Discovery of Amide (Peptide) Bond Synthetic Activity in Acyl-CoA Synthetase*

Tomoko Abe<sup>1,2</sup>, Yoshiteru Hashimoto<sup>2</sup>, Hideaki Hosaka<sup>2</sup>, Kaori Tomita-Yokotani, and Michihiro Kobayashi<sup>1</sup>

From the Institute of Applied Biochemistry, and Graduate School of Life and Environmental Sciences, The University of Tsukuba, 1-1-1 Tennodai, Tsukuba, Ibaraki 305-8572, Japan

Acyl-CoA synthetase, which is one of the acid-thiol ligases (EC 6.2.1), plays key roles in metabolic and regulatory processes. This enzyme forms a carbon-sulfur bond in the presence of ATP and Mg<sup>2+</sup>, yielding acyl-CoA thiosteres from the corresponding free acids and CoA. This enzyme belongs to the superfamily of adenylate-forming enzymes, whose three-dimensional structures are analogous to one another. We here discovered a new reaction while studying the short-chain acyl-CoA synthetase that we recently reported (Hashimoto, Y., Hosaka, H., Oinuma, K., Goda, M., Higashibata, H., and Kobayashi, M. (2005) J. Biol. Chem. 280, 8660–8667). When l-cysteine was used as a substrate instead of CoA, N-acyl-L-cysteine was surprisingly detected as a reaction product. This finding demonstrated that the enzyme formed a carbon-nitrogen bond (EC 6.3.1 acid-ammonia (or amide) ligase (amide synthase); EC 6.3.2 acid-amino acid ligase (peptide synthase)) comprising the amino group of the cysteine and the carboxyl group of the acid. N-Acyl-d-cysteine, N-acyl-DL-homocysteine, and N-acyl-L-cysteine methyl ester were also synthesized from the corresponding cysteine analog substrates by the enzyme. Furthermore, this unexpected enzyme activity was also observed for acetyl-CoA synthetase and firefly luciferase, indicating the generality of the new reaction in the superfamily of adenylate-forming enzymes.

Acyl-CoA synthetase is an important enzyme in living organisms (1, 2). The reaction mechanism and structures of acyl-CoA synthetase have been extensively investigated (3–7). This enzyme catalyzes the ligation of an acid with CoA in the presence of ATP and Mg<sup>2+</sup> through two-step reactions. The initial half-reaction catalyzed by acyl-CoA synthetase comprises the formation of an acyl-AMP intermediate with the release of inorganic pyrophosphate (step 1, acid + ATP → acyl-AMP + PP<sub>i</sub>). Next, the AMP of the acyl-CoA synthetase-bound acyl-AMP intermediate is displaced rapidly by CoA yielding acyl-CoA (step 2, acyl-AMP + CoA → acyl-CoA + AMP). Although there have been a few reports describing investigation of thiols instead of CoA (8, 9), identification of the reaction products and activity toward l-cysteine as a substrate have never been reported. Therefore, we further investigated thiols instead of CoA.

We recently reported acyl-CoA synthetase, which plays an essential role in acid utilization in the nitrile-degradative pathway of Pseudomonas chlororaphis B23 (10). The enzymes involved in the biological metabolism of nitriles have received much attention in applied (11, 12) as well as academic (13–15) fields. One of the fruits of our application-oriented nitrile studies is the current industrial production of acrylamide and nicotinamide using the nitrile hydratase of Rhodococcus rhodochrous J1 (16). On the other hand, the nitrile hydratase of P. chlororaphis B23 (17), which was previously used as a catalyst for acrylamide manufacture (11), is now used for the industrial production of 5-cyanovaleramide (18). Industrial microorganisms often have provided us with interesting enzymes (19, 20) and phenomena (21, 22), and here we discovered a new function of the P. chlororaphis B23 acyl-CoA synthetase (AcS).

Acyl-CoA synthetase (1, 2, 23) belongs to the superfamily of adenylate-forming enzymes that includes acetyl-CoA synthetase (24, 25), Coleoptera luciferase (26, 27), and the adenylation domains of nonribosomal peptide synthetases (28, 29). The x-ray crystal structures of all reported enzymes are very analogous to one another and are composed of the same domains: a large N-terminal domain and a smaller C-terminal domain (10–15, 26–28). The enzymes of the adenylate-forming superfamily have been well characterized; acetyl-CoA synthetase is an enzyme central to metabolism in prokaryotes and eukaryotes (24), and firefly luciferase has been widely used in molecular and cell biology (30). In this study, a new function of acetyl-CoA synthetase and firefly luciferase was revealed.

**EXPERIMENTAL PROCEDURES**

**Materials**

All acyl-CoA compounds, amino acids, N-isobutyryl-L-cysteine, N-isobutyryl-D-cysteine, and N-acetyl-L-cysteine were purchased from Sigma. Acids and ATP were purchased from Nakalai Tesque (Kyoto, Japan) and Kishida Chemical Co., Ltd. (Osaka, Japan), respectively. Saccharomyces cerevisiae acetyl-CoA synthetase and Photinus pyralis luciferase, highly purified, were purchased from Sigma and Promega, respectively. Commercially available beetle luciferin (also known as p-luciferin; Promega) was used as a substrate for luciferase.
Preparation of Enzymes

Expression plasmid pET-acsA was used for the expression of the intact acyl-CoA synthetase from *P. chlororaphis* B23 (AcsA; accession number, BAD90933), and its purification was performed by the method previously reported. To express C-terminally histidine-tagged AcsA (AcsAHis), the fragment of the *acsA* gene was amplified by PCR using pET-acsA (10) as a template. The following two oligonucleotide primers were used: 5’-GAATTCTAAGGAGGAATAGCATATGGCGAGATTATGGAACACGTGTGTTG-3’ (the Ndel recognition site is underlined) and 5’-CACTCGAGACCAAGCGCTGTGGCTTTGACA-3’ (the XhoI recognition site is underlined). The PCR product was digested with Ndel and XhoI and then inserted into the corresponding sites of pET-24a (+). The resultant plasmid was designated as pET-acsAHis. *E. coli* BL21-CodonPlus (DE3)-RIL was transformed with pET-acsAHis. The transformed cells were grown in 2× YT medium containing kanamycin (50 µg/ml) and chloramphenicol (34 µg/ml) at 37 °C. When $A_{460\text{nm}}$ reached 0.6, the incubation temperature was reduced to 18 °C, and protein expression was induced with 1mM isopropyl D-thiogalactoside. After a 12-h culture, the cells were harvested by centrifugation, washed twice, and then suspended in 20 mM potassium phosphate buffer (KPB) (pH 7.4) containing 0.5 M NaCl. The resulting supernatant was applied to a nickel-chelating column of HisTrap™ HP (5 ml) (GE Healthcare UK Ltd.) and was washed with 20 mM sodium phosphate buffer (pH 7.4) containing 0.5 M NaCl. The eluted protein was eluted with a linear gradient (0.01–0.4 M) of imidazole. The active fractions were collected and dialyzed, and then the enzyme solution was loaded onto a Resource Q column (6 ml) (GE Healthcare UK Ltd.) equilibrated with 20 mM KPB (pH 7.5). The enzyme was eluted by increasing the ionic strength of KCl in a linear manner from 0 to 0.5 M in the same KPB. The active fractions were mixed and dialyzed against 20 mM KPB (pH 7.5). The homogeneity of the purified AcsAHis was confirmed by SDS-PAGE. The CD spectrum of the purified AcsAHis was confirmed to be identical to that of AcsA (data not shown). Also, both AcsAHis and intact AcsA showed almost the same specific activity (isobutyryl-CoA synthetase activity: 16.3 and 15.1 units/mg; isobutyrate-cysteine synthetic activity: 0.012 and 0.013 units/mg, respectively). Therefore, AcsAHis was used in all experiments in this study.

Detection of Isobutyrate-cysteine or Acetate-cysteine Compounds and Their Structural Determination

The reaction mixture, comprising 5 mM isobutyrate or acetate, 10 mM L-cysteine, 5 mM ATP, 8 mM MgCl$_2$, 100 mM (NH$_4$)$_2$SO$_4$, and an appropriate amount of the purified enzyme in 200 mM Tris-HCl (pH 7.5), was incubated at 20 °C, and the reaction was stopped by adding 5% formic acid solvent (final 0.7%, pH 3.0). Then the mixture was analyzed by HPLC with a Shimadzu LC-6A system equipped with a Shim-Pack SCR-102H column (8.0 × 300 mm; Shimadzu, Kyoto, Japan). The mobile phase solvent system comprised 0.1% (v/v) formic acid solvent (pH 3.0), and chromatographic separation was performed at 40 °C at a flow rate of 0.8 ml/min. The amount of each sample as a product was measured by monitoring the column effluent at 192 nm. The amount of isobutyrate was measured by the same procedure.

For LC-tandem mass spectrometry (MS/MS) analysis, an Agilent1100 HPLC system (Agilent Technologies) equipped with a Shim-pack SCR-102H column was used. The MS/MS analysis data were acquired with a QSTAR® XL mass spectrometer (Applied Biosystems) with TurboIonSpray® (ESI) in the positive and negative modes. The following conditions were used. An ion spray voltage of ±5.5 kV or −4.5 kV was applied to the emitter, and the desolvation temperature was 500 °C. The product ion scan mode was used.

NMR spectra were measured with an Avance 600 spectrometer (Bruker, Ettlingen, Germany). Samples were prepared by dissolution in D$_2$O, which was used as an internal standard.

Detection and MS Analysis of Other Compounds

Determination of the molecular masses of the products (*i.e.* N-acyl-L-cysteine, N-acyl-DL-homocysteine, and N-acyl-L-cysteine methyl ester) was performed by LC-ESI-MS with a Shimadzu LCMS-2010EV system equipped with a Shim-pack SCR-102H column. The reaction mixture for these products, comprising 5 mM various acids, 10 mM L-cysteine, 5 mM ATP, 8 mM MgCl$_2$, 100 mM (NH$_4$)$_2$SO$_4$, and an appropriate amount of the purified enzyme in 200 mM Tris-HCl (pH 7.5), was incubated at 20 °C. The products were eluted with 0.1% formic acid solvent (pH 3.0), and chromatographic separation was performed at 40 °C at a flow rate of 0.8 ml/min. In the case of N-luciferyl-L-cysteine, the reaction mixture (10 mM D-luciferin, 100 mM L-cysteine, 10 mM ATP, 16 mM MgCl$_2$, and an appropriate amount of the purified luciferase in 200 mM Tris-HCl (pH 7.8)) was incubated at 20 °C and then analyzed by LC-ESI-MS with a Shimadzu LCMS-2010EV system equipped with a Cosmosil 5C$_{18}$-MS-II column (4.6 × 150 mm; Nacalai Tesque, Kyoto, Japan). The mobile phase solvent comprised 20 mM ammonium acetate buffer (pH 4.9) containing 10% acetonitrile, and chromatographic separation was performed at 50 °C at a flow rate of 1 ml/min. The product was quantitated by monitoring at 190–600 nm. The MS analysis data were acquired with the following conditions: the curved desolvation line and heat block temperatures were set at 250 and 200 °C, respectively. The nebulizer gas flow rate and drying gas pressure were set at 1.5 liters/min and 0.2 megapascals, respectively.

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*The abbreviations used are: KPB, potassium phosphate buffer; IC, isobutyrate-cysteine; AcsAHis, histidine-tagged AcsA; HPLC, high pressure liquid chromatography; LC, liquid chromatography; MS, mass spectrometry; MS/MS, tandem mass spectrometry; ESI, electrospray ionization.*
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The detector voltage was set at +4.5 or −3.5 kV, and the ion source polarity was set in the positive and negative modes.

Enzyme Assays

Acyl-CoA Synthetase Activity—Acyl-CoA synthetase activity was measured by HPLC on a Cosmosil 5C18-AR-II column (4.6 × 150 mm; Nacalai Tesque) using the previously reported assay system by quantitating acyl-CoA products (10, 31). The reactions were performed under linear conditions with regard to protein (−1.5 μg/ml) and time (−4 min). One unit of acyl-CoA synthetic activity was defined as the amount of enzyme that catalyzed the formation of 1 μmol of acyl-CoA/min under these assay conditions.

N-Isobutyryl-L-cysteine Synthetic Activity—The reaction mixture (60 μl) for N-isobutyryl-L-cysteine synthetic activity comprised 5 mM isobutyrate, 0.03–600 mM L-cysteine, 5 mM ATP, 8 mM MgCl2, 100 mM (NH4)2SO4, and the purified AcsA in 200 mM Tris-HCl (pH 7.5). The reaction was started by adding the enzyme (0.1 mg/ml), followed by incubation at 20 °C for 4 min. The reaction was stopped by adding 5% formic acid (0.5 μl, final 0.7%) to the reaction mixture, and a supernatant was obtained by centrifugation (15,000 × g, 10 min). The N-isobutyryl-L-cysteine synthetic activity was measured by HPLC on a CROWNPAK CR(+) column (4.0 × 150 mm; Dai- cell Chemical Industries, Ltd.). The mobile phase solvent comprised perchloric acid solvent (pH 1.5), and chromatographic separation was performed at 35 °C at the flow rate of 0.8 ml/min. The amount of the product was determined by monitoring the column effluent at 192 nm with authentic N-isobutyryl-L-cysteine as a standard (retention time, 8.9 min). One unit of N-isobutyryl-L-cysteine synthetic activity was defined as the amount of enzyme that catalyzed the formation of 1 μmol of N-isobutyryl-L-cysteine/min under these assay conditions.

Results

Detection of the Isobutyrate-cysteine (IC) Product—In order to further investigate the substrate specificity of AcsA, some thiols were used instead of CoA. Because isobutyrate was a suitable substrate among the acids, judging from the fact that it showed the highest catalytic efficiency (kcat/Km) for acyl-CoA synthesis (10), isobutyrate was usually used as an acid substrate. When the reaction mixture comprising AcsA, ATP, isobutyrate, and L-cysteine was analyzed by HPLC on a Titrainsphere TiO HPLC column, a significant increase in AMP was observed. In addition, when the same reaction mixture was analyzed on a Shim-pack SCR-102H ion exclusion chromatography column, a new product peak (retention time, 15 min) other than that of AMP appeared. The amount of the product was in proportion to the reaction time and the enzyme concentration. Without AcsA, the new product and AMP were not detected. We also performed the reaction using a blank sample, as follows. A fraction was prepared from the Escherichia coli transformant carrying the intact pET-24a(+) vector (i.e. the transformant harboring no acsA gene) by the same procedure as that used for the purification of AcsA from the E. coli transformant, but it did not exhibit the production of the new product or an increase in AMP. These results suggested that AcsA itself catalyzes the new reaction using L-cysteine and isobutyrate, with the decomposition of ATP to AMP.

There have been a few reports describing investigation of thiols instead of CoA (8, 9). The results of the spectrometric assay, where the amount of AMP produced in the reaction was determined through the coupling reaction with adenylate kinase, pyruvate kinase, and lactate dehydrogenase, and the following oxidation of NADH at 334 nm (8), indicate that the long chain acyl-CoA synthetase acts on dephospho-CoA, Nα-etheno-CoA, 4′-phosphopantetheine, and pantetheine. However, identification of these reaction products other than AMP has never been reported, and L-cysteine and related compounds (e.g. N-acetylcysteamine, glutathione, and diithiothreitol) are inactive as substrates. The new method we used here (described under “Experimental Procedures”) enabled the measurement of a small amount of AMP, resulting in the discovery of the reactivity of L-cysteine as a substrate. Therefore, we performed analysis of the resultant reaction product.

High resolution fast atom bombardment-MS analysis of the isolated resultant product indicated a molecular formula of C11H13O2NS (molecular ion [M + H]+ m/z 192.0713; mass calculated for C11H12O2NS). Because this formula shows that the compound ligates isobutyrate and cysteine are ligated through dehydration ((CH3)2CH-CO-NH-CH(COOH)CH2-SH (N-isobutyryl-cysteine)) or (CH3)2CH-CO-S-CH2-CH(NH2)COOH (5′-isobutyryl-cysteine)) this product was designated as the IC.

N-Acylation by Acyl-CoA Synthetase: New Amide Bond Formation—Authentic N-isobutyryl-cysteine (32) was eluted at exactly the same time as the IC compound from the Shim-pack SCR-102H column, to which the reaction mixture had been applied. Similarly, authentic N-isobutyryl-cysteine and the IC compound were eluted at the same retention time (9.0 min) from an HPLC CROWNPAK® CR (+) column, respectively. The structure of the IC compound was also determined by LC-
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We previously described salt activation by ammonium sulfate of the acyl-CoA synthetic activity of AcsA (10). Hence, the effect of ammonium sulfate on N-isobutyryl-L-cysteine synthesis was compared with that on isobutyryl-CoA synthesis, the following results being obtained: for isobutyryl-CoA synthesis, 0 mM (NH₄)₂SO₄, 30%; 5 mM (NH₄)₂SO₄, 46%; 50 mM (NH₄)₂SO₄, 88%; 100 mM (NH₄)₂SO₄, 100%, respectively; for N-isobutyryl-L-cysteine synthesis, 0 mM (NH₄)₂SO₄, 33%; 5 mM (NH₄)₂SO₄, 41%; 50 mM (NH₄)₂SO₄, 73%; 100 mM (NH₄)₂SO₄, 100%, respectively. These findings demonstrated that the presence of the ammonium sulfate activates not only isobutyryl-CoA synthesis (thioester bond synthesis) but also N-isobutyryl-L-cysteine synthesis (amide bond synthesis); the mode of activation by ammonium sulfate for N-isobutyryl-L-cysteine synthesis is similar to that for isobutyryl-CoA synthesis. Moreover, the addition of 100 mM ammonium sulfate never inhibited both the isobutyryl-CoA synthesis and the N-isobutyryl-L-cysteine synthesis. Therefore, 100 mM ammonium sulfate was added to all of the reaction mixtures used in the assays.

Stoichiometry—The stoichiometry of substrate consumption and product formation during the synthesis of N-isobutyryl-L-cysteine was examined in a reaction mixture consisting of 5 mM ATP, 5 mM isobutyrate, 400 mM L-cysteine, 8 mM MgCl₂, 100 mM ammonium sulfate, 200 mM Tris-HCl buffer (pH 7.5), and an appropriate amount of the enzyme, in a final volume of 700 μl. The reaction was carried out at 20 °C. At several time points, the molar amounts of the formed products (N-isobutyryl-L-cysteine and AMP) and accompanying decreases in the substrates (isobutyrate and ATP) were measured using four authentic compounds as standards (Fig. 2). The formation of no other compounds was observed. The results indicated that N-isobutyryl-L-cysteine and AMP were formed with the consumption of isobutyrate and ATP in a 1:1:1 stoichiometry.

Kinetics Analysis of N-Isobutyryl-L-Cysteine Synthetic Activity of AcsA—The N-isobutyryl-L-cysteine synthetic activity value calculated from Hanes-Woolf plots (Fig. 3) was compared with the isobutyryl-CoA synthetic activity (Table 1). The Vₘ₉₉ value of the N-isobutyryl-L-cysteine synthetic activity (0.197 units/mg) was one-fifteenth that of the isobutyryl-CoA synthetic activity (2.77 units/mg). The Kₘ values for isobutyrate of the two reactions were nearly the same (0.88 ± 0.09 mm for N-isobutyryl-L-cysteine synthesis; 0.86 ± 0.13 mm for isobu-
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FIGURE 2. Stoichiometry analysis of the N-isobutyryl-L-cysteine synthesis reaction. •, ATP; ○, isobutyrate; ●, AMP; ●, N-isobutyryl-L-cysteine. The methods for the analysis of reactants and products are described under “Experimental Procedures.” All data points represent the mean values ± S.D. for three experiments.

FIGURE 3. Hanes-Woolf plots of N-isobutyryl-L-cysteine synthetic activities (A) or isobutyryl-CoA synthetic activities (B). All data points represent the mean values ± S.D. for three experiments. Lines through the mean values represent a mathematical fit of the data on linear least squares regression analysis.

TABLE 1
Kinetics comparison between isobutyryl-CoA synthesis and N-isobutyryl-cysteine synthesis by AcsA

|                         | V_max (units/mg) | K_m (mM) | k_cat/K_m (s⁻¹·mM⁻¹) |
|-------------------------|------------------|----------|------------------------|
| N-Isobutyryl-L-cysteine synthesis | 0.197 ± 0.013    | 71.1 ± 4.3 | 0.00283 ± 0.00003     |
| Isobutyryl-CoA synthesis   | 2.77 ± 0.29      | 0.0789 ± 0.0034 | 35.8 ± 2.2            |

tyryl-CoA synthesis). In contrast, there was a significant difference between the K_m values for CoA and l-cysteine (Table 1).

Synthesis of Various N-Acyl-L-cysteines—The ability of AcsA to catalyze the N-acyl-cysteines from various acids was examined in a reaction mixture that included l-cysteine. An increase in AMP was detected when the following acids were utilized as the substrate: acetate (74%), propionate (108%), n-butyrate (89%), valerate (90%), hexanoate (46%), heptanoate (9%), acrylate (138%), and crotonate (94%), compared with isobutyrate (100%). In the cases of the reactions with propionate, n-butyrate, and acrylate as an acid substrate, the reaction products other than AMP were separated on Shim-pack SCR-120 (retention times, 15.0, 17.9, and 17.8 min, respectively), and their molecular masses were determined by LC-ESI-MS. The results for the products were consistent with the corresponding estimated molecular masses for acrylyl-L-cysteine, 177 M_r; N-butyryl-L-cysteine, 191 M_r; N-acrylyl-L-cysteine, 175 M_r (Fig. 4), demonstrating that AcsA catalyzed various N-acyl-L-cysteine synthetic reactions.

FIGURE 4. Mass spectra on LC-ESI-MS in the positive (a) and negative (b) ion modes of the AcsA reaction products when acids and L-cysteine were used as substrates. Acids were as follows: propionate (A), n-butyrate (B), and acrylate (C). The reactions were carried out by the methods described under “Experimental Procedures.” The major mass peaks at m/z 178 and 176 correspond to [M + H]^+ and [M − H]^- of N-propionyl-L-cysteine (A), respectively. Those at m/z 192 and 190 correspond to [M + H]^+ and [M − H]^- of N-butyryl-L-cysteine (B), respectively. Those at m/z 176 and 174 correspond to [M + H]^+ and [M − H]^- of N-acrylyl-L-cysteine (C), respectively.
Synthesis of Various N-Isobutyryl Compounds—When D-cysteine was used as the substrate instead of L-cysteine, the production of N-isobutyryl-D-cysteine was confirmed on a CHIRALPAK® QN-AX column (data not shown). A $V_{\text{max}}$ value of 0.071 ± 0.005 units/mg and a $K_m$ value of 78.92 ± 4.14 mM for D-cysteine were calculated using N-isobutyryl-D-cysteine as the standard. The $V_{\text{max}}$ value of the N-isobutyryl-D-cysteine synthetic activity of AcsA was 3-fold lower than that for N-isobutyryl-L-cysteine. Furthermore, when isobutyrate and DL-homocysteine (H$_2$N-CH(CH$_2$COOCH$_3$)CH$_2$SH) or L-cysteine methyl ester (H$_2$N-CH(CH$_2$COOCH$_3$)CH$_2$SH) were used as substrates instead of L-cysteine, the reaction products other than AMP were separated on Shim-pack SCR-120 (retention times, 19.1 and 28.7 min, respectively). Then, the molecular masses determined on LC-ESI-MS were both 205 $M_r$ (Fig. 5). This value was consistent with the corresponding estimated molecular masses (N-isobutyryl-DL-homocysteine, 205 $M_r$; N-isobutyryl-L-cysteine methyl ester, 205 $M_r$). In a similar manner to N-isobutyryl-L-cysteine, these findings indicate that N-isobutyryl-DL-homocysteine or N-isobutyryl-L-cysteine methyl ester would be produced. This new reaction would use some cysteine analogs as substrates and could assess substrate chirality. When cysteamine (2-mercaptoethyamine), glutathione, L-penicillamine (3,3-dimethyl-L-cysteine), β-chloro-L-alanine, L-2-aminobutyrate, 2,3-diaminopropionate, L-norvaline, L-methionine, D-methionine, L-serine, L-alanine, N-acetyl-L-cysteine, N-isobutyryl-L-cysteine, and N-isobutyryl-D-cysteine were used as substrates, reactions were not observed at the screening concentration (10 mM). Because an amino group in N-acetyl-L-cysteine, N-isobutyryl-L-cysteine, and N-isobutyryl-D-cysteine (reaction products) is protected by the corresponding acyl group, it is logical for these compounds to be inert as substrates.

Generality of the N-Acylation (Amide Bond Formation) Reaction—We investigated whether acetyl-CoA synthetase and firefly (P. pyralis) luciferase catalyze the N-acetyl-L-cysteine synthetic reaction (amide bond synthesis). First, acetyl-CoA synthetase from S. cerevisiae (33) was reacted with acetyl and L-cysteine as substrates. For the reaction mixture, a new product (acetate-cysteine compound) peak was observed with the Shim-pack SCR-120 column (retention time, 14.9 min). LC-MS/MS analysis revealed that the molecular mass and fragmentation of the new material (Fig. 6) were in accordance with those of authentic N-acetyl-L-cysteine (data not shown). A $V_{\text{max}}$ value of 0.020 ± 0.00002 units/mg and a $K_m$ value of 17.56 ± 0.13 mm for L-cysteine were calculated using N-acetyl-L-cysteine as the standard. When acetyl and either DL-homocysteine or L-cysteine methyl ester were used as substrates, new products were also detected on Shim-pack SCR-120 (retention times, 20.5 and 28.7 min, respectively), and LC-ESI-MS analysis revealed the molecular masses predicted for the corresponding products (177 $M_r$; Fig. 7). These results indicate that acetyl-CoA synthetase and acyl-CoA synthetase from not only prokaryotes but also eukaryotes would exhibit N-acylation activity generally.

Firefly luciferase is one of the enzymes belonging to the adenylate-forming superfamily, like acyl-CoA synthetase. Although the two enzymes catalyze different reactions, their three-dimensional structures are analogous (26). Thus, the N-acylation reaction (N-luciferyl-L-cysteine synthesis) of firefly luciferase was next investigated. It is interesting to note that we observed a new product peak with a Cosmosil 5C$_{18}^{-}$ MS-II column (retention time, 38.0 min) and determined the molecular mass of N-luciferyl-L-cysteine (383 $M_r$; Fig. 8). N-Acetyl-L-cysteine can possibly be synthesized not only by acyl-CoA synthetase family enzymes but also by other adenylate-forming superfamily enzymes; other adenylate-forming superfamily enzymes may also synthesize both S-acylation and N-acylation products.

**DISCUSSION**

The physiological importance of acyl- and acetyl-CoA synthetase has been recognized for decades; therefore, they have been extensively studied worldwide. However, there have been few studies on thiol substrates instead of CoA (8, 9), and the possibility of L-cysteine being a substrate has never been reported.

During investigation of the substrate specificity of AcsA, we observed that L-cysteine was active as a substrate instead of CoA. When the molecular mass (191 $M_r$) of the product of the reaction with AcsA using isobutyrate and L-cysteine as substrates was found to be consistent with that of a dehydration product (N-isobutyryl-cysteine or S-isobutyryl-cysteine) derived from cysteine and isobutyrate, we at first...
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![Amide Bond Synthesis by S-Acyl-CoA Synthetase](image)

**FIGURE 6.** MS/MS spectra of the acetate-cysteine compound produced by acetyl-CoA synthetase from *S. cerevisiae* in the positive (A) and negative (B) ion modes. The structural formulas show N-acetyl-L-cysteine. The dotted lines show the structures deduced from the peaks at *m/z* 122.0265 (A) and 84.0457 (B), respectively.

It is guessed that AcsA ligates the thiol group of cysteine and the carboxyl group of isobutyrate, yielding a thioester bond like acyl-CoA. However, structural determination of the reaction product revealed that AcsA actually yields an amide bond (-NH-CO-). Namely, we discovered the amide bond synthetic activity of AcsA, which ordinarily catalyzes thioester bond formation. AcsA stoichiometrically produces N-isobutyryl-l-cysteine and AMP from isobutyrate, ATP, and l-cysteine. AcsA was able to form a variety of N-acyl-compounds when various acids and cysteine analogues were used as substrates. Comparison of the reactions between the original acyl-CoA synthesis (thioester bond synthesis) and the new N-acyl-l-cysteine synthesis, both of which are catalyzed by acyl-CoA synthetases, is shown in Fig. 9.

Acyl-CoA synthetase belongs to the superfamily of adenylate-forming enzymes. These enzymes are involved in many catabolic and anabolic processes. Scientists from many disciplines have studied this family of enzymes because of their involvement in the synthesis of antibiotics and anti-cancer agents and the degradation of pollutants (29, 34). Firefly luciferase, which also belongs to this superfamily, has had a long history of use in biology, especially for the detection of ATP. The firefly luciferase gene expression in cells from different organisms has generated a great deal of interest as to possible applications of the gene as a tool in biological studies (30). Luciferase originally catalyzes the oxidation of luciferin in the presence of ATP and O₂ to generate oxy-luciferin and light (hv), as shown in Fig. 10A. This bioluminescence (monooxygenase) reaction catalyzed by luciferase differs from the N-luciferyl-l-cysteine synthetic reaction (Fig. 10B). Recently, luciferyl-CoA or long chain acyl-CoA synthetic activity of firefly luciferase was reported (35, 36). The monooxygenase reaction of firefly luciferase also differs from the acyl-CoA synthesis reaction. Additionally, it was recently reported that the adenylation domain of nonribosomal peptide synthetase catalyzes aminoacyl-CoA synthesis (37). The adenylation domain of nonribosomal peptide synthetase normally transfers reactive aminoacyl-adenylates onto the covalently attached 4'-phosphopantetheine moiety of another domain (38). This reaction comprises thioesterification (S-acylation) as well as acyl-CoA synthesis. Considering the finding that luciferase catalyzes the N-luciferyl-l-cysteine synthetic reaction, enzymes belonging to the adenylate-forming superfamily may possibly catalyze N-acyl compound production; these enzymes would thus have another reaction mechanism. Enzymes of the adenylate-forming superfamily (catalyzing various types of reactions at present) may share a common ancestor that originally catalyzed the N-acylation reaction (amide bond synthesis) discovered in this study or acyl-CoA synthesis. Further studies on the adenylate-forming superfamily could provide information on this evolutionary implication. This new N-acyl-l-cysteine synthetic reaction opens the door for the development of a new procedure for the production of new peptide/amide compounds. For example, other types of acyl-CoA synthetases (e.g. long-chain fatty acyl-CoA synthetase (39), 4-coumarate and cinnamate-CoA ligase (7, 40), and 3-chlorobenzoate-CoA ligase (34)) can synthesize a new acyl-amino acid.

During the synthesis of N-isobutyryl-l-cysteine, AMP was formed stoichiometrically with the consumption of ATP (Fig. 2). Furthermore, the *Kₘ* value for isobutyrate in N-isobutyryl-l-cysteine synthesis was almost the same as that for isobutyrate in isoat-byuryl-CoA synthesis. These findings strongly indicate that the first half-reaction (i.e. acyl-AMP synthesis) conserved in adenylate-forming enzymes would proceed even in N-isobutyryl-l-cysteine synthesis and that the reaction mechanism (6, 7) for the first half-reaction of acyl-CoA synthesis would be identical to that of N-acyl-l-cysteine synthesis. In consequence, the acid recognition
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(binding) site for N-isobutryl-L-cysteine synthesis would also be identical to that for acyl-CoA synthesis. On the other hand, considering the $K_m$ value for L-cysteine (71.1 mM) in N-isobutryl-L-cysteine synthesis, N-isobutryl-L-cysteine synthesis may not proceed physiologically. However, our discovery demonstrating that an acyl-CoA synthetase yields not only a thioester bond but also an amide bond is very surprising, considering that an acyl-CoA synthetase is a ubiquitous and important enzyme that all living organisms possess. In order to obtain information on the L-cysteine recognition (binding) site involved in the second half-reaction, the effect of L-cysteine on the acyl-CoA synthetic reaction was examined. When the reaction mixture contained 1 mM CoA, the isobutyryl-CoA synthetic reaction was inhibited by the addition of L-cysteine: 0 mM L-cysteine, 100%; 1 mM L-cysteine, 86%; 2 mM L-cysteine, 66%; 3 mM L-cysteine, 43%; 10 mM L-cysteine, 0% relative residual activity, respectively. The concentrations of L-cysteine involved in the inhibition of isobutyryl-CoA synthesis are lower than the $K_m$ value for L-cysteine (71.1 mM). Taken together, these results raise the possibility that acyl-CoA synthetases, which play a critical role in the carbon metabolism of all living organisms, are physiologically controlled by L-cysteine. To date, the role of L-cysteine as a regulatory molecule for acid and fatty acid metabolic pathways in vivo has not been reported to the best of our knowledge. Fig. 11 shows the Hanes-Woolf plots for the isobutyryl-CoA synthesis in the absence of L-cysteine and the presence of 1, 2, and 3 mM L-cysteine. Although each $V_{max}$ value in isobutyryl-CoA synthesis decreased, each $K_m$ value for CoA did not shift upon the addition of L-cysteine. The plots reveal that L-cysteine acted as a noncompetitive inhibitor of CoA. Accordingly, the L-cysteine recognition (binding) site in AcsA would be different from the CoA rec-
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FIGURE 11. Inhibition of isobutyryl-CoA synthesis by L-cysteine. The isobutyryl-CoA synthetic reaction rate in the absence (●) of L-cysteine, and the presence of 1 mM (●), 2 mM (●), and 3 mM (●) L-cysteine are shown by Hanes-Woolf plots. L-Cysteine is a noncompetitive inhibitor of CoA. The \( K_i \) value measured from Dixon plots (41) was 1.19 ± 0.02 mM. All data points represent the mean values ± S.D. for three experiments. Lines through the mean values represent a mathematical fit of the data on linear least squares regression analysis.

Ognition (binding) site. Based on a lot of structural and functional information on adenylate-forming enzymes, a domain alternation mechanism for these enzymes, including acyl-CoA synthetases, has been proposed; upon completion of the initial adenylation reaction, the C-terminal domain of these enzymes undergoes a 140° rotation to perform the second thioester-forming half-reaction (3, 4). For AcsA, the initial adenylation reaction would proceed during \( N \)-isobutyryl-L-cysteine synthesis. Because of the difference between the L-cysteine recognition (binding) site and the CoA recognition (binding) site in AcsA, the rotation of the C-terminal domain may be inhibited by L-cysteine, resulting in that of a reaction yielding an amide bond (or \( N \)-acyl-L-cysteine synthesis) may proceed.

Here, we found \( N \)-acyl-L-cysteine synthesis by AcsA and the generality of this unique reaction in the adenylate-forming superfamily. However, it is difficult to investigate the mechanism underlying the second half-reaction in \( N \)-acyl-L-cysteine synthesis in detail even if a lot of structural and functional information on adenylate-forming enzymes is available, because the L-cysteine recognition (binding) site in acyl-CoA synthetase is unclear. Thus, further analyses, including three-dimensional structural analysis of the acyl-CoA synthetase with L-cysteine, are in progress in order to understand the reaction mechanism underlying amide bond formation in detail.

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