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Functional Expression and Characterization of C-terminal Mutant of 4-Aminobutyrate Aminotransferase

Bo Kyung Sung, Jung Jong Cho and Young Tae Kim*
Department of Microbiology, Pukyong National University, Pusan, 608-737, Korea

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4-Aminobutyrate aminotransferase plays an essential role in the 4-aminobutyric acid shunt, converting 4-aminobutyrate to succinic semialdehyde. Recombinant 4-aminobutyrate aminotransferases were overexpressed as their catalytically active forms in *E. coli* by coproduction with thioredoxin and their solubilities were also dramatically increased. In order to study the structural and functional aspects of the C-terminal domain of brain 4-aminobutyrate aminotransferase, we have constructed a C-terminal mutant of pig brain 4-aminobutyrate aminotransferase and analyzed the functional and structural roles of C-terminal amino acids residues on the enzyme. The deletion of five amino-acid residues from C-terminus did not interfere with the kinetic parameters and functional properties of the enzyme. Also, the deletion did not affect the dimeric structure of the protein aligned along the subunit interface at neutral pH. However, the deletion of the C-terminal region of the protein changed the stability of its dimeric structure at acidic pH. The dissociation of the enzyme acidic, facilitated by the deletion of five amino acids from C-terminus, abolished the catalytic activity.

Keywords: 4-Aminobutyrate aminotransferase, Dimeric structure, Expression, Mutagenesis, Stability.

Introduction

4-Aminobutyric acid has long been recognized for its role as the major inhibitory neurotransmitter in mammalian brain. The concentration in brain is controlled by two pyridoxal 5′-P-dependent enzymes, i.e., glutamate decarboxylase and 4-aminobutyrate aminotransferase. The first enzyme catalyzes the synthesis of 4-aminobutyrate, whereas the second enzyme catalyzes the conversion of 4-aminobutyrate to succinic semialdehyde in a transamination reaction. Further progress in the design of effective drugs for the treatment of convulsive disorders depends upon detailed information on the structure of the target enzymes.

4-Aminobutyrate aminotransferase (4-aminobutyrate: 2-oxoglutarate aminotransferase, EC 2.6.1.19) from pig brain contains one equivalent of pyridoxal 5′-phosphate (PLP) firmly bound to the dimeric form of molecular weight 100 kDa, and catalyzes the reversible transamination of 4-aminobutyrate to yield succinic semialdehyde and pyridoxamine 5′-phosphate (Churchich and Moses, 1981). The mitochondrial enzyme has been highly purified from pig brain (Beeler and Churchich, 1981). The peptide bearing the cofactor PLP covalently attached to a lysine residue has been sequenced (Kim and Churchich, 1989). Also, several cysteine residues critical for enzyme function have been identified (Kim and Churchich, 1991).

The full-length cDNA encoding the pig brain GABA-T has been isolated (Kim *et al.*, 1991) and sequenced (Kwon *et al.*, 1992). The amino acid sequence predicted from the cDNA sequence shows that the precursor protein consists of the mature enzyme of 473 amino acid residues and an amino-terminal signal peptide composed of 27 amino acids (Kwon *et al.*, 1992; Park *et al.*, 1993). When recombinant 4-aminobutyrate aminotransferase was overexpressed in *E. coli*, the protein formed insoluble inclusion bodies (Lee *et al.*, 1996). However, overproduced proteins regained their catalytic activity after renaturation (Lee *et al.*, 1996; Kim *et al.*, 1997). Also, the lysyl residue at the 330 position of the amino acid sequence has been verified to serve as the catalytic site of the enzyme by site-specific mutagenesis (Kim *et al.*, 1997).

We describe here the construction, functional expression, and characterization of the C-terminal deletion mutant of 4-aminobutyrate aminotransferase.

* To whom correspondence should be addressed.
Tel: 82-51-620-6366; Fax: 82-51-628-7432
E-mail: ytkim@dolphin.pknu.ac.kr

Materials and Methods

Materials *E. coli* strains BL21(DE3) and HB101 have been described previously (Kim and Richardson, 1993; 1994). Dr. S. Tabor (Harvard Medical School) kindly provided the pT7-7 expression vector. Restriction enzymes were purchased from New England Biolabs Inc. (Cambridge, USA) and United States Biochemicals Corp. (Cleveland, USA). T4 DNA ligase and *Taq* DNA polymerase were purchased from Promega (Madison, USA). Isopropyl-β-D-thiogalactopyranoside (IPTG) was purchased from Boehringer Mannheim (Mannheim, Germany). Hydroxylamine and 4-aminobutyric acid were obtained from Kodak (Boston, USA). Gabase, succinic semialdehyde, 2-oxoglutarate, pyridoxal 5′-P, and other chemicals were obtained from Sigma Chemical Co. (St. Louis, USA). Oligonucleotide primers were synthesized by Genetech (Seoul, Korea).

Construction of mutant 4-aminobutyrate aminotransferases Cloning of the premature (pYKG1) and mature (pYKG2) forms of 4-aminobutyrate aminotransferase was as previously described (Kim *et al.*, 1997). The premature form of the carboxy-terminal mutant (pYKG1-Δ5C), with 5 amino acids deleted from the C-terminus, was amplified by the polymerase chain reaction (PCR) (Innis and Gelfand, 1990) using the 5′-end primer (GT-1; CGGGATCCATATGGCTTCCGTGTTGCTC) and the 3′-end primer (GT-6; CGTAGGATCCGGCTAAGATGTCGC) containing *Nde*I and *Bam*HI sites, respectively. The PCR-generated fragment was then cloned into the *Nde*I and *Bam*HI site of pT7-7. pT7-7 vector contained the T7 RNA polymerase promoter Ø10 as well as a strong translation initiation region prior to the polylinker (Tabor, 1990; Kim *et al.*, 1992a; 1992b). Also, the mature form of the carboxy-terminal mutant (pYKG2-Δ5C) was constructed by PCR amplification with the 5′-end primer (GT-2; GGATGGCATATGATCAGTCAGGCCGCAG) and the 3′-end primer (GT-6; CGTAGGATCCGGCTAAG-ATGTCGC) containing *Nde*I and *Bam*HI sites, respectively. The PCR-generated fragment was also cloned into the *Nde*I and *Bam*HI sites of pT7-7 and the resulting plasmid was named pYKG2-Δ5C. pYKG1-Δ5C and pYKG2-Δ5C clones were transformed to HB101.

All the clones generated from the PCR-amplified DNA were sequenced and were found to have the correct sequences.

Functional expression of recombinant 4-aminobutyrate aminotransferases pT-Trx, the plasmid in which the T7 promoter was linked to the *E. coli* thioredoxin gene (*trx*) and the aspA transcription terminator were kindly provided by Dr. S. Ishii (Yasukawa *et al.*, 1995). As shown in Fig. 1, the plasmids to produce various recombinant 4-aminobutyrate aminotransferases were constructed using the appropriate pT expression vector containing the T7 promoter (Kim and Richardson, 1993, 1994; Kim *et al.*, 1997). To generate bacteria producing both recombinant 4-aminobutyrate aminotransferase and thioredoxin, pYKG plasmids encoding various forms of 4-aminobutyrate aminotransferase were transformed into *E. coli* BL21(DE3) harboring pT-Trx. Cells were grown in LB broth at 37°C containing 50 μg/ml of ampicillin and 25 μg/ml of chloramphenicol. At a cell density corresponding to A_{590} = 0.7, Isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM to induce the expression of T7 RNA polymerase (Tabor and Richardson, 1985; Studier and Moffat, 1986) and thioredoxin (Yasukawa *et al.*, 1995). After induction, the cells were incubated for three additional hours, harvested as described previously (Kim *et al.*, 1997), and then frozen in liquid N_2 and stored at −80°C.

Purification of recombinant 4-aminobutyrate aminotransferases Frozen cells (2 g) were suspended in 10 ml of 0.01 M potassium phosphate (pH 7.4), containing 1 mM 2-oxoglutarate, 1 mM β-mercaptoethanol, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM EDTA, and 10 μM pyridoxal 5′-phosphate (PLP). Lysozyme (egg white) was added to the cells to a final concentration of 0.25 mg/ml, and the suspension was stirred on ice for 15 min. The lysed cells were sonicated with a Branson Sonifier and centrifuged in a Sorvall SS 34 rotor at 10,000 × *g* for 30 min. Purification of the expressed protein was performed as previously described (Kim *et al.*, 1997).

Enzyme assays A coupled assay system consisting of 4-aminobutyrate aminotransferase and succinic semialdehyde dehydrogenase was used to study the catalytic conversion of 4-aminobutyrate to succinic semialdehyde. Enzyme assays were performed in 0.1 M sodium pyrophosphate (pH 8.4) containing 5 mM NAD^+, 30 mM 4-aminobutyrate, and 10 mM 2-oxoglutarate. Initial rate measurements were carried out by monitoring changes in absorbance at 340 nm for at least 2 min. The reaction rate of transamination of β-alanine was determined by a fluorometric method (Beeler and Churchich, 1978).

Synthesis of AP4-PL Adenosine tetraphosphate pyridoxal (AP4-PL) was synthesized according to the procedure of Tagaya *et al.* (1985). Samples (20 μM) of premature and C-terminal deletion forms of 4-aminobutyrate aminotransferase were each preincubated with 100 μM AP4-PL, respectively. A control experiment for measuring the catalytic activity was performed with native enzyme without adding ATP analogs. Experiments were conducted in 0.1 M phosphate buffer (pH 7.0). Aliquots withdrawn from the incubation mixtures at the indicated times were tested for enzymatic activity.

Determination of free cysteinyl groups Iodosobenzoate, which reacts with free cysteinyl groups, inactivates the enzymatic activity of 4-aminobutyrate aminotransferase. The inactivation reaction was carried out in a 2 ml reaction volume containing 0.7 mg of enzyme/ml and iodosobenzoate at an initial concentration of 100 μM in 0.1 M phosphate buffer (pH 7.0). The reaction was stopped after a 80 min incubation at 25°C, and unreacted sulfhydryl reagent was removed by chromatography at 4°C on a Sephadex G-25 column (1 × 20 cm) that had been previously equilibrated with 0.1 M potassium phosphate (pH 7.0). The column eluate was monitored at 280 nm and fractions with the greatest protein concentrations were combined. The enzymatic activity and protein concentration of fractions were determined. Aliquots of the combined fractions were mixed with guanidinium-HCl to a final concentration of 5 M, and titrated with 5,5′-dithiobis(nitrobenzoic acid) (DTNB). The concentration of free sulfhydryl groups was determined spectrophotometrically at 412 nm by employing an extinction coefficient of 13,600 M^{-1} cm^{-1}.

Protein characterization Protein concentration was determined by the Bradford method (Bradford, 1976). Sodium

dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on 12% polyacrylamide slab gels according to Laemmli (1970). Spectrophotometric measurements were carried out using a Kontron UV-932 spectrophotometer (Cho *et al.*, 1997). Fluorescence spectra were measured with a Perkin Elmer LS 50B spectrofluorometer.

Results

Construction of recombinant 4-aminobutyrate aminotransferases To overproduce recombinant 4-aminobutyrate aminotransferases using the T7 RNA polymerase expression system and *E. coli* thioredoxin (*trx*), we constructed several plasmids coding for various forms of recombinant 4-aminobutyrate aminotransferase as shown in Fig. 1. pYKG plasmids (Figs. 1A and 1B) were introduced into the *E. coli* strain BL21(DE3) harboring pT-Trx. To coproduce thioredoxin at a level similar to that of foreign proteins of interest, the Trx-coding region was also linked to the T7 promoter and inserted into the pACYC vector, which contains a p15A replicon and a chloramphenicol resistance marker as shown in Fig. 1C

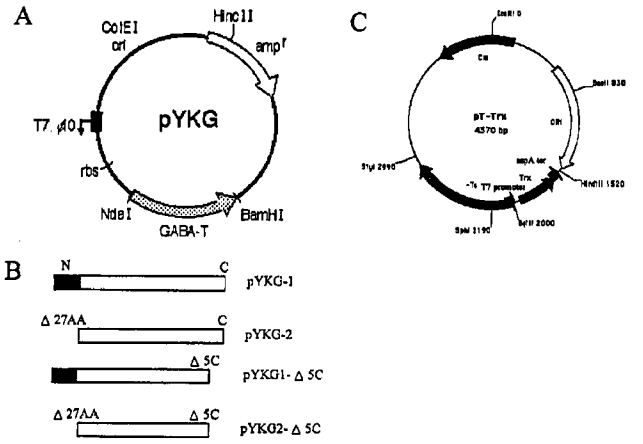

Fig. 1. Schematic diagram of the expression plasmid, recombinant 4-aminobutyrate aminotransferase, and *E. coli* thioredoxin. A. pT7-7 expression plasmid. DNA fragments containing 4-aminobutyrate aminotransferases were obtained from PCR method and ligated into the T7 transcription/expression region of pT7-7 after digestion with *Nde*I and *Bam*HI, yielding pYKGs. B. pYKG-1, pYKG-2, pYKG1-Δ5C, and pYKG2-Δ5C were designed for the expression of polypeptides of various forms of 4-aminobutyrate aminotransferase. pYKG-1 was designed to express the wild-type premature protein; pYKG-2 was designed to express the wild-type mature protein; pYKG1-Δ5C was designed to express the C-terminal mutant protein of premature enzyme in which 5 amino acids from the C-terminal end of protein were deleted; pYKG2-Δ5C was designed to express the C-terminal mutant protein of mature enzyme in which 5 amino acids from the carboxy-terminal end of the protein were deleted. C. *E. coli* thioredoxin expression vector. Trx-coding region was linked to the T7 promoter and inserted into pACYC vector containing the chloramphenicol resistance marker.

(Yasukawa *et al.*, 1995). The generated plasmid (pT-Trx) allowed for cotransformation with the pYKG plasmids to produce various 4-aminobutyrate aminotransferases based upon plasmid compatibility.

Functional expression and purification of 4-aminobutyrate aminotransferase After IPTG induction, the cells were incubated for three additional hours and then harvested by centrifugation. To analyze the solubility of the expressed proteins, the cells were disrupted by sonication and the supernatant was rescued as the soluble fraction. The fraction was loaded onto SDS-PAGE. As shown in Fig. 2, the solubility of 4-aminobutyrate aminotransferase was dramatically increased by coproduction with thioredoxin. In order to examine whether the aminotransferase, produced in soluble form by coproduction with thioredoxin, have a native conformation, we analyzed the catalytic activity of the enzymes (Table 1). The proteins produced in soluble form by coproduction with thioredoxin appear to have the native protein conformation. Approximately 30 mg of soluble form protein was produced per liter of culture.

As indicated in Table 1, the kinetic parameters of premature and mature forms of 4-aminobutyrate

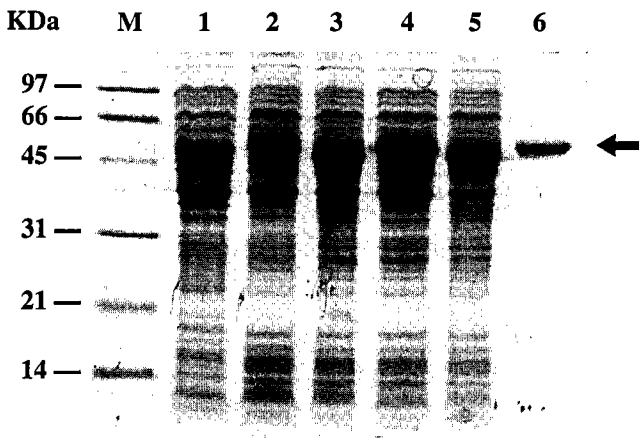

Fig. 2. Analysis of the expressed proteins by SDS-PAGE. After IPTG induction, cells were incubated for three additional hours and then harvested by centrifugation. To analyze the solubility of the expressed proteins, pellets were disrupted by sonication and supernatants were rescued as the soluble fractions. The fractions were analyzed by 12% SDS-PAGE. Lane M indicates low molecular weight standard proteins; lane 1, proteins from uninduced cell extracts; lane 2, proteins from the supernatant of induced cell extracts containing pYKG-1 (premature enzyme); lane 3, proteins from the supernatant of induced cell extracts containing pYKG-2 (mature enzyme); lane 4, proteins from the supernatant of induced cell extracts containing pYKG1-Δ5C (premature protein of C-terminal deletion enzyme); lane 5, proteins from the supernatant of induced cell extracts containing pYKG2-Δ5C (premature protein of C-terminal deletion enzyme); lane 6, purified proteins from pig brain. The gel was stained with Coomassie Brilliant Blue.

Table 1. Kinetic properties of wild-type and C-terminal mutant 4-aminobutyrate aminotransferases. The activity of 4-aminobutyrate aminotransferase was determined using a coupled enzymatic assay system consisting of succinic semialdehyde dehydrogenase (NAD^+ dependent) as auxiliary enzyme.

Enzyme forms	K_m [a] (mM)	K_m [b] (mM)	V_{max} (μmol/min)	Specific activity (units/mg)
Wild-type				
YKG-1	0.20	6.2	1.2	8.8
YKG-2	0.19	5.9	1.3	9.1
Δ5C mutant				
YKG1-Δ5C	0.21	6.0	1.2	8.4
YKG2-Δ5C	0.18	6.6	1.4	9.3

[a] Kinetic constants for 2-oxoglutarate.
[b] Kinetic constants for 4-aminobutyrate.

aminotransferase are identical. This result suggests that the amino terminal signal peptide does not affect the functional properties of the enzyme.

C-terminal mutant of 4-aminobutyrate aminotransferase In order to confirm whether the C-terminal region is critical for the catalytic function and structural stability of 4-aminobutyrate aminotransferase, we constructed the deletion mutant in which five amino acids from the C-terminus were deleted. The mutant protein was also overexpressed into the soluble fraction (Fig. 2, lanes 4 and 5). Purification of the C-terminal-deleted protein (YKG1-Δ5C) was performed by the same method used in the purification of native enzyme (YKG-1) and is shown in Fig. 3.

Characterization of C-terminal mutant protein The catalytic activity of aminotransferases was determined using a coupled enzymatic assay system consisting of succinic semialdehyde dehydrogenase (NAD^+ dependent) as the auxiliary enzyme. Kinetic parameters of both native and mutant 4-aminobutyrate aminotransferases were determined and are summarized in Table 1. The deletion mutants displayed K_m and V_{max} values identical to those of the native enzyme for substrates, 4-aminobutyrate and 2-oxoglutarate (Table 1). This result suggests that the deletion of five amino acids residues from the C-terminus may not interfere with the kinetic parameters and functional properties of the enzyme.

We also tested the catalytic activity of 4-aminobutyrate aminotransferase using ATP analogues and a chemical modification method. The time course of inactivation of various 4-aminobutyrate aminotransferases by ATP analogs was performed as described in Materials and Methods. When samples (20 μM) of premature and C-terminal-deleted forms of aminotransferase were treated with

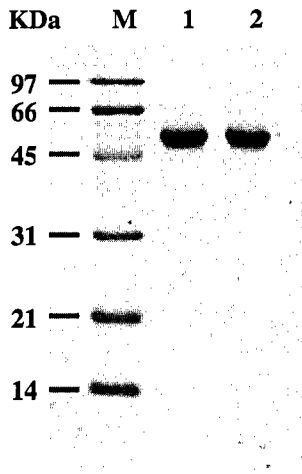

Fig. 3. Analysis of purified recombinant 4-aminobutyrate aminotransferases (YKG-1 and YKG1-Δ5C) by SDS-PAGE. Purified proteins were analyzed by 12% PAGE in the presence of 0.1% SDS. Lane M, low molecular weight standard proteins; lane 1, purified premature enzyme (YKG-1); lane 2, purified premature enzyme with deleted C-terminus (YKG1-Δ5C).

100 μM AP4-PL in 0.1 M phosphate buffer (pH 7.0), it was found that the catalytic activity decayed as shown in Fig. 4. A loss of 50% of the original catalytic activity was detected after preincubating the premature amino-transferase with a 10-fold excess of AP4-PL at 25°C for

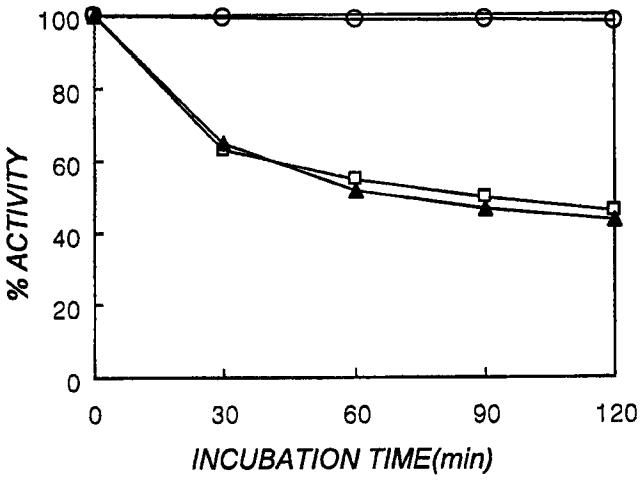

Fig. 4. Time course of inactivation of 4-aminobutyrate aminotransferase by ATP-analogs. Results obtained from when samples of wild-type premature form (□) and C-terminal deletion form (▲) of 4-aminobutyrate aminotransferase (20 μM) were preincubated with 100 μM AP4-PL. Control experiment (○) for measuring the catalytic activity was performed with native enzyme without adding ATP analogs. Experiments were conducted in 0.1 M phosphate buffer (pH 7.0). Aliquots withdrawn from the incubation mixtures at the indicated times were tested for enzymatic activity.

30 min. The loss of catalytic activity was also detected at a similar extent in the C-terminal-deleted form of the enzyme, indicating that the deletion of five amino acids from the C-terminus does not interfere with binding of the substrate analogs.

When the purified deletion protein (YKG2-5ΔC) was applied to high performance liquid chromatography (HPLC) using a gel filtration column (LKB; TSK G3000 SW) and eluted with 0.1 M phosphate buffer (pH 7.0), the elution profile of the C-terminal deleted enzyme was identical to that of native enzyme. These results indicate that the dimeric state of the protein had not been perturbed by the release of five amino acids at the C-terminal end of the protein (Figs. 5A and 5B). In addition, no changes in the shape of the emission spectra and in the relative fluorescence yield were detected when the C-terminal deleted enzyme was compared to native 4-aminobutyrate aminotransferase (Fig. 5C). Fluorescence spectra also indicate that the immediate microenvironment surrounding the tryptophyl residues of the dimeric proteins was not perturbed by the release of five amino acids from the COOH terminal portion.

Vicinal sulfhydryl groups as probes of conformational changes Iodosobenzoate which reacts with vicinal sulfhydryl (SH) groups in proteins to form disulfide bonds was shown to blocked SH groups of 4-aminobutyrate aminotransferase and reversibly inhibited catalytic activity (Choi and Churchich, 1985; Kim and Churchich, 1989).

To investigate the reaction of the vicinal SH groups of the C-terminal deleted protein, enzyme samples (2 mg/ml) were allowed to react with iodosobenzoate for 15 min at 25°C and were then passed through a Sephadex G-25 gel filtration column. The protein was then dissolved in 5 M guanidinium-HCl and titrated with DTNB. The results, as shown in Table 2, indicate that one pair of SH residues in the carboxy-terminal deleted enzyme had been oxidized by iodosobenzoate. Thus, the deletion of five amino acids from the C-terminal portion of aminotransferase did not affect the dimeric structure of the protein aligned along the subunit interface.

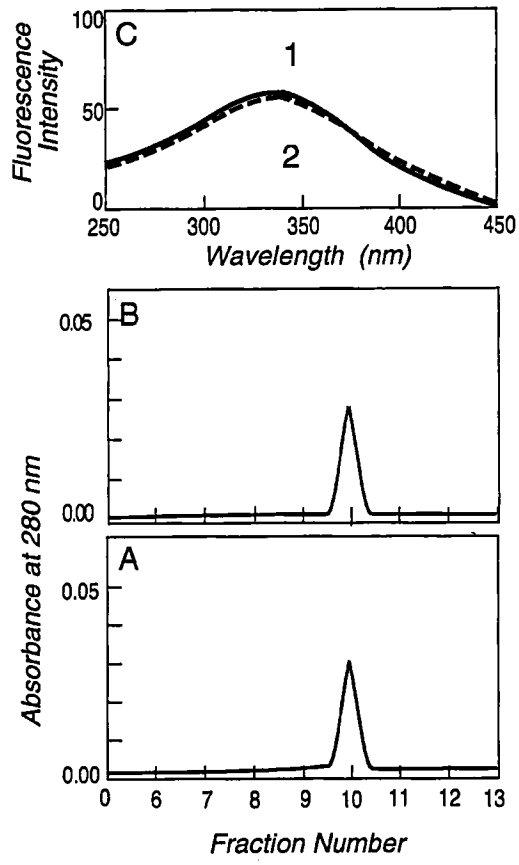

Fig. 5. HPLC elution profiles of native and mutant enzymes using a gel filtration column at pH 7.0. Panel A, The elution profile of native 4-aminobutyrate aminotransferase eluted with 0.1 M phosphate buffer (pH 7). Panel B, The elution profile of the carboxy-terminal mutant enzyme eluted with 0.1 M phosphate buffer (pH 7). Panel C, The emission spectra of native (1) and mutant (2) 4-aminobutyrate aminotransferase in 0.1 M phosphate buffer (pH 7.0). Excitation wavelength was at 280 nm.

Effects of C-terminus on the dimeric structure The effect of the C-terminal region on the stability of the dimeric structure was investigated by HPLC using a gel filtration column (LKB: TSK G3000 SW) at a flow rate of 0.2 ml/min. As shown in Fig. 6A, the elution profile of the

Table 2. Inactivation of wild-type (WT) and C-terminal deletion mutant (Δ5C-mutant) 4-aminobutyrate aminotransferase by iodosobenzoate and determination of SH residues by titration with DTNB in 5 M guanidinium HCl.

Reaction mixture	Activity (%)	SH groups /dimer	Disulfide bonds /dimer
WT-enzyme	100	5.9	0
Δ5C-mutant	100	5.8	0
WT-enzyme + iodosobenzoate[a]	5	3.8	1
Δ5C-mutant + iodosobenzoate[a]	4	3.9	1

[a] 10 μM of the enzyme and 1 mM of iodosobenzoate were preincubated in 0.1 M phosphate buffer (pH 7) at 25°C for 15 min.

wild-type protein remained identical when the pH was changed from pH 7.0 to pH 5.1. In marked contrast to the wild-type enzyme, the elution profile of the C-terminal mutant protein changed as a function of pH (Fig. 6B). Two peaks were clearly observed at pH 5.1. The large component of the C-terminal mutant protein eluted with 0.1 M Na-Citrate (pH 5.1). At acidic pH, the mutant protein was dissociated into monomeric subunits. The net effect of the deletion of five amino acids was to change the stability of the enzyme at acidic pH. The UV absorbance of the native enzyme was decreased by the addition of 4-aminobutyrate, whereas only a very small change in the absorbance of the mutant enzyme was detected under similar conditions (Fig. 7, top). Also, the UV emission of pyridoxamine 5′-P (PMP) was higher in the native than in the deleted enzyme (Fig. 7, bottom). It should be noted that at acidic pH, dissociation of the enzyme facilitated by the deletion of five amino acids from the C-terminal end abolished the catalytic activity of the enzyme.

Discussion

We have overproduced the cloned aminotransferases in their catalytically active forms by coexpressing *E. coli* thioredoxin. When recombinant 4-aminobutyrate aminotransferases were overproduced in *E. coli* using the T7 promoter and RNA polymerase expression system only, the expressed proteins were insoluble and formed inclusion bodies resulting in catalytically-inactive proteins (Lee *et al.*, 1996; Kim *et al.*, 1997). Induction of the cells at lower temperature, vigorous shaking, and changes in the treatment for optimal expression did not prevent the formation of insoluble inclusion bodies. Therefore, we tried to examine the effect of coproduction with *E. coli* thioredoxin on the solubility of overexpressed 4-aminobutyrate aminotransferase in *E. coli*. The solubility of 4-aminobutyrate aminotransferase was dramatically increased by coproduction with thioredoxin. Increase in the solubility of aminotransferase by overproduction of Trx strongly suggests that the redox state affects the solubility and native conformation of the aminotransferase. This could induce formation of abnormal intramolecular disulfide bonds that aggregate the proteins. This enzyme contains the catalytically important SH groups (Kim and Churchich, 1989). In particular, vicinal sulfhydryl (SH) groups in 4-aminobutyrate aminotransferase form disulfide bonds, and blocking the thiol groups of 4-aminobutyrate aminotransferase reversibly inhibited to catalytic activity (Choi and Churchich, 1985).

We have described the purification of catalytically-active enzymes from an overproducing bacterial strain by coproduction with *E. coli* thioredoxin. Coproduction of free thioredoxin dramatically increases the solubility of cloned aminotransferases in *E. coli*. This finding provides us with a useful tool in preparing the large amount of active proteins necessary for structural and molecular biological studies.

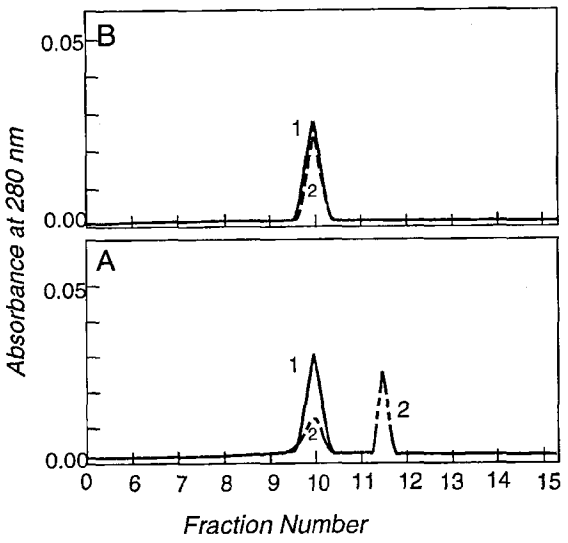

Fig. 6. HPLC elution profiles of native and mutant enzymes using a gel filtration column at pH 7.0 and 5.1. A. The elution profiles of the mutant enzyme eluted with 0.1 M phosphate buffer, pH 7.0 (1) and 0.1 M Na-Citrate, pH 5.1 (2). B. The elution profiles of native enzyme eluted with 0.1 M phosphate buffer, pH 7.0 (1) and 0.1 M Na-Citrate buffer, pH 5.1 (2).

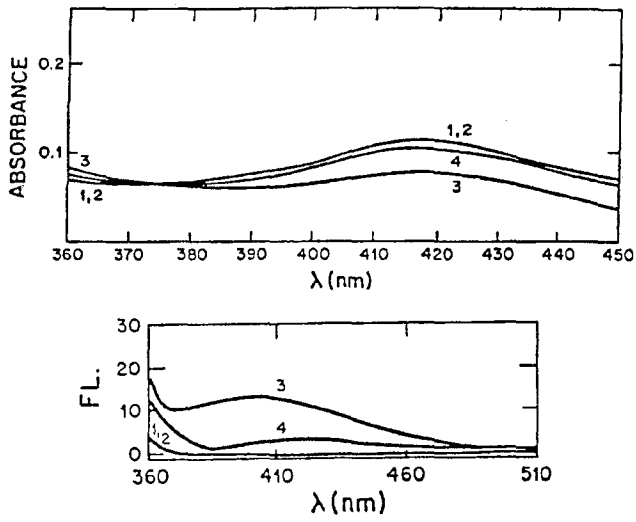

Fig. 7. Absorption and emission spectra of native and mutant enzymes. Top, Absorption spectra in 0.1 M Na-Citrate (pH 5.1). (1) indicates native enzyme (7 μM), (2) carboxy-terminal mutant enzyme (7 μM), (3) native enzyme (7 μM) reacted with 4-aminobutyrate (1 mM), and (4) mutant enzyme (7 μM) reacted with 4-aminobutyrate (1 mM). Bottom, Emission spectra in 0.1 M Na-Citrate (pH 5.1) excited at 340 nm. (1) and (2) indicate native (7 μM) and deleted mutant (7 μM) enzymes, respectively. (3) Native enzyme (7 μM) reacted with 4-aminobutyrate (1 mM) and (4) mutant enzyme (7 μM) reacted with 4-aminobutyrate (1 mM)), respectively.

C-terminal residues have been recognized as being important for quaternary structure, serving as a critical role for enzyme function. In order to investigate how the C-terminal domain of the enzyme contributes to the structural stability and catalytic activity of 4-aminobutyrate aminotransferase, we constructed the C-terminal mutant with five amino acids deleted from the C-terminus. The deletion mutant at the C-terminus displayed identical enzymatic activity to the native enzyme. This result suggests that the deletion of five amino acids residues from the C-terminus did not interfere with the kinetic parameters and functional properties of the enzyme. It was also shown that the deletion did not affect the dimeric structure of the protein aligned along the subunit interface. In marked contrast to neutral pH, the mutant protein was dissociated into monomeric subunits at acidic pH. The net effect of the deletion of the five amino acids was to change the stability of the enzyme at acidic pH. Spectroscopic studies also showed that the dimeric structure of the enzyme was dissociated at acidic pH. It should be noted that, at acidic pH, the facilitated dissociation of the enzyme abolished the catalytic activity.

What is the catalytic function of the C-terminal mutant enzyme at acidic pH? The enzymatic reaction of 4-aminobutyrate aminotransferase is normally performed at pH 8 in a coupled assay. However, in order to determine the catalytic function of this enzyme at pH 5.1, we decided to monitor the change in absorption spectra elicited by addition of the substrate, 4-aminobutyrate. If at acidic pH, the following reaction (half transamination) takes place,

Enzyme-PLP + 4-aminobutyrate →

Enzyme-PMP + Succinic Semialdehyde

then the absorption band at 420 nm corresponding to pyridoxal 5′-P should be decreased. Also, the formation of pyridoxamine 5′-P in the half reaction at acidic pH can be detected by fluorescence spectroscopy. The signal of pyridoxamine 5′-P is higher in frequency than that of pyridoxal 5′-P.

The native enzyme reveals a degree of subunit interaction at acidic pH not found with the mutant enzymes, suggesting that the five amino acids residues play an important role in the structural organization of the two subunits. Finally, the results obtained after deletion of the C-terminal group indicate that thiol groups located at the subunit interface serve as sensitive probes for changes in the dimeric state of the active enzyme. Judging from the results obtained with the C-terminal mutant of 4-aminobutyrate aminotransferase, it appears that amino acids from the C-terminal region of the enzyme are critical for the dimeric structure of 4-aminobutyrate aminotransferase.

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