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# Mechanistic Studies of the Long Chain Acyl-CoA Synthetase Faa1p from *Saccharomyces cerevisiae*

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## Abstract

Long chain acyl-CoA synthetase (ACSL; fatty acid CoA ligase: AMP forming; EC 6.2.1.3) catalyzes the formation of acyl-CoA through a process, which requires fatty acid, ATP and coenzymeA as substrates. In the yeast *Saccharomyces cerevisiae* the principal ACSL is Faa1p (encoded by the *FAA1* gene). The preferred substrates for this enzyme are *cis*-monounsaturated long chain fatty acids. Our previous work has shown Faa1p is a principal component of a fatty acid transport/activation complex that also includes the fatty acid transport protein Fat1p. In the present work hexameric histidine tagged Faa1p was purified to homogeneity through a two-step process in the presence of 0.1% η-dodecyl-β-maltoside following expression at 15°C in *Escherichia coli*. In order to further define the role of this enzyme in fatty acid transport-coupled activation (vectorial acylation), initial velocity kinetic studies were completed to define the kinetic parameters of Faa1p in response to the different substrates and to define mechanism. These studies showed Faa1p had a $V_{max}$ of 158.2 nmol/min/mg protein and a $K_m$ of 71.1μM oleate. When the concentration of oleate was held constant at 50μM, the $K_m$ for CoA and ATP were 18.3μM and 51.6μM respectively. These initial velocity studies demonstrated the enzyme mechanism for Faa1p was Bi Uni Uni Bi Ping Pong.

### Keywords

long chain acyl-CoA synthetase mechanistic enzymology fatty acid transport

## INTRODUCTION

Long chain acyl-CoA synthetase (ACSL; fatty acid CoA ligase: AMP forming; EC 6.2.1.3) occupies a central position in the metabolism of fatty acids through oxidative and non-oxidative pathways including the synthesis of complex membrane lipids and the synthesis and mobilization of storage lipids including triglycerides and sterol esters. It has become increasingly apparent that separate ACSL isoforms provide unique roles in these pathways including the transport of exogenous fatty acids into the cell [1], the targeting of fatty acids into specific metabolic pathways [2], and in fatty acid-mediated transcriptional control [3,4]. Under-expression of certain ACSL isoforms can compromise the ability of a cell to store or

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oxidize fatty acids leading to decreased cellular function. Overexpression, on the other hand, can lead to excess accumulation of toxic lipids and cell death [5].

In the yeast *Saccharomyces cerevisiae*, four long chain ACSL isoforms with unique expression profiles, cellular localizations and functions have been described [6,7]. In addition, yeast cells also have short chain and very long-chain acyl-CoA synthetases [8–10]. The very long chain acyl-CoA synthetase has also been described as a fatty acid transport protein, which functions in concert with ACSL in the transport-coupled activation of exogenous fatty acids (vectorial acylation) [8,11]. Faa1p is the major ACSL in yeast accounting for nearly 90% of the long chain activity [3,7,12]. While a second ACSL, Faa4p, can partially compensate for lack of Faa1p activity, many cellular functions are compromised in cells harboring a deletion of the *FAA1* gene including fatty acid transport [3], maintenance of intracellular acyl-CoA pools [3], and fatty acid dependent regulation of transcription mediated by Spe23p and Mga2p [3]. Localization studies and proteomic approaches have shown this enzyme is both cytosolic and membrane-associated (including on the plasma membrane [13], lipid particle [14], mitochondria [15], and ER [16]) suggesting it may move to the membrane to facilitate catalysis, particularly in the context of vectorial acylation. This membrane association is consistent with studies by Bar-Tana *et al*. [17], who showed acyl-CoA synthetase activity in the rat is maximal when the fatty acid substrate is presented in the presence of either nonionic detergent or phospholipid vesicle. The preferred substrates for Faa1p are *cis*-monounsaturated long chain fatty acids; this enzyme also activates *trans*-monounsaturated, tetradecenoic fatty acids but with more selectivity [18].

Long chain acyl-CoA synthetases are proposed to catalyze the formation of fatty acyl-CoA by a two-step process proceeding through the hydrolysis of ATP to yield pyrophosphate. A central feature of the catalysis is the formation of an enzyme-bound adenylated intermediate [19]. This activation step initially involves an acyl-phosphate bond formation between the carboxyl group of the fatty acid and the α-phosphate of ATP. Subsequently a transfer of the fatty acyl group to the sulfhydryl group of coenzyme A occurs thereby releasing AMP.

fatty acid+ATP => fatty acyl-AMP + $PP_i$

fatty acyl-AMP+CoA => fatty acyl-CoA + AMP

In the present work, we have purified the ACSL Faa1p to homogeneity as part of our efforts to define the biochemical mechanisms operational in the vectorial transport and activation of exogenous long chain fatty acids. Following over expression, Faa1p was purified in the presence of 0.1% η-dodecyl-β-maltoside and 1mM ATP. This is the first work, which describes the purification and comprehensive characterization of a eukaryotic ACSL. Previous studies have defined the kinetic mechanism of a partially purified acyl-CoA synthetase from rat microsomes and inferred the kinetic mechanism from the structure of the acyl-CoA synthetase of *Thermus thermophilus* (likely has specificity for medium chain fatty acids) [17,20,21]. In the first study, total acyl-CoA synthetase activity was purified from rat microsomes and characterized kinetically; this is likely the contribution of several different ACSL isoforms as opposed to a highly purified single enzyme ([17]; see also [22]). In the second study the kinetic mechanism was inferred based on the structure of the bacterial enzyme in lieu of mechanistic studies [21]. The present work demonstrates the highly purified ACSL Faa1p behaves as a dimer as measured on an analytical gel filtration column and has an obligate requirement for ATP and $Mg^{+2}$ or $Mn^{+2}$ for activity. Further, initial velocity studies completed on highly purified Faa1p demonstrate the enzyme reaction is Bi Uni Uni Bi Ping Pong, consistent with other adenylate-forming enzymes.

# MATERIALS AND METHODS

## Construction of FAA1 Expression System and Over Expression of Faa1p in Escherichia coli

The *FAA1* gene was amplified by PCR from plasmid pBB319 (gift from Dr. Jeffrey Gordon, Washington University, St. Louis MO) using a pair of oligonucleotide primers (50μM; forward – 5'-CGACGGTCCATATGGTTGCTCAATATACCGT-3'; reverse - 5'-CATAGTTGGAGCTCTCAGTGATGGTGATGGTG-3') designed to contain *Nde*I and *Sca*I sites at the 5'- and 3'-ends respectively. PCR products were purified using a QIAQuick spin column (Qiagen, Inc., Valencia CA), digested with the restriction enzymes *Nde*I and *Sac*I and subsequently ligated into the T7-RNA responsive expression plasmid pET21-a (Novagen, Madison, WI) to yield pDB222. The DNA sequence of *FAA1* in pDB222 was verified by dye-terminator sequencing. For expression of Faa1p-6×His, the plasmid pDB222 was transformed into *E. coli* strain LS2226 (BL21-CodonPlus (DE3) Δ*fadD*::$Kn^R$). Transformants were grown in 2×YT (1.6% tryptone, 1% yeast extract, 0.5% NaCl) supplemented with ampicillin (100μg/ml) and kanamycin (45μg/ml) in a gyratory incubator/shaker at 22°C until they reached an absorbance of $A_{600}$ 0.6–0.8 (3–4hr). Isopropyl-β-D-thiogalactopyranoside (IPTG; 1mM, final concentration) was added to induce Faa1p-6×His and incubation continued at 15°C for 16hr. Cells were harvested by centrifugation (4,000 ×*g*, 15min), washed with 1X PBS and frozen at −80°C until use.

## Purification of Faa1p

Faa1p-6×His was purified from soluble crude extract by chromatographic methods. *E. coli* cells (1L) were suspended in 100ml of extraction buffer (50mM Hepes/NaOH, pH 7.4, 0.5M NaCl, 15% glycerol, 0.1% η-dodecyl-β-maltoside (DDM), 1mM PMSF, 1mM ATP, 5mM $MgCl_2$) and were lysed by sonication in an ice water bath (5min; 30s on/off intervals). The cell lysate was spun at 27,000 ×g for 25min at 4°C to yield a soluble cell-free extract. All subsequent purification processes were accomplished at 4°C. The soluble cell extract was applied to a gravity column (1.5 × 10 cm; Bio-Rad, Hercules, CA) packed with 5ml Ni-NTA resin (Qiagen, Inc., Valencia CA) and pre-equilibrated with buffer A (50mM Hepes/NaOH, pH 7.4, 0.5M NaCl, 10% glycerol, 0.05% η-dodecyl-β-maltoside (DDM), 1mM ATP, 5mM $MgCl_2$). The column was washed sequentially with 120ml of buffer A and 50ml of buffer A containing 20mM imidazole to elute unbound protein. Bound Faa1p was eluted with 25ml buffer A containing 250mM imidazole. Fractions containing active Faa1p were pooled (4–5 ml) and applied to a Sephacryl S-200 HR size exclusion column (1.6 × 60cm; GE Healthcare, Piscataway, NJ) pre-equilibrated with buffer A attached to an FPLC system (Biologic HR workstation, Bio-Rad). The Sephacryl S-200 HR column was run at a flow rate of 0.5ml/min and 1ml fractions were collected. Fractions containing Faa1p activity were pooled, concentrated to 6–8mg/ml and subsequently used for biochemical analysis.

Purity of the different protein samples was evaluated by SDS polyacrylamide gel electrophoresis. Apparent molecular weight ($M_r$) was determined by comparison to BenchMark™ pre-stained protein ladder (Invitrogen, Carlsbad, CA). Protein concentrations were measured using the Bradford total protein assay with bovine serum albumin as the standard.

The sequence and monomeric molecular weight of purified Faa1p was determined using ion trap-nanoelectrospray mass spectrometry performed on a ThermoFinnigan LTQ MS at the Biological Mass Spectrometry and Proteomics Facility (Wadsworth Center, Albany NY). Protein identification and molecular weight was determined by querying the DNA/protein databases with MS/MS spectra using the Turbo SEQUEST.

### Analytical Size Exclusion Chromatography

Size exclusion chromatography was performed at 4°C on a Superose™ 6 10/300 GL column (1.0 × 30cm; GE Healthcare). The column was equilibrated with buffer A and run with a flow rate of 0.4ml/min. Protein standards (Bio-Rad) were used to calibrate the sizing column and included bovine thyroglobulin (670,000), bovine gamma globulin (158,000), chicken ovalbumin (44,000), horse myoglobin (17,000) and vitamin B-12 (1,350). Faa1p was detected by absorbance at 280nm and by measuring acyl-CoA synthetase activity.

### Determination of Acyl-CoA Synthetase Activity

Acyl-CoA synthetase activity was measured using standard conditions as previously described [23]. The standard reaction mixtures contained 50mM Tris/HCl, pH 8.0, 10mM ATP, 10mM $MgCl_2$, 0.3mM dithiothreitol (DTT), 0.01% Triton X-100, 50μM [$^3$H] oleate (or other fatty acids substrates) and 200μM coenzyme A (CoA). Fatty acid stocks (free acid) were prepared in 10mg/ml α-cyclodextrin. The reaction was initiated by the addition of CoA at 30°C, continued for 15min and terminated by the addition of 2.5ml of isopropanol/η-heptane/1M $H_2SO_4$ (40:10:1). The free fatty acid was removed by sequential organic extractions using η-heptane. Acyl-CoA formed during the reaction remained in the aqueous fraction and was quantified by scintillation counting. Data were analyzed by analysis of variance using PRISM software (GraphPad Software Inc., San Diego, CA).

To test the heat stability, purified Faa1p was incubated for 30min at temperatures ranging from 4°C to 50°C prior to assay of acyl-CoA synthetase activity as detailed above. The effect of pH on enzyme activity was evaluated using a three-component buffer system containing 75mM *bis*-Tris, 38mM Hepes and 38mM CHES; the pH was adjusted to 5.5–9.5 using either NaOH or HCl. The requirement for divalent metal cations was evaluated using $MgCl_2$, $MnCl_2$ and $CoCl_2$. Nucleotide (ATP, AMP) or nucleotide analogs (adenosine 5'-methylenetriphosphate (AMP-CPP) and adenylyl methylenediphosphate (AMP-PCP)) were used to assess ATP requirement and provide insights into the nature of the nucleotide-binding site within the enzyme.

### Kinetic Studies and Determination of Enzyme Reaction Mechanism

The kinetic properties of purified Faa1p were determined by monitoring oleoyl-CoA synthetase activity; initial reaction rates were measured under standard assay conditions at various concentrations of ATP (0–1mM) or CoA (0–200μM) or oleate (0–100μM) as detailed in the figures and figure legends. The curves from the Michaelis-Menten equation were fit through the initial rate data by nonlinear regression using the PRISM software (GraphPad Software Inc.).

Initial velocities were measured by choosing enzyme concentrations ensuring a linear response during measurement. For these studies, Faa1p was purified in the absence of ATP. All experiments were carried out for the overall reaction in which the concentrations of one substrate was varied in the presence of different levels of a second substrate, while the concentration of the third substrate was held constant [24,25]. Kinetic results were analyzed using double-reciprocal plots followed by replots of slopes and intercepts as a function of the reciprocal concentration of the changing fixed substrate in the initial-velocity studies or the inhibitor concentration in the product-inhibition studies [26]. The concentrations of the product inhibitors is noted within the text and figures in Results. The data reported were representative sets of experiments carried out at least three times.

### Materials

Enzymes required for DNA manipulations were from New England Biolabs, Promega, or Roche Molecular Biochemicals. [$^3$H] labeled fatty acids were obtained from Perkin-Elmer. Antibiotics and other supplements for bacterial growth were obtained from Difco and Sigma. All other chemicals were obtained from standard suppliers and were of reagent grade.

## RESULTS

### Purification and characterization of the ACSL Faa1p

Faa1p was purified from *E. coli* as a hexameric histidine tagged protein following expression as detailed in *Experimental Procedures*. Following the Sephacryl S-200 HR size exclusion column, the final protein samples were concentrated to 6–8mg/ml in 50mM Hepes, pH7.4; 1mM ATP, 5mM $MgCl_2$, 0.5M NaCl, 10% glycerol, 0.05% DDM (Figure 1A). In a typical purification experiment, there was routinely a 200-fold enrichment of acyl-CoA synthetase activity with a yield of 8–10mg pure Faa1p. Given the activity of the enzyme in both the crude extract and in a highly purified form, we conclude that heterologous expression in *E. coli* at 15°C results in a properly folded protein. The sequence of the highly purified Faa1p was confirmed using ion trap-nanoelectrospray mass spectrometry, which also defined a molecular weight of 77,817. The protein is stable and retains activity and homogeneity for up to 7 days at 4°C; the protein also retains activity for at least 4 months when flash-frozen in liquid nitrogen in aliquots and stored at −80°C. When ATP was eliminated from the buffers used in purification, peptide fragments were noted after 48hr of storage at 4°C or when flash frozen, which was also accompanied by a loss of activity. For the mechanistic studies detailed below, Faa1p purified in the absence of ATP was used immediately. Protein purified using these methods were used in all of the kinetic experiments.

Like other ACSLs, Faa1p appears to be a homodimer based on its elution patterns from an analytical size exclusion column in the presence of 0.05% DDM (Figure 1B). Purified Faa1p elutes from the Superose 6 10/300 column at 17min, which is consistent with a native MW of ~145,000, with the caution that this column was run using a buffer system containing 0.05% DDM. As noted above, the inclusion of 0.05% DDM maintained both the stability and activity of Faa1p. Considerable effort was expended to define these conditions, including studies evaluating enzyme activity and stability in the presence of several other nonionic detergents including η-octyl-β-D-glucopyranoside (OG), lauryldimethylamine-oxide (LDAO), dodecyl nonaoxyethylene ether (C12E9), and tetraethylene glycol monooctyl ether (C8E4). We found for example when 1% or 0.5% OG was used, Faa1p eluted from the analytical sizing column as a monomer; 0.2% LDAO on the other hand maintained the protein primarily as a dimer. In both cases purified Faa1p lost 50–60% original activity within 24hr, which was accompanied by the appearance of peptide fragments (data not shown). Under conditions where the concentrations of OG or LDAO were reduced to 0.05%, Faa1p eluted as high molecular weight soluble aggregates that also lost activity (<50% original activity) when stored at 4°C for 24hr. We also evaluated the detergents C12E9 and C8E4 (0.2% final concentrations) and found that Faa1p eluted as a dimer but had 20–30% original enzymatic activity when maintained at 4°C for 24hr. The optimal conditions for purification also included the addition of ATP (1mM, final concentration), which we suspect enhances the stability of the ACSL Faa1p.

### Enzymatic properties of the ACSL Faa1p

The kinetic parameters of Faa1p were initially evaluated using both oleate and ATP as variable substrates. For these studies, ATP was not included in the purification buffers; given the stability issues noted above, these experiments were completed immediately following purification of Faa1p. Using standard assay conditions, we evaluated total oleoyl-CoA synthetase activity as a function of enzyme concentration (Figure 2A). The reaction was linear

from 1–3μg of purified Faa1p; above 3μg purified Faa1p, the reaction was not linear, which may be in part due to is association with the membrane. For all of the kinetic studies detailed below, 1.5μg Faa1p was used. The oleoyl-CoA synthetase activities of Faa1p using varying concentrations of oleate (holding ATP and CoA concentrations at 10mM and 200μM respectively) in the reaction cocktail demonstrated this enzyme has typical Michaelis-Menten profiles, which when displayed as a double-reciprocal plot gave a calculated $V_{max}$ and a $K_m$ of 158.2 nmol/min/mg protein and 55.1μM respectively (Figure 2B). When ATP was used as the variable substrate, similar Michaelis-Menten profiles were obtained giving a calculated $V_{max}$ of 185.1 nmol/min/mg protein and a $K_m$ for ATP of 55.0μM. The enzyme required $Mg^{+2}$ for activity although $Mn^{+2}$ provides equivalent function. (Table I). When $Co^{+2}$ was use as the divalent cation, the enzyme activity was reduced to less than 40% the levels defined using $Mg^{+2}$; the addition of $Co^{+2}$ also had an apparent inhibitory effect as activity was reduced when added to the reaction mixture along with $Mg^{+2}$.

The oleoyl-CoA synthetase activity of purified Faa1p was essentially unchanged over a range of pH (7.0 – 8.5) but was markedly reduced at a pH below 6.5 (Figure 3A). This is consistent with the notion that the fatty acid must be in an anionic state for maximal catalysis. Purified Faa1p (in the presence of ATP) was incubated at the temperatures noted in Figure 3B for 30min, warmed or cooled to room temperature and activity measured. These data showed that Faa1p was able to tolerate changes in temperature from 4°C to 40°C; the enzyme lost essentially all of its activity when heated to 50°C and above.

The two non-hydrolyzable ATP analogues AMPCPP and AMPPCP were able to bind to purified Faa1p and essentially block all acyl-CoA synthetase activity. In these experiments, the oleoyl-CoA synthetase activity of purified Faa1p was monitored under standard conditions in the presence of 5mM AMPCPP or AMPPCP (100-fold excess when compared to ATP, which was held at 50μM). At these concentrations of analogue, activity was depressed over 90% indicting both compounds compete for ATP binding thus blocking catalysis (data not shown). These results, along with the kinetic studies using ATP as the variable substrate are consistent with the conclusion the long chain acyl-CoA synthetase Faa1p has an absolute requirement for ATP.

## Mechanistic studies of the ACSL Faa1p

Acyl-CoA synthetase requires three substrates: fatty acid, ATP and CoA; the mechanistic studies detailed below used Faa1p, which was purified in the absence of ATP. Initial velocity experiments of the forward reaction were conducted by keeping the concentration of one substrate constant and varying the concentration of the remaining two (Figure 4). In the first set of experiments the concentration of CoA was held constant at 200μM and the concentrations of ATP and oleate varied (Figure 4A). The double-reciprocal pots of these data show a pattern that is suggestive of mixed inhibition indicating the ATP and oleate binding sites are distinct, which are likely to overlap during the formation of the acyl-adenylate intermediate. When ATP was held at saturating concentrations (10mM) and the concentrations of oleate and CoA varied, patterns of uncompetitive inhibition were observed (Figure 4B). These data argue the oleate and CoA binding sites within Faa1p are distinct. Moreover these data are consistent with the notion that ATP and oleate bind to the enzyme, which then catalyzes the formation of the acyl-adenylate, followed by the binding of CoA. When the concentration of oleate was held constant and ATP and CoA concentrations varied, the same pattern of uncompetitive inhibition was observed (Figure 4C). These data also support the ordered binding of substrates (oleate and ATP followed by CoA) in the catalytic cycle of the ACSL Faa1p. These data are consistent with the initial velocity rate law for a Bi Uni Uni Bi Ping Pong mechanism [26].

Product inhibition studies were completed to discern the order in which products were released from the enzyme (Figure 5). When evaluated in combination with varying concentrations of

ATP, AMP functioned as a competitive inhibitor of the enzyme (Figure 5A). The patterns of inhibition by pyrophosphate in combination with varying concentrations of oleate were mixed, but the kinetic behavior was more competitive in nature (Figure 5B). When varying concentrations of AMP and CoA were used in the product inhibition studies, the pattern was non-competitive (Figure 5C). Collectively these data are consistent with the release of $PP_i$ from a site on the enzyme, which overlaps the site occupied by the acyl-adenylate. CoA appears to bind in a site distinct from AMP and thus its binding site is likely to be distinct from that of the acyl-adenylate. From these studies, in the absence of either AMP or $PP_i$ as a product inhibitor, the $V_{max}$ calculated for the enzyme ranged between 13.5nmol/min/mg protein to 27.3 nmol/min/mg protein (Table II). The $K_i$ of AMP was in the low mM range (5.2 mM); due to the mixed nature of inhibition by $PP_i$, both $K_i$ and $K_i$' were defined (2.1mM and 1.4mM respectively). In the initial velocity experiments varying $PP_i$ and oleate, the $K_m$ of oleate was 83μM; this value is consistent with the $K_m$ for oleate calculated from the Lineweaver-Burke plot in Figure 2.

The initial velocity and product inhibition studies are consistent with a Bi Uni Uni Bi Ping Pong mechanism. The initial velocity experiments using varying concentrations of oleate and ATP and the results from the product inhibition studies using varying concentrations of $PP_i$ and oleate were indicative of competitive inhibition, but still rather mixed. In the case of the forward reaction experiments using oleate and ATP as the variable substrates, we suspect this mixed pattern of inhibition is due to overlap between the fatty acid and ATP binding sites overlapping following ligand binding to facilitate the formation of the acyl-adenylate. In the case of the product inhibition studies, the same type of mixed inhibition was seen when using $PP_i$ and oleate as the variable substrates. Here again there is some indication the sites are distinct, but the pattern is more similar to competitive inhibition, which we suggest is the result of independent sites that become overlapped during catalysis.

## DISCUSSION

These studies provide the first comprehensive kinetic evaluation of the highly purified ACSL Faa1p from *S. cerevisiae*. This enzyme catalyses the formation of acyl-CoA by a two step process that proceeds through the formation of an acyl-adenylate intermediate; initial velocity studies are consistent with a Bi Uni Uni Bi Ping Pong mechanism. The sequence and monomeric molecular weight of 77,817 of purified Faa1p was confirmed using ion trap-nanoelectrospray mass spectrometry. Maximal activity of the enzyme was retained when purified in the presence of 0.05% η-dodecyl-β-maltoside (DDM). Moreover, the enzyme retained stability when purified in the presence of ATP and $Mg^{+2}$. In the presence of 0.05% DDM, the enzyme is a homodimer as analyzed using both an analytical sizing column and analytical ultracentrifugation. The linear range of enzyme activity was from 0.5–3μg purified Faa1p. The loss of linearity above 3μg of enzyme may be the consequence of the requirement of this enzyme to associate with the membrane during catalysis. The enzyme has a high affinity for long chain fatty acids and is active over a range of pH consistent with the fatty acid anion as the preferred substrate. Previous work has shown Faa1p has a preference for fatty acid substrates ranging from $C_{10}$-$C_{18}$. The preferred substrates for Faa1p are *cis*-monounsaturated long chain fatty acids; this enzyme also activates *trans*-monounsaturated, tetradecenoic acids, but with more selectivity. For Faa1p, the activity toward *E4* and *E6* compounds were comparable to the *cis* isomers, while *E2* and *E5*were not activated [7]. While Faa1p is insensitive to triacsin C, the AMP-palmitate analogue adenosine 5'-hexadecylphosphate (AMPC16) inhibits activity [27]. AMPC16 mimics the adenylated intermediate formed in the enzyme reaction and in addition results in the accumulation of free fatty acids and disruption of membrane integrity [28]. In the present work we showed oleoyl-CoA synthetase activity is essentially eliminated in the presence of the nucleotide analogues AMPCPP and AMPPCP. These studies collectively support the conclusion that Faa1p requires ATP for catalysis and

that this reaction proceeds through the formation of the acyl-adenylate. Using a rat microsomal preparation of acyl-CoA synthetase Bar-Tana et al. [20] concluded the reaction was Bi Uni Uni Bi Ping Pong, a mechanism that is generally accepted for both acetyl and acyl-CoA synthetases. In the context of ACSLs, the present work used a highly purified preparation of Faa1p, which was extensively characterized and verified the enzyme reaction mechanism. While the work is fundamentally descriptive in nature, this work is essential to direct studies in understanding how these enzymes function in the vectorial transport and activation of exogenous fatty acids.

The purification of Faa1p posed a considerable challenge as this enzyme could not be purified and maintained in a homogeneous state in the absence of a nonionic detergent. Our early attempts to purify Faa1p in the absence of detergent resulted in the formation of higher order aggregates, which based on analytical ultracentrifugation were generally 8-mers, 16-mers and 32-mers as opposed to dimers. This type of behavior is reminiscent of membrane-bound and–associated proteins. Like several other ACSLs that have been described, Faa1p is likely to be membrane associated. Total acyl-CoA synthetase activity from rat microsomes is maximal when assayed in the presence of nonionic detergent or phospholipid [29]. The *E. coli* ACSL FadD also prefers a hydrophobic environment for maximal activity [30]. We have previously demonstrated Faa1p functions in the vectorial acylation of exogenous long chain fatty acids [3,13]. Of particular note was the finding that Faa1p and the fatty acid transport protein Fat1p form a functional complex at the plasma membrane. We suggest dimeric Faa1p functions at the membrane surface to abstract fatty acids as part of its catalytic cycle.

This work has defined the kinetic parameters of the highly purified ACSL Faa1p, which are essential to understand the catalytic patterns of this enzyme and moreover how it functions in the activation and trafficking of long chain fatty acids. Current studies stemming from our ability to purify Faa1p and maintain the enzyme in a monodispersed state are directed towards defining the structure of this long chain acyl-CoA synthetase.

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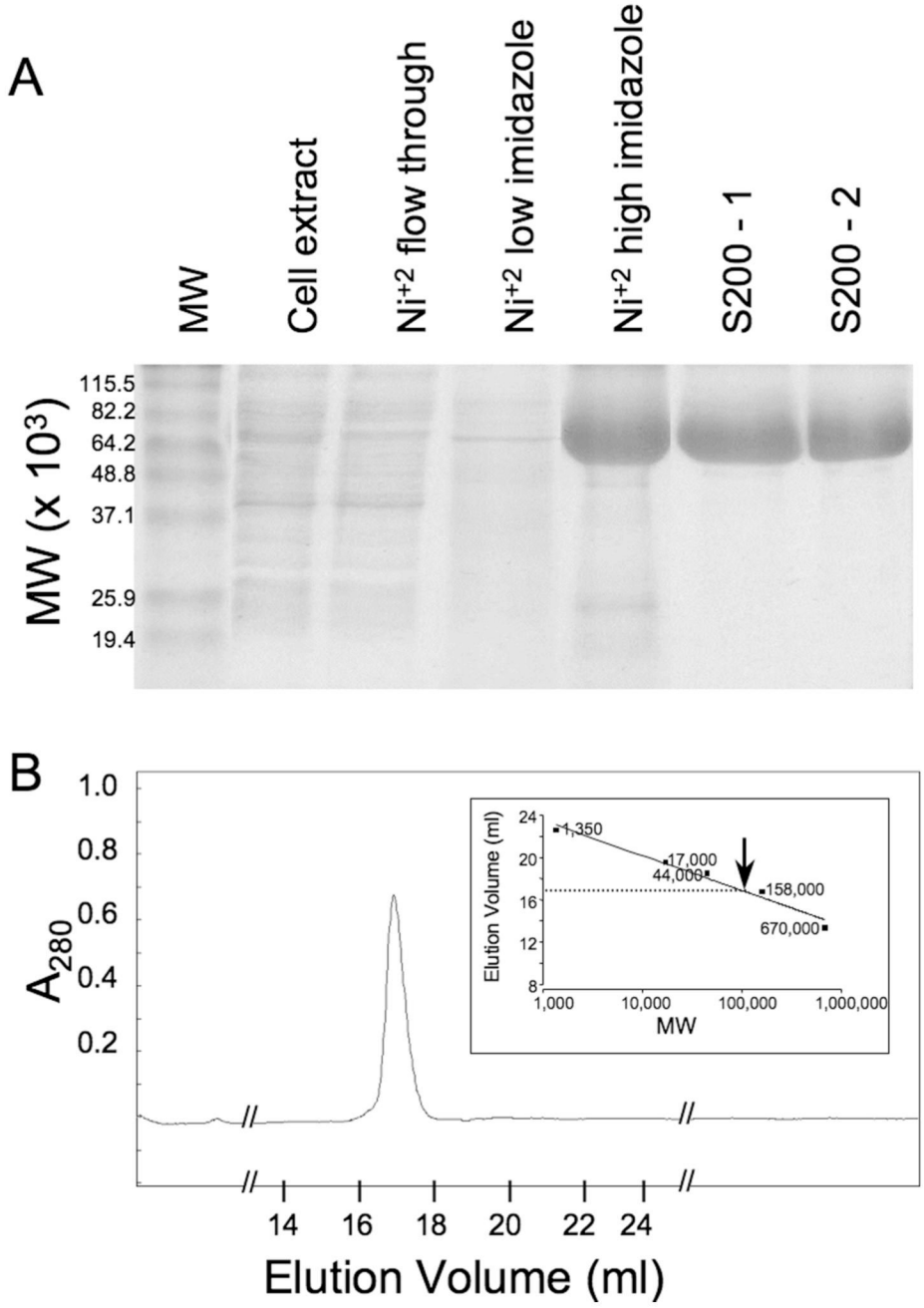


**Figure 1. Purification of the long chain acyl-CoA synthetase Faa1p following expression in E. coli**

A. SDSPAGE of protein during the different steps of purification (S200-1 and S200-2 represents combined and concentrated fractions from the Faa1p peak from the S200 column); MW – molecular weight standards (indicated on the left). B. Determination of the native molecular weight of purified Faa1p in 50mM Hepes, pH7.4; 1mM ATP, 5mM $MgCl_2$, 0.5M NaCl, 10% glycerol, 0.05% DDM using an analytical size exclusion column (Superose™ 6 10/300 GL). The insert shows the elution position of Faa1p at ~17ml (arrow) relative to the standards used to calibrate the column (bovine thyroglobulin (670,000), bovine gamma globulin (158,000), chicken ovalbumin (44,000), horse myoglobin (17,000) and vitamin B-12 (1,350).

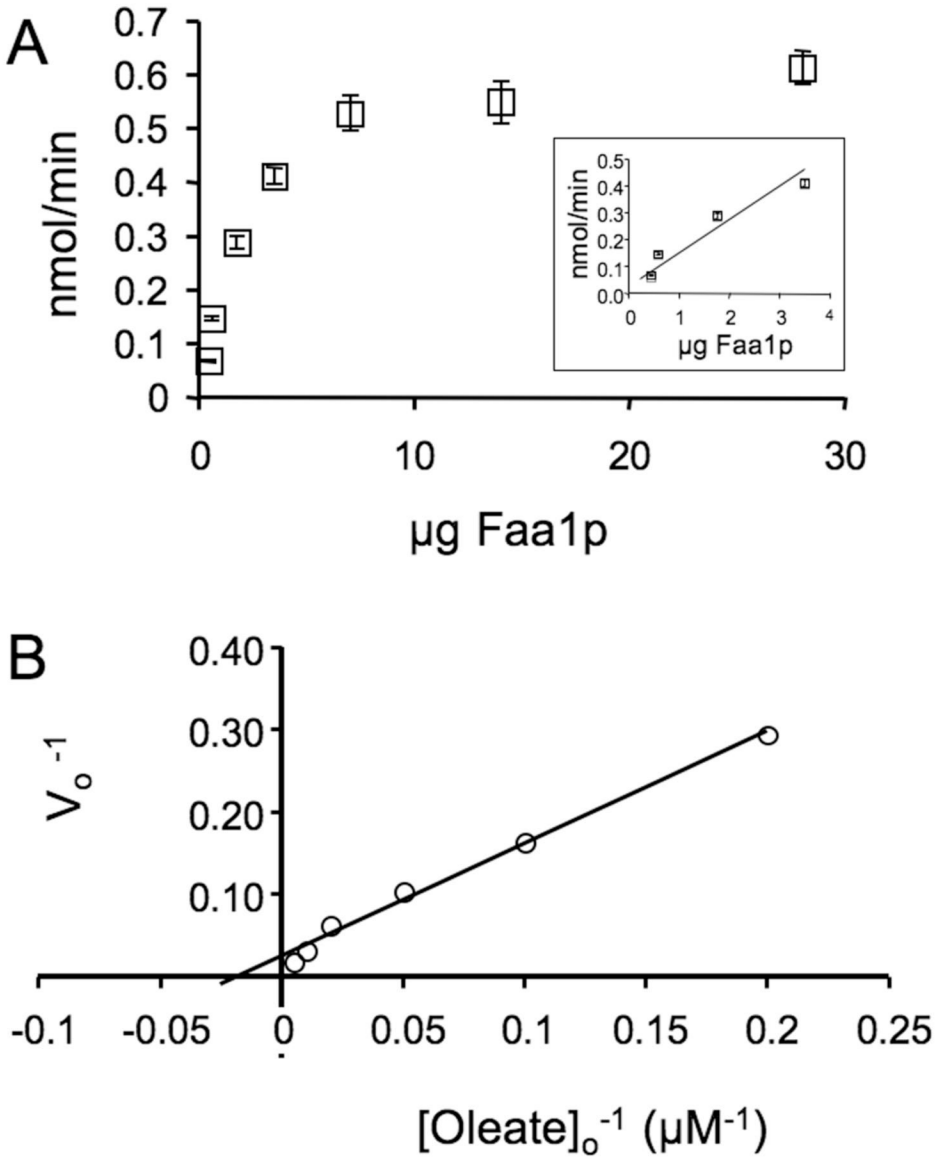


**Figure 2. Characteristics of purified Faa1p**

A. Acyl-CoA synthetase activity as a function of enzyme concentration; insert illustrates the linearity of the reaction between 0.5 and 3µg of enzyme. B. Lineweaver-Burke plot of the enzyme reaction as a function of varying oleate ($C_{18:1}$) concentration (5–200µM) using 1.5µg of purified enzyme. ATP and CoA concentrations were held constant at 10mM and 200µM respectively. $V_o^{-1}$ values are $(nmol/min/mg\ protein)^{-1}$.

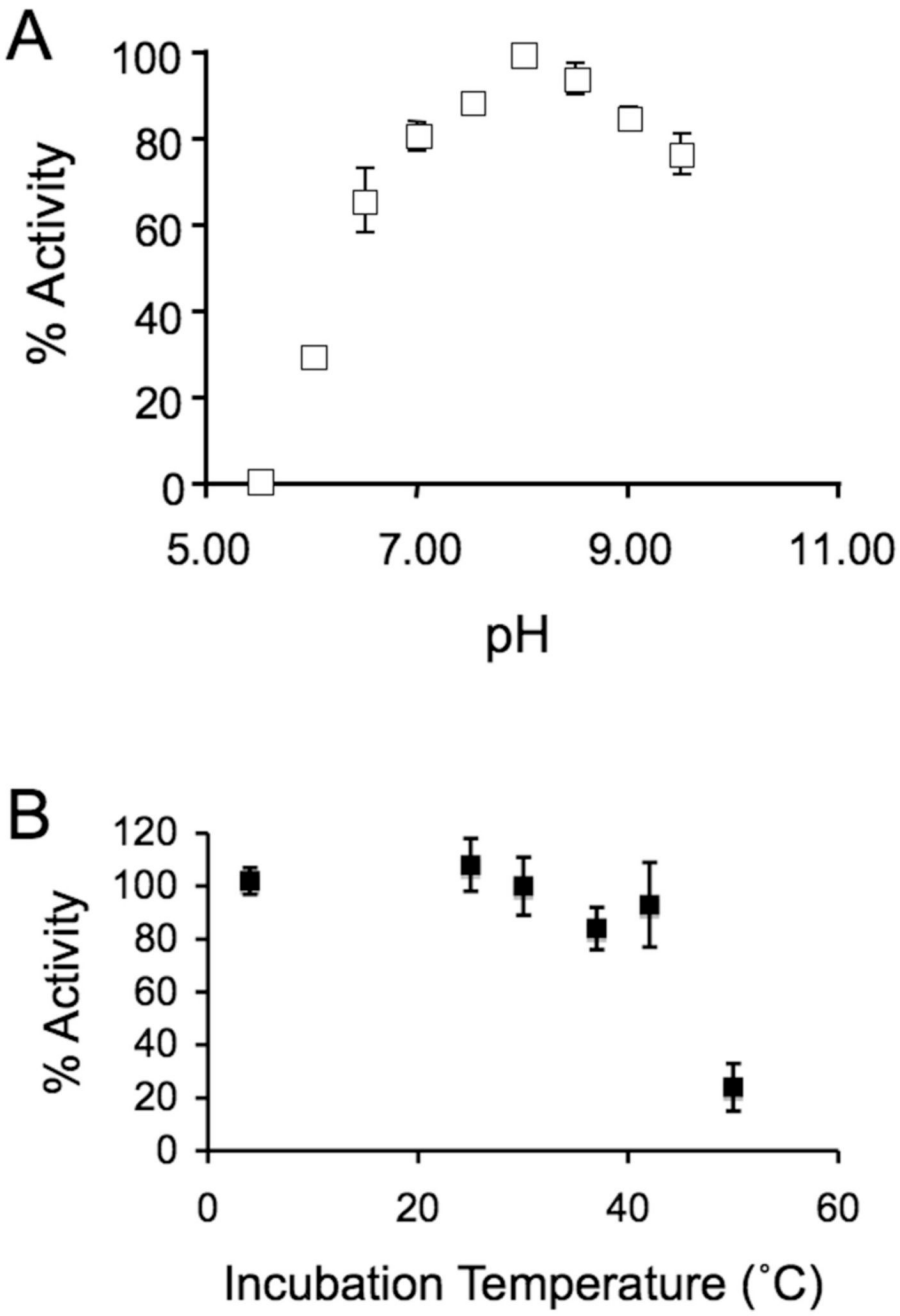


**Figure 3. Activity and stability of purified Faa1p**

A. Enzyme activity was measured over a range of pH values using 1.5μg purified Faa1p; the activity at pH of 8.0 was set to 100% (163.7±12.9 nmol/min/mg protein). B. The thermal stability of Faa1p (1.5μg) was determined following incubation at the noted temperatures for 30min at which time activity was measured; the activity of Faa1p when maintained at 30°C was set to 100% (157.5±17.3 nmol/min/mg protein). The error bars are the standard error of the mean (n=3).

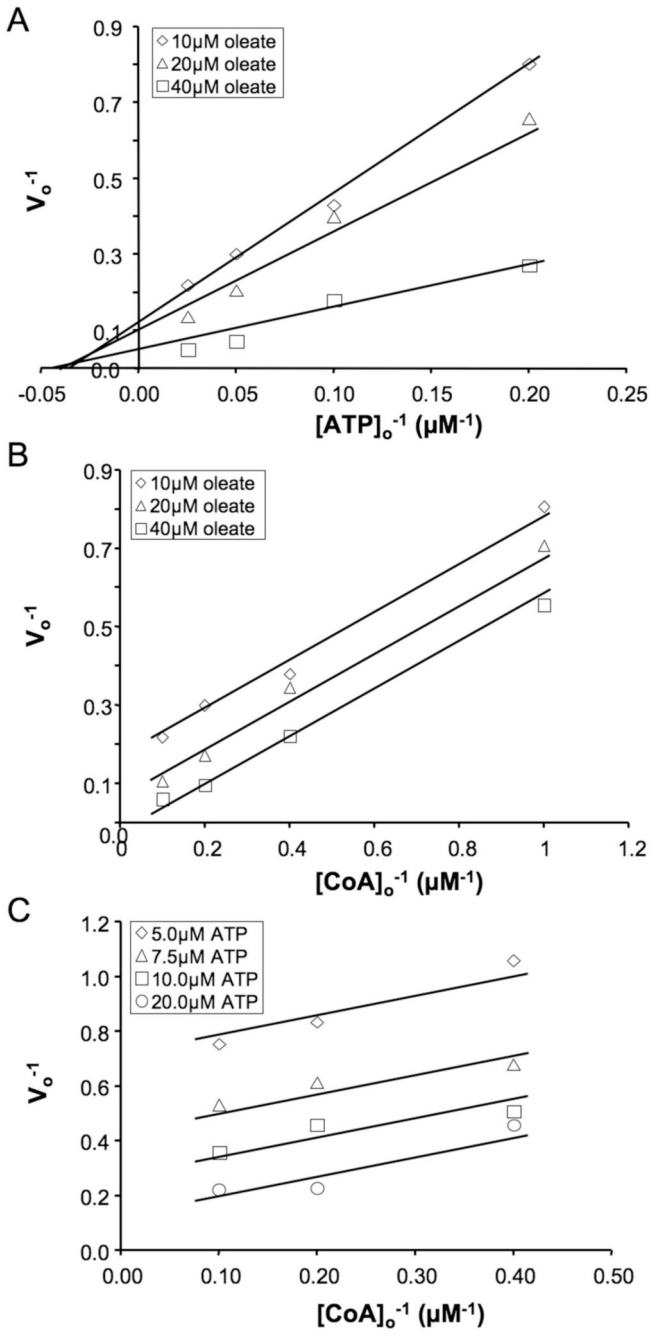


**Figure 4. Initial velocity studies of the acyl-CoA synthetase reaction using 1.5μg purified Faa1p**

Double reciprocal plots where A. CoA concentrations were held constant at 200μM and ATP ($\mu M^{-1}$) and oleate (μM) concentrations varied as indicated; B. ATP concentrations were held constant at 10mM and CoA ($\mu M^{-1}$) and oleate (μM) concentrations varied as indicated; and C. Oleate concentrations were held constant at 200μM and CoA ($\mu M^{-1}$) and ATP (μM) concentrations varied as indicated. $V_o^{-1}$ values are (nmol/min/mg protein)$^{-1}$. Shown are representative experiments of an n=4.

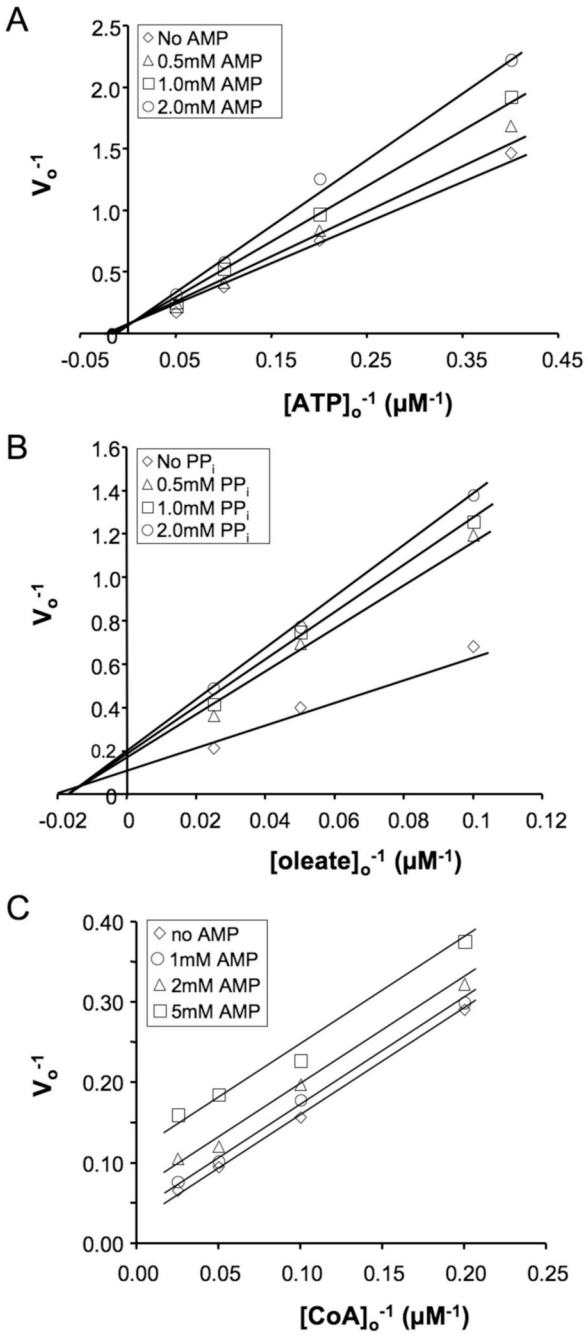


**Figure 5. Initial velocity studies of the acyl-CoA synthetase reaction in the presence of end products using 1.5μg purified Faa1p**

Double reciprocal plots where A. CoA and oleate concentrations were held constant at 20μM and 50μM respectively and the concentrations of ATP ($\mu M^{-1}$) and AMP (mM) varied as indicated; B. CoA and ATP concentrations were held constant at 20μM and 5μM respectively and the concentrations of oleate ($\mu M^{-1}$) and pyrophosphate ($PP_i$) (mM) varied as indicated; and C. ATP and oleate concentrations were held constant at 100μM and 50μM respectively and the concentrations of CoA ($\mu M^{-1}$) and AMP (mM) varied as indicated. $V_o^{-1}$ values are $(nmol/min/mg\ protein)^{-1}$. Shown are representative experiments of an n=4.

**Table I**

Cation requirement for long chain acyl-CoA synthetase activity

| Added Cation | Specific Activity (±SE)[1] (nmol/min/mg protein) | %[2] |
|---|---|---|
| 10mM $MgCl_2$ (normal assay) | 145.5 (12.5) | 100 |
| 10mM $MnCl_2$ | 121.9 (9.1) | 84 |
| 10mM $CoCl_2$ | 56.3 (3.8) | 39 |
| 5mM $MgCl_2$ + 5mM $MnCl_2$ | 141.2 (6.8) | 97 |
| 5mM $MgCl_2$ + 5 mM $CoCl_2$ | 62.1 (9.0) | 42 |

[1] SE – standard error of the mean, n=4

[2] Compared to the activity under normal assay conditions (50mM Tris/HCl, pH 8.0, 10mM ATP, 10mM $MgCl_2$, 0.3mM DTT, 0.01% Triton X-100, 50μM [$^3$H] oleate and 200μM CoA).

**Table II**

Kinetic parameters of Faa1p from initial velocity experiments

| | |
|---|---|
| *Forward reaction* | |
| Varying CoA and ATP concentrations; concentration of oleate (50μM) held constant | |
| $K_m$ | 18.3μM CoA |
| $K_m$ | 51.6μM ATP |
| $V_{max}$ | 22.5nmol/min/mg |
| *Product inhibition* | |
| Varying $PP_i$ and oleate concentrations; ATP (5μM) and CoA (20μM) concentrations held constant | |
| $K_m$ | 83.0μM oleate |
| $V_{max}$ | 13.5nmol/min/mg (in the absence of $PP_i$) |
| $K_i$ | 2.1mM $PP_i$ |
| $K_i$' | 1.4mM $PP_i$ |
| Varying AMP and CoA concentrations; ATP (100μM) and oleate (50μM) concentrations held constant | |
| $K_m$ | 34.4μM CoA |
| $V_{max}$ | 27.3nmol/min/mg (in the absence of AMP) |
| $K_i$ | 5.2mM AMP |