Catalytic and Structural Properties of Trypsin-treated 4-Aminobutyrate Aminotransferase*

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4-Aminobutyrate aminotransferase (EC 2.6.1.19, 4-aminobutyrate:2-oxoglutarate aminotransferase) is cleaved by trypsin, yielding an enzymatically active species which can be separated from the split peptides by gel filtration. The shortened enzyme derivative gives one band ($M_r = 95,000$) on polyacrylamide gradient gel electrophoresis. Changes in protein conformation induced by trypptic digestion were studied by fluorescence spectroscopy. The native enzyme tagged with the chromophore fluorescein yields a rotational relaxation time of 108 ns, whereas the trypsin-digested enzyme gives a rotational relaxation time of 33 ns. The decrease in rotational relaxation time is attributed to flexibility of the polypeptide chain with enhanced rotational freedom of the probe covalently linked to one thiol group. The reactivity of sulfhydryl groups toward 5,5'-dithiobis(2-nitrobenzoic acid) also decreases in the shortened enzyme derivative. The reactivity of sulfhydryl groups toward 5,5'-dithiobis(2-nitrobenzoic acid) is affected by tryptic cleavage. More ---SH groups (2.6/dimer) become reactive toward 5,5'-dithiobis(2-nitrobenzoic acid) as a result of trypsin digestion. Local conformational fluctuations are induced as a result of tryptic cleavage, but the catalytic sites remain intact.

The peptides released from 4-aminobutyrate aminotransferase were characterized by fingerprint analysis and their amino acid composition determined.

It is the purpose of this work to report the effect of trypsin digestion on the structural and catalytic properties of 4-aminobutyrate aminotransferase.

EXPERIMENTAL PROCEDURES

Purification of Enzymes—4-Aminobutyrate aminotransferase from pig brain was purified according to a procedure previously described (1). This preparation has a specific activity of 20 units/mg at 25°C, and it migrates as a single protein band on polyacrylamide gel electrophoresis.

Protein concentration was determined by the colorimetric method of Lowry et al. (4). The pyridoxal-5-P content of the purified aminotransferase was determined by the method of Wada and Snell (5). The enzyme succinic semialdehyde dehydrogenase from pig brain was purified by a method already described (6).

Enzymatic Assays—A coupled assay system consisting of two purified enzymes, i.e. 4-aminobutyrate aminotransferase and succinic semialdehyde dehydrogenase, was used to study the catalytic conversion of 4-aminobutyrate to succinic semialdehyde. Enzymatic assays were performed in 0.1 M sodium pyrophosphate (pH 8.4) containing 5 mM NADH, 30 mM 4-aminobutyrate, and 10 mM 2-oxoglutarate. Initial rate measurements were carried out by monitoring the changes in absorbance at 340 nm for at least 2 min. A unit of enzyme activity is defined as the amount of enzyme which produces 1 µmol/min of succinic semialdehyde at 25°C.

Titration of Thiol Groups—The number of reactive thiol groups was determined by reaction with DTNB (7) using the procedure of Ellman (7). A molar extinction coefficient of 13,600 M⁻¹ cm⁻¹ at 412 nm was used for the determination of the concentration of the anion 2-nitro-5-mercaptopentanote.

Labeling of the Enzyme—4-Aminobutyrate aminotransferase at a concentration of 5 mg/ml was allowed to react with 1 mM IAF in 0.1 M potassium phosphate (pH 7) at 4°C. The reaction was allowed to proceed for 5 h at 4°C. Excess of free reagent was removed by gel filtration through Sephadex G-25 equilibrated with 0.1 M potassium phosphate (pH 7) containing 0.1 mM 2-mercaptoethanol. The degree of labeling of the enzyme was determined spectrophotometrically using an extinction coefficient of 3.4 × 10⁻³ M⁻¹ cm⁻¹ at 490 nm. The incorporation of 0.92 mol of dye/mol of enzyme does not affect the catalytic activity.

Polyacrylamide Gel Electrophoresis—Polyacrylamide gel electrophoresis was performed according to the procedure of Davis (8). Discontinuous sodium dodecyl sulfate-polyacrylamide gel electrophoresis was carried out at 25°C as described by Laemmli (9). The gel (15% acrylamide) contained 0.1% sodium dodecyl sulfate.

Polyacrylamide gradient gel electrophoresis (4–30%) was performed at 30°C using a voltage of 125 V for 15 h. Standards of known molecular weight, bovine serum albumin, catalase, and cytochrome c, were used in the plots of 1/XL (where XL is the distance [millimeter] of the protein from the origin) versus molecular weight 1/3 following the procedure of Manwell (10).

Protein bands were detected by staining with Coomassie blue dye for 1 h and subsequently destained overnight in a solution containing 10% methanol and 7% acetic acid in water.

Tryptic Digestion of 4-Aminobutyrate Aminotransferase—4-Aminobutyrate aminotransferase (5 mg/ml) was incubated with trypsin (0.025 mg/ml) in 50 mM ammonium bicarbonate (pH 7.6) at 37°C for 2 h. At the end of the incubation, the mixture was filtered through

*This work was supported by National Institutes of Health Grant GM27639-03. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The abbreviations used are: DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); IAF, 6-iodoacetamide fluorescein.
a column of Sephadex G-75 (2 × 100 cm) equilibrated with 50 mM ammonium bicarbonate (pH 7.6) at 4 °C.

The fractions eluted from the column were monitored by absorbance measurements at 280 nm. The protein, which eluted in the void volume of the column, was dialyzed against 0.1 M phosphate buffer (pH 7) containing 0.1 mM 2-mercaptoethanol and used for further characterization.

All the fractions eluted between the excluded and included volumes of the column were combined and lyophilized.

Amino Acid Analysis—Acid hydrolysis was carried out in 4 N methanesulfonic (11) acid and in 6 N HCl at 110 °C in sealed evacuated tubes for 24 h. The amino acid composition of the peptide mixture was determined in a Jeol-6-AH autoanalyzer using the expansion scale of 0.15 absorbance as full scale.

Fingerprint Analysis—This procedure was performed using the method of Schiltz and Reimbolt (12). The sample was applied to a micromerelcrystalline cellulose plate (Polygram-cel 400), and electrophoresis in the first dimension was conducted at 600 V using the solvent system pyridine/acetic acid/H2O (2:4:79, v/v) (pH 4.4). Chromatography in the second dimension was performed using the solvent system n-butyl alcohol/pyridine/acetic acid/H2O (15:10:3). The thin layer sheets were sprayed with fluorescamine (o.01%, v/v) in acetone and then sprayed with 10% (v/v) triethylamine in dichloromethane.

Analytical Ultracentrifugation—Sedimentation velocity experiments were conducted in the Sánczo Model E analytical ultracentrifuge at constant temperature in the range of 8-10 °C. For sedimentation velocity experiments, the ultracentrifuge was operated at 80,000 rpm, and the sedimentation coefficients were corrected for the density and viscosity of water at 20 °C. Samples of enzyme at concentrations varying between 3 and 10 μg/ml in 0.1 M potassium phosphate (pH 7.0) were studied in the analytical ultracentrifuge equipped with a photoelectric scanner (13).

Spectroscopy—Absorption spectra were recorded in a Cary Model 15 spectrophotometer. Fluorescence emission spectra were recorded on a spectrofluorimeter equipped with two Bausch and Lomb monochromators. The slits of the monochromators were set to give a band width of 3 nm.

Polarization of fluorescence measurements were performed on a SLM 4800 spectrofluorimeter. The excitation was set at 440 nm, and fluorescence polarized light emitted by the sample was passed through a Corning Glass filter C.S.-3-69.

Fluorescence decay measurements were made using the monochromator technique with an Ortec Model 9200 ns spectrofluorimeter. A free running lamp operating in air at 1 atm pressure was the exciting light source. The lamp was pulsed at 10 kHz. Excitation was set at 440 nm and the emission was filtered through a Corning Glass filter C.S.-3-60. Deconvolution of the data was performed with a computer program based on the least square method of Ward et al. (14).

Materials—Fig brains were obtained from East Tennessee Packing Co., Knoxville, TN. Trypsin was purchased from Worthington. Sephadex G-25, G-200, and G-75, DEAE-Sephadex, CM-Sephadex, and polyacrylamide gradient gels (PAA 4/30) were purchased from Pharmacia Fine Chemicals. Acrylamide, N,N'-methylenebisacrylamide, and standards for protein molecular weight were from Bethesda Research Laboratories, DTNB from Aldrich, and IAF from Molecular Probes.

RESULTS

Digestion of 4-Aminobutyrate Aminotransferase—4-Aminobutyrate aminotransferase (2 mg/ml) was incubated with trypsin (10 μg/ml) in 50 mM ammonium bicarbonate (pH 7.6) at 37 °C. After various incubation times, samples were removed for enzyme assay and polyacrylamide gel electrophoresis. The overall catalytic activity of 4-aminobutyrate aminotransferase after 2 h of incubation at 37 °C was observed to be unaffected by trypsin addition, whereas the polyacrylamide gel electrophoresis patterns revealed the presence of mainly one band displaying different mobility than the native enzyme (Fig. 1). If digestion with trypsin had taken place, the peptides produced would be of small molecular weight and they would have migrated rapidly in the gel electrophoresis system used and therefore would be undetectable.

When the experiment was repeated with samples of enzyme (2 mg/ml) incubated with trypsin concentrations of 20 and 30 μg/ml for 2 h, the same results were obtained, i.e. the overall catalytic activity remained constant. No change in the catalytic behavior of 4-aminobutyrate aminotransferase was detected when the incubation with trypsin was conducted at pH 7 or 7.4 (Fig. 1). At pH 8, the aminotransferase loses a good deal of its catalytic activity when maintained at 37 °C for 1 h.

Separation of Peptides—For characterization of the action of trypsin on 4-aminobutyrate aminotransferase, the native enzyme in 0.1 M potassium phosphate buffer (pH 7) containing 0.1 mM 2-mercaptoethanol was dialyzed against 50 mM ammonium bicarbonate (pH 7.6). The dialyzed enzyme (100 mg/20 ml) was incubated with 0.5 mg of trypsin at 37 °C for 2 h.

At the end of the incubation, the trypsin-treated enzyme was applied to Sephadex G-75 as described under “Experimental Procedures.” The fractions eluted between the excluded and included volumes of the column were combined and lyophilized. Approximately 4.6 mg of material were recovered in the lyophilized fractions. The void volume of the column (93 mg) was dialyzed against 0.1 M potassium phosphate (pH 7) containing 1 mM 2-mercaptoethanol and used for further experiments.

The lyophilized material (4.6 mg) was used for sodium dodecyl sulfate-polyacrylamide gel electrophoresis, fingerprint analysis, and quantitative determination of the amino acid content.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis, performed on gels containing 15% acrylamide, failed to detect the presence of peptides of molecular weight greater than 3000. Fingerprint analysis showed the presence of at least 10 spots after the cellulose sheets were sprayed with fluorescamine (Fig. 2).

The amino acid composition of the peptides released by tryptic cleavage from 4-aminobutyrate aminotransferase was determined on samples hydrolyzed in 6 N HCl or 4 N methanesulfonic acid. The results of eight independent measure-
were not detected when unhydrolyzed samples of peptides
Amino acid composition of the peptides derived from 4-aminobutyrate
The aromatic acids, were titrated with DTNB in the presence and absence of
are also present in the hydrolyzed samples. Cysteinyl residues
unhydrolyzed samples to amino acid analysis.

| Amino acid | Calculated value | Nearest integer |
|------------|------------------|----------------|
| Arg        | 4.17             | 4              |
| Lys        | 5.30             | 5              |
| His        | 0.84             | 1              |
| Phe        | 1.75             | 2              |
| Tyr        | 1.12             | 1              |
| Leu        | 3.9              | 4              |
| Ile        | 1.73             | 2              |
| Met        | 1                | 1              |
| Val        | 2.85             | 3              |
| Half-cystine | 0.0             | 0              |
| Ala        | 2.92             | 3              |
| Gly        | 2.80             | 3              |
| Pro        | 3.10             | 3              |
| Glu        | 4.65             | 4              |
| Ser        | 1.20             | 1              |
| Thr        | 1.10             | 1              |
| Asp        | 2.92             | 3              |
| Trp        | 1.01             | 1              |

* Determined from the amount of cysteic acid found in the performic acid oxidized sample, and from titrations of the unhydrolyzed material by DTNB.

| Amino acid | Calculated value | Nearest integer |
|------------|------------------|----------------|
| Arg        | 4.17             | 4              |
| Lys        | 5.30             | 5              |
| His        | 0.84             | 1              |
| Phe        | 1.75             | 2              |
| Tyr        | 1.12             | 1              |
| Leu        | 3.9              | 4              |
| Ile        | 1.73             | 2              |
| Met        | 1                | 1              |
| Val        | 2.85             | 3              |
| Half-cystine | 0.0             | 0              |
| Ala        | 2.92             | 3              |
| Gly        | 2.80             | 3              |
| Pro        | 3.10             | 3              |
| Glu        | 4.65             | 4              |
| Ser        | 1.20             | 1              |
| Thr        | 1.10             | 1              |
| Asp        | 2.92             | 3              |
| Trp        | 1.01             | 1              |

* Determined on samples hydrolyzed in 4 N methanesulfonic acid.

The concentration dependence of the sedimentation coefficients of native and modified enzymes was examined in the analytical ultracentrifuge over the concentration ranges 8–3 μM (Fig. 4).

At a concentration of 8 μM, the sedimentation coefficient of the native enzyme (5.15 S) is decreased to a value of 5.0 S as a result of tryptic digestion. This reduction in sedimentation coefficient was also detected at protein concentrations around 3 μM, the lowest concentration studied in the analytical ultracentrifuge equipped with optical scanner. As illustrated in Fig. 4, there is little variation in the sedimentation values of both species when the concentration is decreased, indicating that none of the enzyme species tend to dissociate into subunits of smaller molecular weight at pH 7.

Catalytic Properties—The absorption spectra of the shortened enzyme derivative displayed the characteristic features of the cofactor pyridoxal-5-P covalently bound to the catalytic site. Two absorption bands centered at 330 and 415 nm were detected over the spectral range 300–500 nm at pH 5.8, 6, and 7.0. The intensity of the absorption bands are not affected by variations in the pH of the solution.

The specific activity of the modified enzyme is, within experimental error, identical with that of the native enzyme. Addition of pyridoxal-5-P, followed by preincubation at 25 °C for 1 h prior to enzymatic assays, did not enhance the specific

FIG. 2. Fingerprint pattern given by the peptides released from the native enzyme. Electrophoresis was conducted at pH 4.4 in the solvent system pyridine/acetic acid/H2O/acetonitrile (2:4:78:15, v/v). Chromatography in the second dimension was performed using the solvent system n-butyl alcohol/acetic acid/H2O (15:10:3:12).

FIG. 3. Determination of the molecular weight of trypsin-treated 4-aminobutyrate aminotransferase on polyacrylamide gradient gel electrophoresis. XL is the migration distance (millimeter) of the protein from the origin. The following standards were used: catalase (1), ceruloplasmin (2), bovine serum albumin (3), and 4-aminobutyrate aminotransferase (5). The molecular weight of trypsin-treated 4-aminobutyrate aminotransferase is 95,000.

The plot is linear, and from this plot the molecular weight

FIG. 4. Sedimentation coefficients of native (○) and trypsin-treated (□) enzymes 0.1 M potassium phosphate (pH 7). The ultracentrifuge was operated at 60,000 rpm.
activity of the shortened enzyme derivative. Hence, the modified enzyme behaves as the native aminotransferase, and limited proteolysis did not abolish the apparent negative cooperativity between subunits. The results of these experiments are summarized in Table II.

Reactivity of Sulfhydryl Groups—4-Aminobutyrate aminotransferase is easily inactivated by DTNB; the extent of chemical modification was measured by monitoring the release of 2-nitro-5-mercaptobenzoate at 412 nm. Under denaturing conditions, five —SH groups/dimer are titrable with DTNB (15). The time course for a typical reaction under pseudo-first order conditions is given in Fig. 5.

The reaction of approximately 1.2 sulphydryl groups/dimer proceeds with an observed rate constant of 0.05 min⁻¹ leading to 90% loss of catalytic activity (Fig. 5). Addition of 2-mercaptoethanol (1 mM), followed by dialysis against 0.1 M potassium phosphate (pH 7) containing 1 mM 2-mercaptoethanol, readily restores 90% of the original enzymatic activity (results not shown). This well behaved kinetic system can be exploited to probe the reactivity of the sulphydryl groups of the shortened enzyme derivative.

When the trypsin-treated enzyme was allowed to react with DTNB under pseudo-first order conditions, the reaction of approximately 2.5 sulphydryl groups/mol of enzyme has taken place with an observed rate constant of 0.08 min⁻¹. This increased accessibility of —SH groups to the attacking reagent DTNB reflects local conformational changes of the enzyme elicited by trypsic cleavage.

Fluorescence Polarization—Protein conformational changes elicited by trypsin action were examined using a fluorescent probe covalently linked to the protein.

IAF was selected for these studies for two reasons. First, because it displays absorption and emission properties distinct from the cofactor pyridoxal-5-P; and second, because IAF is a potential modifier of sulphydryl groups which are not released during proteolytic cleavage (Table I).

In marked contrast to DTNB, IAF and iodoacetamide have no effect on the catalytic activity of the aminotransferase. The enzyme was reacted with IAF under the conditions described under “Experimental Procedures.” The labeled enzyme exhibits an absorption band at 495 nm and emission band centered at 515 nm which can be unambiguously assigned to fluorescein (Fig. 6). Using an extinction coefficient of 3.4 × 10⁴ M⁻¹ cm⁻¹ for bound fluorescein, a degree of labeling of 0.9 chromophores/dimer was determined for the reacted enzyme.

In order to ascertain whether IAF has blocked sulphydryl groups, samples of native and IAF aminotransferase were allowed to react with DTNB under denaturing conditions (6 M guanidinium HCl).

The results of those measurements indicated that 5 —SH groups/dimer of native enzyme are blocked by DTNB, whereas only 3.9 —SH/dimer of IAF-aminotransferase have reacted with DTNB (Table III). Thus, these results support the concept that there is one class of sulphydryl groups in the enzyme which can be modified by the alkylating reagent IAF without any concomitant loss of catalytic activity. The spectroscopic properties of bound fluorescein can be used conveniently to detect large structural fluctuations induced by reaction with trypsin. When IAF-aminotransferase (2 mg/ml) was incubated with trypsin (40 g/ml) at pH 7, and the polar-

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**Table II**

| Enzyme samples | Absorption* (nm) | pH | Specific enzyme activity* (units/mg) |
|----------------|------------------|----|-------------------------------------|
| Native         | 330, 415         | 7  | 20                                  |
| Trypsin-treated| 330, 415         | 7  | 20                                  |
| Native         | 330, 415         | 5.8| 20                                  |
| Trypsin-treated| 330, 415         | 5.8| 19.2                                |
| Native + PLP   |                  |    | 20                                  |
| Trypsin-treated + PLP |   |    | 20.2                                |

* Absorption spectra recorded at protein concentrations of 5 mg/ml at the indicated pH values.
* Specific activity of the enzymes exposed to the indicated pH values and assayed at pH 8.4.
* Samples preincubated with 20-fold molar excess of pyridoxal-5-P (PLP) prior to enzymatic assays.
TABLE III

| Enzyme samples | Number of SH/mol of enzyme | Life-time ns | Polarization | Rotational relaxation time ns | Catalytic activity units/mg |
|----------------|---------------------------|--------------|--------------|------------------------------|---------------------------|
| IAF-enzyme     | 3.9                       | 2.7          | 0.22         | 106                          | 20                        |
| Trypsin-digested IAF-enzyme | 3.8                   | 4            | 0.15         | 33                           | 19.2                      |

1. Titrated with DTNB in GM guanidinium HCl at pH 7.
2. IAF-aminotransferase (2 mg/ml) incubated with trypsin (40 μg/ml) for 1 h at 25 °C in 0.1 M potassium phosphate (pH 7). At the end of the incubation, the digested sample was run through Sephadex G-25 equilibrated with 0.1 M potassium phosphate (pH 7). The protein eluted in the void volume of the column was used for —SH titration and polarization measurements.
3. Enzymatic activity determined on the shortened enzyme derivative after passage through a Sephacryl column G-25 (1 × 15 cm) equilibrated with 0.1 M potassium phosphate (pH 7) containing 1 mM 2-mercaptoethanol.

**DISCUSSION**

The results reported in this paper have direct bearing on the effect of conformational fluctuations on the catalytic parameters of the enzyme, 4-aminobutyrate aminotransferase.

Recently, the subject of limited proteolysis of aminotransferase has attracted the attention of two independent laboratories. Hargrove et al. (16) have shown that endopeptidase cathepsin T generates multiple forms of tyrosine aminotransferase. Upon incubation of tyrosine aminotransferase I with cathepsin, tyrosine aminotransferase II and a peptide of about 4500 D are generated without release of single amino acids.

On the other hand, Sandmeier and Christen (17) have reported that mitochondrial aspartate aminotransferase is cleaved selectively by trypsin at two peptide bonds, yielding enzyme derivatives devoid of catalytic activity.

The studies reported in this paper have demonstrated that limited proteolysis of 4-aminobutyrate aminotransferase does not impair its catalytic function. The shortened enzyme derivative generated by trypsin action is not only catalytically competent, but also preserves negative cooperativity between subunits.

As a consequence of trypsic digestion, a heterogeneous population of small molecular weight peptides are released from the native enzyme. The mechanism by which trypsin cleaves the aminotransferase remains unclear on the basis of the present data, and it is the subject of further investigations aimed at the elucidation of the sequence of COOH and NHNH terminus of the enzyme.

Research conducted in several laboratories indicate that protein molecules display conformational fluctuations in the nanosecond, millisecond, and second time ranges (19–19). More frequently considered are those fluctuations where a given structural part of the macromolecule is destroyed and a new configuration is built up. The shortened enzyme derivative of 4-aminobutyrate aminotransferase can be used to investigate the effect of large conformational fluctuations on the catalytic power of an enzyme.

The results obtained using chemical and biophysical methods indicate that conformational changes have taken place in 4-aminobutyrate aminotransferase as a result of trypsic action. Thus, the increased reactivity of thiol groups toward DTNB reflects structural fluctuations in the microenvironment surrounding sulphydryl residues critically connected with catalytic activity.
On the other hand, the spectroscopic properties of a fluorescent probe covalently linked to another class of sulfhydryl groups are influenced by local conformational changes in the protein.

The polypeptide chains of the shortened enzyme derivative display a high degree of flexibility as revealed by polarization of fluorescence measurements. Indeed, the decrease in rotational relaxation time from 106 to 33 ns, the values corresponding to native and trypsin-digested enzymes, respectively, is more than can be expected for macromolecules exhibiting such small differences in molecular weight.

These conformational changes, however, did not prevent the development of the steps required for activation of the enzyme substrate complex, suggesting that structural fluctuations detected at the level of sulfhydryl groups did not perturb the catalytic site domain.

Acknowledgments—We thank Dr. Davis K. Lin of The Memorial Research Center (University of Tennessee) for his help in the amino acid analysis.

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Catalytic and structural properties of trypsin-treated 4-aminobutyrate aminotransferase.
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J. Biol. Chem. 1983, 258:11768-11773.

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