Determination of the native form of FadD, the *Escherichia coli* fatty acyl-CoA synthetase, and characterization of limited proteolysis by outer membrane protease OmpT

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Several studies have described FadD, the *Escherichia coli* fatty acyl-CoA synthetase [also known as fatty acid :CoA ligase (AMP-forming); EC 6.2.1.3], as a 42–50 kDa enzyme. Based on sequencing and expression data from the *fadD* gene, other reports have suggested that FadD is a 62 kDa protein and represents the sole fatty acyl-CoA synthetase in *E. coli*. We report that the 62 kDa FadD enzyme is a substrate for the outer membrane protease OmpT *in vitro*, producing a 43 kDa C-terminal fragment and a 19 kDa N-terminal fragment. Immunoblotting with a FadD antibody revealed that only the 62 kDa form of the enzyme is present *in vivo*, but we utilized the proteolytic sensitivity of FadD to investigate its structure. Photoaffinity labelling experiments revealed that both intact FadD and the 43 kDa fragment bound a long-chain fatty acid. Intact and cleaved FadD were also purified to determine the effect of cleavage on function. When using oleate as a substrate, cleaved FadD displayed 2-fold higher K_m and V_{max} values compared with intact FadD, but the catalytic efficiencies (k_{cat}/K_m) of the two forms were similar. This indicated that cleavage did not adversely affect enzyme activity. Proteolysis of FadD by OmpT was altered by the presence of oleate or ATP, both of which are ligands for the fatty acyl-CoA synthetase. This suggested that FadD undergoes ligand-induced conformational changes and implies that the region surrounding the cleavage site is mobile, a common characteristic of linker domains.

Key words: fatty acid uptake, linker domain, photoaffinity labelling.

INTRODUCTION

Fatty acyl-CoA synthetase enzymes activate fatty acids for metabolism by catalysing the formation of fatty acyl-CoA thioesters, which are substrates for β-oxidation [1], phospholipid biosynthesis [2] and cellular signalling [3–5]. FadD, encoded by the *fadD* gene, is an *Escherichia coli* fatty acyl-CoA synthetase with substrate specificity for medium-chain fatty acids and long-chain fatty acids (LCFAs) [6–8]. FadD is associated with the plasma membrane [6], but is a soluble protein that is activated by bacterial membranes and Triton X-100 detergent *in vitro*, suggesting that the enzyme requires a lipid surface for activity [9,10]. Previous work from our laboratory proposed that LCFA uptake is regulated by translocation of FadD from the cytosol to the plasma membrane [10].

Growth of *E. coli* on LCFAs requires de-repression of the transcription factor FadR, which represses transcription of genes from the *fad* (fatty acid degradative) regulon [2]. De-repression is achieved by dissociation of FadR from its operator sites upon binding long-chain fatty acyl-CoA molecules [2,5]. Therefore by catalysing the formation of fatty acyl-CoA molecules, FadD plays a pivotal role in the regulation of lipid catabolism. Furthermore, FadD and FadL, an outer membrane fatty-acid binding protein [11,12], are both required for LCFA uptake. FadL is thought to facilitate the permeation of medium-chain fatty acids and LCFAs across the outer membrane [13], which are then sequestered inside the cell by vectoral thio-esterification catalysed by FadD [14]. The periplasmic protease Tsp has also been implicated in LCFA uptake, but its precise role remains unclear [15].

Animal, plant and yeast cells express multiple forms of fatty acyl-CoA synthetase [16–21]. Sequencing of the *Mycobacterium tuberculosis* genome revealed that it devotes a large number of genes to lipid metabolism, including 36 putative fatty acyl-CoA synthetases [22]. In contrast, *fadD* represents the only fatty acyl-CoA synthetase gene in *E. coli* [6–8,14], although fatty acyl-CoA synthetase isoforms have been described [23,24]. Purified native FadD has been reported to exhibit a molecular mass ranging from 42 to 50 kDa [6,23,25]. However, the *fadD* gene sequence predicts a 62 kDa protein [7,8], which is consistent with expression studies [7,26]. There have also been reports of multiple *E. coli* fatty acyl-CoA synthetase isoforms [6], which can be distinguished by isoelectric point [23] and substrate [24] specificities.

The nature of these different forms of FadD and their relationship to each other has remained unclear. In the present study we report that FadD is normally present as a 62 kDa protein *in vivo*, which becomes cleaved during cell lysis producing 43 and 19 kDa fragments that remain tightly associated. Intact and nicked FadD were purified and characterized, and only minor differences were apparent between the two forms of the enzyme. Our results showed that the periplasmic protease Tsp is not involved in cleavage of FadD and instead implicate the outer membrane protease, OmpT. We made use of FadD sensitivity to OmpT-mediated proteolysis to demonstrate that FadD undergoes ligand-induced conformational changes. Our results suggest the region surrounding the OmpT cleavage site may represent a linker domain, connecting the C-terminal FadD fragment, possessing the putative ATP and LCFA binding sites, to the N-terminal FadD fragment of unknown function.

Abbreviations used: IPTG, isopropyl β-D-thiogalactoside; LB, Luria–Bertani; LCFA, long-chain fatty acid; MBP, maltose-binding protein.

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EXPERIMENTAL

Bacterial strains, growth conditions, media and materials

The following strains of *E. coli* were used for expression and proteolysis experiments: JM105, K27, BL21(DE3), W3110 and XL-1 Blue. Genomic DNA for PCR amplification of the *fadD* gene was isolated from the *E. coli* strain ML308, using the procedure described by Marmur [27]. Unless noted otherwise, bacteria were grown aerobically at 37 °C with shaking in Luria–Bertani (LB) medium or M9 minimal medium salts [1 % (w/v); Sigma, St Louis, MO, U.S.A.] supplemented with 1 mM $MgSO_4$, 0.1 mM $CaCl_2$, 5 μg/ml thiamin and either glucose (0.5 %) or KOH-neutralized oleic acid (5 mM in 0.5 % Brij detergent) as carbon sources. When required, ampicillin was added to the media to a final concentration of 150 μg/ml. Cell number was estimated by measuring attenuance at 600 nm with a UV–visible spectrophotometer (Varian–Cary, Palo Alto, CA, U.S.A.).

Construction of FadD and maltose-binding protein (MBP)–FadD expression vectors

All basic molecular biology techniques were performed following the procedures given by Sambrook et al. [28]. We constructed the following PCR primers based on the published nucleotide sequence of the *fadD* gene by Black et al. [7]. The 5′ primer used was 5′-TGC TCT AGA AGG AGA TAT ACA CAT GAA GAA GGT TTG GCT TAA CCG-3′, which contained a *Xba*I site, a ribosome binding site and an ATG translation initiation codon. The 3′ PCR primer used was 5′-TGA CAT AAG CTT ATT ATT GTC CAC TTT GGC GCG C-3′, which contained a *Hin*DIII site to facilitate cloning. The PCR primers were used to isolate the *fadD* gene and ligated into the expression plasmid pKK223-3. The cloned gene was then sequenced and a *fadD* mutant cell line K27 was transformed with a low expression vector to ensure that the cloned gene was able to complement the *fadD* phenotype. The MBP–FadD fusion protein expression vector was constructed by subcloning the *fadD* gene into the pMal-p2 expression plasmid (New England Biolabs).

Preparation of bacterial whole cell and lysate samples

When analysing whole cell *E. coli*, cultures were spun in a Microfuge for 2 min at maximum speed. The pellets were resuspended with 1 × SDS/PAGE sample buffer [62.5 mM Tris/HCl (pH 6.8), 5 % (w/v) SDS, 10 % (v/v) 2-mercaptoethanol, 10 % (w/v) glycerol and 0.01 % Bromophenol Blue] to 2.4×10^9 cells/ml and immediately boiled for 4 min prior to analysis by SDS/PAGE [28]. Cell lysates were prepared by using a modified method by Witholt et al. [29], which involved spinning *E. coli* cells at 10000 ***g*** for 10 min at 4 °C and resuspension of the pellets with 200 mM Tris/HCl (pH 8.0)/1 mM EDTA to 2×10^{10} cells/ml. Lysozyme was added to a final concentration of 100 μg/ml and the suspension was incubated at 25 °C for 20 min with periodic mixing. Ice-cold solution (3 vols.) containing 100 μg/ml DNase I and 10 mM magnesium sulphate was added and mixed, before incubation at 37 °C for a further 20 min. The resulting cell lysate typically contained 2 mg of cell protein/ml.

Purification of intact and cleaved FadD protein

JM105 cells transformed with the FadD expression plasmid were grown to a cell density of 4.8×10^8 cells/ml and induced with 1 mM isopropyl β-D-thiogalactoside (IPTG) for 75 min. The cultures were then centrifuged at 10000 ***g*** for 10 min at 4 °C and washed once with cold 50 mM potassium phosphate buffer, pH 7.5 (buffer A). The cells were resuspended in buffer A and passed through a French pressure cell three times. PMSF (1 mM) in isopropyl alcohol and EDTA (1 mM) were added to the cell lysate immediately after passage through the French pressure cell. Cell debris and unlysed cells were cleared by centrifugation at 12000 ***g*** for 15 min at 4 °C. The crude cell extract was then centrifuged at 100000 ***g*** for 60 min. After the supernatant was discarded, the total membrane pellet was resuspended in cold buffer A and stored at −80 °C. For purification of intact FadD, total membrane pellets were resuspended in 100 mM Tris/HCl (pH 7.5), 0.2 % Triton X-100 and 1 mM neutralized oleic acid, and incubated on ice for 10 min. These samples were centrifuged at 100000 ***g*** for 60 min at 4 °C and the supernatant (detergent soluble extract) was applied on to a Mono-Q column that was pre-equilibrated with 100 mM Tris/HCl, pH 7.5 (buffer B). Buffer B (5 column vols.) was used to wash the loaded column of excess oleate/Triton X-100. A linear gradient was then established using buffer B and 100 mM Tris/HCl (pH 7.5)/400 mM NaCl to elute proteins from the column. Purification of cleaved FadD was carried out in essentially the same manner except for the following. Oleate was not added during detergent extraction of total membranes and was incubated at 25 °C.

Fatty acyl-CoA synthetase and proteolysis assays

Acyl-CoA synthetase activity was monitored by measuring the formation of radiolabelled [9,10-^{3}H]oleoyl-CoA from [9,10-^{3}H]oleic acid, ATP and CoA with some modifications as previously described [9].

Detergent-soluble membrane fractions prepared prior to application on to ion-exchange columns were used as the substrate for the proteolysis assays. Total membrane and supernatant fractions used for testing protease activity were prepared by centrifugation of cell lysates at 100000 ***g*** for 60 min at 4 °C (see above). These fractions were incubated with partially purified FadD (0.25 μg/μl in 25 mM sodium phosphate, pH 7.0) and incubated at 37 °C for 60 min. For proteolysis with whole cells, *E. coli* cultures were grown to mid-exponential phase in LB medium, harvested and washed with 1 × M9 minimal medium and resuspended to 0.25 μg/μl with 25 mM sodium phosphate, pH 7.0. Typically, 5 μl of the bacterial suspension was incubated with 20 μl of the FadD substrate at 37 °C for 60 min. These reactions were then centrifuged in a Microfuge for 1 min and the supernatant was saved.

Purification of MBP–FadD fusion protein

BL21(DE3) *E. coli* transformed with the MBP–FadD expression plasmid were grown in LB medium and induced at mid-exponential phase with 1 mM IPTG for 90 min. Soluble fractions from total membrane pellets resuspended in buffer C [25 mM Tris (pH 7.5), 25 mM NaCl and 0.25 % Triton X-100] were isolated from these cells in a manner similar to that described for isolation of cleaved FadD (see above). The supernatant containing MBP–FadD was then incubated with amylose resin and eluted as instructed by the manufacturer using batch purification (New England Biolabs). The samples from the first 5 mM maltose elution were kept and used as a source of purified MBP–FadD.

Photoaffinity labelling of FadD

Photoaffinity labelling was carried out as described previously [11]. In brief, bacterial cell lysates were typically diluted to a total protein concentration of 1 mg/ml in 50 mM Tris/HCl (pH 7.0)

and kept on ice. Equal volumes of cell lysate and 11-diazirinophenoxy-[11-^{3}H]undecanoate solution were mixed and incubated for 2 min at 37 °C and then photolysed using a 1000 W Xenon–Mercury arc lamp. The samples were then analysed by SDS/PAGE, stained with Coomassie Brilliant Blue stain solution, soaked in Amplify (Amersham), dried and placed against X-ray film (Kodak XAR) at −80 °C before being developed.

Immunological methods

Immunoblotting was performed as described by Burnette [30]. Polyclonal serum containing antibodies raised against the C-terminal FadD fragment was produced using the following procedure. The antigen was prepared by eluting purified 45 kDa FadD from a SDS/10 % (w/v) polyacrylamide gel (visualized by brief staining with Coomassie Brilliant Blue dye) by excision and suspension in 0.85 % (w/v) NaCl solution. The suspension was then injected into specific pathogen-free rabbits, and boosts were repeated at intervals of at least 6 weeks with subsequent suspensions also containing Freund's adjuvant. Blood was collected 12 days post-injection and the serum was stored at −20 °C after clotting. Antigen specificity was then tested by immunoblotting intact and cleaved FadD samples.

Miscellaneous

Protein concentrations were determined using the bicinchoninic acid ('BCA') protein assay as described by the manufacturer (Pierce), and electrophoresis was performed as described by Laemmli [31]. For N-terminal protein sequencing, FadD fragments were isolated from an SDS/polyacrylamide gel in a manner similar to that described for preparation of antibodies, except the protein samples were transferred on to PDVF membrane. These samples were then sent to the protein sequencing facility at the Hospital for Sick Children (Toronto, Ontario, Canada) for analysis.

RESULTS

Expression of 62 kDa FadD and the appearance of cleaved FadD

To facilitate studies of FadD, we constructed an IPTG-inducible, FadD overexpression system (see the Experimental section). Figure 1(A) shows overexpression of FadD from *E. coli* transformed with the overexpression plasmid, treated with IPTG, and lysed by one of two methods. When cells were lysed by boiling (4 min) in the presence of 5 % SDS (SDS/PAGE sample buffer) and immediately analysed by SDS/PAGE, an induced 62 kDa protein was observed [Figure 1(A), lane 3, arrow labelled 'intact FadD'; compare with lane 1]. In contrast, when cells were lysed with lysozyme following exposure to Tris/EDTA (as a standard protocol for cell lysis [29]), the 62 kDa band was not observed. Instead, two polypeptides (45 and 19 kDa in size, labelled 'FadD fragments 1 and 2' in Figure 1A, lane 4) appeared to be induced. We reasoned that the incubation time required for the Tris/EDTA + lysozyme method probably led to proteolytic digestion of the 62 kDa FadD protein, resulting in formation of the 45 and 19 kDa fragments. To verify that the 45 kDa band was derived from the 62 kDa band, we generated antibodies against the 45 kDa fragment (see below for details on the purification of cleaved FadD). Immunoblotting of cell lysates and whole cells revealed that the antiserum detected both the 62 and 45 kDa fragments (Figure 1B). Furthermore, when *E. coli* were grown on oleate minimal medium, to induce expression of endogenous FadD, only a 62 kDa band was observed in cells boiled in the

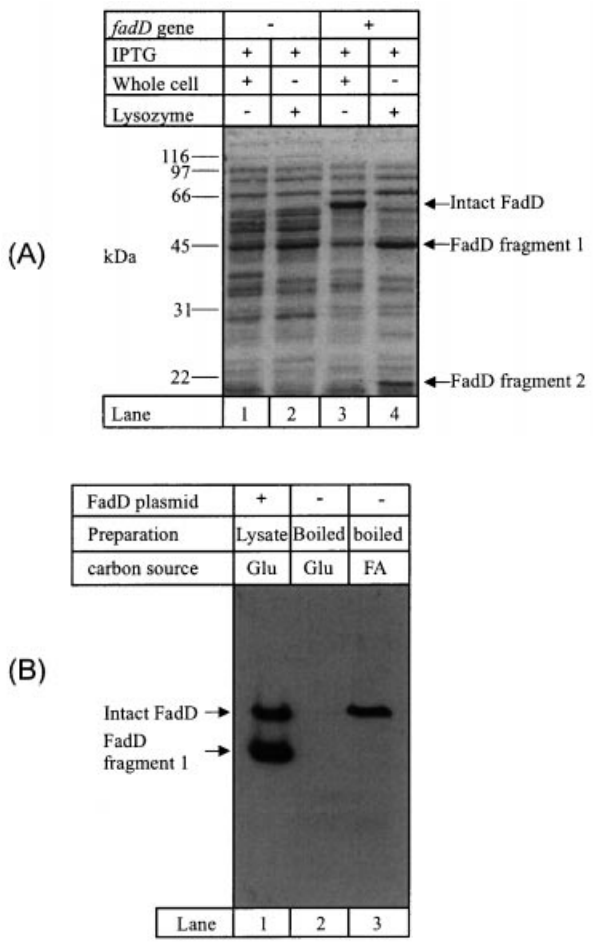

Figure 1 SDS/PAGE analysis of the FadD overexpression system and immunoblot analysis of wild-type *E. coli* grown in glucose or oleate minimal media

(**A**) SDS/PAGE. JM105 *E. coli* were transformed with the pKK223-3 plasmid containing the *fadD* gene (+; lanes 3 and 4) or the plasmid alone (−; lanes 1 and 2). These cells were grown and induced with 1 mM IPTG before being boiled in SDS/PAGE sample buffer (lanes 1 and 3) or boiled in SDS/PAGE sample buffer after lysis with Tris/EDTA + lysozyme (lanes 2 and 4). The gel was visualized by Coomassie Brilliant Blue staining. (**B**) Immunoblot. ML308 *E. coli* cells were grown in glucose (Glu; lane 2) or oleate (FA; lane 3) minimal media, boiled and analysed by SDS/PAGE. These samples were then transferred on to nitrocellulose and immunoblotted with FadD antibody. A sample of cell lysate from JM105 cells overexpressing FadD was also analysed, to indicate the position of intact and cleaved enzyme (lane 1).

presence of SDS (lane 3). These results suggested that only the 62 kDa band is present *in vivo* and represents the native form of FadD.

Photoaffinity fatty acid labelling of intact and cleaved FadD

To determine if cleavage affected LCFA binding activity, we performed photoaffinity labelling experiments with intact and cleaved FadD using the photoreactive fatty acid analogue, 11-diazirinophenoxy-[11-^{3}H]-undecanoate [32]. This photoreactive reagent has previously been used to identify and characterize both membrane bound and soluble fatty-acid binding proteins from *E. coli* and mammalian cells [11,33–35]. *E. coli* cell lysates containing intact and cleaved FadD were incubated with the photoreactive fatty acid, subjected to photolysis and analysed by SDS/PAGE and fluorography. Intense labelling of 62 and

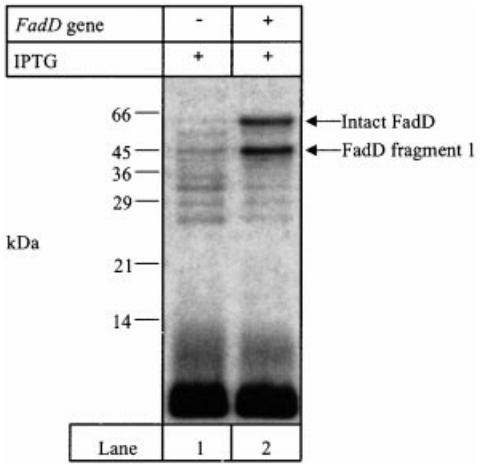

Figure 2 Photoaffinity labelling of intact and cleaved FadD with a photoreactive fatty acid

Cell lysates were prepared from JM105 *E. coli* cells transformed with the pKK223-3 plasmid (lane 1) or pKK223-3 with the *fadD* gene insert (lane 2), incubated with 11-diazirinophenoxy-[11-^{3}H]undecanoate and exposed to high intensity 365 nm radiation. Samples were then analysed by SDS/PAGE and fluorography.

45 kDa proteins was observed (Figure 2, lane 2), suggesting that both intact and cleaved FadD bound LCFAs. It appeared from the above results that cleavage of FadD did not affect the ability of FadD to bind LCFAs.

Purification and characterization of intact and cleaved FadD

To determine the effect of proteolysis on FadD activity, we proceeded to purify intact and cleaved forms of the enzyme. Overexpressed FadD was initially purified by fractionation of cell lysates using centrifugation, and retention of the membrane pellet, which under these growth conditions contained most of the enzyme. During the development of the purification scheme, we noted that the addition of Triton X-100 detergent resulted in complete cleavage of intact FadD, allowing efficient isolation of cleaved FadD (Figure 3A, lane 4). The two fragments appeared to be tightly associated, since the fragments co-eluted during ion-exchange chromatography (Figure 3A, lane 4) and gel filtration (results not shown). Purification of intact FadD was inherently more difficult, since significant proteolysis occurred, even in the presence of various protease inhibitors. However, we made the unexpected discovery that addition of millimolar amounts of oleate to the lysis buffer partially inhibited proteolysis [Figure 3, compare Figure 3A (lane 3) with Figure 3B (lane 1)]. This allowed us to purify intact FadD with only trace amounts of the cleaved form (Figure 3B, lane 2).

We proceeded to compare the activities of intact and cleaved FadD *in vitro*. When using oleate as the substrate, cleaved FadD exhibited over 2-fold higher V_{max} and K_m (app) values than intact FadD (Table 1). However, the k_{cat}/K_m values for intact and cleaved FadD were similar, indicating that cleavage did not significantly affect its catalytic efficiency. Bacterial membranes and Triton X-100 detergent activate FadD *in vitro*, suggesting that FadD requires a lipid interface for activity [9]. The *E. coli* lipid-dependent enzyme, pyruvate oxidase, can be constitutively activated by limited proteolysis [36,37]. We therefore investigated whether proteolysis of FadD affected its lipid-dependent activation, by performing the fatty acyl-CoA synthetase activity assays in the presence or absence of Triton X-100 detergent.

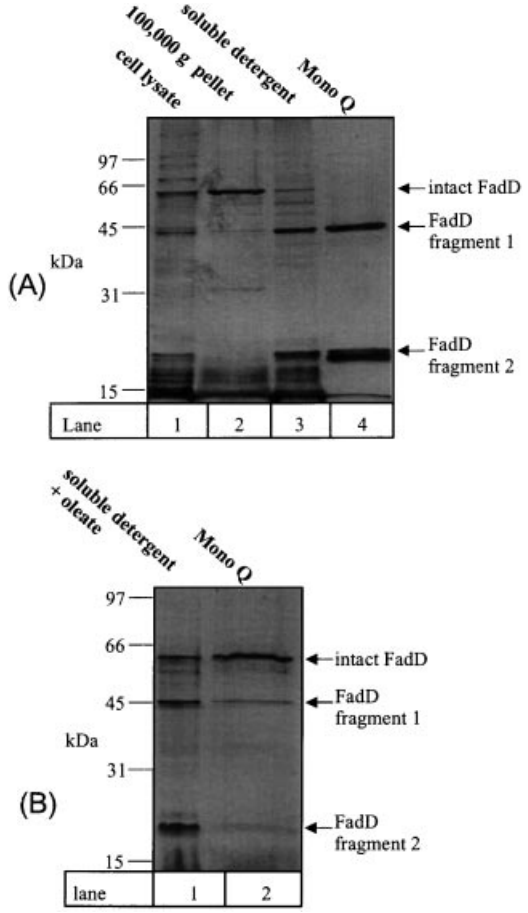

Figure 3 SDS/PAGE analysis of purifed cleaved and intact FadD

(**A**) Cleaved FadD. Cell lysates were prepared from JM105 *E. coli* transformed with the FadD expression plasmid and induced with 1 mM IPTG (lane 1). The lysate was then subjected to centrifugation at 100000 ***g*** for 60 min and the pellet was resuspended in 100 mM Tris/HCl (pH 7.5)/0.2% Triton X-100 (lane 2). The suspension was subjected to centrifugation again at 100000 ***g*** for 60 min and the supernatant (lane 3) was applied on to a Mono-Q column pre-equilibrated with 100 mM Tris/HCl (pH 7.5). A linear gradient using 100 mM Tris/HCl (pH 7.5)/400 mM NaCl was used to elute the fraction containing cleaved FadD (lane 4). (**B**) Intact FadD. Intact FadD was isolated in a similar manner to cleaved FadD, except that 1 mM oleate was added to all the solutions before application on to the Mono-Q column. Both gels were silver stained.

Table 1 Kinetic properties of purified intact and cleaved FadD

FadD	K_m (μM oleate)	V_{max} (nmol of oleoyl-CoA/min per mg of potein)	k_{cat}/K_m
Cleaved	85 ± 18	1550 ± 165	18.2
Intact	38 ± 12	633 ± 79	16.7

Figure 4 shows that unlike pyruvate oxidase, proteolytic cleavage of FadD did not result in lipid-independent activation or affect lipid activation.

Role of outer membrane protease OmpT

Tsp protease had been implicated in *E. coli* LCFA uptake, but its precise role is unclear [15]. To determine if the Tsp protease was

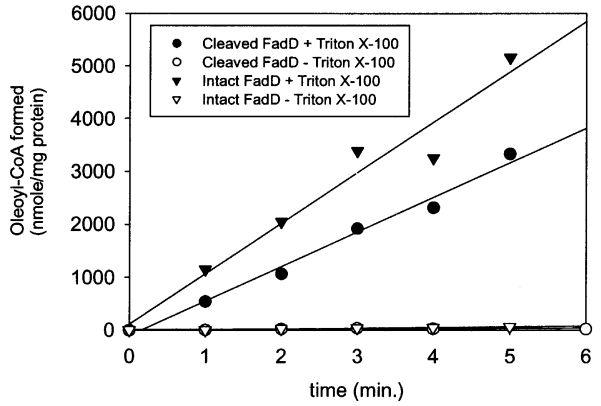

Figure 4 Effect of Triton X-100 on long-chain fatty acyl-CoA synthetase activity of intact and cleaved FadD

A time course of oleoyl-CoA formation by purified cleaved FadD or purified intact FadD was monitored in the presence or absence of 0.1% Triton X-100, as described in the Experimental section.

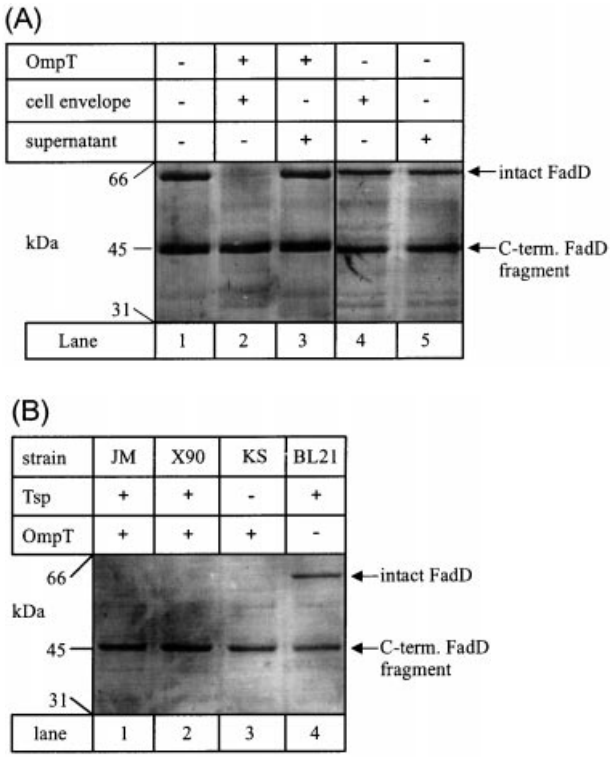

Figure 5 Incubation of partially purified FadD with cell envelope fractions or whole *E. coli* cells

(**A**) Partially purified FadD (lane 1) was incubated with cell envelope or supernatant fractions from wild-type (JM105) (lanes 2 and 3) or *ompT*-deficient [BL21(DE3); lanes 4 and 5] cells and analysed by SDS/PAGE and Coomassie Brilliant Blue staining. (**B**) Partially purified FadD was also incubated with various intact *E. coli* cells. The reactions were subjected to centrifugation and the supernatants were analysed by SDS/PAGE and Coomassie Brilliant Blue staining. Tsp, genotype for the periplasmic protease; JM, JM105; KS, KS1000; BL21, BL21(DE3); C-term., C-terminal.

responsible for cleavage, we overexpressed FadD in the *E. coli* strain KS1000, a *tsp* deletion strain [38], and the isogenic wild-type strain, X90. Lysates prepared from these cells using the Tris/EDTA+lysozyme method [29] were analysed by SDS/PAGE. Cleaved FadD was observed in both the Tsp-deficient, as well as the wild-type, *E. coli* cells (results not shown), indicating that Tsp protease was not required for FadD cleavage.

To gain insight into the protease responsible for FadD cleavage, we performed N-terminal protein microsequencing on purified FadD fragments to determine the N-terminal amino acid sequences (results not shown). The smaller 19 kDa fragment could not be sequenced, suggesting that its N-terminus was blocked and therefore represented the N-terminal FadD fragment. The amino acid sequence of the 45 kDa fragment indicated that cleavage of FadD occurred between amino acid residues Lys^{172} and Arg^{173} (see Figure 7). Cleavage at this site predicted the formation of a 42.97 kDa C-terminal fragment and a 19.39 kDa N-terminal fragment. Although the C-terminal fragment appeared to migrate as a 45 kDa polypeptide on an SDS/polyacrylamide gel, MS performed on this fragment indicated that it was 42.95 kDa, in line with the predicted size. MS performed on the 19 kDa fragment revealed that there were actually two fragments present, with approximate molecular masses of 19.38 and 18.98 kDa. The former polypeptide displayed the expected mass for the N-terminal FadD fragment, whereas the latter fragment appeared to be formed by a second cleavage event occurring between residues Lys^{169} and Tyr^{170} (see Figure 7). These data suggested that the protease(s) responsible displayed a preference for cleavage sites containing basic residues.

As indicated previously, cleavage of overexpressed FadD only occurred after cell lysis (see Figure 1). This suggested that FadD and the unidentified protease were located in different subcellular compartments. To test this, *in vitro* cleavage assays were performed by incubating partially purified FadD with either cell envelope or supernatant fractions from *E. coli* cell lysates. As shown in Figure 5(A), cleavage of FadD only occurred when cell envelope fractions were added (compare lanes 2 and 3), indicating that the protease(s) responsible for cleavage was associated with the cell envelope. It should be noted that there was a significant amount of the cleaved 43 kDa FadD fragment already present in the partially purified preparation used for these experiments (Figure 5A, bottom arrow); this was the result of cleavage that occurred during purification, since oleate (which inhibits lysis, see Figures 3 and 6A) was not included to avoid potential inhibition of cleavage during the subsequent *in vitro* proteolysis assays.

Investigation of known *E. coli* membrane-associated proteases suggested that OmpT, a serine protease with specificity for dibasic residues [39–43], might be a likely candidate as the protease responsible for FadD cleavage. To test this, cell envelope or supernatant fractions from the OmpT-deficient *E. coli* cell line BL21(DE3) were incubated with partially purified FadD. Cell envelope fractions from the OmpT-negative cells were unable to cleave intact FadD (Figure 5A, compare lanes 2 and 4), showing that OmpT was required for cleavage. OmpT is an outer membrane protein with an active site that is thought to reside on the cell surface [41,42]. Based on this, we found that FadD could be cleaved when incubated with intact *E. coli* cells expressing OmpT (Figure 5B, lanes 1–3), but not with intact, OmpT-deficient cells [BL21(DE3) cells] (lane 4). These results strongly suggested that OmpT was responsible for cleavage of the 62 kDa FadD, resulting in an enzymically active complex of a 19 kDa N-terminal fragment and a 43 kDa C-terminal fragment. In addition, the localization of OmpTs proteolytic activity at the cell surface, suggested that FadD cleavage did not represent *in vivo*

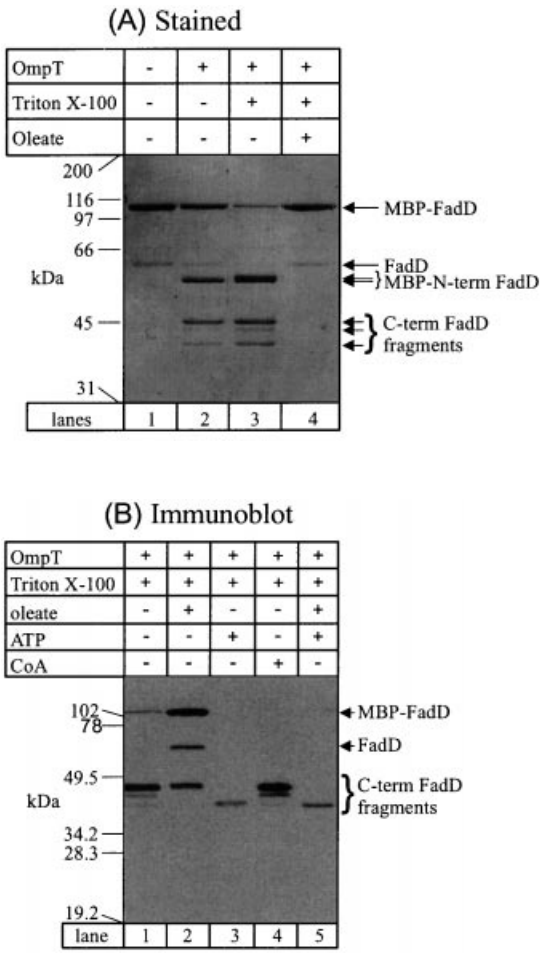

Figure 6 Effect of detergents and FadD ligands on the proteolysis of the MBP–FadD fusion protein

Purified MBP–FadD fusion protein was incubated with various ligands and Triton X-100 before incubation with intact JM105 *E. coli* cells. The reactions were subjected to centrifugation and the supernatants were analysed by SDS/PAGE and Coomassie Brilliant Blue staining (**A**) or immunoblotting (**B**) with antibodies raised against the C-terminal FadD fragment. term, terminal.

processing. In support of this, we found that OmpT was not required for *E. coli* growth on minimal medium using oleate as the sole carbon source (results not shown).

Effect of FadD ligands on the limited proteolysis of FadD

The implication of OmpT in FadD cleavage prompted us to attempt to overexpress FadD in the OmpT-deficient cell line BL21(DE3) as a means of (1) confirming that OmpT was responsible for FadD cleavage and (2) purifying intact FadD free of the cleaved form. Attempts at overexpressing FadD in the OmpT-deficient cell line using the above expression vector, were unsuccessful. However, we succeeded in constructing and overexpressing an MBP–FadD fusion protein in these cells. The MBP–FadD fusion protein remained uncleaved after cell lysis and during the purification process. The fusion protein also displayed high levels of long-chain fatty acyl-CoA synthetase activity (results not shown). Analysis by SDS/PAGE revealed that purified MBP–FadD fusion protein consisted of a major band of approx. 105 kDa (the expected size for the fusion protein, since processed MBP is 43 kDa [44]) and minor amounts of a 62 kDa band (Figure 6A, lane 1). Both proteins were detected with the anti-(FadD C-terminal) antibody, confirming that the 105 kDa protein was a FadD fusion protein (Figure 6B, lane 2). The 62 kDa band appeared to represent intact FadD from native sources or from non-specific cleavage of the fusion protein, which co-eluted with MBP–FadD during purification.

Much like native intact FadD, purified MBP–FadD was cleaved after incubation with intact *E. coli* cells expressing OmpT (Figure 6A, lane 2), yielding two prominent protein bands, 45 and 60 kDa in size. The 45 kDa band represented the C-terminal FadD fragment, since it was detected with the antibody specifically raised against that fragment (Figure 6B, lane 1). The 60 kDa band could not be detected with the FadD antibody (compare Figure 6B, lane 1 with Figure 6A, lane 3) suggesting that it represented MBP (43 kDa) fused to the 19 kDa N-terminal FadD fragment. Smaller, faint bands below the 45 kDa band were also visible by Coomassie Brilliant Blue staining and immunoblotting, suggesting that they represented minor degradation products from the C-terminal FadD fragment. None of these bands were observed when purified MBP–FadD was incubated with OmpT-deficient cells (results not shown), confirming that OmpT was required for FadD cleavage.

Limited proteolysis is a commonly used technique to gain insight into the structure–function relationship of a protein [45], and purification of intact MBP–FadD allowed us to test the effects of Triton X-100, oleate, ATP and CoA on OmpT-mediated cleavage *in vitro*. The addition of Triton X-100 detergent to the digestion reactions resulted in more efficient cleavage of MBP–FadD (Figure 6A, lane 3), while the addition of oleate prevented proteolysis (Figure 6A, lane 4). Furthermore, the presence of ATP overcame oleate-mediated inhibition, and actually led to more extensive cleavage of the 45 kDa FadD fragment (Figure 6B, lanes 3 and 5). Finally, addition of CoA, another substrate of FadD, did not affect cleavage. Triton X-100, oleate and ATP did not affect the ability of intact OmpT-expressing cells to cleave T7 RNA polymerase (a previously characterized OmpT substrate [42]; results not shown), showing that their affects on FadD cleavage were not due to effects on the protease itself. These results suggest that interaction of FadD with detergents, oleate or ATP results in the exposure or protection of OmpT cleavage sites, caused by conformational changes in FadD upon interaction with detergents or specific substrates.

DISCUSSION

In our attempt to overexpress and characterize FadD, we discovered that the fatty acyl-CoA synthetase is subjected to proteolysis by OmpT during cell lysis. This observation probably accounts for the discrepancies in the size predicted by the nucleic acid sequence [7,8] and that of the purified protein from wild-type *E. coli* [6,25]. Proteolysis appears to be mediated by the outer membrane protease OmpT, and does not play a role in the processing of FadD *in vivo*. Moreover, this cleavage may also explain the reported isolation of different substrate-specific [24] and charge-specific [23] isoforms of FadD.

FadD cleavage was subsequently used to gain insight into the structure of the enzyme, by utilizing OmpT as a probe for detecting changes in FadD structure. OmpT-mediated cleavage of FadD occurs between amino acids Lys^{172} and Arg^{173}, resulting in the conversion of the 62 kDa enzyme into an associated complex comprised of a 19 kDa N-terminal fragment and a 43 kDa C-terminal fragment (Figure 7). In spite of proteolysis, the nicked FadD enzyme retained enzymic activity at levels comparable with wild-type.

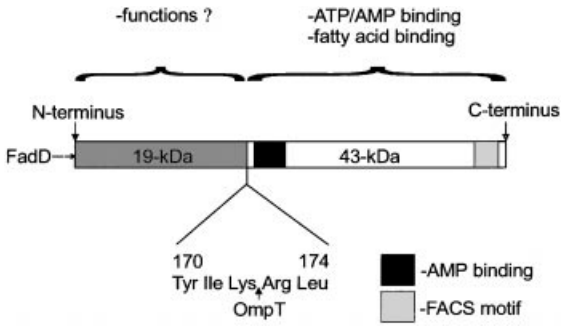

Figure 7 Domain structure diagram of FadD

Diagram depicting the OmpT cleavage site and proposed functions contained within the two fragments of cleaved FadD. FadD contains signature motifs found in other AMP-binding proteins, the first of which appears between amino acids 213–224 [2,7,8]. FadD also contains a highly conserved sequence found in other fatty acyl-CoA synthetases called the fatty acyl-CoA synthetase signature motif, which is located at amino acids 431–445 [26,46]. The AMP-binding sequence is thought to be involved in binding nucleotides, whereas the fatty acyl-CoA synthetase motif is involved in forming a binding pocket for LCFAs [46]. Our data suggests that the region surrounding the OmpT cleavage site is a linker domain.

The use of photoaffinity labelling with a photoreactive fatty acid revealed that the hydrocarbon chain of the fatty acid interacts with the 43 kDa FadD fragment, suggesting that the fatty-acid binding site resides in this portion of FadD. Black et al., using a different photoaffinity fatty acid probe, reached a similar result [46]. They showed that the fatty acid probe mostly cross-linked to FadD near the C-terminus, just adjacent to the fatty acyl-CoA synthetase signature motif. The fatty acyl-CoA synthetase signature motif is a 25-amino-acid consensus sequence present in all fatty acyl-CoA synthetases, that is thought to play a role in fatty acid substrate specificity and binding [26]. FadD and other fatty acyl-CoA synthetases also belong to the AMP-binding protein family, which are characterized by specific signature motifs that are thought to represent nucleotide binding regions [7,8] (Figure 7) and found within the 43 kDa FadD fragment.

Previous studies from our laboratory showed that FadD is activated in the presence of Triton X-100 and bacterial membranes [9]. Similarly, *E. coli* pyruvate oxidase is another lipid-surface activated enzyme that is sensitive to limited proteolysis by chymotrypsin [36,37]. Proteolysis of pyruvate oxidase results in lipid-independent activation, but cleavage of FadD by OmpT did not have a similar effect. However, the presence of Triton X-100 increased the efficiency of FadD cleavage, which may be due to interaction of the enzyme with detergent, and conversely we also noted that proteolysis could be inhibited by LCFAs. These data suggest that the OmpT cleavage site is located within an accessible and mobile region of FadD, since sensitivity of specific regions to proteolysis have been correlated with increased mobility and flexibility [45,47,48]. This region appears to undergo conformational changes upon binding LCFAs, such that the OmpT cleavage site is no longer accessible and/or more structurally rigid (Figures 6A and 6B). ATP also altered the proteolytic pattern of FadD, suggesting that ATP binding also induces conformational changes, but in a manner different from oleate. In the presence of ATP it appears that the region surrounding the OmpT cleavage site becomes less structured and/or more accessible to the protease. CoA had no effect on FadD cleavage, which was expected since CoA is thought to be bound after fatty acid and ATP binding [1]. It is tempting to speculate from our data that the region containing the OmpT cleavage site represents a flexible hinge region or domain, linking the 19 kDa N-terminal fragment to the 43 kDa C-terminal fragment.

Using computer modelling, Black et al. proposed a partial structural model for FadD (starting at Thr^{213} of the native enzyme) in which the ATP/AMP and fatty-acid binding domains reside within the 43 kDa C-terminal FadD fragment (Figure 7) [46]. Their proposed structure places the OmpT cleavage site near the boundary between the N-terminal FadD fragment and the ATP/AMP binding domain. In light of this, we were interested in determining whether the enzymic activity of FadD could be observed with only the purified 43 kDa fragment. Preliminary attempts to address this involved expression of the 43 kDa C-terminal fragment alone or fused to MBP, but our initial analyses revealed that expression levels of both proteins were very low. This suggested that the C-terminal FadD fragment expressed alone or as a fusion protein is unstable. It is possible that the C-terminal FadD fragment requires the N-terminal fragment for structural stability, which is consistent with our observations that the two fragments remain associated.

Our results show that the 62 kDa FadD enzyme represents the native fatty acyl-CoA synthetase in *E. coli* and contains an OmpT-sensitive site in a region of the enzyme that undergoes conformational changes upon binding ligands or detergents. The proposed linker domain appears to connect the C-terminal end of FadD, containing the nucleotide and fatty-acid binding domains, to the 19 kDa N-terminal region of unknown function. Future work is focused towards determining what function(s) is contained within the N-terminal fragment and its functional relationship with the C-terminal FadD fragment.

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