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Enzymatic and Genetic Characterization of Firefly Luciferase and *Drosophila* CG6178 as a Fatty Acyl-CoA Synthetase

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Recently we found that firefly luciferase is a bifunctional enzyme, catalyzing not only the luminescence reaction but also long-chain fatty acyl-CoA synthesis. Further, the gene product of *CG6178* (CG6178), an ortholog of firefly luciferase in *Drosophila melanogaster*, was found to be a long-chain fatty acyl-CoA synthetase and dose not function as a luciferase. We investigated the substrate specificities of firefly luciferase and CG6178 as an acyl-CoA synthetase utilizing a series of carboxylic acids. The results indicate that these enzymes synthesize acyl-CoA efficiently from various saturated medium-chain fatty acids. Lauric acid is the most suitable substrate for these enzymes, and the product of lauroyl CoA was identified with matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS). Phylogenetic analysis indicated that firefly luciferase and *CG6178* genes belong to the group of plant 4-coumarate:CoA ligases, and not to the group of medium- and long-chain fatty acyl-CoA synthetases in mammals. These results suggest that insects have a novel type of fatty acyl-CoA synthetase.

Key words: acyl-CoA synthetase; bifunctional enzyme; bioluminescence; lauric acid; ortholog gene

In firefly, the bioluminescence reaction is catalyzed by luciferase (monooxygenase [EC 1.13.12.7]) with firefly luciferin in the presence of ATP, Mg^{2+}, and O_2.[1–3] The luminescence reaction consists of two steps, the adenylation of luciferin (Eq. 1) and the oxygenation of luciferyl adenylate (LH_2-CO-AMP) (Eq. 2).

$$LH_2\text{-COOH} + \text{ATP} \longrightarrow LH_2\text{-CO-AMP} + \text{PPi} \quad (1)$$

$$LH_2\text{-CO-AMP} + O_2 \longrightarrow \text{Oxyluciferin} + CO_2 + \text{Light} \quad (2)$$

Recently, we found that firefly luciferase has a catalytic function of acyl-CoA (R-CO-S-CoA) synthesis from some unsaturated long-chain fatty acids (R-COOH, C18∼C20) in the presence of ATP, coenzyme A (CoA-SH), and Mg^{2+} (Eq. 3 and 4).[4]

$$\text{R-COOH} + \text{ATP} \longrightarrow \text{R-CO-AMP} + \text{PPi} \quad (3)$$

$$\text{R-CO-AMP} + \text{CoA-SH} \longrightarrow \text{R-CO-S-CoA} + \text{AMP} \quad (4)$$

Thus firefly luciferase is a bifunctional enzyme, not only monooxygenase but also long-chain fatty acyl-CoA synthetase (ACSL). A suitable substrate for luciferase from the North American firefly *Photinus pyralis* was arachidonic acid (C20:4), which showed about 50% catalytic efficiency with firefly luciferin.[4]

Further, we demonstrated that the gene product of *CG6178* (CG6178), an ortholog of firefly luciferase in *Drosophila melanogaster*, has an acyl-CoA synthetic activity. Unsaturated long-chain fatty acids (C18∼C20) were good substrates for CG6178, as in the case of firefly luciferase. α-Linolenic acid (C18:3n-3) was the best as far as we examined.[5] But no luminescence activity or acyl-CoA formation with firefly luciferin were detected.

Various acyl-CoA synthetases (EC 6.2.1.-, Eq. 3 and 4) have been characterized, and were classified by the substrate specificities of carboxylic acids: acetyl-CoA synthetase (AceCS; C2), medium-chain fatty acyl-CoA synthetase (ACSM; C4∼C14), ACSL (C10∼C20), very-long-chain fatty acyl-CoA synthetase (VLCS; ≥ C22), 4-coumarate:CoA ligase (4CL), phenylacetyl-CoA synthetase, *etc.* (http://www.chem.qmul.ac.uk/iubmb/enzyme/).

To identify the type of acyl-CoA synthetase for firefly luciferases (from *P. pyralis* and the Japanese firefly *Luciola cruciata*) and CG6178, the substrate specificities of these enzymes were examined utilizing various carboxylic acids. The results indicate that they have similar specificity of ACSM or ACSL from mammals. On the other hand, phylogenetic analysis of acyl-CoA synthetases suggests that firefly luciferase and CG6178

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Abbreviations: AceCS, acetyl-CoA synthetase; ACSL, long-chain fatty acyl-CoA synthetase; ACSM, medium-chain fatty acyl-CoA synthetase; 4CL, 4-coumarate:CoA ligase; EPA, *cis*-5,8,11,14,17-eicosapentaenoic acid; HETE, hydroxyeicosatetraenoic acid; MALDI-TOF-MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; PTS, peroxisomal targeting signal; VLCS, very-long-chain fatty acyl-CoA synthetase

are not to be classified in the group of ACSM or ACSL. From these results, we concluded that insects have a novel type of fatty acyl-CoA synthetase.

Materials and Methods

Materials. The following materials were obtained from commercial sources: [1-^{14}C]lauric acid (60.0 mCi/mmol, Amersham Biosciences, Buckinghamshire, UK); lauric acid sodium salt, myristic acid sodium salt, propionic anhydride, thiazolidine-2-carboxylic, acid and *n*-hexanoic anhydride (Tokyo Kasei Kogyo, Tokyo); D-proline, benzoic acid sodium salt, and 2-naphthylacetic acid (Aldrich, Milwaukee, WI); linoleic acid, sodium acetate, L-phenylalanine, ferulic acid, 1-naphthylacetic acid, and 2-quinolinecarboxylic acid (Wako, Osaka, Japan); octanoic acid sodium salt, decanoic acid sodium salt, oleic acid sodium salt, palmitoleic acid, arachidonic acid sodium salt, *cis*-5,8,11,14,17-eicosapentaenoic acid (EPA) sodium salt, D-cysteine, thiaproline, *p*-coumaric acid, caffeic acid, γ-linolenic acid, and lauroyl CoA lithium salt (Sigma, St. Louis, MO); L-proline, D-luciferin (firefly luciferin) sodium salt, and DL-α-lipoic acid (Nacalai Tesque, Kyoto, Japan); recombinant *P. pyralis* luciferase (Promega, Madison, WI); [α-^{32}P]ATP (3000 Ci/mmol, NEN Life Science Products, Boston, MA); palmitic acid sodium salt and L-cysteine (Kanto Chemical, Tokyo); α-linolenic acid (NOF, Tokyo); recombinant *L. cruciata* luciferase (Funakoshi, Tokyo); (6*E*,8*Z*,11*Z*,14*Z*)-(±)-5-hydroxyeicosatetraenoic acid (5-HETE), (5*Z*,9*E*,11*Z*,14*Z*)-(±)-8-hydroxyeicosatetraenoic acid (8-HETE), (5*E*,7*Z*,11*Z*,14*Z*)-(±)-9-hydroxyeicosatetraenoic acid (9-HETE), (5*Z*,8*Z*,12*E*,14*Z*)-(±)-11-hydroxyeicosatetraenoic acid (11-HETE), (5*E*,8*Z*,10*Z*,14*Z*)-(±)-12-hydroxyeicosatetraenoic acid (12-HETE), and (5*E*,8*Z*,11*Z*,13*Z*)-(±)-15-hydroxyeicosatetraenoic acid (15-HETE) (Cayman Chemical, Ann Arbor, MI). His$_6$-tagged CG6178 expressed in *Escherichia coli* was purified by Ni-chelating chromatography, as previously described.[5)]

Assay for adenylation activity. Adenylation activity for carboxylic acids was determined by ^{32}P-AMP generated from [α-^{32}P]ATP using TLC analysis.[4,5)] The reaction mixture (20 µl) containing [α-^{32}P]ATP (0.33 µCi), ATP (100 µM), CoA (250 µM), $MgCl_2$ (5 mM), and enzyme (50 nM) in 100 mM Tris–HCl (pH 7.8) was incubated at 25 °C. After 30 min, the reaction was terminated by addition of 20 µl of ethanol, and 2 µl was spotted on a TLC plate (100 × 50 mm, Silica gel 60 F_{254}, Merck). First development was performed in dioxane/50 mM acetic acid (4:1). After drying, second development was in dioxane/ammonium hydroxide/water (6:1:5).[6)] The R_f values for [α-^{32}P]ATP and ^{32}P-AMP formed were identified as 0.17 and 0.51 respectively. The radioactivity of ^{32}P-AMP was measured using an imaging analyzer (BAS 2500, Fuji Film, Tokyo) after exposing for 2 h. Relative intensity of the radioactivity was obtained by subtracting the background value without substrate. Palmitoleic acid, *p*-coumaric acid, caffeic acid, ferulic acid, linoleic acid, α-linolenic acid, γ-linolenic acid, 1-naphthylacetic acid, 2-naphthylacetic acid, and lipoic acid were neutralized by 5 N NaOH before dissolving in water. *n*-Hexanoic acid was dissolved in ethanol to a concentration of 100 mM and then diluted with water for assay. The final concentration of ethanol in the reaction mixture was 0.1%. The concentration of ethanol at 2% in the reaction mixture did not affect the activity (data not shown). Hydroxyeicosatetraenoic acids (HETEs) were dissolved in 2% DMSO, and the assay was performed at 0.2%. The concentration of DMSO at 3% in the reaction did not affect the activity (data not shown). Other substrates were dissolved in water. For sparingly soluble or unstable compounds such as arachidonic acid and EPA, concentrations and purity were analyzed using HPLC before assay (data not shown).

Detection of ^{14}C-lauroyl CoA synthesis. The reaction mixture (20 µl) containing [1-^{14}C]lauric acid (12.0 nCi = 10 µM), ATP (250 µM), CoA (250 µM), $MgCl_2$ (5 mM), and enzyme (50 nM) in 100 mM Tris–HCl (pH 7.8) was incubated at 25 °C. After 1 h, the reaction was terminated by addition of 20 µl of ethanol, and 2 µl was applied to TLC analysis (50 × 50 mm, Silica gel 60 F_{254}). The development was performed in dioxane/ammonium hydroxide/water (3:0.5:2). The radioactivity of ^{14}C-lauroyl CoA was measured on BAS 2500 after exposing for 17 h. Authentic lauroyl CoA on TLC was detected using a 254 nm UV lamp.

Identification of lauroyl CoA by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS). The reaction mixture (500 µl) contained lauric acid (10 µM), ATP (250 µM), CoA (250 µM), $MgCl_2$ (5 mM), and enzyme (50 nM) in 100 mM Tris–HCl (pH 7.8). The reaction was started by addition of the enzyme and incubated at 25 °C for 2 h. It was terminated by adding 333 µl of acetonitrile, and 750 µl was applied to HPLC. Reversed-phase HPLC was performed using a Capcell Pak C18 column (4.6 × 150 mm, Shiseido, Japan) with a linear gradient of 40–70% acetonitrile in 25 mM KH_2PO_4 from 5 to 17 min, followed by 70% acetonitrile for 8 min at a flow rate of 0.8 ml/min. The fractions were monitored using a multi-wavelength detector (195–650 nm; MD-2010 plus, Jasco). The elution time for lauroyl CoA was 11.3 min. MALDI-TOF-MS analysis was performed with AutoFLEX (Bruker Daltonics, Billerica, MA) using 3-hydroxypicolinic acid as a matrix. The data were acquired in the negative reflector mode of operation.

Effect of lauric acid concentration on acyl-CoA synthesis. The reaction and separation conditions were same as those described above section, except for the concentrations of substrate (0 to 100 µM) and enzyme

(5 nM) and the reaction time (10 min). The amount of lauroyl CoA produced was determined by the peak area of HPLC analysis at 260 nm. The linearity between peak area and amount was verified from 0 to 1,000 pmol, using authentic lauroyl CoA.

Phylogenetic analysis. Amino acid sequences were acquired from NCBI Database (http://www.ncbi.nlm.nih.gov/) and FlyBase (http://flybase.bio.indiana.edu/). The phenylalanine-activating subunit of gramicidin synthetase 1 in *Brevibacillus brevis* (PheA)[7,8] and the A domain of δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine synthetase in *Penicillium chrysogenum* (acvA)[7,9] were designated as outgroups. Multiple alignment was achieved with the CLUSTAL X program.[10] The Gonnet series was selected for the protein weight matrix. All sites containing gap were excluded, and 295 characters were included for analysis. Neighbor joining tree was constructed using PAUP* 4.0beta10 (http://paup.csit.fsu.edu/).

Results

Substrate specificity

The substrate specificity of enzymes for acyl-CoA synthesis was determined using a series of carboxylic acids (Fig. 1). The results showed that firefly luciferases of *P. pyralis* and *L. cruciata* exhibited significant activity only with saturated medium-chain and unsaturated long-chain fatty acids. The profiles of specificity were similar to each other (Fig. 2). The most suitable substrate was lauric acid (C12:0), which showed about 4 times higher activity than firefly luciferin. Myristic acid (C14:0) and decanoic acid (C10:0) were also good substrates among saturated medium-chain fatty acids, while octanoic acid (C8:0) and palmitic acid (C16:0) were not used for firefly luciferase. With unsaturated long-chain fatty acids, the longer and more highly unsaturated fatty acids were better substrates for firefly luciferase, except for palmitoleic acid (C16:1).

For the substrate specificity of CG6178, significant activities were found only with saturated medium-chain fatty acids and unsaturated long-chain fatty acids. As with firefly luciferase, lauric acid (C12:0) was the most suitable substrate for CG6178, and several fatty acids (C8∼C20) were used broadly as substrates. HETEs are monohydroxyl fatty acids, produced enzymatically and non-enzymatically from arachidonic acid (C20:4).[11,12] They contain a hydroxyl group in their structure, like firefly luciferin, but they were poor substrates (0 to 30% activity with arachidonic acid) for firefly luciferases. In contrast, some HETEs (5-HETE, 12-HETE, 15-HETE) were good substrates (150 to 180% activity with arachidonic acid) for CG6178 (Fig. 3). The catalytic site of CG6178 for adenylation may have interactive residues with the hydroxyl group of HETE.

All other carboxylic acids were poor substrates for firefly luciferase of *L. cruciata* and *P. pyralis* and

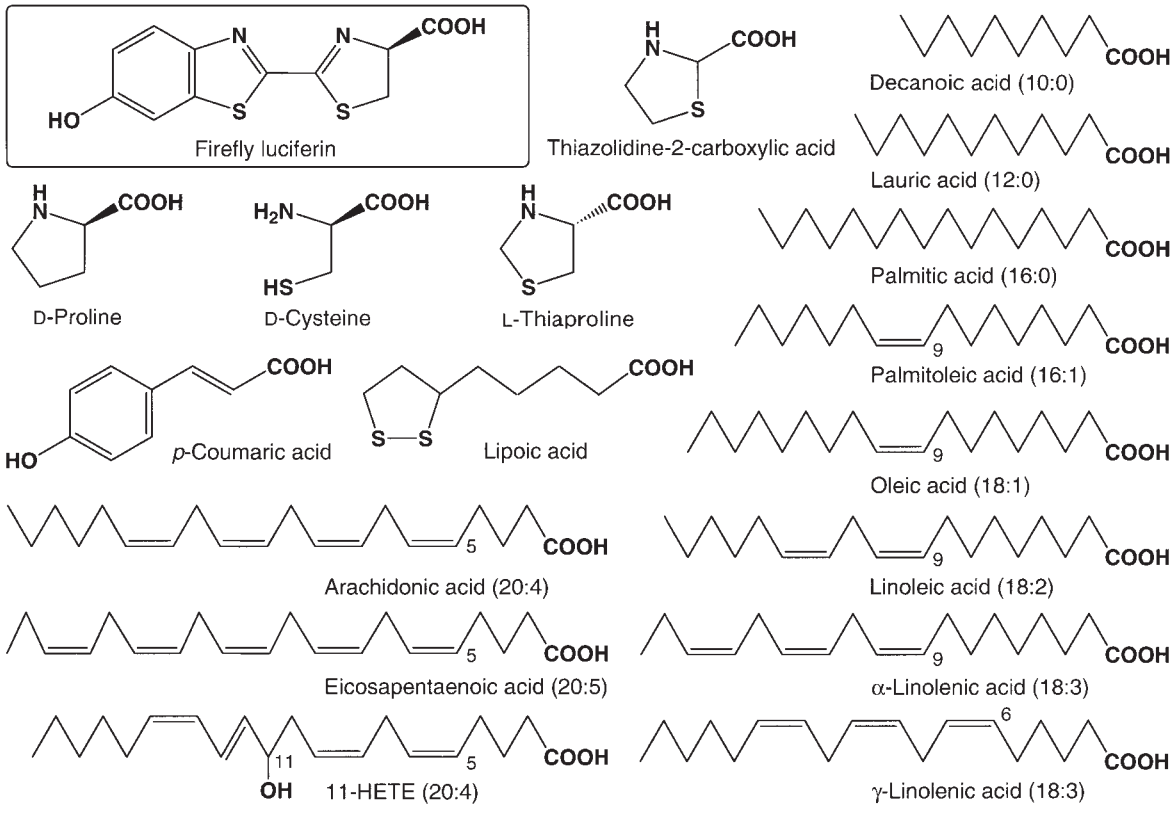

Fig. 1. Structure of Carboxylic Compounds for Substrate Specificity Analysis in Firefly Luciferases and CG6178.

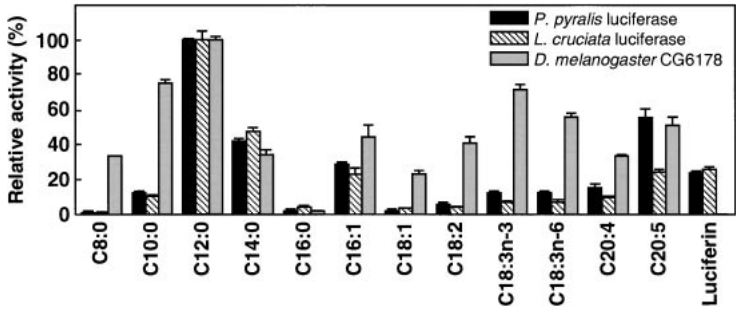

Fig. 2. Substrate Specificity of Firefly Luciferases and CG6178 for Fatty Acids.

Fatty acyl-CoA synthetic activity was determined by the formation of acyl-adenylate from a series of fatty acids. Fatty acyl-adenylate formation was monitored by detection of released ^{32}P-AMP from [α-^{32}P]ATP with TLC analysis. The data represent the means ± SEM for triplicate determinations. Relative activity for each enzyme is expressed as a percentage with respect to lauric acid. Abbreviations are as follows: C8:0, octanoic acid; C10:0, decanoic acid; C12:0, lauric acid; C14:0, myristic acid; C16:0, palmitic acid; C16:1, palmitoleic acid; C18:1, oleic acid; C18:2, linoleic acid; C18:3n-3, α-linolenic acid; C18:3n-6, γ-linolenic acid; C20:4, arachidonic acid; C20:5, EPA; luciferin, firefly luciferin.

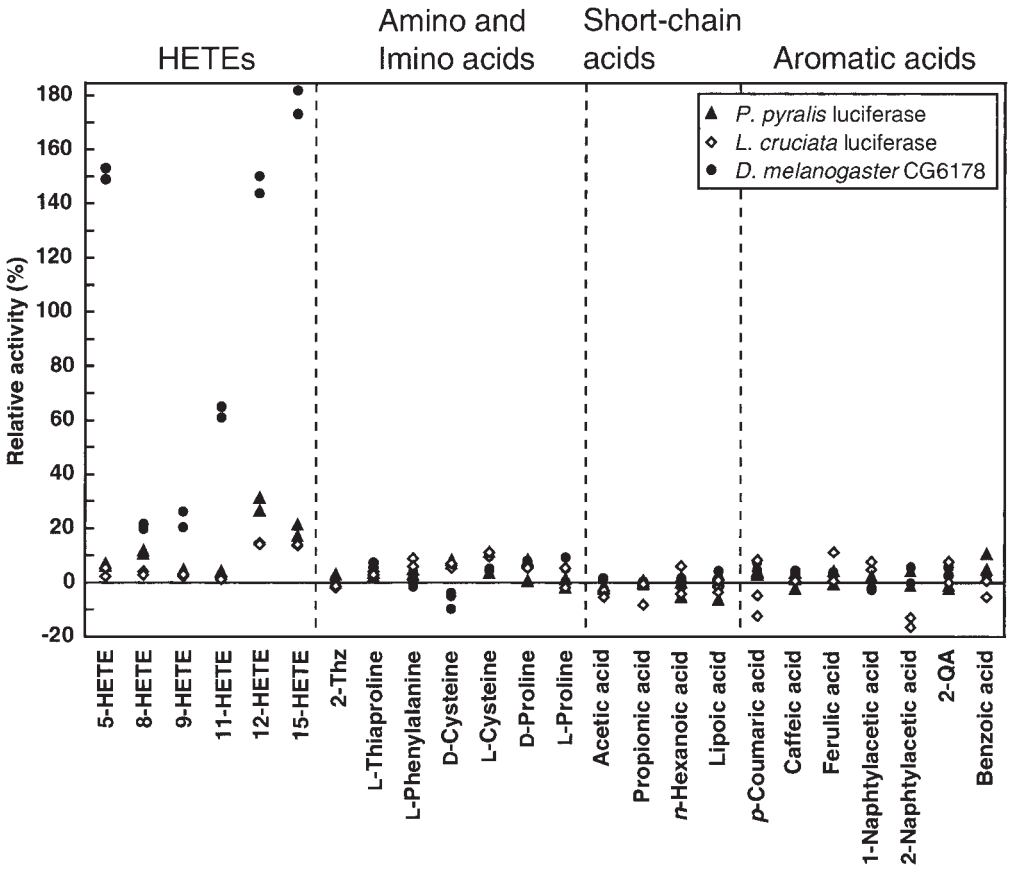

Fig. 3. Substrate Specificity of Firefly Luciferases and CG6178 for Other Carboxylic Acids.

The assay method was same as that described in Fig. 2. Relative activity is expressed as a percentage with respect to arachidonic acid. Each point represents the value of independent experiments (n = 2 or 3). Closed triangle, *P. pyralis* luciferase; open diamond, *L. cruciata* luciferase; closed circle, *D. melanogaster* CG6178. 2-Thz, thiazolidine-2-carboxylic acid; 2-QA, 2-quinolinecarboxylic acid.

CG6178 (Fig. 3). Very weak activities (0 to 10% activity with arachidonic acid) were observed for amino and imino acids (L-proline, D-proline, L-cysteine, D-cysteine, L-phenylalanine, L-thiaproline, and thiazolidine-2-carboxylic acid). These results suggest that the structural similarity to the thiazole moiety of firefly luciferin is not essential for substrate recognition in acyl-CoA synthesis. No significant activity was observed (less than 5% activity with arachidonic acid) for short chain acids (acetic acid, propionic acid, *n*-hexanoic acid, and lipoic acid) or aromatic acids (*p*-coumaric acid, caffeic acid, ferulic acid, 1-naphtylacetic acid, 2-

naphtylacetic acid, 2-quinolinecarboxylic acid, and benzoic acid) in firefly luciferase or CG6178.

Lauroyl CoA production by enzyme reaction

The reaction product of lauric acid with firefly luciferases and CG6178 was identified as lauroyl CoA by TLC analysis using [1-^{14}C]lauric acid and MALDI-TOF-MS analysis. TLC analysis showed that the *R*f value (= 0.38) of the radioactive spot generated from the enzymatic reaction was identical to that of authentic lauroyl CoA (Fig. 4). Also, the product from the enzymatic reaction with lauric acid was isolated by HPLC and applied to MALDI-TOF-MS analysis. The mass values of the product corresponding to $[M - H]^-$ (calculated mass 948.3) and $[M + K - 2H]^-$ (calculated mass 986.3) of lauroyl CoA were detected, and the authentic sample of lauroyl CoA exhibited values of $[M - H]^-$ ($m/z = 948.3$) and $[M + Li - 2H]^-$ ($m/z = 954.3$). Thus the reaction product of lauric acid with firefly luciferase and CG6178 was lauroyl CoA (Table 1).

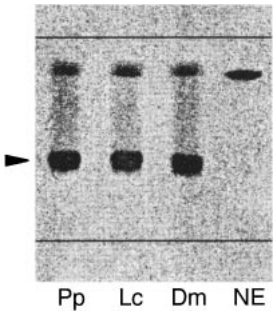

Fig. 4. Autoradiography of ^{14}C-Lauroyl CoA Produced by Enzymatic Reaction.

[1-^{14}C]Lauric acid was incubated with enzyme in the presence of ATP, CoA, and Mg^{2+}. The product was separated by TLC and the radioactivity was measured with an imaging analyzer. The arrow indicates the position corresponding to authentic lauroyl CoA. Pp, *P. pyralis* luciferase; Lc, *L. cruciata* luciferase; Dm, *D. melanogaster* CG6178; NE, no enzyme.

Table 1. MALDI-TOF-MS Analyses of Lauroyl CoA Produced by Enzymatic Reaction

P. pyralis	*L. cruciata*	CG6178	Lauroyl CoA	Lauroyl CoA
	m/z			Calcd.
948.2	948.3	948.5	948.3	948.3 $[M - H]^-$
—	—	—	954.3	954.3 $[M + Li - 2H]^-$
986.1	986.4	986.3	—	986.3 $[M + K - 2H]^-$

Enzymatic properties of firefly luciferases and CG6178 for lauric acid

The effects of the concentration of lauric acid on acyl-CoA synthetic activity in firefly luciferase and CG6178 were investigated (Fig. 5). With *P. pyralis* luciferase and CG6178, strong inhibition of acyl-CoA synthetic activity was observed at high concentrations (over 20 μM) of long-chain fatty acids (C18∼C20), as reported.[4,5] However, no inhibition by lauric acid was observed at a concentration of 40 μM. A weak inhibitory effect was detected only over 80 μM of lauric acid in CG6178. The K_m, V_{max}, and k_{cat} values of these enzymes for lauric acid at a concentration of 5–50 μM were determined from Lineweaver–Burk plots (Table 2). The K_m values of *P. pyralis* luciferase, *L. cruciata* luciferase, and CG6178 were 7.41, 16.3, and 1.68 μM respectively. The affinity of lauric acid for *P. pyralis* luciferase was about 2 times higher than that for *L. cruciata* luciferase. The affinity of lauric acid was about 2 times

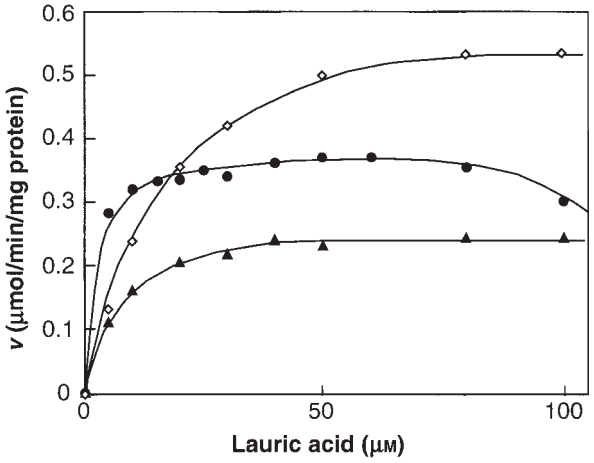

Fig. 5. Effect of Lauric Acid Concentration on Fatty Acyl-CoA Synthesis.

The formation of lauroyl CoA was determined by HPLC procedures, as described in "Materials and Methods". The vertical axis represents the initial velocity (*v*) of the reaction (μmol/min/mg of enzyme). Closed triangle, *P. pyralis* luciferase; open diamond, *L. cruciata* luciferase; closed circle, *D. melanogaster* CG6178.

Table 2. Comparison of Kinetic Parameters

Enzyme	Substrate	K_m (μM)	V_{max} (μmol/min/mg)	k_{cat} (s^{-1})	k_{cat}/K_m ($\mu M^{-1} \cdot s^{-1}$)
P. pyralis luciferase	Lauric acid	7.41	0.273	0.278	0.0375
	α-Linolenic acid[a]	13.6	0.127	0.130	0.0096
L. cruciata luciferase	Lauric acid	16.3	0.640	0.651	0.0399
CG6178	Lauric acid	1.68	0.373	0.402	0.2393
	α-Linolenic acid[a]	1.88	0.490	0.530	0.2819

[a]Ref. 5

higher than that of α-linolenic acid in *P. pyralis* luciferase. The K_m value of lauric acid for CG6178 was almost same as that of α-linolenic acid.

Discussion

In the present study, we found that *P. pyralis* luciferase, *L. cruciata* luciferase, and *D. melanogaster* CG6178 showed significant activity of fatty acyl-CoA synthesis from saturated medium-chain fatty acids (C8∼C14) and unsaturated long-chain fatty acids (C16∼C20) (Fig. 2). Among the fatty acids examined, lauric acid (C12:0) was the best substrate for acyl-CoA synthesis in firefly luciferases and CG6178. At the present time, the relationship between the carbon length of lauric acid and the structure of firefly luciferin has not been explained clearly. Other carboxylic acids, short-chain acids (< C6), aromatic acids, amino acids, and imino acids were not utilized as the substrate for the adenylate formation in acyl-CoA synthesis. Based on these results, firefly luciferase and CG6178 will be classified into ACSM or ACSL.

Previously, Ueda and Suzuki[13] and Matsuki *et al.*[14] reported that long-chain fatty acids (C14:0, C16:0, C18:0, and C20:4) strongly inhibited the luminescence activity of firefly luciferase (IC_{50} = 0.62–0.68 μM), that fatty acids of C10:0 and C12:0 were weaker inhibitors (IC_{50} = 13.2 and 1.2 μM, respectively), and that carboxylic acids of C4:0∼C8:0 showed low inhibition (IC_{50} = 3–14 mM). Lipoic acid is also a strong inhibitor (IC_{50} = 0.05 μM),[15] but it was not used for adenylation (Fig. 3). Thus the carboxylic acid, which strongly inhibits the luminescent activity of firefly luciferase, is not always a good substrate for acyl-CoA synthesis.

Several genes having significant homology with those coding for ACSM and ACSL were found in the insect genome databases of *D. melanogaster*, *Apis mellifera*, and *Anopheles gambiae*, but their gene products have not been characterized enzymatically. In addition, no genes for ACSM and ACSL were identified in insects. In mammals, ACSM gene products have been studied.[16] These enzymes are localized in mitochondria and exhibited substrate specificity for C6∼C12 saturated acids. The best substrates are *n*-hexanoic acid (C6:0) and octanoic acid (C8:0). Some aromatic acids, such as benzoic acid and naphthylacetic acid, showed significant activity for purified bovine ACSM.[17] These characteristics of ACSM in mammals are different from that of firefly luciferases and CG6178 (Figs. 2 and 3). On the other hand, ACSL gene products have been well characterized from various organisms. Five genes were cloned from rat, mouse, and human,[18] and the substrate specificities of these enzymes are different from each other.[19–21] Interestingly, the substrate specificity of rat Acsl3 (rAcsl3 in Table 3 and Fig. 6) is similar to that of firefly luciferases and CG6178. The efficient substrates for rat Acsl3 are lauric acid (C12:0), myristic acid (C14:0), arachidonic acid (C20:4), and EPA (C20:5).[19] Furthermore, rat ACSL activity was observed in peroxisomes,[22,23] as in firefly luciferase.[24] Firefly luciferases and CG6178 possess a peroxisomal targeting signal sequence at the C-terminus.[25,26] Thus the substrate specificity and the cell localization of firefly luciferase are similar to that of mammalian ACSL rather than ACSM.

Studies of the X-ray structure analysis of firefly luciferase[27] and the mutagenesis analysis[28] have been reported, and the binding residues of firefly luciferin in luciferase have been proposed by molecular modeling. Recently, the crystal structures of ACSL from *Thermus thermophilus*, including complexes with ATP analog and myristoyl-AMP, were first resolved.[29] This report suggests that firefly luciferase and ACSL are similar in structure, consisting of a large N-terminal domain and a small C-terminal domain. The catalytic site was formed at the junction between the two domains. Furthermore, three motifs specific for ACSLs were also conserved in firefly luciferase.[29] The structural similarities and the substrate specificities support the conclusion that both firefly luciferase and CG6178 are to be classified as ACSL.

Phylogenetic analysis of acyl-CoA synthetase (Table 3) suggests that these genes are mainly classified into four different families: AceCS, ACSM, ACSL, and VLCS.[16] In this study, we constructed a phylogenetic tree of acyl-CoA synthetase genes including beetle luciferases, *CG6178*, plant 4CLs, unknown genes of *D. melanogaster*, and fungi (Fig. 6). It indicates that beetle luciferases and *CG6178* form a clade with unknown genes of fungi and plant 4CLs. This group is independent of the AceCS, ACSM, ACSL, and VLCS families, suggesting that beetle luciferases (and CG6178) are phylogenetically different from the type of ACSL in mammals and yeast. Interestingly, the gene products possessing the peroxisomal targeting signal sequence 1 (PTS1, marked by asterisks in Fig. 6) were all located in this clade (referred to as the PTS1 family). Furthermore, no gene grouped in the PTS1 family was found in vertebrate genomes (including Fugu, zebrafish, human, and mouse genomes, searched by genomic BLAST: http://www.ncbi.nlm.nih.gov/). This suggests that the ortholog of the PTS1 family has been lost in the vertebrate lineage. We postulate that the PTS1 family originates from ACSL (not AceCS), because a gene product of *FadD* in *E. coli* (see Fig. 6) has been identified as ACSL.[30]

In insects, studies of the content of medium-chain fatty acids (C8∼C14) were not characterized in detail. In *D. melanogaster*, C12:0 is not abundant, as compared to long-chain fatty acids.[31] In Coleoptera, including Lampyridae, the amounts of fatty acids of C14:0 and C14:1 are trace or not detected.[32] On the contrary, the content of long-chain fatty acids (C16∼C20) in insects has been well determined, and the main components are C16 and C18 (reviewed in ref. 32). Nor Aliza *et al.*[33] reported that the proportions of arachidonic acid (C20:4)

Table 3. The Members of Acyl-CoA Synthetase

Name	Access. No.	Organism	Function of gene product	PTS1 signal[a]	Reference
Mammal					
mAcsm	NP_473435	*Mus musculus*	ACSM	−	16
mSA protein	NM_016870	*Mus musculus*	ACSM	−	16
mVLCS	AAB87982	*Mus musculus*	VLCS	−	35
rAcsl1	NP_036952	*Rattus norvegicus*	Rat ACSL	−	19
rAcsl3	NP_476448	*Rattus norvegicus*	Rat ACSL	−	19
rAcsl4	NP_446075	*Rattus norvegicus*	Rat ACSL	−	20
rAcsl5	NP_446059	*Rattus norvegicus*	Rat ACSL	−	21
rAcsl6	NP_570095	*Rattus norvegicus*	Rat ACSL	−	36
hSA protein	NM_005622	*Homo sapiens*	Human SA gene	−	
hACSM	NM_052956	*Homo sapiens*	Putative ACSM	−	
hVLCS	NP_003636	*Homo sapiens*	VLCS	−	37
BAA91273	BAA91273	*Homo sapiens*	Unknown	−	
hAceCS1	AAF75064	*Homo sapiens*	AceCS	−	38
hAceCS2	Q9NUB1	*Homo sapiens*	Putative AceCS	−	
Insect					
P. pyralis	AAA29795	*Photinus pyralis*	Luciferase and ACSL	+	4, 39
L. cruciata	M26194	*Luciola cruciata*	Luciferase and ACSL	+	4, 40
Railroad worm	AF139645	*Phrixothrix hirtus*	Railroad worm luciferase	+	41
Click beetle	AAQ11720	*Pyrophorus plagiophthalamus*	Jamaican click beetle luciferase	+	42
CG3961	NP_649067	*Drosophila melanogaster*	Unknown	−	
CG4830	NM_141903	*Drosophila melanogaster*	Unknown	−	
CG6178	NM_142964	*Drosophila melanogaster*	ACSL	+	5
CG7400	NM_079984	*Drosophila melanogaster*	Putative fatty acid transporter	−	
CG8732	NP_724696	*Drosophila melanogaster*	Unknown	−	
CG8834	NM_136935	*Drosophila melanogaster*	Unknown	−	
CG9390	S52154	*Drosophila melanogaster*	Putative AceCS	−	
CG9993	NP_611518	*Drosophila melanogaster*	Unknown	±	
CG11407	NM_142574	*Drosophila melanogaster*	Unknown	−	
CG18586	NM_139736	*Drosophila melanogaster*	Unknown	−	
Nematode					
F11A3.1	CAA94751	*Caenorhabditis elegans*	Unknown	+	
Plant					
At4CL1	U18675	*Arabidopsis thaliana*	*Arabidopsis* 4CL	−	43
At4CL2	AF106085	*Arabidopsis thaliana*	*Arabidopsis* 4CL	−	43
At4CL3	AF106087	*Arabidopsis thaliana*	*Arabidopsis* 4CL	−	43
At5g63380	AY250835	*Arabidopsis thaliana*	*Arabidopsis* 4CL-like protein	+	
At4g05160	AY250839	*Arabidopsis thaliana*	*Arabidopsis* 4CL-like protein	+	
At4g19010	AY250834	*Arabidopsis thaliana*	*Arabidopsis* 4CL-like protein	+	
Fungi					
Ustilago 1	EAK81955	*Ustilago maydis*	Unknown	−	
Ustilago 2	EAK85592	*Ustilago maydis*	Unknown	+	
Aspergillus 1	EAA57739	*Aspergillus nidulans*	Unknown	+	
Aspergillus 2	EAA61507	*Aspergillus nidulans*	Unknown	+	
yAceCS1	AAC04979	*Saccharomyces cerevisiae*	Yeast AceCS	−	44
yAceCS2	P52910	*Saccharomyces cerevisiae*	Yeast AceCS	−	44
yVLCS	NP_009597	*Saccharomyces cerevisiae*	Yeast VLCS (Fat1p)	−	45
yACSL (FAA1)	NP_014962	*Saccharomyces cerevisiae*	Yeast ACSL (Faa1p)	−	46
yACSL (FAA2)	NP_010931	*Saccharomyces cerevisiae*	Yeast peroxisomal ACSL (Faa2p)	−	46
yACSL (FAA4)	NP_013974	*Saccharomyces cerevisiae*	Yeast putative ACSL (*FAA4*)	−	
Bacteria					
PheA	1AMUA	*Brevibacillus brevis*	(See in Materials and methods)	−	
acvA	P19787	*Penicillium chrysogenum*	(See in Materials and methods)	−	
eAceCS	NP_418493	*Escherichia coli* K12	Putative AceCS	−	
ttLC-FACS	AB126656	*Thermus thermophilus* HB8	ACSL	−	29
FadD	P29212	*Escherichia coli* K12	ACSL	−	30
ScCCL	NP_628552	*Streptomyces coelicolor* A3(2)	Cinnamate:CoA ligase	−	47

[a]The PTS1 signal was searched by the PTS1 predictor (http://mendel.imp.univie.ac.at/mendeljsp/sat/pts1/PTS1predictor.jsp). These genes were classified as "targeted" which possess the PTS1 signal (+), "not targeted" which do not possess the PTS1 signal (−), or "twilight zone" which the predictor could not classify (±).

and EPA (C20:5) are high in tissue lipids of adult *P. pyralis*, especially in the light organ. These facts are of interest in connection with our present results that arachidonic acid and EPA are good substrates for *P. pyralis* and *L. cruciata* luciferases. Palmitoleic acid (C16:1) is found in small amounts in a Lampyridae,[32,33] but is abundant in *D. melanogaster*.[31,32] There is much evidence that the β-oxidation pathway of fatty acids is

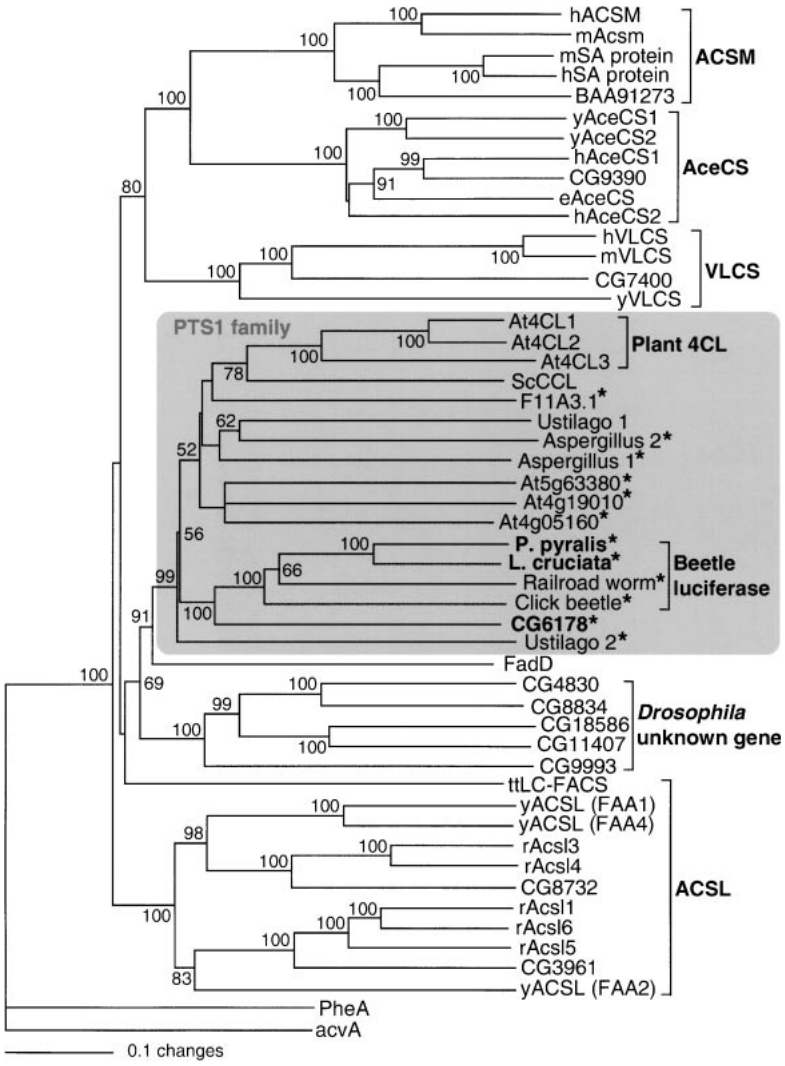

Fig. 6. Phylogenetic Tree of Acyl-CoA Synthetase Genes.

The tree was constructed by the neighbor-joining method using amino acid sequences. Abbreviated names of the genes are listed in Table 3. Numbers indicate bootstrap values from 1,000 replicates. Only the bootstrap values over 50% are indicated on the nodes. Horizontal branch lengths indicate genetic distances. The genes marked by an asterisk possess the PTS1 signal at the C-terminus. A shaded clade shows the PTS1 family.

present in insects, and ACSL activity in peroxisomes has also been studied in several insect species.[34] Firefly luciferase might have another physiological role, such as the β-oxidation of fatty acids.

In conclusion, our results suggest that beetle luciferase is evolved from a novel type of fatty acyl-CoA synthetase, which differs from ACSM and ACSL in mammals and yeast.

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