Structure basis of the unidirectional catalysis of long chain fatty acyl-CoA synthetase.
- Toward the vectorial movement across the membrane

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Abstract
Due to the biological importance of long chain fatty acyl-CoA synthetase (ACSL), ACSL has been studied extensively in the past quarter century. ACSL is a ubiquitous enzyme from bacteria to mammal, and its product, long chain fatty acyl-CoA, is well known to be utilized for the energy metabolism. Furthermore ACSL participates in the cellular uptake of the exogenous...
fatty acids. The unique catalytic mechanism, by which the fatty acyl-CoA as the final product is formed unidirectionally via the stable intermediate fatty acyl-AMP, is behind the biological functions of ACSL. In this manuscript, the unique catalytic mechanism is reviewed with respect to 3-dimensional structure of ACSL.

Introduction

Organisms have four types of acyl-CoA synthetase (ACS) deferent in the substrate specificity; acetyl-CoA synthetase (ACSS) (EC 6.2.1.1) preferring C2-C4 fatty acid [1], medium chain fatty acyl-CoA synthetase (ACSM) (EC 6.2.1.2) preferring C4-C12 fatty acid [2,3], long chain fatty acyl-CoA synthetase (ACSL) (EC 6.2.1.3) preferring C12-C22 fatty acid [4], and very long chain fatty acyl-CoA synthetase (ACSVL/FATP) preferring fatty acids with C22 or longer aliphatic chain [5]. Each ACS is reported to have separate role each other. ACSS participates in biosynthesis of fatty acid and cholesterol through the production of acetyl-CoA [6], ACSM catalyzes the conjugation of xenobiotic carboxylic acid with CoA in a biological detoxification mechanism [7]. Long chain fatty acyl-CoA synthesized by ACSL not only participates in the energy metabolism but also acts as a lipid mediator regulating various cellular functions [8-15]. Furthermore ACSL is reported to be a target of therapeutic agent for type 2 diabetes [16]. ACSVl is a membrane protein and has two separate enzyme activities [5]. One is the activity as ACSVl which catalyzes the esterification between very long chain fatty acid and CoA. The other is the fatty acid transport activity to absorb exogenous free fatty acid beyond cell membrane. Interestingly the objective fatty acid of the fatty acid transport activity of ACSVl is not for very long chain fatty acid but for long chain fatty acid [17-19]. Thus it is suggested that the fatty acid transport activity of ACSVl enhances synthesis of the long chain fatty acyl-CoA by ACSL synergistically at the extracellular environment with low concentration of long chain fatty acids [20,21].

The biological functions of ACSLs would be based on the molecular functions of ACSL in various cells, and thus the molecular functions of ACSL has been studied extensively in a passed quarter century. These studies shows ACSL belongs to the adenylate forming enzyme superfamily which has an acyl-adenylate intermediate in the enzyme catalysis commonly. The formation of the fatty acyl-CoA by ACSL proceeds by two reaction steps with the Bi Uni Uni Bi Ping-Pong mechanism, in which the fatty acyl-adenosine monophosphate (AMP) as the intermediate is synthesized and used unidirectionally, in other words the externally added fatty acyl-AMP is utilized for neither the forward nor reverse reactions [4,22,23]. Furthermore the fatty acyl-AMP had not been isolated experimentally. The unidirectional catalysis of ACSL is the
characteristic feature of ACSL catalysis in contrast to the catalytic features of ACSS and ACSM [3,24-26].

X-ray crystallographic analysis of ACSL from Thermus thermophilus HB8 (ttACSL) has elucidated that ttACSL consists of the large N-terminal and the small C-terminal domains connected by a short linker peptide, and the crystal structure provides the structure basis of the unidirectional proceeding of the two step reaction of ACSL [27]. The essence of the unidirectional two step catalysis is that the structural changes of ACSL upon the ATP binding. The prominent structural changes are that the C-terminal domain becomes the closed conformation and the gate opening of the fatty acid binding tunnel. The fatty acid binding tunnel pass through the large N-terminal domain, and the unidirectional movement of the fatty acid appear to be an appropriate feature for the biological function of ACSL, such as the fatty acid uptake of cells [28-30].

**Biological roles of ACSL**

ACSL exists widely in various organisms from bacteria to mammal, and plays pivotal roles through production of long chain fatty acyl-CoA. The well known role of ACSL is that ACSL catalyses the first step of fatty acid degradation for energy production, such as β-oxidation [31,32]. At the first step of β-oxidation ACSL synthesizes fatty acyl-CoA from fatty acid substrate, and subsequently adenosine tri-phosphates (ATP) are generated concomitantly with the degradation of the aliphatic chain of the fatty acyl-CoA by other enzymes participating in β-oxidation [31]. The available molecule for β-oxidation is not fatty acid itself but fatty acyl-CoA ester synthesized by ACSL. Fatty acyl-CoA synthesized by ACSL is also utilized to store excess energy, in which fatty acyl-CoA is recomposed to stable energy storage materials in organisms, such as triglyceride, phospholipids, and cholesterols [33,34]. Fatty acyl-CoA is not only the energy storage material but also the regulatory molecule for the energy provision by β-oxidation. In Escherichia coli, binding of fatty acyl-CoA to FadR, a long chain acyl-CoA-responsive transcription factor, regulates the DNA binding activity of FadR with result that the expression of nine genes involved in fatty acid degradation and biosynthesis are modulated depending on the concentration of fatty acyl-CoA in cells [14]. Furthermore ACSL is involving in the physiological regulation of various cellular functions through the production of fatty acyl-CoA ester, which affects protein transport, enzyme activation, protein acylation, cell signaling [8-10,11,14,35-39], and ACSL is reported to be the target protein of some therapeutic agents to the type 2 diabetes [16]. ACSL is a ubiquitous and essential enzyme to cellular energy metabolism of various organisms and physiological regulation of cellular functions.
There are several isozymes of ACSL distinguished by the tissue distribution and the substrate specificity, and the expression pattern of ACSL isozymes in a tissue would be highly relevant to the tissue specific fatty acid adsorption [40]. A eukaryote cell is reported to have several kinds of genes encoding ACSL isozymes which are different in their substrate specificity and in the tissue dependent expression level. In mammalian cells, five isozymes of ACSL (ACSL1, ACSL3, ACLS4, ACSL5, and ACSL6) are known [41-44]. *Saccharomyces cerevisiae* is reported to have four ACSLs at least; Faa1p, Faa2p, Faa3p, and Faa4p [45-47]. The genome projects on *T. thermophilus* reveals that *T. thermophilus* HB8 contains five genes annotated to encoding long chain fatty acyl-CoA synthetase (TTHA0448, TTHA0586, TTHA0604, TTHA1430, and TTHA1463) [48], and three genes (TTC0079, TTC1065, and TTC1099) for *T. thermophilus* HB27 [49]. The protein corresponding to TTHA0604 (ttACSL, ttLC-FACS) from *T. thermophilus* HB8 has been confirmed to be a long chain fatty acyl-CoA synthetase by the substrate specificity determined experimentally (Fig. 1) [27]. With respect to having isozymes, *T. thermophilus* is similar to *S. cerevisiae* and mammalian cell than *E. coli*, which has only ACSL, FadD [14].

The five ACSLs in mammalian cell are divided into two sub-families depending on the amino acid sequence similarity and the substrate specificity. ACSL1, ACSL5 and ACSL6 are in a sub-family preferring saturated fatty acids with broad chain length [41,44]. ACSL3 and ACSL4 are in the other

![Figure 1. Acyl chain length specificity of ttACSL. Acyl chain length specificity of ttACSL was measured with an enzyme-coupled assay at 25°C. Saturated fatty acids of a variety of chain lengths; C8 (caproate: 8), C10 (decanoate: 10), C12 (laurate: 12), C14 (myristate: 14), C16 (palmitate: 16), C18 (stearate: 18) and C20 (arachidate: 20) were assessed as well as the C14 (myristoleic acid: 14u) and C16 (palmitoleic acid: 16u) unsaturated fatty acids at concentrations of 250 µM, 500 µM and 750 µM. The mean value +/- standard deviation from n=3 experiments is shown on the graph.]
sub-family preferring highly unsaturated fatty acids [42,43]. ACSL3 accepts more wide range of unsaturated fatty acids than ACSL4 preferring arachidonate and eicosapentaenate. The tissue distribution of each ACSL isozyme is as follow. ACSL1 with broad substrate specificity is expressed abundantly in the liver, heart, and adipose tissue in which various fatty acids are used for energy production and storage [50]. ACSL3 and ACSL6 are expressed highly in brain, and the amount of mRNA of these two ACSLs change in the course of the development of rat brain [42,51]. ACSL4 preferring arachidonate and eicosapentaenate is expressed abundantly in tissues responsible for steroid synthesis, especially adrenal gland and ovary [43]. ACSL5 utilizing wide range of unsaturated fatty acid exists much abundantly in the small intestine absorbing various type of fatty acid from diet [44]. Together with these information cell appears to selectively express ACSL isozymes with appropriate substrate specificity to utilize both endogenous and exogenous essential fatty acids. Thus the biological functions of ACSL are closely relevant to the molecular function for the esterification catalysis and the structural feature determining the substrate specificity.

Three dimensional structure of ACSL

The crystal structure of ACSL from *T. thermophilus* HB8 (ttACSL) corresponding to TTHA0604 gene have been determined by X-ray crystallographic analysis at SPring-8 [27]. The determined structures were the uncomplexed form (PDB ID: 1ULT), the adenosine 5’-(β,γ-imido) triphosphate (AMP-PNP) bound (PDB ID: 1V25) and the myristoyl-AMP bound (PDB ID: 1V26) forms. In all structures two ttACSLs form a tight dimer with much large contact surface and domain swapping structure (Fig. 2A and 2B). The monomers of the dimer interacts at their N-terminal domains with much large contact surface area of 3,600Å² for each monomer in a manner of two fold symmetry. Each ttACSL monomer swaps the eight residues from Ala8 to Glu16 into a trench on the surface of the other N-terminal domain (Fig. 2C). Two of the eight swapped amino acid residues participate in the intermolecular bonding with the other N-terminal domain. Asp15 forms an intermolecular salt bridge with Arg176. The main chain carbonyl oxygen of Glu16 forms an intermolecular hydrogen bond with Arg199. Furthermore Glu175 and Arg199 form an intermolecular salt bridge. The amino acid residues involved in the inter-molecular bonding are conserved in ACSLs but not in the other adenylate forming enzymes (Fig. 3). The dimer would be the functional unit, as described below. These results suggest that the formation of tight homo dimer would be characteristic feature common in ACSLs.
Figure 2. Crystal structure of ttACSL. (A) Ribbon representation of the ttACSL dimer are shown. In the panel, the secondary structure of the C-terminal domain are in green. In the N-terminal domain, $\alpha$-helix and $\beta$-sheet are colored in blue and red, respectively, and the swapped peptide in the N-terminal domain is in yellow. (B) The electrostatic potential surface of ttACSL in the same orientation of panel A. Blue and red surfaces represent the positively and negatively charged surface. (C) The amino acid residues with pink carbons and cyan surface interact to the trench on the other monomer with yellow surface. The inter-domain bonds are shown by white dashed lines (D) The monomer of ttACSL with each secondary structure feature is labeled according to the scheme of Fig. 3B.

At the center of the opposite side to the swapped peptide of the homo dimer, there is a positively charged valley, due to twelve basic amino acid residues (six amino acid residues per each monomer in the two fold dimer: Lys219, Arg296, Arg297, Arg321, Lys350, and Lys354). The complexed crystal structures show that the entrance of the fatty acid binding tunnel opens at the positively charged central valley. It implies that the attractive force between the positive charges in the central valley and the negative charge of the carboxyl head group of a fatty acid involves in the substrate uptake to the fatty acid binding tunnel, and the homo dimer is a biological functional unit.
Figure 3. Amino acid sequence alignments of ACSLs and adenylate forming enzymes.
A: Sequence alignment based on the known structures of adenylate forming enzymes; DhbE (pdb code: 1mdb), PheA (1amu), ACS (ACSS; 1pg3), Luci (luciferase; 1lci) and ttACSL (ttACSL; this work). The shaded and underlined residues correspond to α-helical and β-strand, respectively. The residues conserved among all the sequences are indicated by the boxed regions. The bar above the sequence corresponds to the linker region. G-, A- and L-motifs in ttACSL and luciferase are indicated by the gray boxes, and the filled triangle under the sequence denotes the gating residue of Trp234 of ttACSL. B: Multi-sequence alignment of ttACSL (ttACSL: this work), human (LCFA_HUMAN: P41215).
yeast (LCF1_YEAST: P30624) and E. coli (LCFA_ECOLI: P29212). Regions marked with thin bars and shaded bars correspond to β-strand and α-helix respectively. The boxed areas denoted with bold letters correspond to conserved motifs of ACSLs; Gate, Adenine and Linker motifs as well as P-loop. Square dots, open circles, filled circles and filled triangles indicate residues believed to be involved in dimer formation, fatty acid binding, magnesium ion binding and adenylate binding respectively.

Each monomer of ttACSL is composed of a large N-terminal domain (residues 1-431) and a small C-terminal domain (residues 438-541) that are connected by a six amino acid peptide linker (residues 432-437) (Fig. 2D). The N- and C-terminal domains adopt open and close conformations depending on ATP binding at the ATP binding site between the N- and C-terminal domains.

Although the overall amino acid sequence identity between ttACSL and other ACSL is about 20% or less, there are highly three conserved regions, of which the regions from Thr184 to Lys192 and from Gly323 to Thr329 in the N-terminal domain are the P-loop as the phosphate binding motif and the Adenine(A)-motif as the ATP binding motif, respectively (Fig. 3B) [27]. These two motifs are commonly observed in the adenylate forming enzymes which form adenylated intermediate in their catalysis (Fig. 3A), and are called the ATP-AMP signature motif collectively [52]. The crystal structure of AMP-PNP bound ttACSL complex shows that the amino acid residues belonging to the motifs are essential functionally with respect to the ATP binding (Fig. 4). The side chains of Thr184 from the P-loop and Glu328 from the A-motif are the binding site of an essential Mg$^{2+}$ ion. The bound Mg$^{2+}$ ion and the side chain hydroxyl group and main chain nitrogen of Thr327 bind to the oxygen of α-phosphate of bound AMP-PNP in the complex structure. Furthermore the main chain carbonyl of Gly323 forms a hydrogen bond to the amino group of the adenine ring, and the adenine ring interacts to the aromatic side chain of Tyr324 directly. The other conserved region from Asp432 to Leu437 is the linker region by which the two domains of ttACSL are connected, and is designated as the Linker(L)-motif. The L-motif is a C-terminal part of the fatty acyl-CoA synthetase (FACS) signature motif [52]. The side chain of Lys435 in the L-motif interacts to the hydroxyl group at C3 position of ribose ring in the AMP-PNP complex structure (Fig. 4). This interaction between the L-motif and the bound nucleotide would be responsible for that the C-terminal domain adopts the open and close conformations depending on the AMP-PNP binding as described below. The crystal structure of ttACSL reveals the roles of the characteristically conserved regions in ACSLs.
Figure 4. Schematic diagram of the polar interactions between ttACSL and the bound AMP-PNP. The crystal structure of the AMP-PNP bound ttACSL has been determined at 2.3Å resolution. The polar interactions participating in the binding of AMP-PNP are shown by dashed lines.

Two step catalysis of ACSL

ACSL catalyzes a magnesium dependent multi-substrate reaction, resulting in the formation of fatty acyl-CoA [4,53]. The reaction requires ATP, fatty acid, and CoA with an overall reaction scheme as described in reaction 1.

\[
\text{fatty acid} + \text{ATP} + \text{CoA} \rightarrow \text{fatty acyl-CoA} + \text{PPi} + \text{AMP} \quad \text{reaction 1}
\]

The ACSL family catalyzes the formation of fatty acyl-CoA in two discrete steps: 1) the formation of a fatty acyl-AMP as an enzyme bound intermediate (reaction 2), and 2) the formation of a fatty acyl-CoA molecules as the final product (reaction 3).

\[
\text{fatty acid} + \text{ATP} \rightarrow \text{fatty acyl-AMP} + \text{PPi} \quad \text{reaction 2}
\]

\[
\text{fatty acyl-AMP} + \text{CoA} \rightarrow \text{fatty acyl-CoA} + \text{AMP} \quad \text{reaction 3}
\]

The esterification of fatty acids by ACSL has been proposed to proceed via Bi Uni Uni Bi Ping-Pong mechanism based on extensive kinetic studies of rat enzyme [22,54], and the proposed mechanism would be common in ACSs [6,55].
Interestingly the two step fatty acyl-CoA synthesis catalyzed by ACSL proceeds unidirectionally [23,56]. In another word the external fatty acyl-AMP intermediate has not been utilized as substrate for the second step (reaction3) or as product for the first step (reaction2) in reverse catalysis in constant to the acetyl-AMP or butyryl-AMP for ACSS or ACSM [24-26]. In fact, the AMP-PNP bound ttACSL crystals should be dipped into the solution containing myristate in order to synthesize myristoyl-AMP from the bound AMP-PNP per se in the crystalline ttACSL, resulting in formation of the myristoyl-AMP complex ttACSL crystals [27]. The crystal structures of ttACSL with and without ligand show that the structural changes to the closed-form of ACSL induced by the ATP binding would be the structural determinant of the unidirectional fatty acyl-CoA synthesis by ACSL. The structural changes of ACSL upon the ATP binding are the fixation of the C-terminal domain with the closed conformation and the gate opening of the fatty acid binding tunnel.

Fixation of the C-terminal domain with ATP-binding

The ATP dependent closed conformation would be maintained through the whole catalytic reactions (Fig. 5A), and it means that the bound ATP and fatty acyl-AMP intermediate are unlikely to be released from ACSL until the completion of the two step catalyses. It is consistent with that the fatty acyl-AMP intermediate has not been isolated experimentally for the last quarter century. The C-terminal domains of the AMP-PNP complex ttACSL and the myristoyl-AMP complex ttACSL are in the same closed conformation with direct interaction formed between the C- and N-terminal domains, in contrast, the C-terminal domains in the apo-form crystal were two types of open conformations without direct interactions between the C- and N-terminal domains (Fig. 5A). The minimal unit of the ttACSL crystal called “crystallographic asymmetric unit” contains a homo-deim er of ttACSL, thus there are two N-terminal and two C-terminal domains in the minimal unit of the crystal. The superimposition of the four N- and C-terminal domains of the AMP-PNP complex structure and myristoyl-AMP complex structure yields the average root mean square deviations of 0.34Å (486 Cα atoms) and 0.57Å (57 Cα atoms), respectively. It support strongly that the closed conformation is maintained through the two-step catalyses. The accessibility of the bound ATP molecule is quite limited in the closed conformation. One path is the funnel cavity newly formed between the N- and closed C-terminal domains (Fig. 5B). The other one is the fatty acid binding tunnel passing through the N-terminal domain described below.

A few hydrogen bonds formed upon the ATP binding would have a dominant role for the closed conformation of the C-terminal domain. The bound AMP-PNP molecule holds the N- and C-terminal domains together through an
Figure 5. Conformational changes induced by ATP binding in ttACSL. (A) The open and closed conformations of the C-terminal domain in the uncomplexed and two complex ttACSL structures. Panels 1 and 2 show the open conformation in the uncomplexed structure. The C-terminal domain conformation is slightly different for the two monomers of the dimer. Panels 3 and 4 show the identical closed conformation of the C-terminal domain for both the AMP-PNP and myristoyl-AMP structures. The back-bone structures of the large N- and small C-terminal domains are presented in cartoon and wire models colored in silver and yellow green, respectively. The bound ligands of AMP-PNP and myristoyl-AMP are shown as space-filled CPK representations. To facilitate the orientation of the C-terminal domain, Lys439 is shown as a stick model, and the β-strand 22 as a cartoon model. (B) The Cα trace of the AMP-PNP complex ttACSL dimer. The N-terminal domains are colored in cyan and blue, and the C-terminal domains in orange and yellow, respectively. The bound AMP-PNP and the fatty acid binding tunnel are represented by a red space filling model and a solid magenta surface, respectively. The entrance of the fatty acid binding tunnel opens in the central valley at the dimer interface. The fatty acid binding tunnel extends through the N-terminal domain from the central concave region to the ATP binding site. The funnel cavity formed between the N-and C-terminal domains and the central valley at the dimer interface is shown in yellow green and orange, respectively.
extensive hydrogen bond networks (Fig. 4). In the C-terminal domain, only two amino acid residues of Lys439 and Trp444 form hydrogen bonds directly to the bound AMP-PNP, whereas the many amino acid residues of the P-loop, A- and L-motifs. Lys439 is well conserved whereas Trp444 is substituted by the similar amino acid residue tyrosine in almost ACSLs (Fig. 3B). Concomitant with the closed conformation of the C-terminal domain, the six linker peptide (L-motif: Asp432-Arg-Leu-Lys-Asp-Leu437) changes its conformation from open one to close one (Fig. 6). Lys435 in the L-motif is conserved in ACSLs and forms hydrogen bond with the 3'-hydroxyl group of the bound ribose ring (Fig. 3A, 4 and 6). Lys435 and Lys439 would be a responsible amino acid reside for the ATP dependent closure of the C-terminal domain.

In the closed conformation three amino acid residues, Glu443, Glu475, and Lys527 in the C-terminal domain stabilize the closed conformation by forming non-covalent interactions with residues of the L-motif (Arg433 and Asp436) and the N-terminal domain (His230) (Fig. 6). In these amino acid residues, Glu443 of the C-terminal domain and His230 of the N-terminal domain are conserved in ACSLs (Fig. 3B) and would form a conserved inter-domain

Figure 6. Open and close conformation of the linker peptide. The linker peptides in the uncomplexed ttACSL and in the ligand complexes ttACSL are shown in blue (open conformation) and violet (close conformation) thick sticks, respectively. The amino acid residues participating in the inter-domain interaction and the ATP binding are presented by ones with cyan carbons respectively, and green carbons, respectively, and the polar interactions are indicated by dashed lines.
hydrogen bond in ACSLs. This inter-domain hydrogen bond between His230 and Glu443 would participate in not only the stable closed conformation but also the ATP dependent opening of the fatty acid binding tunnel as one of the structural determinants of the characteristic unidirectional catalysis of ACSL.

**Fatty acid binding tunnel with open gate upon ATP binding**

A fatty acid binding tunnel passing through the large N-terminal domain is identified in the crystal structure of ligand bound ttACSL (Fig. 5B). There is a positively charged central valley over the ttACSL homo dimer on the other side of the domain swapping (Fig. 2B). The entrance of the fatty acid binding tunnel opens in the central valley. The exit opens at the ATP binding site, and is plugged by the bound AMP-PNP as the end cap of the tunnel (Fig. 5B and 7). The tunnel is composed of a large central pathway that is divided into the distinct two paths, the “ATP path” and “center path” by the indole ring of Trp234. Additionally there is another pocket that branches from the central pathway named the “dead end branch”. All three paths are separated by the indole ring of Trp234 as a gate closing the fatty acid binding tunnel in the apo-form ttACSL. The amino acid residues with a large side chain are the putative gate amino acid residues commonly in the amino acid sequence alignment of ACSL G-motifs (Fig. 3B), meaning that the gate opening would be the commonly essential event to the enzyme activity of ACSLs.

The conformational changes of the side chain of Trp234 and the peptide back bone of seven amino acid residues (Gate(G)-motif: His230-Val-Asn-Ala-Trp-Cys-Leu236) are key events for the gate opening with the AMP-PNP binding. By the AMP-PNP binding the peptide back-bone shifts by 0.9Å in a direction by which the dead end branch becomes wider than that of the apo-form (Fig. 7). Concomitantly with the back-bone shift, the hydrogen bonds are formed between the G-motif and the C-terminal domain. One is the direct inter-domain hydrogen bonds between His230 from the G-motif and Glu443 from the C-terminal domain, and the other is the hydrogen bond between the ring nitrogen of Trp234 and a oxygen of β-phosphate of bound AMP-PNP (Fig. 6 and 7). The amino acid residues of His230 and Glu443 would be the responsible amino acid residues for the conformational change of the G-motif. Because the inter-domain hydrogen bond between His230 and Glu443 is newly formed upon the ATP binding and is the only direct interaction between the C- and N-terminal domains other than the bound ATP mediated interactions (Fig. 3B).
Figure 7. The fatty acid binding tunnel consists of three paths. (1) The ATP path (violet envelop) connects with the ATP binding site. In the AMP-PNP complex structure, the bound AMP-PNP molecule blocks the exit of the ATP path. (2) The center path opens at the central valley of the two-fold dimer and is the entrance pathway for the fatty acid (Fig. 2B and 5B). (3) The dead end branch extends to $\alpha$-helix h. The three paths join near Trp234 (W234). In the absence of ligand, the indole ring of Trp234 closes the junction (green), whereas in the presence of ligand the indole ring (red) opens the fatty acid binding tunnel.

**Unidirectional binding and release of fatty acid and its product fatty acyl-CoA**

The substrate fatty acid should come into the opened fatty acid binding tunnel from the entrance in the positively charged central valley, and the bulkier final product fatty acyl-CoA should be released from the exit of the fatty acid binding tunnel opening at the ATP binding site, that is, the movement of a fatty acid is unidirectional from the central valley to the ATP binding site. The ATP binding is precedent event to the fatty acid binding, because the fatty acid binding tunnel is closed by the indole ring of tryptophane as the gate in the apo-form, which opens upon the ATP binding (Fig. 7). Thus the ATP binding structure, in which the C-terminal domain is the stably closed conformation, is concluded to be the catalytically active structure. In the ATP binding structure, the only entrance of the fatty acid binding tunnel opens at the positively charged central valley and the tunnel penetrates the N-terminal domain, whereas the exit of the fatty acid binding tunnel is plugged by the bound ATP. Once the fatty acyl-AMP or fatty acyl-CoA are formed in the closed conformation of ACSL, the intermediate and final product could not pass back the fatty acid binding tunnel due to the bulky adenine moiety of them, resulting in the release of the final product, fatty acyl-CoA, from the exit at the ATP binding site after reopening of the C-terminal domain.
The electrostatically positive surface of the central valley lined many basic amino acid residues (Lys219, Arg296, Arg297, Arg321, Lys350, and Lys354) (Fig. 2) would contribute to attract fatty acid with a negative charge to the tunnel. The negative-charged carboxyl of the substrate fatty acid would head into the fatty acid binding tunnel in the aligned direction to form a fatty acyl-AMP in the closed-form enzyme, because the fatty acid does not appear to be able to change front in the narrow fatty acid binding tunnel.

**Fatty acid binding tunnel as the determinant of the substrate specificity**

In the myristoyl-AMP bound structure, the aliphatic chain of the myristoyl-AMP kinks at the C9 carbon with a torsion angle of 59°, resulting in that the C1 to C9 carbons and C10 to C14 carbons occupies the ATP path and the dead end branch, respectively, and a water molecule exists at the space between the bottom of the dead end branch and the C14 carbon of the myristoyl-AMP (Fig. 8). The size of the space is about 3.5Å. Thus the depth of the dead end branch meets palmitate longer than myristate by 2.7Å. In fact the substrate specificity of ttACSL is that myristate and palmitate is the best and the second best substrate, respectively (Fig. 1). Laurate shorter than myristate by two carbons and stearate longer than palmitate by two carbons are acceptable but in reduced activities significantly. It is the consistent with the previous result for rat enzyme [4,41]. The depth of the dead end branch would be the determinant of the substrate specificity of ttACSL.

The dead end branch may be contributed to the release of the final product, fatty acyl-CoA. Interestingly the environment of the bottom of the dead end branch is hydrophilic caused by the hydrophilic amino acid residues, His204, Ser209, and Thr214 (Fig. 8). In fact, there were two water molecules in the AMP-PNP bound structure. The hydrophilic surface of the dead end branch surrounds the hydrophobic aliphatic chain of the bound myristate-AMP, and would promote the release of the final product fatty acyl-CoA enthalpically.

The diameter of the entrance of the center path opening at the central valley may be a selectivity filter distinguishing saturate fatty acids from unsaturated ones. The unsaturated fatty acids, myristoleate and palmitoleate, were not the substrate for ttACSL under the assay condition at 25°C (Fig. 1). The diameter of the tunnel appropriate for unsaturated fatty acids would be wider than for saturated fatty acids, because the aliphatic chain of unsaturated fatty acids are more rigid and bulkier than that of saturated ones due to the 9-cis double bond. However, it can not be excluded that ttACSL from extreme thermophilic bacterium T. thermophilus catalyze unsaturated fatty acids at higher temperature expected growing these organism.
Figure 8. Electron density map of bound myristoyl-AMP and Schematic drawing of the polar interactions in ttACSL and bound myristoyl-AMP. (A) The myristoyl-AMP as the intermediate are synthesized in ttACSL and the intermediate is clearly identified in the electron density map contoured at 3σ level. (B) The aliphatic chains from C1 to C9 and from C10 to C14 occupy the ATP path and the dead end branch of the fatty acid binding tunnel, respectively.

Structure bases of ttACSL acylation catalysis

Based on the three high resolution crystal structures including the apo-form and two complexes, the molecular mechanism of ttACSL is proposed to be consistent with the Bi Uni Uni Bi Ping-Pong reaction mechanism. The reaction scheme is summarized as follows.

Firstly, ATP binding dependent induction of the stably closed conformation of the C-terminal domain and the gate opening of the fatty acid binding tunnel (Fig. 9A and 9B). Lys435 in the L-motif and His230 in the G-motif, Lys439 and Glu443 in the C-terminal domain are the key amino acid residues responsible for the ATP dependent closure of the C-terminal domain and the gate opening of fatty acid binding tunnel (Fig. 6). The formation of the direct hydrogen bonds between the amino acid residues (Lys435 in the linker peptide and Lys439 in the C-terminal domain) and the bound ATP would induce a large conformational change of the linker peptides, resulting in the closed conformation of the C-terminal domain. The formation of the inter-domain hydrogen bond between the two amino acid residues, His230 and Glu443 changes the back-bone structure of the G-motif and the side chain conformation of Trp234, resulting in opening the gate of the fatty acid binding tunnel.
Figure 9. The schematic mechanism for the uni-directional ordered catalysis by ttACSL. In the proposed overall catalysis of the acylation, the substrate fatty acid is processed uni-directionally through the fatty acid binding tunnel in the N-terminal domain and funnel cavity at the interface of N- and C-terminal domains from the central valley of the dimer interface. This schematic is based on Fig. 5B. The binding of ATP to ttACSL is the initial event in the catalysis process (A); it is the trigger for both closing the C-terminal domain and the opening and widening of the gated fatty acid binding tunnel (B). The fatty acid binding tunnel conveys the substrate molecule uni-directionally from the positively charged concave in the central valley of the dimer interface to the ATP binding site (B). The pyrophosphate is released after the formation of a fatty acyl-AMP. A CoA then binds to the fatty acyl-AMP complex (C). The thiol group of the bound CoA attacks the acyl carbon of the fatty acyl-AMP (D). Opening the N- and C-terminal domains again, the fatty acyl-CoA and AMP products are released from the ttACSL (E). Over-all catalysis is represented after Cleland\textregistered expression (F).

Secondly, the binding of a fatty acid and the formation of the fatty acyl-AMP intermediate (Fig. 9B). The substrate fatty acid comes into the fatty acid binding tunnel from the central valley due to the attractive force between the counter-charges of the central valley surface and the carboxylate of the substrate. Twelve basic amino acid residues in the total involves in the positive electrostatic surface of the central valley. In the first catalysis to form the fatty
acyl-AMP intermediate, Thr327 and Thr184, Glu328, and Lys439, Trp444 would stabilize the putative penta-valent transition state at the α-phosphorus (Fig.4). Thr327 from the A-motif forms hydrogen bonds with an α-phosphate oxygen by its back-bone nitrogen and side chain hydroxyl group. Thr184 from the P-loop and Glu328 from the A-motif are the binding site of the essential Mg\(^{2+}\) ion, and the bound Mg\(^{2+}\) forms a charge-charge interaction with an α-phosphate oxygen. Lys439 and Trp444 from the C-terminal domain form hydrogen bonds with an α-phosphate oxygen. These polar interactions delocalize the negative charge growing on the transition state, resulting in the lower energy barrier of the transition state. The pyrophosphate as a product releases from the funnel cavity (Fig.9C). The acyl oxygen of fatty acyl-AMP instead of the oxygen of β-phosphate of AMP-PNP forms a hydrogen bond with Lys439 (Fig. 8). Thus the number of the hydrogen bonds between the C-terminal domain and the bound ligand is the same before and after the first half reaction (reaction2).

Thirdly, binding of a CoA molecule and formation of the final products. The thiol group of CoA comes in the funnel cavity as the only path connecting the intermediate and bulk solvent, and attacks the C1 carbon of the fatty acyl-AMP in the second half reaction (reaction3) (Fig.9C and 9D). Lys439 is the only amino acid residue forming a hydrogen bond directly with the carboxyl oxygen with 2.7Å distance (Fig. 8), and would be catalyze the reaction3 by delocalization of the negative charge on the transition state and stabilization of the negative charge on the products. In the catalysis the intermediate in the enzyme divides into the two products as the fatty acyl-CoA and the AMP.

Finally, re-open the C-terminal domain to release of the products (Fig. 9E). After the completion of the catalysis, the C-terminal domain becomes the open conformation again and the fatty acyl-CoA and the AMP as the final products are released from the ATP binding site. The reduced number of the hydrogen bonds between the C-terminal domain and the AMP as the final product and the steric hindrance between the newly formed acyl group of fatty acyl-CoA and α-phosphate of AMP may be the reason of the re-opening of the C-terminal domain.

This scheme is compatible with the results of the kinetic assays (Fig. 9F). The deeply buried long chain fatty acyl-AMP intermediate is not released from the tightly closed conformation of ttACSL, in contrast to the short and medium chain fatty acyl-CoA synthetases.

**Vectorial transmembrane movement of long chain fatty acid**

One of the important functions of ACSL is the cellular uptake of the exogenous long chain fatty acid by consuming ATP, and the function is called
“vectorial acylation” after the “vectorial phosphorylation” coined by H. R. Kaback for the sugar transport system of *E. coli* [57]. The ACSL of *E. coli*, FadD, is the most extensively studied ACSL with respect to the vectorial acylation [58]. FadD was reported to stimulate the cellular uptake of external fatty acids in ATP dependent manner [59], and the amino acid residues in the ATP/AMP signature motif of FadD are shown to participate in ATP dependent fatty acid uptake [60]. FadD function for the fatty acid uptake was summarized to abstract and activate the exogenous fatty acid [61].

Enzymes involved in the vectorial acylation spread over various organisms, e.g. yeast ACSLs, Faa1p and Faa4p of *S. cerevisiae* [29]; ACSL5 of mammalian cells [30,62]. Faa1p and Faa4p are reported to form a physical complex with fatty acid transport protein, Fat1p, to import exogenous fatty acid, and furthermore Faa1p is shown to be able to substitute for the function of Fat1p [29]. Expression of ACSL5 in glioma cell stimulates cellular uptake of external fatty acids [62], and expression of ACSL5 in *E. coli* knocked-down FadD restores growth of the transformed *E. coli* on oleate as the sole carbon source [30]. These results for ACSL5 are compatible to that ACSL5 expresses in the cells involving in the fatty acid uptake such as intestinal epithelial cells and proliferating preadipocytes [44]. ACSLs should be one of the key enzymes for the cellular uptake of external fatty acids.

The homo-dimer of ttACSL, forming the positively charged central valley at the center of the dimer, appears to be much appropriate for the efficient abstraction of fatty acids from the cell membrane. The un-ionized free fatty acid at a physiological concentration is reported to across the lipid bilayer readily in a manner of free diffusion depending on the concentration gradient of free fatty acids between the interior and exterior against cell membrane [63]. The basic environment would be formed locally between the cytoplasmic surface of cell membrane and the central valley by the adsorption of the ttACSL on the cytoplasmic surface of cell membrane due to the positive charges at the central valley. The basic environment accelerates to deprotonate carboxylic acid of the fatty acid partitioned in the cytoplasmic surface, resulting in the reduction of the re-diffusion rate of the un-ionized fatty acid from the cytoplasmic to the extra-cellular side leaf of cell membrane. Furthermore the attractive force between the counter charges of the carboxylate and the central valley surface also should accelerate suction of fatty acids as the substrate from the cytoplasmic surface leaf of the cell membrane.

**References**