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Purification, Characterization and Gene Cloning of Thermostable *O*-Acetyl-L-Homoserine Sulfhydrylase Forming γ-Cyano-α-Aminobutyric Acid

HIRONORI OMURA,[1,2]* MASAHIRO IKEMOTO,[2] MICHIHIKO KOBAYASHI,[3] SAKAYU SHIMIZU,[3] TOYOKAZU YOSHIDA,[1] AND TORU NAGASAWA[1]

Department of Biomolecular Science, Gifu University, Yanagido, Gifu 501-1193, Japan,[1] Ikeda Food Research Co., Ltd., Minooki-cho, Fukuyama, Hiroshima 721-0956, Japan,[2] and Agricultural Sciences, Kyoto University, Kyoto 606-8502, Japan[3]

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A thermophilic and cyanide ion-tolerant bacterium, *Bacillus stearothermophilus* CN3 isolated from a hot spring in Japan, was found to produce thermostable γ-cyano-α-aminobutyric acid synthase. The enzyme was purified and characterized. The purified enzyme has a molecular mass of approximately 180 kDa and consists of four identical subunits. It was stable in the pH range of 6.0 to 10.5 and up to 60°C. The enzyme catalyzed the γ-replacement reaction of *O*-acetyl-L-homoserine with cyanide ions. The gene encoding the γ-cyano-α-aminobutyric acid synthase was isolated from *B. stearothermophilus* CN3. Sequence homology analysis revealed that the γ-cyano-α-aminobutyric acid synthase from the bacterium is *O*-acetyl-L-homoserine sulfhydrylase. A recombinant plasmid, constructed by ligation of the cloned gene and an expression vector, was introduced into *Escherichia coli* JM109. The transformed *E. coli* cells overexpressed γ-cyano-α-aminobutyric acid synthase. The heat stable γ-cyano-α-aminobutyric acid synthase can be applied to the synthesis of [5-^{11}C]L-glutamic acid used as a tracer for positron emission tomography.

[**Key words:** positron emission tomography, *O*-acetyl-L-homoserine sulfhydrylase, γ-cyano-α-aminobutyric acid, *Bacillus stearothermophilus* CN3, cyanide ion-tolerant bacterium]

Via the metabolism of a certain substance in brain, various tissues or cells can be visually observed by positron emission tomography (PET), and the application of PET has attracted considerable attention in the field of medical diagnosis. The use of positron-emitting radionuclides is advantageous due to their high specific radioactivity. However, labeled compounds should be rapidly prepared due to their short half-lives. For the rapid and specific synthesis of labeled compounds, enzymatic synthesis has a clear advantage.

We have explored and identified useful enzymes for the synthesis of labeled compounds for PET (1–5). In our previous study, β-cyano-L-alanine synthase was found in a thermophilic and cyanide ion-tolerant bacterium, *Bacillus stearothermophilus* CN3 (6). The β-cyano-L-alanine synthase can be used for the positron-labeling of [4-^{11}C]L-2,4-diaminobutyric acid as shown in Fig. 1A. In the course of studies on β-cyano-L-alanine synthase, we found, by chance, the presence of a novel enzyme, γ-cyano-α-aminobutyric acid synthase, in the cell-free extract of the same CN3 strain. The enzyme catalyzes the synthesis of γ-cyano-α-aminobutyric acid from *O*-acetyl-L-homoserine and cyanide ions. Therefore, we designed a synthetic scheme for positron-labeled L-glutamic acid and L-glutamine (Fig. 1B) (7, 8). The present paper describes the purification and characterization of γ-cyano-α-aminobutyric acid synthase from a thermophilic and cyanide ion-tolerant bacterium, *B. stearothermophilus* CN3. We also studied the overexpression of this enzyme in *Escherichia coli* cells.

MATERIALS AND METHODS

Materials *O*-Acetyl-L-homoserine and γ-cyano-α-aminobutyric acid were synthesized as described previously (9, 10). DEAE–Cellulofine A-500m was purchased from Seikagaku Kogyo (Tokyo). Phenyl-Toyopearl 650M and TSK G-3000 SW were obtained from Tosoh (Tokyo). Sephacryl S-200 HR and low molecular weight markers for SDS–PAGE were purchased from Amersham Pharmacia Biotech (Tokyo). Molecular marker proteins for HPLC were purchased from Oriental Yeast (Osaka). All other reagents were of analytical grade and available from commercial sources.

Microorganism and cultivation *B. stearothermophilus* CN3, isolated from a hot spring in Matsue, Shimane, Japan (6), was used for the present study. The seed cultivation was carried out at 58°C for 18 h with reciprocal shaking at 120 strokes/min in a 500-ml Erlenmeyer flask containing 100 ml of medium containing 1 g of soluble starch, 0.5 g of yeast extract, 0.1 g of L-glutamine, 0.05 g of $MgSO_4 \cdot 7H_2O$, 1.0 mg of $FeSO_4 \cdot 7H_2O$ and 0.1 g of K_2HPO_4 (pH 7.2). The subculture was then transferred into a 2-*l* jar fermentor containing 1.6 *l* of the same medium supplemented with 0.01% (v/v) antifoam (Adekanol LG-109; Asahi Denka Kogyo, Tokyo).

* Corresponding author. e-mail: omura.hironori@nifty.com
phone: +81-(0)84-957-3411 fax: +81-(0)84-957-3421
Abbreviation: PET, positron emission tomography.

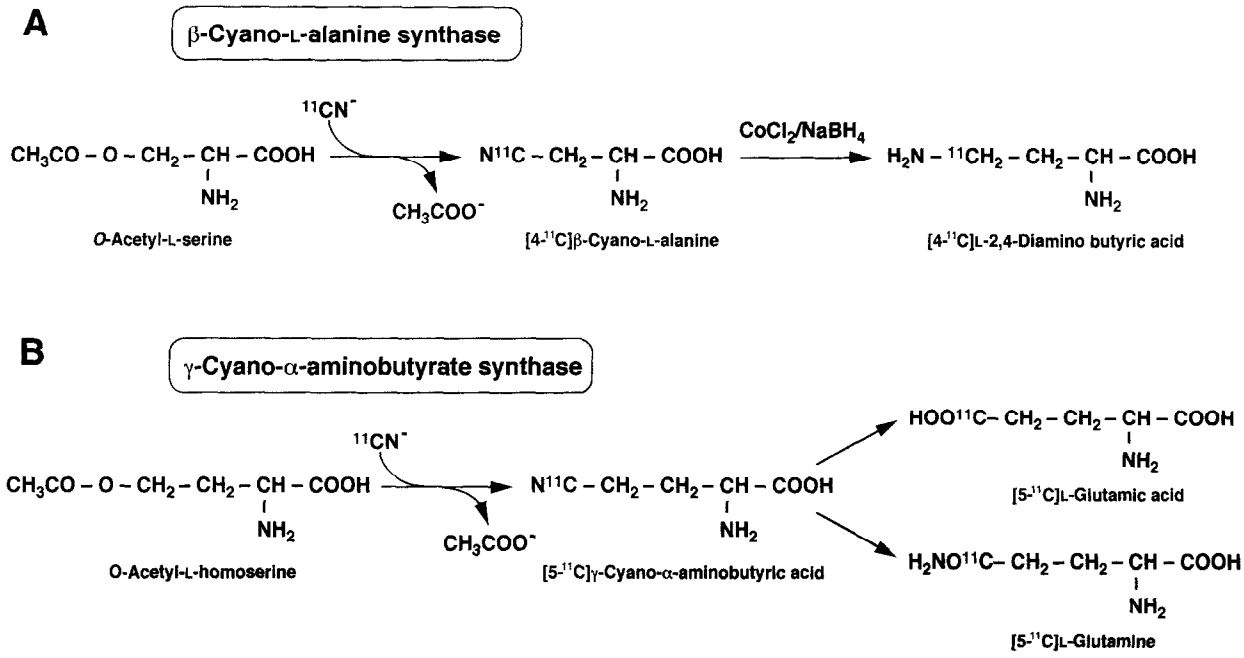

FIG. 1. (A) Synthesis of [4-^{11}C]L-2,4-diamino butyric acid via [4-^{11}C]β-cyano-L-alanine. β-Cyano-L-alanine synthase catalyzes the synthesis of β-cyano-L-alanine from cyanide ions and *O*-acetyl-L-serine. [4-^{11}C]L-2,4-Diamino butyric acid is obtained by the selective reduction of β-cyano-L-alanine with cobalt dichloride and sodium borohydride. (B) Synthesis of [5-^{11}C]L-glutamic acid and [5-^{11}C]L-glutamine via [5-^{11}C]γ-cyano-α-aminobutyric acid. γ-Cyano-α-aminobutyric acid synthase catalyzes the synthesis of γ-cyano-α-aminobutyric acid from cyanide ions and *O*-acetyl-L-homoserine.

The cultivation was carried out with aeration (1.6, v/v/m) and agitation (300 rpm) at 58°C for 18 h. The 1.6 *l* of culture was then transferred into a 200-*l* jar fermentor containing 160 *l* of the same medium supplemented with 0.01% (v/v) antifoam with aeration (1.0, v/v/m) and agitation (200 rpm) at 58°C for 18 h. Cells were harvested by centrifugation (20,000×*g*).

Enzyme assay The standard assay mixture for γ-cyano-α-aminobutyric acid synthase contained 50 mM potassium phosphate buffer (pH 7.5), 5 mM *O*-acetyl-L-homoserine, 10 mM potassium cyanide, 0.08 mM pyridoxal 5'-phosphate and enzyme solution in a total volume of 200 μl. The reaction was carried out at 45°C for 10 min and stopped by boiling for 2 min. The γ-cyano-α-aminobutyric formed acid was determined by HPLC after pretreatment of the reaction mixture (6). One unit of the enzyme was defined as the amount of enzyme catalyzing the formation of 1 μmol of γ-cyano-α-aminobutyric acid per minute under the standard assay conditions.

Enzyme purification All purification steps were performed at 4°C, and 20 mM potassium phosphate buffer (pH 7.5) containing 0.1 mM pyridoxal 5'-phosphate and 0.01 mM dithiothreitol (KP buffer) was used unless otherwise stated. The cells harvested from 160 *l* of culture broth (660 g wet weight) were suspended in 2.5 *l* of KP buffer. The cell suspension was subjected to treatment with a DYNO-Mill disintegrator (W.A. Bachofen, Basel, Switzerland) to disrupt the cells. Cell-free extract was obtained after the removal of cell debris by centrifugation at 20,000×*g* for 30 min. The cell-free extract was subjected to heat treatment at 60°C for 30 min. The denatured protein was removed by centrifugation at 20,000×*g* for 30 min. Solid ammonium sulfate was added to the supernatant solution to 90% saturation, and the suspension was centrifuged at 20,000×*g* for 30 min. The precipitate was dissolved in 20 mM KP buffer and dialyzed overnight against the same buffer. The enzyme solution was loaded onto a DEAE–Cellulofine A-500m column (8×22 cm) equilibrated with 20 mM KP buffer. After the column was washed with 100 mM KP buffer, the enzyme was eluted with a linear gradient of 0–0.4 M KCl in 100 mM KP buffer. The active fractions were combined and fractionated with ammonium sulfate at 60–75% saturation. The precipitate was dissolved in 20 mM KP buffer and dialyzed against 20 mM KP buffer containing ammonium sulfate at 30% saturation. This solution was applied to a phenyl-Toyopearl 650M column (2.5×8.5 cm) equilibrated with 20 mM KP buffer containing ammonium sulfate at 30% saturation, and the enzyme was eluted with a linear gradient of ammonium sulfate (30–0% saturation) in 20 mM KP buffer. The active fractions were combined and loaded onto a Sephacryl S-200 HR column (2.0×106 cm) equilibrated with 50 mM KP buffer containing 0.2 M NaCl. The enzyme was eluted with the same buffer at a flow rate of 2.0 ml per hour. The active fractions were combined and dialyzed against 20 mM KP buffer.

Amino acid sequence The digest of the purified enzyme following treatment with TPCK–trypsin was subjected to SDS–PAGE, and the resulting peptides were transferred onto PVDF membrane. Amino acid sequences were determined by a protein sequencer, model 476A (PE Biosystems, Tokyo). To identify the lysine residue binding pyridoxal 5'-phosphate, the purified enzyme was reduced by $NaBH_4$, and the tryptic peptide containing ε-(*N*-phosphopyridoxyl)lysine was isolated and purified as described by Nagasawa *et al.* (11).

Cloning and analysis of the gene for γ-cyano-α-aminobutyric acid synthase from *B. stearothermophilus* CN3 The genomic DNA was prepared after cell lysis with lysozyme and *Achromobacter* peptidase (Wako Pure Chemicals Industries, Osaka) according to the method of Saito and Miura (12). *B. stearothermophilus* CN3 genomic DNA (100 ng) was subjected to amplification with upstream primers, 5'-TCGCIATCCAYGCIGGICA RAARCCIGAYGCNGARAC-3' (corresponding to LAIHAGQKP DAET) and downstream primers, 5'-AGTCAGCTGYTCYTCIGG IGTNARYTGYTCRTG-3' (corresponding to HEQLTPEEQLS). Here, R indicates A or G, Y indicates C or T, N indicates, A, C, G or T and I indicates inosine. Cycling under the control of a MiniCycler™ model PTC-150 (MJ Research, Waltham, MA, USA) was performed as follows: annealing at 55°C for 1 min,

extension at 70°C for 3 min and denaturation at 98°C for 1 min, for a total of 30 cycles. A 1.2-kb DNA fragment, the only product amplified by PCR, was sequenced. On the basis of the sequence, an oligonucleotide (5′-GCAAAAACCGGATGCGGAAACGGGC GCGCGGGCGGTGCCG-3′) was synthesized and utilized as a probe DNA.

The genomic DNA of *B. stearothermophilus* CN3 was digested by *Bam*HI, *Eco*RI, *Fba*I, *Hin*dIII or *Xho*I, separated on a 1.5% agarose gel and transferred to a Hybond-N$^+$ membrane filter (Amersham Pharmacia Biotech). Southern-blot analysis was performed by the method of Maniatis *et al.* (13) using the ECL 3′-Oligolabelling and Detection System (Amersham Pharmacia Biotech). A *B. stearothermophilus* CN3 partial genomic library was prepared with genomic DNA digested with *Fba*I on the basis of the results of Southern-blot analysis. The DNA fragments corresponding to a positive band in the size region of 4 kb on the Southern blot were recovered by EASYTRAP™ ver. 2 (Takara Shuzo, Shiga) and ligated into the *Bam*HI site of pUC19. The *E. coli* JM109 cells transformed with the plasmids were screened with the labeled probe DNA described above. Colony hybridization was carried out under the same conditions as used for Southern-blot analysis.

DNA sequencing was performed from both DNA strands by the dideoxy chain termination method using an ALF DNA sequencing system (Amersham Pharmacia Biotech). The nucleotide sequence and deduced primary structure were compared with the protein sequences available from the GenBank and SWISS-PROT databases, and analyzed using the GENETYX MAC program (Software Development, Tokyo).

Expression of γ-cyano-α-aminobutyric acid synthase gene in *E. coli* JM109 The γ-cyano-α-aminobutyric acid synthase gene of *B. stearothermophilus* CN3 was amplified by PCR with the following two primers: 5′-GAGCCCGGGATGAGCAATGAACAAA CTTTCCGCCCGGAGA-3′ and 5′-TTCGCTGCAGCTACTTCA CCCCCACCGTCTGGCTTTGGCG-3′, in which *Xma*I and *Pst*I linkers were contained, respectively. The PCR product with a size of 1.3 kb was digested with *Xma*I and *Pst*I, and ligated into the *Xma*I-*Pst*I gap of pKK223-3. *E. coli* transformed with the resulting plasmid (pGCS20) was grown at 37°C in 2×YT broth containing 50 μg/ml ampicillin and 0.1 mM pyridoxine. When the absorbance at 600 nm of the culture reached around 0.65, isopropyl-β-thiogalactopyranoside was added to the culture broth at the final concentration of 1 mM, and the cultivation was further continued for 154 h. The cells were harvested by centrifugation at 6000×*g* for 10 min and subjected to enzyme purification.

Purification of recombinant γ-cyano-α-aminobutyric acid synthase The *E. coli* JM109 cells transformed with pGCS20 (39 g wet weight), cultivated in 8 *l* of 2×YT broth, were washed with 20 mM KP buffer, suspended in 80 ml of the same buffer and disrupted with an ultrasonic oscillator (Insonator 201M; Kubota, Tokyo) at 100 W for 30 min. The cell debris was removed by centrifugation at 20,000×*g* for 15 min. The cell-free extract was subjected to heat treatment at 60°C for 20 min, and the denatured protein was removed by centrifugation at 20,000×*g* for 15 min. The supernatant solution was applied to a DEAE–Cellulofine A-500m column (4.2×15 cm) equilibrated with 20 mM KP buffer. After the column was washed with 100 mM KP buffer, the enzyme was eluted with a linear gradient of 0–0.4 M KCl in 100 mM KP buffer. The active fractions were combined and fractionated with ammonium sulfate at 60–90% saturation. The pellet was dissolved in 20 mM KP buffer and dialyzed against 20 mM KP buffer containing ammonium sulfate at 30% saturation. The dialyzed enzyme solution was loaded onto a butyl-Toyopearl 650M column (2.0×6.6 cm) equilibrated with 20 mM KP buffer containing ammonium sulfate at 30% saturation, and the enzyme was eluted by a linear gradient of ammonium sulfate (30–0% saturation) in 20 mM KP buffer. The active fractions were combined and concentrated by a centrifugal filter, Centriprep YM-10 (Millipore Corporation, Bedford, MA, USA).

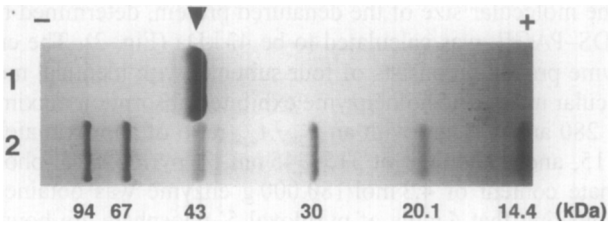

FIG. 2. SDS–PAGE of the purified γ-cyano-α-aminobutyric acid synthase. Lane 1, 10 μg of purified γ-cyano-α-aminobutyrate synthase; lane 2, molecular mass marker proteins (phosphorylase *b*, 94 kDa; bovine serum albumin, 67 kDa; ovalbumin, 43 kDa; carbonic anhydrase, 30 kDa; soybean trypsin inhibitor, 20.1 kDa; α-lactalbumin, 14.4 kDa).

Analytical methods SDS–PAGE was performed using 12.5% polyacrylamide slab gels (14). Protein concentration was determined by the method of Bradford (15) using bovine serum albumin as the standard. The molecular mass of the enzyme was estimated by HPLC on a TSK G-3000 SW gel filtration column (0.75×60 cm) at a flow rate of 0.7 ml/min with 0.1 M KP buffer (pH 7.5) containing 0.2 M NaCl. The following proteins were used for the calibration: glutamate dehydrogenase (M_r 290,000), lactate dehydrogenase (M_r 142,000), enolase (M_r 67,000), adenylate kinase (M_r 32,000) and cytochrome *c* (M_r 12,400) (Oriental Yeast). The pyridoxal 5′-phosphate content of the holoenzyme was determined by the methods reported by Wada and Snell (16).

RESULTS

Purification of γ-cyano-α-aminobutyric acid synthase from *B. stearothermophilis* CN3 The purified enzyme gave only one band upon SDS–PAGE (Fig. 2). The overall purification of γ-cyano-α-aminobutyric acid synthase from 160 *l* of culture broth was 632-fold with a yield of 46% (Table 1). The purified enzyme catalyzed the formation of γ-cyano-α-aminobutyric acid from *O*-acetyl-L-homoserine and cyanide ions at 17.2 μmol min^{-1} (mg protein)$^{-1}$ under the standard assay conditions. The apparent molecular mass of the purified enzyme was estimated to be 180 kDa by gel-permeation HPLC in comparison with standard proteins.

TABLE 1. Purification of γ-cyano-α-aminobutyric acid synthase from *B. stearothermophilus* CN3

Step	Total activity (units)	Total protein (mg)	Specific activity (units/mg)	Purification (-fold)	Yield (%)
Cell-free extract	1040	38100	0.0272	1	100
Heat treatment	1460	30500	0.0479	1.76	140
DEAE–Cellulofine A-500m	1470	3970	0.370	13.6	141
Phenyl-Toyopearl 650M	932	64.4	14.5	533	90
Sephacryl S-200 HR	479	27.9	17.2	632	10

The molecular size of the denatured protein, determined by SDS–PAGE, was calculated to be 43 kDa (Fig. 2). The enzyme probably consists of four subunits with identical molecular mass. The holoenzyme exhibited absorption maxima at 280 and 428 nm, with an A_{280}/A_{428} ratio of approximately 5.15, and a shoulder at 315–345 nm. A pyridoxal 5′-phosphate content of 4.3 mol/180,000 g enzyme was obtained, indicating that 4 mols of pyridoxal 5′-phosphate are bound to 1 mol of holoenzyme. Since the enzyme appears to be a homotetramer consisting of four identical subunits, each subunit may contain pyridoxal 5′-phosphate at a stoichiometric ratio of unity.

Catalytic properties of γ-cyano-α-aminobutyric acid synthase

Effect of pH and temperature The enzyme was stable on incubation at 60°C for 30 min in the pH range of 6.0–10.5 (Fig. 3a). After the enzyme was incubated at temperatures up to 60°C for 30 min at pH 7.5, no loss of activity was observed (Fig. 3b). When the enzyme reaction was carried out for 10 min in various buffers at 50 mM and at various temperatures, the maximum activity was observed at pH 8.0 using Tris–HCl buffer (Fig. 3c) and 60°C (Fig. 3d).

Substrate specificity The purified enzyme catalyzed the γ-replacement reaction of *O*-acetyl-L-homoserine to form γ-cyano-α-aminobutyric acid in the presence of cyanide ions. The apparent K_m values for *O*-acetyl-L-homoserine and L-homocystine were 1.87 mM and 4.17 mM, respectively. *O*-Acetyl-L-serine, *O*-phospho-L-serine, *O*-succinyl-L-homoserine, L-glutamic acid, L-glutamine, L-serine, L-cystine and L-homoserine were inert as the substrate when used instead of *O*-acetyl-L-homoserine. The enzyme does not catalyze the degradation (α,γ- or α,β-elimination reaction) of the following compounds: *O*-acetyl-L-homoserine, L-homocystine, *O*-acetyl-L-serine, *O*-phospho-L-serine, *O*-succinyl-L-homoserine, L-glutamic acid, L-glutamine, L-serine, L-cystine and L-homoserine. Cyanide ions could be replaced as cosubstrates by hydrogen sulfide, methylmercaptan, ehtylmercaptan and phenylmercaptan; the formation of L-homocysteine, L-methionine, L-ethionine and *S*-phenyl-L-homocysteine was detected using an amino acid analyzer (K-101; Kyowa Seimitsu, Tokyo) (data not shown).

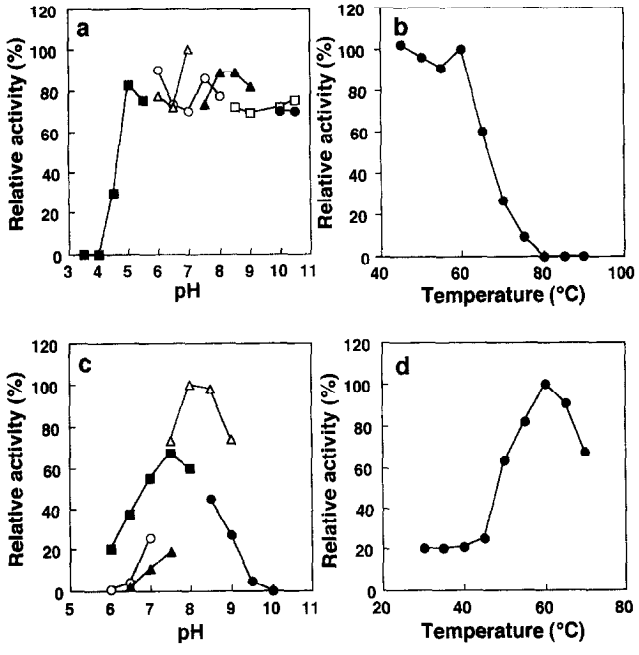

FIG. 3. Effects of pH and temperature on the enzyme. (a) The enzyme was incubated at 60°C for 30 min in various buffers at the indicated pHs, and the residual activity was measured. The following buffers (50 mM) were used; closed squares, citrate–citrate Na; open triangles, MES; open circles, KH_2PO_4–K_2HPO_4; closed triangles, Tris–HCl; open squares, NH_4Cl–NH_4OH; closed circles, glycine–KOH. (b) The enzyme was incubated for 30 min at the indicated temperatures in 20 mM potassium phosphate buffer (pH 7.5), and the residual activity was measured. (c) Relative activity was measured in the following buffers (50 mM): open circles, 2-(*N*-morpholino)ethanesulfonic acid; closed squares, KH_2PO_4–K_2HPO_4; closed triangles, 3-(*N*-morpholino)propanesulfonic acid; open triangles, Tris–HCl; closed circles, NH_4Cl–NH_4OH. (d) The reaction was carried out for 10 min at the indicated temperatures.

Cloning and nucleotide sequence analysis of the gene for γ-cyano-α-aminobutyric acid synthase The peptide fragments obtained by digestion of the purified enzyme with TPCK–trypsin were sequenced as ALSSGQAAVFY, SLIIHPASTTHEQLTPEEQLSAGVTPGLVR and FLEEE EAVESVNYPGLPSHPSHELAK. The amino terminal sequence of the purified enzyme was MSNEQTFRPETLAI HAGQKPDAETGARXVP. A nucleotide fragment of the γ-cyano-α-aminobutyric acid synthase gene of *B. stearothermophilus* CN3 was amplified by PCR with primers designed based on the partial amino acid sequences (see Materials and Methods). The amplified gene fragment was verified by nucleotide sequencing; two internal amino acid sequences were found in the amino acid sequence deduced from the nucleotide sequence. Southern-blot analysis with a probe DNA showed a single band on each restriction-enzyme digestion of genomic DNA. On the basis of the results of Southern-blot analysis, approximately 4.0-kb *Fba*I fragments containing the γ-cyano-α-aminobutyric acid synthase gene were screened by colony hybridization. From positive clones, the plasmid DNA (pGCS10) was isolated and characterized.

The γ-cyano-α-aminobutyric acid synthase gene consisted of 1302-bp and encoded a protein of 434 amino acids with a calculated molecular mass of 46,847 (Fig. 4). This value is in agreement with the molecular mass of 43 kDa of the purified enzyme determined by SDS–PAGE. All the amino acid sequences determined for peptide fragments of the purified enzyme exactly matched with the amino acid sequence predicted from the nucleotide sequence. The amino acid sequence of a peptide, which was isolated as a pyridoxyl peptide containing the lysine residue of the active site, was determined to be SATXFIGGHGNSIGGVIVDS GK. Therefore, Lys210 is involved in the formation of a Schiff base with pyridoxal 5′-phosphate (Fig. 4).

Expression of γ-cyano-α-aminobutyric acid synthase gene in *E. coli* JM109 and the purification of the recombinant enzyme The γ-cyano-α-aminobutyric acid synthase gene was inserted into an expression vector, pKK223-3, under the control of the *tac* promoter. The resulting plasmid (pGCS20) was introduced into *E. coli* JM109 cells, which showed no γ-cyano-α-aminobutyric acid synthase activity. The expressed γ-cyano-α-aminobutyric acid synthase was purified according to the method outlined in Materials and

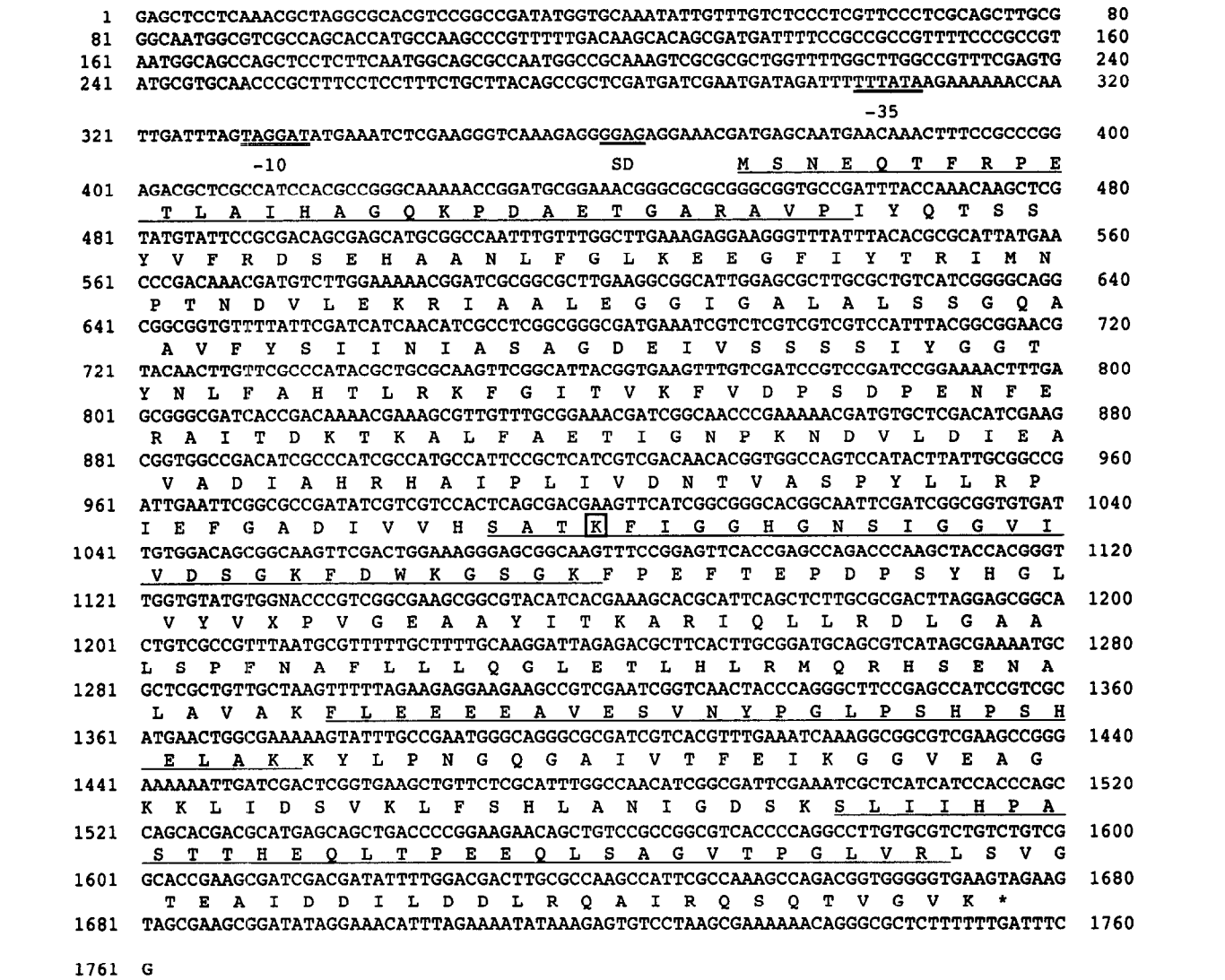

FIG. 4. Nucleotide sequence of the γ-cyano-α-aminobutyrate synthase gene and its flanking regions, and deduced amino acid sequence. The putative sites for −35 and −10 regions of the promoter sequence, and for the ribosomal-binding site (SD, Shine–Dalgarno sequence) are indicated by double underlining. The partial amino acid sequences determined using the purified enzyme are underlined. A presumed lysine residue involved in the active site, Lys210, is boxed. The sequence data have been submitted to the DDBJ/ENBL/GenBank databases under accession no. E16859.

Methods. The purification achieved was approximately 11-fold with a yield of 9.0%; the specific activity of the purified enzyme was 29.9 units/mg. The purified enzyme, which gave only one band upon SDS–PAGE, showed the same physicochemical properties, substrate specificity and stability as the γ-cyano-α-aminobutyric acid synthase purified from *B. stearothermophilus* CN3.

DISCUSSION

γ-Cyano-α-aminobutyric acid was found for the first time in the culture filtrate of *Chromobacterium violaceum* strain D341 grown on medium containing cyanide ions (10). Ressler *et al.* (17) reported the nonenzymatic formation of γ-thiocyano-α-aminobutyric acid from L-homocystine and cyanide ions. They also found that the γ-cyano-α-aminobutyric acid synthase of the bacterium catalyzes the formation of γ-cyano-α-aminobutyric acid and SCN^- from γ-thiocyano-α-aminobutyric acid and cyanide ions (17). The γ-cyano-α-aminobutyric acid synthase from *B. stearothermophilus* CN3 was different from that of *C. vialaceum* strain D341 in terms of the molecular mass and substrate specificity. The partially purified enzyme of *C. vialaceum* strain D341 had a molecular mass of 130 kDa, and efficiently catalyzed the γ-replacement reaction of L-homocystine. The activity with *O*-acetyl-L-homoserine is 5% of that with L-homocystine (17). The molecular mass of *B. stearothermophilus* CN3 γ-cyano-α-aminobutyric acid synthase is 180 kDa, and the enzyme clearly prefers *O*-acetyl-L-homoserine to L-homocystine.

The primary structure of the γ-cyano-α-aminobutyric acid synthase exhibited 60%, 68%, 63% and 59% identity with those of the *O*-acetyl-L-homoserine sulfhydrylases of *Acremonium chrysogenum*, *B. halodurans*, *Thermotoga maritima* and *Leptospira meyeri*, respectively. The result of the homology search demonstrates that the *B. stearothermophilus* CN3 γ-cyano-α-aminobutyric acid synthase corresponds to the *O*-acetyl-L-homoserine sulfhydrylase of vari-

ous microorganisms. Actually, the enzyme from *B. stearothermophilus* CN3 shows *O*-acetyl-L-homoserine sulfhydrylase activity. We investigated the distribution of similar γ-cyano-α-aminobutyric acid synthases in various *B. stearothermophius* strains (data not shown), since the enzyme productivity of *B. stearothermophilus* CN3 was not high. However, the γ-cyano-α-aminobutyric acid synthase activities in the cell-free extract were even considerably lower and ranged between 0.001–0.008 unit (mg protein)$^{-1}$. The growth of the tested strains was completely inhibited by adding 4 mM cyanide ions to the nutrient medium, whereas *B. stearothermophilus* CN3 grew well even in the presence of 5 mM cyanide ions (data not shown). These results suggest that the cyanide ion resistance of *B. stearothermophilus* CN3 might derive from the trapping of cyanide ions by some enzymes such as β-cyano-L-alanine synthase (6) and the present γ-cyano-α-aminobutyric acid synthase.

We previously reported on the synthesis of positron-labeled L-glutamic acid and L-methionine from *O*-acetyl-L-homoserine and ^{11}C-labeled HCN using the γ-cyano-α-aminobutyric acid synthase of *B. stearothermophilus* CN3 (7, 8). Furthermore, the γ-cyano-α-aminobutyric acid synthase can be applied to the determination of L-homocysteine, which is a diagnostic marker of arteriosclerosis. L-Homocysteine is quantified from the formation of hydrogen sulfide in the presence of γ-cyano-α-aminobutyric acid synthase and an appropriate nucleophilic reagent (Ebinuma and Ushizawa, Japan Kokai Tokkyo Koho, 166597 [2000]; Hama and Yabuuchi, Japan Kokai Tokkyo Koho, 228998 [2000]; Ebinuma and Ushizawa, Japan Kokai Tokkyo Koho, 270895 [2000]). The overexpression system of a stable γ-cyano-α-aminobutyric acid synthase, established in this study, will extend the availability of the enzyme for use in radionuclide synthesis and clinical diagnosis. The productivity of the enzyme in *E. coli* is 646 units/*l* of culture medium, which is about 100 times that of *B. stearothermophilus* CN3.

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