATP binding as is the case for the comparable residue in the mammalian fatty acid transport protein FATP1, which also was a member of the adenylate-forming family of enzymes (31, 32). With the phenylalanine activating subunit (PheA) of gramicidin synthetase in Bacillus brevis, Glu<sub>61</sub> was essential for the catalytic activity of the enzyme. The homologous amino acid in PheA was a glutamate, which appears to function in concert with a neighboring tyrosine to coordinate the binding of Mg<sup>2+</sup>-AMP (33). In this regard, Glu<sub>61</sub> may specifically contribute to ATP binding.

Three different fadD alleles (fadDT214A, fadDY216A, and fadDY221A) resulted in enzyme activities that had k<sub>cat</sub> values 2–15-fold lower than wild-type, whereas the K<sub>m</sub> values for ATP were relatively unchanged. This suggests that the primary role of these residues was for catalysis as opposed to ATP binding. Two other alleles (fadDT217A and fadDE222A) affected both K<sub>m</sub> and k<sub>cat</sub>. Both mutants had k<sub>cat</sub> values that were reduced nearly 4-fold and K<sub>m</sub> values that were increased when compared with the wild type. These results were consistent with the notion that these two residues contribute to ATP binding. On the basis of previous work on PheA, the positive charge on the comparable lysine residue was thought to function by directing ATP to the binding site (33).

Analysis of Long-chain Fatty Acid Transport—In view of the compromised acyl-CoA synthetase activities in the fadD alleles described in the preceding sections, we postulated that this would lead to reduced levels of long-chain fatty acid import. Therefore, we selected a subset of these fadD mutants and defined the patterns of fatty acid transport using [3H]oleate as the variable substrate (Fig. 4). When fatty acid transport was measured using different concentrations of oleate, a subset of mutants (fadDY213A, fadDT214A, and fadDE361A), the same patterns of activity were found (Fig. 5) indicating the changes in transport rate were the direct result of changes in the catalytic efficiency of FadD. Kinetic constants extracted from these experiments indicated that the K<sub>m</sub> of oleate transport resulting from FadDT217A was essentially equivalent to the wild-type, whereas FadDE361A resulted in a 3-fold reduction (Table IV). The changes in V<sub>max</sub> were more
dramatic indicating that a reduction in the catalytic activity of FadD resulted in a commensurate reduction in oleate transport. The addition of exogenous ATP to the fatty acid transport assay mixture had no effect on the measured fatty acid transport rates of strains containing the wild-type FadD or mutant forms of FadD (data not shown), which suggested that the intracellular pools of ATP were required and sufficient for enzymatic activity as previously suggested (45). These data, in conjunction with those presented above, argue that there was specific coupling between fatty acid import and activation of exogenous long-chain fatty acids in *E. coli*.

**Lipid Activation of Fatty Acyl-CoA Synthetase**—The *E. coli* FACS FadD appears to be an essential component of the long-chain fatty acid transport apparatus, perhaps by acting to metabolically trap the fatty acid as a CoA thioester. A central question that remains to be answered is how this enzyme was specifically involved in this process. Indeed, there is increasing evidence that in higher eukaryotic systems, this enzyme also plays a role in long-chain fatty acid transport (46). The first studies on the subcellular localization of a long-chain FACS were carried out on mammalian small intestinal mucosa where the enzyme was predominantly localized in the microsomal fraction (34, 35). A mouse isoform of FACS was found within the plasma membrane and was thought to function in concert with a fatty acid transport protein (36). Previous work on FadD has shown that it was present in both the particulate and soluble fractions of the cell (18, 19, 29). These observations suggest that FadD may partition into the membrane during its catalytic cycle and suggests this enzyme functions to abstract fatty acids from the membrane concomitant with esterification with CoA.

FACS activity from *E. coli* was routinely monitored in the presence of low concentrations of nonionic detergent. When detergent was removed, FACS activity either cannot be measured or was extremely low indicating that either the substrate requires delivery in a micellar form and/or that FACS prefers a lipophilic environment for activity. Total *E. coli* lipid can functionally replace the nonionic detergent in restoring enzyme activity (Fig. 6) implying that the enzyme requires a lipophilic environment to be active. In this regard, FadD may indeed be lipid activated as it associates with the membrane.

**DISCUSSION**

The present studies were initiated to identify residues within fatty acyl-CoA synthetase required for catalysis and the vectorial import of exogenous long-chain fatty acids. Protein sequence homology searches have identified a number of amino acid residues as active site candidates, and in particular, identified residues common to the more limited family of fatty acyl-CoA synthetases and to the larger family of ATP/AMP-binding proteins. Site-directed mutagenesis of the *fadD* gene was undertaken to specifically evaluate the contribution of amino acid residues identified in the region called the ATP/AMP signature motif in the *E. coli* FACS, FadD. The crystal-
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Table IV

| FACS            | Kinetic parameters a |
|-----------------|----------------------|
|                 | \( V_{\text{max}} \) | \( K_m \) |
| FadD            | 1187.83              | 98.36    |
| \( \Delta \text{fadD} \) | 6.185                | ND c     |
| FadD T312A      | 10.41                | ND c     |
| FadD T217A      | 41.33                | 60.65    |
| FadD D361A      | 66.80                | 28.43    |

a Kinetic constants \( K_m \) and \( V_{\text{max}} \) were defined using the Lineweaver-Burk equation.

b Picomole of oleate transported/min/mg of protein.

c Micromolar oleate.

The \( \Delta \text{fadD} \) strain has no FadD and serves as the negative control.

The first sequence element of the ATP/AMP signature motif

![Diagram](image)

Fig. 7. Model illustrating the role of FadD in the vectorial transport of exogenous fatty acids. 1, free fatty acids become protonated in the periplasmic space following FadL-dependent transport across the outer membrane (not illustrated here) and then partition into the inner membrane. 2, the protonated fatty acid flips from the periplasmic face of the inner membrane to the cytoplasmic face. 3, free fatty acids within the membrane signal FadD to partition into the inner membrane, presumably in an ATP-bound form. 4, FadD functions to abstract fatty acids from the inner membrane concomitant with the formation of fatty acyl-CoA for further metabolism.

is the highly conserved sequence of amino acids in the superfamily of adenylate-forming enzymes (39, 40). Although this sequence \((\text{YTSG(ST)}\text{GXPKGV})\) has poor homology to the Walker A type motif \((\text{GXXXXGK(TS)})\), it is rich in glycine and contains a lysine. The Walker A motif forms a phosphate-binding loop found in a large number of nucleotide-binding proteins (41). The lysyl residue in this motif is thought to be responsible for binding the \(\gamma\)-phosphate of the nucleoside triphosphate. A comparable Lys has been identified in the ATP-binding site of SecA, a protein involved in protein transport across the plasma membrane of \(E. coli\) (42). In \(B. brevis\), the comparable Lys is critical for ATP-PPi exchange in the nonribosomal peptide synthetase tyrocidine synthetase I (43). Substitution of this homologous residue to methionine in 4-chlorobenzoate:CoA ligase results in a nearly 3-fold increase in the \(K_m\) for ATP and a 4-fold drop in catalytic activity indicating the importance of this residue in ATP binding (44). In the present work, steady state kinetic results from whole cells expressing the \(\text{fadD}_{112}^{222}\) allele demonstrated a 3-fold increase in \(K_m\) for ATP and a 4-fold decrease in catalysis, which supports the hypothesis that \(\text{Lys}^{222}\) of the \(E. coli\) FACS was involved in ATP binding. The predicted three-dimensional model of FadD places \(\text{Lys}^{222}\) in a disordered loop connecting two antiparallel \(\beta\)-strands that faces the ATP phosphate residue to the catalytic site of the enzyme (38).

Other potential key residues within this loop that may contribute to ATP binding were \(\text{Tyr}^{213}\) and \(\text{Thr}^{214}\). The data presented here suggests that both \(\text{Tyr}^{213}\) and \(\text{Thr}^{214}\) were important for the catalytic competence of the enzyme. Whereas nearly 10% of the members of the adenylate-forming family of enzymes contain a Tyr at the position comparable with \(\text{Tyr}^{213}\) in FadD, this work was the first to document a potentially important role for this residue in catalytic activity. We suggest that \(\text{Tyr}^{213}\) contributes to the catalytic integrity of the enzyme as the mutant form \(\text{fadD}_{213}^{214}\) was devoid of any enzymatic activity even though it was expressed at wild type levels and
had comparable localization patterns. The homologous tyrosine (Tyr<sup>189</sup>) in PheA of gramicidin S synthetase does not form specific contacts with AMP, perhaps indicative of a role in catalysis (33). On the other hand, there is considerable evidence, which supports the role of the adjacent Thr<sup>214</sup> in nucleotide binding. In the crystal structure of PheA, Thr<sup>190</sup> (corresponding to Thr<sup>214</sup> in <i>E. coli</i>) lies adjacent to Tyr<sup>189</sup> and contacts the α-phosphate of AMP. Our data show that Thr<sup>214</sup> in the <i>E. coli</i> FadD is critical for function. This interpretation suggests that this residue may form contacts with AMP to stabilize the fatty acyl-AMP intermediate.

The crystal structure of PheA complexed with AMP shows a Mg<sup>2+</sup> bridge between the invariant glutamate of the second sequence element of the ATP/AMP signature (corresponding to Glu<sup>361</sup> of FACS) and the O-1 phosphate of AMP. Substitution of this glutamate to alanine in FadD results in complete loss of enzyme activity that results in an inability to transport long-chain fatty acids. The predicted three-dimensional structure for FACS, which we have generated, suggests that this glutamate lies in proximity to Thr<sup>213</sup> and Tyr<sup>214</sup>, which is presumed to be involved in nucleotide binding (33).

An additional goal in the present work was to provide evidence linking the catalytic activity of FadD to long-chain fatty acid transport. Overath and colleagues (18) first showed that FadD was partially membrane associated and required for the conversion of exogenous long-chain fatty acids to CoA thioesters thereby rendering fatty acid transport unidirectional. This enzyme is clearly required for fatty acid import and activation in <i>E. coli</i> as revealed in the present studies showing that the import of [<sup>14</sup>CH<sub>3</sub>]oleate is abolished in a Δ<sup>fadD</sup> strain. Additionally, specific mutations corresponding to the ATP/AMP signature motif that reduced or abolished enzyme activity likewise reduced or abolished the import of exogenous long-chain fatty acids. Indeed, these data fully support Overath’s hypothesis that vectorial acylation is one underlying biochemical mechanism that governs fatty acid transport.

We have previously shown that the long-chain fatty acid transport system in <i>E. coli</i>: 1) is partially shock sensitive, 2) requires ATP generated by either substrate-level or oxidative phosphorylation, and 3) requires the proton electrochemical gradient across the inner membrane for maximal proficiency (45). More specifically, the transport of oleate requires ATP generated through the substrate level or oxidative phosphorylation, which reflects the ATP requirement of FadD as a component of this transport apparatus. In addition, these previous studies demonstrate that the process of long-chain fatty acid transport is linked to the proton electrochemical gradient across the inner membrane. In other words, the long-chain fatty acid transport system of <i>E. coli</i> requires both intracellular pools of ATP and an energized inner membrane.

Fig. 7 shows a model of how we hypothesize FadD functions in the vectorial transport of exogenous fatty acids. FadD is found both within the particulate and soluble fractions of the cell (18, 19, 29). There is evidence that the enzyme is recruited to the membrane although the signal that promotes this association remains elusive (19). We have shown that an energized plasma membrane is necessary for optimal fatty acid transport and have suggested that uncharged fatty acids simply flip across the inner membrane by diffusion and are then abstracted from the membrane by FadD (45). It is tempting to speculate that FadD specifically associates with the membrane in response to exogenous protonated long-chain fatty acids in the membrane. In the present work, we show that the mutant forms of FadD containing substitutions within the ATP/AMP signature motif are expressed at levels equivalent to the wild type and are found within both the particulate and soluble fractions of the cell. These data argue that there must be regions of FadD outside the catalytic core, which are responsible for membrane association. In addition, whereas the signal that promotes membrane association is undefined, it seems plausible that an increase in free fatty acid concentration within the inner membrane was sufficient to promote the association of ATP-bound FadD to the membrane. The formation of the fatty acyl adenylate effectively pulls the free fatty acids from the membrane for subsequent thioesterification to coenzyme A. The net result is the metabolic trapping of fatty acyl-CoA within the cell rendering the process unidirectional. Upon release of fatty acyl-CoA, the enzyme becomes ATP-bound and the cycle repeats.

The present work has established a platform of information, which serves for our current investigations toward understanding the mechanism and specificity of substrate and cofactor binding of this enzyme further. Clearly, this line of investigation is essential to understand the mechanisms that underpin the general role of fatty acyl-CoA synthetases and the particular role of the <i>E. coli</i> FadD in the coupled import and activation of exogenous long-chain fatty acids.

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