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Discovery of amino acid amides as new substrates for α-amino-ε-caprolactam racemase from *Achromobacter obae*

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Abstract

Amino acid amide racemizing activity was discovered in α-amino-ε-caprolactam (ACL) racemase (EC 5. 1. 1. 15) from *Achromobacter obae*. The nucleotide sequence of 1305 bp ACL racemase gene was designed for expression in *Escherichia coli* and synthesized in a reaction with 46 oligonucleotides by assembly PCR technique. The gene was expressed under the control of the *lac* promoter, and ACL racemase was purified from *E. coli* JM109/pACL60 harboring the gene for the enzyme. The enzyme catalyzed the racemization of newly discovered substrates such as 2-aminobutyric acid amide, alanine amide, threonine amide, norvaline amide, norleucine amide, leucine amide, methionine amide, serine amide, and phenylalanine amide. The relative activity towards L-2-aminobutyric acid amide was 2.7% that for L-ACL (350 U/mg), followed by L-alanine amide by 2.1%. Kinetic parameters for the two D- and L-alanine-isomers of amino acid amides (2-aminobutyric acid amide and alanine amide) and L-ACL were obtained from Lineweaver–Burk plots. The K_{cat} values for L-ACL, D- and L-2-aminobutyric acid amide, and D- and L-alanine amide were calculated to be 360, 40, 13, 9.8 and 7.2 S^{-1}, respectively. The K_m values for L-ACL, D- and L-2-aminobutyric acid amide, and D- and L-alanine amide were shown to be 10.1, 3.5, 1.1, 3.4 and 2.5 mM, respectively. The L-enantiomer of 2-aminobutyric acid amide (22.5 μmol) was completely racemized by ACL racemase (136 μg) in 100 min, and the same amount of L-alanine amide in 120 min. The catalytic efficiency of the newly discovered racemization reaction of 2-aminobutyric acid amide was calculated for be fairly high at about 33% that for L-ACL, while that for alanine amide was about 8.1%. Here, we report that amino acid amides act as new substrates for the ACL racemase.

Keywords: Amino acid amide; α-Amino-ε-caprolactam; Racemase; Assembly PCR

1. Introduction

A number of studies have been made on amino acid racemases acting on amino acid derivatives [1], these include alanine racemase (EC 5.1.1.1) from *Salmonella typhimurium* [2], glutamate racemase (EC 5.1.1.3) from *Pediococcus pentosaceus* [3], threonine racemase (EC 5.1.1.6) from *Pseudomonas putida* [4], arginine racemase (EC 5.1.1.9) from *Pseudomonas graveolens* [5], amino acid racemase (EC 5.1.1.10) from *P. putida* [6] and *Aeromonas punctata* [7], and aspartate racemase (EC 5.1.1.13) from *Streptococcus faecalis* [8].

α-Amino-ε-caprolactam (ACL) racemase (EC 5. 1. 1. 15) from *Achromobacter obae* catalyzes racemization of D- and L-ACL [9,10], and the enzyme was used in combination with L-ACL hydrolase from *Cryptococcus laurentii* for the industrial L-lysine production from DL-ACL in Japan [11]. Soda et al. reported the enzymatic properties of this ACL racemase and found that the substrates for the enzyme are only ACL, α-amino-δ-valerolactam, and α-amino-3-thio-ε-caprolactam; neither α-H-amino acids nor α-H-amino acid amides appeared to be substrates. They even reported that the enzyme does not act on tryptophan amide and leucine amide [12,13].

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D-Amino acids and their derivatives are important commercial products with applications in feed supplements, medicines, cosmetics, and as building blocks for compounds with biological or physiological activities [14]. Most of the proteinogenic L-amino acids are efficiently manufactured by fermentation, but D-amino acids are hardly produced by fermentation, with an exception of D-alanine [15].

We previously reported that newly discovered D-stereospecific hydrolases [14,16–19] can be used for the preparation of D-amino acids by an asymmetric hydrolysis of racemic amino acid amides, which are prepared by hydration of the corresponding amino nitriles synthesized from an aldehyde and cyanide by the Strecker synthesis [19,20]. The amino acids can only be obtained in 50% yield, since the enzyme accepts only the D-amino acid amide as a substrate. In this process, if amino acid amide racemase could be used together with D-stereospecific hydrolases, the remaining L-amino acid amide would be racemized and the D-amino acid amide formed could be hydrolyzed by D-stereospecific hydrolases, resulting in the production of optically active D-amino acid in 100% theoretical yield. It would also be possible to synthesize optically active L-amino acids in 100% yield, when amino acid amide racemase is used with L-stereospecific hydrolases.

Knowing the unique properties of ACL racemase which specifically acts on neutral cyclic amides but not on amino acids, we were prompted to examine the detailed catalytic activity of ACL racemase on amino acid amides. We synthesized ACL racemase gene of *A. obae* and transformed *E. coli* with a plasmid harboring the gene, and then selected ACL racemase-positive *E. coli* transformant. The ACL racemase gene was synthesized based on the published gene sequence, using the assembly PCR approach that provides a fast way to synthesize the gene. The synthetic ACL racemase gene was then expressed in *Escherichia coli*. We report the newly discovered activity towards α-H-amino acid amides of the ACL racemase, as one of the first reports on amino acid amide racemizing activity ever shown by an enzyme. After completing our studies, we noticed that α-H-amino acid amide racemizing activities from *Agrobacterium rhizogenes*, *Arthrobacter nicotianae* and *Ochrobactrum anthropi*, and some of the gene sequences were described in a recent patent [21]. Our preliminary report has appeared [22].

2. Materials and methods

2.1. Materials, enzymes, and chemicals

Oligonucleotides were purchased from Hokkaido System Science Co., Ltd. (Hokkaido, Japan). *Takara Ex Taq* DNA polymerase was obtained from Takara Shuzo (Shiga, Japan). Restriction endonucleases were obtained from Takara Shuzo, Toyobo (Osaka, Japan), and New England Biolabs (Beverly, MA, USA). Shrimp alkaline phosphatase was obtained from Roche Diagnostics GmbH (Penzberg, Germany). Ligation Kit ver. 2 was purchased from Takara shuzo. Crownpak CR (+) column was from Daicel Chemical Industries, Ltd. (Tokyo, Japan). DEAE-Toyopearl 650M and Butyl-Toyopearl 650M were purchased from Tosoh Corp. (Tokyo, Japan). Superdex 200 HR 10/30 and MonoQ HR 5/5 were from Amersham Biotech (Uppsala, Sweden). Bacto yeast extract and Bacto tryptone were obtained from Difco (Detroit, USA). All other chemicals were of commercial sources and used without further purification.

2.2. Bacterial strain, plasmids, and culture condition

E. coli JM109 (*recA*1, *endA*1, *gyrA*96, *thi*, *hsdR*17, *supE*44, *relA*1, Δ (*lac-proAB*)/F'[*traD*36, *proAB*$^+$, *lacIq*, *lacZ*ΔM15]) was used as a host for the recombinant plasmid. pUC18 (Takara Shuzo) was used as cloning vector. Recombinant *E. coli* was cultured at 37 °C for 12 h in LB medium containing 10 g/l Bacto tryptone, 5 g/l Bacto yeast extract, and 10 g/l NaCl, pH 7.2, supplemented with a final concentration of 80 μg/ml ampicillin. To induce the *lac* promoter, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.5 mM.

2.3. Substrates

L-ACL was prepared from DL-ACL by the optical resolution using (*S*)-(−)-L-pyrrolidone carboxylic acid according to the method of Brenner et al. [23]. Amino acid methylesters were prepared from the corresponding amino acids with thionylchloride in methanol at −20 °C. Amino acid amides were then obtained by ammonialysis of the corresponding methylesters with dry NH_3 gas in methanol at room temperature. ^{1}H- and ^{13}C-NMR spectra were recorded by JEOL LA-400 spectrometers (Tokyo, Japan) with tetramethylsilane as an internal standard.

2.4. Enzyme assay

The standard reaction mixture (100 μl) for assaying the ACL racemase activity contained 100 mM potassium phosphate buffer (KPB), pH 7.0, 2 μM pyridoxal phosphate (PLP) and 100 mM L-ACL. The reaction was performed at 30 °C for 10 min and stopped by the addition of 20 μl 2N $HClO_4$. ACL racemase activity toward L-ACL was determined by measuring the amount of D-ACL formed. The amount of D-ACL formed was determined with a high-performance liquid chromatography (HPLC) equipped with a Crownpak CR (+) column at a flow rate of 0.6 ml/min, using the solvent system of 60 mM $HClO_4$. Absorbance of the eluate was monitored at 200 nm. One unit of the enzyme activity was defined as the amount of enzyme that catalyzed the formation of 1 μmol of D-ACL from L-ACL per minute.

2.5. Synthesis of ACL racemase gene from A. obae

The ACL racemase gene from *A. obae* is composed of 1305 nucleotides (435 amino acid residues) [24]. Forty-six

oligonucleotides (26 nt × 50 nt, 10 nt × 49 nt, 2 nt × 48 nt, 4 nt × 47 nt, 2 nt × 46 nt, 2 nt × 40 nt) were designed on the basis of the nucleotide sequence of the ACL racemase gene from *A. obae* [25]. Forty-six oligonucleotides configured complementary oligonucleotides, which will overlap by 20 nt (Fig. 1). *Eco*RI and *Sph*I sites were introduced into the flanking region of the ACL racemase gene by using two primers (ACL1 and ACL46 oligonucleotides); ACL1 primer, 5′-

A1 5’ - gccgggaattcgaaggaggcaTAGtggatgacgaaggcgctttacgatcg -3’
A2 5’ - ctgcaggttaccgatggccgcaccgtcgcgatcgtaaagcgccttcg -3’
A3 5’ - gccatcggtaacctgcagaagctgcgcttcttcccgcttgccatctcggg -3’
A4 5’ - ctcttcgatcaggcgggcgcctcggccgcccgagatggcaagcggg -3’
A5 5’ - gcccgcctgatcgaagagaacggacgcgagctgatcgatctttccggcgc -3’
A6 5’ - ggccgtagcctaggctcgccgcgccccaggcgccggaaagatcgatcag -3’
A7 5’ - gcgagcctaggctacggccacccggccatcgtcgccgcggtttccgccgc -3’
A8 5’ - ggatcgttgcgccggccggattggcggcagcggcggaaaccgcggcgac -3’
A9 5’ - ccggccggcgcaacgatcctttcggcgtccaacgcaccggccgtgaccc -3’
A10 5’ - ccgggaaagctggccaacagcctttcggccagggtcacggccggtgcg -3’
A11 5’ - ctgttggccagctttcccggcgaaggaacgcacaagatctggttcggcc -3’
A12 5’ - cgttggcatccgagccggaatggccgaaccagatcttgtg -3’
A13 5’ - ccggctcggatgccaacgaagccgcctatcgggcgatcgtgaaggc -3’
A14 5’ - cggcgaaggcgatgacgccgctgcggccggttgccttcacgatcgcccg -3’
A15 5’ - cggcgtcatcgccttcgccggcgcctatcacggctgcacggtcggttcg -3’
A16 5’ - cggcctgaacgctgtggccggaaaaggccatcgaaccgaccgtgcagccg -3’
A17 5’ - cggccacagcgttcaggccgacgcggccaaagcggacgggctgatccttc -3’
A18 5’ - gataggggcgatagggatcggggtagggcagaaggatcagcccgtccgct -3’
A19 5’ - cgatccctatcgcccctatcggaacgatccgacgggcgatgccatcctc -3’
A20 5’ - gaaccgcagccagcttttccgtgagaagcgtgaggatggcatcgcccgtc -3’
A21 5’ - ggaaaagctggctgcggttccggccggctcgatcggtgcggcctttatcg -3’
A22 5’ - cgatcagcccgccgtccgactggatcggttcgataaaggccgcaccgatc -3’
A23 5’ - gtcggacggcgggctgatcgttcctcgggacggctttctgcgcaagttcg -3’
A24 5’ - cgagaatgccatgggcgcggcagatatcggcgaacttgcgcagaaagccg -3’
A25 5’ - ccgcgcccatggcattctcgtcgtctgcgacgaggtgaaggtgggccttg -3’
A26 5’ - gctcgaagcagtgcaggcggccgctgcgggcaaggcccaccttcacctcg -3’
A27 5’ - ccgcctgcactgcttcgagcatgagggcttcgttcccgatatcctggtgc -3’
A28 5’ - gcggcagcccgccgccaagccctttgcccagcaccaggatatcgggaacg -3’
A29 5’ - gcttggcggcgggctgccgctctcggcggtcatcgcaccggccgagatcc -3’
A30 5’ - gcatggcaaatgcgctcgcgcagtcgaggatctcggccggtgcgatg -3’
A31 5’ - gagcgcatttgccatgcaaaccttgcacggcaacccgatctccgccgctg -3’
A32 5’ - ggtcgatcgtttccagcacggcaaggccggcagcggcggagatcgggttg -3’
A33 5’ - cgtgctggaaacgatcgaccgggacgacctccccgctatggccgagcgg -3’
A34 5’ - ccgacagaccatcacgcaggagcctgcccttccgctcggccatagcgggg -3’
A35 5’ - cctgcgtgatggtctgtcggaactcgcaaaacggcatccgctgatcggcg -3’
A36 5’ - gccgcaggcaagtccgcggccgcggatatcgccgatcagcggatgccg -3’
A37 5’ - ccgcggacttgcctgcggcatggaactcgtatgcgaccggcagagccgcg -3’
A38 5’ - gagctttgccgtctcggctctcgccggttcgcggctctgccggtcgc -3’
A39 5’ - gccgagacggcaaagctcatctaccgcgcctaccagctcggtctcgtcg -3’
A40 5’ - gcacattgccgttcatgccgacataatagacgacgagaccgagctggtag -3’
A41 5’ - cggcatgaacggcaatgtgctggagttcacaccgcccctgacgataacgg -3’
A42 5’ - ggaggtcaagcgccttatggatgtcggtttccgttatcgtcaggggcgg -3’
A43 5’ - ccataaggcgcttgacctcctggacagggctttcagtgaactttctgccg -3’
A44 5’ - cagcgaactgcgcgatttcctcgttcgaaacggcagaaagttcactg -3’
A45 5’ - ggaaatcgcgcagttcgctggctggtaaaacgttccaacacggagtagcc -3’
A46 5’ - tgctcgtggcggctgcatgcggctactccgtgttggaacg -3’

Fig. 1. Oligonucleotides synthesized based on the nucleotide sequence of ACL racemase gene from *A. obae*.

gccgggaattcgaaggaggggaggcaTAGtgg**atg**acgaaggcgctttacg-atcg-3′; ACL46 primer, 5′-tgctcgtggcggctgcatgcggctactccgt-gttggaacg-3′. The ACL1 oligonucleotide contained an *Eco*RI recognition site (underlined sequence), a ribosome-binding site (double underlined sequence), a TAG stop codon (upper case letters) in frame with the *lacZα* gene in pUC18, and the 5′-end of the ACL racemase gene starting with an ATG start codon (bold letters). The ACL46 oligonucleotide contained a *Sph*I site (underlined sequence).

The 46 oligonucleotides were combined and assembled by PCR. The reaction mixture (50 μl) for PCR contained 2 mM Tris/HCl, pH 8.0, 10 mM KCl, 0.01 mM EDTA, 1 mM DTT, 0.05% Tween 20, 0.05% Nonidet P-40, 5% Glycerol, 0.25 mM dNTP, 0.02 pmol/μl of each oligonucleotide, and 0.05 U/μl *Takara Ex Taq* DNA polymerase. The first PCR program consisted of 55 cycles at 94 °C for 30 s, 52 °C for 30 s and 72 °C for 30 s. PCR was carried out on an MJ Research (Watertown, MA, USA) thermal cycler, PTC-200. Then the assembled ACL racemase gene was amplified in a 2nd PCR by using oligonucleotides ACL1 and ACL46 as outside primers. Gene amplification mixture for this 2nd PCR contained 1.3 μl gene assembly reaction mixture, 2 mM Tris/HCl, pH 8.0, 10 mM KCl, 0.01 mM EDTA, 1 mM DTT, 0.05% Tween 20, 0.05% Nonidet P-40, 5% Glycerol, 0.25 mM dNTP, 2 pmol/μl of both outside primers, and 0.05 U/μl *Takara Ex Taq* DNA polymerase. The PCR program was performed with 23 cycles at 94 °C for 30 s, 50 °C for 30 s and 72 °C for 60 s.

2.6. Cloning of ACL racemase gene and construction of pACL

The amplified PCR products were digested with *Eco*RI and *Sph*I, and run on agarose gel electrophoresis, and then extracted using QIAquick™ gel extraction kit. The amplified DNA was inserted downstream of the *lac* promoter in pUC18, and then used to transform *E. coli* JM109 cells. Each colony was inoculated in a tube containing LB medium supplemented with ampicillin and IPTG and shaken at 37 °C for 12 h. The collected cells were resuspended in reaction mixture containing 10 mM KPB, 10 mM D-ACL, and 2 μM PLP. ACL racemase activity towards D-ACL was determined by measuring the amount of L-ACL formed by HPLC. The transformant *E. coli* JM109/pACL60 showed ACL racemase activity. The recombinant plasmid extracted from *E. coli* JM109/pACL60 and the DNA sequence of the inserted fragment was determined. Nucleotide sequencing was performed using the dideoxynucleotide chain-termination method with M13 forward and reverse oligonucleotides. Sequencing reactions were carried out with a Thermo sequence™ cycle sequencing kit (Amersham Pharmacia Biotech, Inc., USA) and dNTP mixture with 7-deaza-dGTP from Amersham Pharmacia Biotech (Uppsala, Sweden), and the reaction mixtures were run on DNA sequencer 4000L (Li-Cor, Lincoln, NE, USA).

2.7. Purification of recombinant ACL racemase from E. coli JM109/pACL60

E. coli JM109 harboring pACL60 was subcultured at 37 °C for 12 h in a test tube containing 5 ml LB medium supplemented with ampicillin. The subculture was then inoculated into a 2 l shaking flask containing 500 ml LB medium supplemented with 80 μg/ml ampicillin and 0.5 mM IPTG. After 8 h incubation at 37 °C with reciprocal shaking, the cells were harvested by centrifugation at 8000 × *g* for 5 min at 4 °C and washed with 0.85% NaCl. The buffer used throughout this study was 10 mM KPB, pH 7.0, containing 250 mM sucrose, 2 μM PLP and 5 mM 2-mercaptoethanol. Washed cells from 10 l culture were resuspended in 10 mM buffer and disrupted by sonication for 10 min (19 kHz; Insonator model 201 M; Kubota, Tokyo, Japan). For the removal of intact cells and cell debris, the lysate was centrifuged at 12,000 × *g* for 30 min at 4 °C. The supernatant was heated at 60 °C for 10 min followed by centrifugation [26]. The supernatant was applied to a DEAE-Toyopearl 650M column equilibrated with 10 mM buffer. After the column was washed thoroughly with 10 mM buffer, the enzyme was eluted by a linear gradient of 0 to 300 mM KCl in 10 mM buffer. The active fractions were combined and then brought to 30% $(NH_4)_2SO_4$ saturation and added to a Butyl-Toyopearl 650M column equilibrated with 10 mM buffer 30% saturated $(NH_4)_2SO_4$. After the column had been washed with the same buffer, the enzyme was eluted by a linear gradient of 30–0% $(NH_4)_2SO_4$ in 10 mM buffer. The active fractions were combined, concentrated by a Centricon YM-10, and applied to a column of Superdex 200 HR 10/30 equilibrated with 10 mM buffer containing 150 mM NaCl and eluted using FPLC system (Amersham Biosciences, Sweden) at 0.4 ml/min, and the active fractions were collected and concentrated by a Centricon YM-10. The enzyme was applied to a MonoQ HR 5/5 column which had been equilibrated with 10 mM buffer. The enzyme was eluted using FPLC system by a linear gradient of 0–300 mM NaCl in 10 mM buffer. The active fractions were combined and concentrated by a Centricon YM-10. Protein concentration was estimated by an absorbance at 280 nm, and determined by the method of Bradford using the dye reagent supplied by Bio-Rad. Bovine serum albumin was used as standard.

2.8. Substrate specificity

The substrate specificity was measured in a reaction mixture containing 2 μM PLP, 100 mM KPB (pH 7.0), 100 mM substrate, and an appropriate amount of enzyme, and the reaction mixture (2 ml) was incubated at 30 °C. The reaction was stopped by the addition of $HClO_4$. The concentration of the products was determined with HPLC.

3. Results

3.1. Synthesis of ACL racemase gene from A. obae, cloning, and construction of pACL

The ACL racemase gene consists of 1305 bp that encodes a protein of 435 amino acid residues [24]. Forty-six oligonucleotides were synthesized on the basis of the nucleotide sequence of the ACL racemase gene from *A. obae*. The *Eco*RI-*Sph*I fragment of the amplified PCR product with a size of 1380 bp was extracted from agarose gel and ligated into pUC18, and then used to transform *E. coli* JM109 cells. *E. coli* JM109/pACL60 showed ACL racemase activity. Plasmid pACL60 containing the fragment coding for the ACL racemase was extracted from *E. coli* JM109/pACL60, and the DNA sequence of the inserted fragment was determined to be identical with the reported sequence (Fig. 2) [24].

3.2. Substrate specificity of ACL racemase

ACL racemase was purified from the cell-free extract of *E. coli* JM109/pACL60 in 16% yield, by a procedure involving heat treatment, and chromatography on DEAE-Toyopearl 650M, Butyl-Toyopearl 650M, Superdex 200 HR 10/30, and MonoQ HR 5/5 columns. The final preparation gave a single band on SDS-PAGE. After purification, 4.2 mg of ACL

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   1  ACGAAGGCGCTTTACGATCGCGACGGTGCGGCCATCGGTAACCTGCAGAAGCTGCGCTTC    60
      T  K  A  L  Y  D  R  D  G  A  A  I  G  N  L  Q  K  L  R  F

  61  TTCCCGCTTGCCATCTCGGGCGGCCGAGGCGCCCGCCTGATCGAAGAGAACGGACGCGAG   120
      F  P  L  A  I  S  G  G  R  G  A  R  L  I  E  E  N  G  R  E

 121  CTGATCGATCTTTCCGGCGCCTGGGGCGCGGCGAGCCTAGGCTACGGCCACCCGGCCATC   180
      L  I  D  L  S  G  A  W  G  A  A  S  L  G  Y  G  H  P  A  I

 181  GTCGCCGCGGTTTCCGCCGCTGCCGCCAATCCGGCCGGCGCAACGATCCTTTCGGCGTCC   240
      V  A  A  V  S  A  A  A  A  N  P  A  G  A  T  I  L  S  A  S

 241  AACGCACCGGCCGTGACCCTGGCCGAAAGGCTGTTGGCCAGCTTTCCCGGCGAAGGAACG   300
      N  A  P  A  V  T  L  A  E  R  L  L  A  S  F  P  G  E  G  T

 301  CACAAGATCTGGTTCGGCCATTCCGGCTCGGATGCCAACGAAGCCGCCTATCGGGCGATC   360
      H  K  I  W  F  G  H  S  G  S  D  A  N  E  A  A  Y  R  A  I

 361  GTGAAGGCAACCGGCCGCAGCGGCGTCATCGCCTTCGCCGGCGCCTATCACGGCTGCACG   420
      V  K  A  T  G  R  S  G  V  I  A  F  A  G  A  Y  H  G  C  T

 421  GTCGGTTCGATGGCCTTTTCCGGCCACAGCGTTCAGGCCGACGCGGCCAAAGCGGACGGG   480
      V  G  S  M  A  F  S  G  H  S  V  Q  A  D  A  A  K  A  D  G

 481  CTGATCCTTCTGCCCTACCCCGATCCCTATCGCCCCTATCGGAACGATCCGACGGGCGAT   540
      L  I  L  L  P  Y  P  D  P  Y  R  P  Y  R  N  D  P  T  G  D

 541  GCCATCCTCACGCTTCTCACGGAAAAGCTGGCTGCGGTTCCGGCCGGCTCGATCGGTGCG   600
      A  I  L  T  L  L  T  E  K  L  A  A  V  P  A  G  S  I  G  A

 601  GCCTTTATCGAACCGATCCAGTCGGACGGCGGGCTGATCGTTCCTCGGGACGGCTTTCTG   660
      A  F  I  E  P  I  Q  S  D  G  G  L  I  V  P  R  D  G  F  L

 661  CGCAAGTTCGCCGATATCTGCCGCGCCCATGGCATTCTCGTCGTCTGCGACGAGGTGAAG   720
      R  K  F  A  D  I  C  R  A  H  G  I  L  V  V  C  D  E  V  K

 721  GTGGGCCTTGCCCGCAGCGGCCGCCTGCACTGCTTCGAGCATGAGGGCTTCGTTCCCGAT   780
      V  G  L  A  R  S  G  R  L  H  C  F  E  H  E  G  F  V  P  D

 781  ATCCTGGTGCTGGGCAAAGGGCTTGGCGGCGGGCTGCCGCTCTCGGCGGTCATCGCACCG   840
      I  L  V  L  G  K  G  L  G  G  G  L  P  L  S  A  V  I  A  P

 841  GCCGAGATCCTCGACTGCGCGAGCGCATTTGCCATGCAAACCTTGCACGGCAACCCGATC   900
      A  E  I  L  D  C  A  S  A  F  A  M  Q  T  L  H  G  N  P  I

 901  TCCGCCGCTGCCGGCCTTGCCGTGCTGGAAACGATCGACCGGGACGACCTCCCCGCTATG   960
      S  A  A  A  G  L  A  V  L  E  T  I  D  R  D  D  L  P  A  M

 961  GCCGAGCGGAAGGGCAGGCTCCTGCGTGATGGTCTGTCGGAACTCGCAAAACGGCATCCG  1020
      A  E  R  K  G  R  L  L  R  D  G  L  S  E  L  A  K  R  H  P

1021  CTGATCGGCGATATCCGCGGCCGCGGACTTGCCTGCGGCATGGAACTCGTATGCGACCGG  1080
      L  I  G  D  I  R  G  R  G  L  A  C  G  M  E  L  V  C  D  R

1081  CAGAGCCGCGAACCGGCGAGAGCCGAGACGGCAAAGCTCATCTACCGCGCCTACCAGCTC  1140
      Q  S  R  E  P  A  R  A  E  T  A  K  L  I  Y  R  A  Y  Q  L

1141  GGTCTCGTCGTCTATTATGTCGGCATGAACGGCAATGTGCTGGAGTTCACACCGCCCCTG  1200
      G  L  V  V  Y  Y  V  G  M  N  G  N  V  L  E  F  T  P  P  L

1201  ACGATAACGGAAACCGACATCCATAAGGCGCTTGACCTCCTGGACAGGGCTTTCAGTGAA  1260
      T  I  T  E  T  D  I  H  K  A  L  D  L  L  D  R  A  F  S  E

1261  CTTTCTGCCGTTTCGAACGAGGAAATCGCGCAGTTCGCTGGCTGG                 1305
      L  S  A  V  S  N  E  E  I  A  Q  F  A  G  W
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Fig. 2. The nucleotide sequence of ACL racemase gene from *E. coli* JM109/pACL60.

Table 1
Summary of purification of ACL racemase from *E. coli* JM109/pACL60

Step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)
Cell-free extract	1200	9100	7.60	100
Heat treatment	210	8600	41.0	95
DEAE-Toyopearl 650M	150	7700	53.0	85
Butyl-Toyopearl 650M	53	4000	76.0	44
Superdex 200 HR 10/30	35	2800	80.0	31
MonoQ HR 5/5	4.2	1500	350	16

racemase was obtained with about 45-fold increase in the specific activity. It was calculated that the enzyme comprised about 2.2% of the total extracted cellular protein. A summary of the purification of the enzyme from *E. coli* JM109/pACL60 is shown in Table 1.

The substrate specificity of the purified enzyme was examined (Table 2). L-Amino acid amides and peptide compounds were tested as substrates for the enzyme and nine compounds were accepted as substrates. The specific activity towards L-2-aminobutyric acid amide was 9.5 U/mg which was 2.7% of the activity towards L-ACL (350 U/mg at 100 mM substrate concentration) and for alanine amide, threonine amide, norvaline amide, norleucine amide, leucine amide, methionine amide, serine amide, and phenylalanine amide, it was 2.1, 1.7, 1.7, 1.5, 1.3, 0.94, 0.47, and 0.052%, respectively.

No racemizing activity was observed with α-H-amino acids, dipeptides containing alanine, and alanine methylester as described in the legend of Table 2.

3.3. Kinetic characterization of ACL racemase

Kinetic parameters for the D- and L-isomers of two amino acid amides (2-aminobutyric acid amide and alanine amide) and L-ACL were calculated from Lineweaver–Burk plots (Table 3). The K_m and K_{cat} values for L-ACL were calculated to be 10.1 mM and 360 S^{-1}, respectively. The K_{cat} values for D- and L-2-aminobutyric acid amide and D- and L-alanine amide were calculated to be 40, 13, 9.8, and 7.2 S^{-1}, respectively. The K_m values for D- and L-2-aminobutyric acid amide and D- and L-alanine amide were shown to be 3.5, 1.1, 3.4, and 2.5 mM, respectively. The K_{cat}/K_m value for L-2-aminobutyric acid amide was calculated to be 11.8 S^{-1} mM^{-1} (V_{max}/K_m: 14.5 μmol min^{-1} mg^{-1} mM^{-1}) and K_{cat}/K_m value for L-alanine amide was 2.88 S^{-1} mM^{-1} (V_{max}/K_m: 3.5 μmol min^{-1} mg^{-1} mM^{-1}). The K_{cat}/K_m values for the D-enantiomers were similar to those for L-enantiomers. The K_{cat}/K_m value for L-ACL was calculated to be 35.6 S^{-1} mM^{-1} (V_{max}/K_m: 43.0 μmol min^{-1} mg^{-1} mM^{-1}). Therefore, the catalytic efficiency of the newly discovered racemization reaction of 2-aminobutyric acid amide is fairly high at about 33% that for L-ACL, while that for alanine amide is about 8.1%. The high affinity (K_m values at 1–3.5 mM) for these aliphatic amino acid amides caused rather good catalytic efficiencies as compared with ACL. When those K_{cat} and K_m values were used, the calculated K_{eq} for 2-aminobutyric acid amide racemization and alanine amide racemization were 1.0 and 1.0, which agrees well with the theoretical value for the chemically symmetric reaction.

$$K_{eq} = \frac{[K_{cat}/K_m]_{D\text{-isomer}}}{[K_{cat}/K_m]_{L\text{-isomer}}} = 1.0$$

Fig. 3 shows the time course of the racemization reaction of L-amino acid amide (L-2-aminobutyric acid amide and L-alanine amide) using ACL racemase. The reaction mixture contained 2 μM PLP, 100 mM KPB (pH 7.0), 40 mM substrate, and ACL racemase, and the reaction mixture was incubated at 30 °C, and controls were run without enzyme at the same condition. After 100 min, L-2-aminobutyric acid

Table 2
Substrate specificity of ACL racemase

Substrate	Relative activity (%)
L-α-Amino-ε-caprolactam	100
L-2-Aminobutyric acid amide	2.7
L-Alanine amide	2.1
L-Threonine amide	1.7
L-Norvaline amide	1.7
L-Norleucine amide	1.5
L-Leucine amide	1.3
L-Methionine amide	0.94
L-Serine amide	0.47
L-Phenylalanine amide	0.052

The enzyme activity was assayed as described in Section 2. The following compounds were not a substrate for the ACL racemase: L-arginine amide, L-lysine amide, L-tyrosine amide, L-tryptophan amide, L-valine amide L-*tert*-leucine amide, L-phenylglycine amide, L-alanine methylester, L-alanyl-D-alanine, D-alanyl-L-alanine, D-alanylglycine, L-alanylglycine, L-2-aminobutyric acid, L-threonine, L-alanine, L-serine, L-norvaline, L-norleucine, L-methionine, L-valine, L-leucine, L-phenylalanine, and L-phenylglycine.

Table 3
Kinetic parameters for ACL racemase

Substrate	K_{cat} (S^{-1})	K_m (mM)	K_{cat}/K_m (S^{-1} mM^{-1})
L-α-Amino-ε-caprolactam	360	10.1	35.6
L-2-Aminobutyric acid amide	13	1.1	11.8
D-2-Aminobutyric acid amide	40	3.5	11.4
L-Alanine amide	7.2	2.5	2.88
D-Alanine amide	9.8	3.4	2.88

The enzyme activity was assayed as described in Section 2. The initial velocities were determined, and the steady-state kinetic parameters were calculated by using Lineweaver–Burk plots.

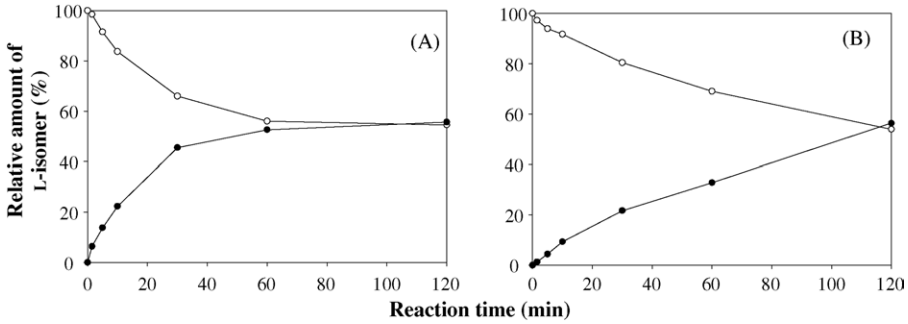

Fig. 3. Enzymatic racemization of D-isomer (●) and L-isomer (○) of different amino acid amide by ACL racemase. The reaction mixture (2.0 ml) contained 200 μmol of potassium phosphate buffer (pH 7.0), 4 nmol PLP, 80 μmol of L-2-aminobutyric acid amide or L-alanine amide and 136 μg of ACL racemase. The reaction was followed by HPLC at the point shown in the graphics. (A) 2-Aminobutyric acid amide; (B) alanine amide.

amide was completely racemized by the enzyme, and L-alanine amide was also completely racemized in 120 min. These facts clearly show that linear α-H-amino acid amides can be racemized by ACL racemase.

4. Discussion

Many different kinds of racemases acting on amino acids and hydroxyl acids, etc. have been reported to occur in some bacteria, however, it is generally accepted that none of them acts on amino acid amides. As for amino acid racemases, alanine racemase from *S. typhimurium* [2], glutamate racemase from *Pediococcus pentosaceus* [3], and aspartate racemase from *Streptococcus faecalis* [8] have very high specificities for the amino acid substrates. Threonine racemase from *P. putida* [4], arginine racemase from *Pseudomonas graveolens* [5], and amino acid racemase from *P. putida* [6] and *Aeromonas punctata* [7] show rather broader substrate specificities on amino acids.

α-Amino-ε-caprolactam is a heterocyclic compound used as the substrate for the enzymatic production of L-lysine in a Japanese chemical company, Toray Industries, Inc.: L-ACL is hydrolyzed to form L-lysine by L-ACL hydrolase of *C. laurentii*, and the unreacted D-isomer is racemized by ACL racemase of *A. obae* [11]. Soda et al. reported that ACL racemase from *A. obae* acts only on cyclic amino lactams such as ACL, α-amino-δ-valerolactam [27,28] and α-amino-3-thio-ε-caprolactam [13]. The properties of the ACL racemase hitherto known and the results of this study are summarized in Table 4 [9,10,12,13,26–29]. There has been no report that ACL racemase shows an activity towards amino acid amides. We predicted that ACL racemase would catalyze the racemization of amino acid amides as well as cyclic amino lactam compounds, based on a consideration in the structural similarity of ACL to simple amino acid amides: ACL has a free amino group adjacent to cyclic amide structure, and can be regarded as a homologue of α-H-amino acid amides with a free amino group on the α-position. Indeed, we have shown in this paper for the first time that the substrate specificity of ACL is rather wide and it acts on several amino acid amides.

Recently, the genetic information has been tremendously expanded and the functions of the proteins are assumed and discussed by homology comparisons of their primary and higher structures. However, it goes without saying that their functions are not fully understood by these methods, until they are proven by experiments. It is one of the surest ways to express the gene and prove their functions by an actual experiment as we did here, if one really would like to use the genetic resources.

Table 4
Properties of ACL racemase from *A. obae*

Property	Value
Specific activity (U/mg)	380 [26]
Molecular weight	
Gel filtration	51000 [12]
Gene sequencing	45568 [29]
No. of subunits	Monomer [12]
pH optimum for	
D-ACL	8.8 [12]
L-ACL	8.8 [12]
L-α-amino-δ-valerolactam	7.0 [10]
L-α-amino-3-thio-ε-caprolactam	10 [13]
Inhibitor	
Gly [12], Ala [12], Glu [12], Pro [12], Leu [12], L-Arg [12], L-His [12], L-Lys [12], L-Met [12], L-Orn [12], L-Phe [28], L-Trp [28], D-Lys [28], D-orn [28], L-α-aminobutyrate [28], D-α-aminobutyrate [28], D-cycloserine [12], ε-caprolactam [12], L-α-amino-δ-valerolactam [28], D-α-amino-δ-valerolactam [28], taurine [28], 2-methyl-3-benzothiazolonohydrazone hydrochloride [12], hydroxylamine [12], NEM [12], PCMB [12], $CuSO_4$ [12], $HgCl_2$ [12], phenylhydrazine [12], sodium borohydride [12]	
prosthetic group pyridoxal 5′-phosphate [12]	
Formation	Inducible [9]

Apparent K_m (mM) and K_{cat} (S^{-1}) values for	K_m	K_{cat}
L-ACL	6 [12]	360 [27]
L-ACL (this study)	10.1	360
D-ACL	8 [12]	–
L-α-Amino-δ-valerolactam	2.9 [27]	520 [27]
L-α-Amino-3-thio-ε-caprolactam	1.5 [13]	–
L-2-Aminobutyric acid amide (this study)	1.1	13
D-2-Aminobutyric acid amide (this study)	3.5	40
L-Alanine amide (this study)	2.5	7.2
D-Alanine amide (this study)	3.4	9.8

Construction of a synthetic gene using assembly PCR provides a fast way to clone and express such genes in *E. coli* without isolating the target gene from biological materials. We have succeeded in the synthesis of the ACL racemase gene from *A. obae* by assembly PCR of oligonucleotides [25], cloning and expression of the gene, purification of the enzyme from *E. coli* JM109/pACL60, and determination of the racemization reaction towards α-H-amino acid amides as substrates. The expression level of the enzyme by the *E. coli* transformant was rather low, although we did not optimize the culture and its induction conditions. The purification procedure of the enzyme may be simplified by adding His-Tag to the protein.

We reported the newly discovered activity of ACL racemase towards amino acid amides with detailed kinetic parameters. This is one of the first reports on amino acid amide racemizing activity ever shown by an enzyme [21]. The enzyme acted on breadth of amino acid amides such as 2-aminobutyric acid amide, alanine amide, threonine amide, norvaline amide, norleucine amide, leucine amide, methionine amide, serine amide, and phenylalanine amide. When the kinetic parameters for 2-aminobutyric acid amide and alanine amide were compared, the K_{cat}/K_m values for 2-aminobutyric acid amide (11.8 S^{-1} mM^{-1} for L-enantiomer, and 11.4 for D-enantiomer) were higher than those for alanine amide (2.88 for L-enantiomer and 2.88 for D-enantiomer). The substrate specificity showed the preference of the enzyme for substrates with shorter aliphatic side chain and the catalytic efficiencies for 2-amino butyric acid amide and alanine amide were 8.1–33% that for ACL. The low K_m values towards these substrates contributed much for the relatively high catalytic efficiencies. We here showed that the racemization reaction of L-2-aminobutyric acid amide and L-alanine amide progressed completely. If the ACL racemase will be used together with a D-stereospecific hydrolase, the remaining L-amino acid amides will be racemized and hydrolyzed by D-stereospecific hydrolases leading to a 100% theoretical yield [14]. With an L-stereospecific hydrolase, it would be possible to synthesize L-amino acids in 100% theoretical yield with high optical purity. Thus, amino acid amide racemase is a key enzyme to realize the dynamic kinetic resolution of amino acid amides when used with stereospecific amide hydrolases. This will result in high yield production of optically active amino acid without loss of substrate, thereby also reducing the production costs. The results of the dynamic kinetic resolution of amino acid amides to form optically active amino acid using ALC racemase and stereospecific amino acid amide hydrolases will be reported elsewhere.

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