4-Aminobutyrate Aminotransferase Reaction of Sulfhydryl Residues Connected with Catalytic Activity*

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4-Aminobutyrate aminotransferase is inactivated by preincubation with N-(1-pyrene)maleimide (mixing molar ratio 10:1) at pH 7. The reaction with N-(1-pyrene)maleimide was monitored by fluorescence spectroscopy and the degree of labeling of the enzyme determined by absorption spectroscopy. The blocking of 2 cysteinyl residues/enzyme dimer is needed for inactivation of the aminotransferase. The time course of the reaction is significantly affected by the substrate α-ketoglutarate, which afforded complete protection against the loss of catalytic activity. Trypsin digestion of pyrene-labeled aminotransferase, followed by gel filtration and “fingerprint” analysis, revealed the presence of only one peptide tagged with the fluorescent probe.

The reaction of approximately 1.9 SH residues/dimer with iodosobenzoate resulted in enzyme inactivation together with a formation of an oligomeric species of $M_r = 100,000$ detectable by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The cross-linked subunits are dissociated by addition of 2-mercaptoethanol which also restores full catalytic activity. Altogether, these observations are consistent with the concept that inactivation of 4-aminobutyrate aminotransferase by iodosobenzoate proceeds through disulfide bond formation between vicinal cysteinyl residues of the protein.

It is postulated that the critical sulfhydryl groups of the enzyme are situated on opposite sides of the dimeric structure at the subunit interfaces.

The amino acid sequence of the P-pyridoxyl peptide pertaining to the catalytic site of 4-aminobutyrate aminotransferase has been determined (1). This peptide which contained 13 different amino acids, did not contain any cysteine residues.

However, two sulfhydryl groups on the enzyme are critically connected with catalytic activity (2). Thus, the blocking of less than 2 SH groups/enzyme dimer by the reagent DTNB abrogates catalytic activity. Although the reactivity of these SH groups with DTNB is markedly influenced by structural fluctuations of the protein (2), their functional role in catalysis remains to be assessed. This paper reports results of investigations aimed at determining whether these SH groups are located on the same or different polypeptide chains.

Our approach consists in measuring the decay of enzymatic activity, the degree of protection afforded by the substrate α-ketoglutarate, and the extent of chemical modification of the inactive enzyme when 4-aminobutyrate aminotransferase is allowed to react with any of several sulfhydryl reagents. N-(1-Pyrene)maleimide, a fluorescent probe, is used to facilitate the location of modified sulfhydryl groups on peptides resulting from trypsin digestion of labeled 4-aminobutyrate aminotransferase, whereas iodosobenzoate is used to oxidize vicinal SH groups to disulfide bonds.

**EXPERIMENTAL PROCEDURES**

**Purification of Enzymes**—4-Aminobutyrate aminotransferase from pig brain was purified according to a procedure previously described (3). 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 pyridine-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 4 mM NAD+, 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 that amount of enzyme which produces 1 μmol/min of succinic semialdehyde at 25 °C.

A second method of enzymatic assays, based on fluorescent measurements of the condensation product of cyclohexene-1,3-dione with succinic semialdehyde (7), was used to determine the catalytic activity of samples of enzyme reacted with N-(1-pyrene)maleimide.

**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 (7.5% acrylamide) contained 0.1% sodium dodecyl sulfate. 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.

**Chemical Modification with PMI**—A fresh stock solution of approximately 40 mM of PMI was prepared in dimethylformamide. Chemical modification of the enzyme at 25 °C was initiated by adding small aliquots of the stock solution of PMI (1–30 μl) to 2 ml of the enzyme (0.2 mg) in 0.1 M potassium phosphate buffer (pH 7). The reaction of PMI with 4-aminobutyrate aminotransferase was monitored by fluorescence intensity measurements at 386 nm (excitation 345 nm) and by enzymatic assays.

In those experiments in which the extent of modification of the enzyme was to be determined, the reaction of the aminotransferase (0.5 mg/ml) with PMI (50 μM) in 2 ml of 0.1 M phosphate buffer (pH 7) was allowed to proceed for 5 min at 25 °C and immediately applied to a Sephadex G-25 column (1 × 20 cm), equilibrated, and eluted at 4 °C with 0.1 M phosphate buffer (pH 7). Fractions (1 ml) collected

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1 The abbreviations used are: DTNB, 5,5'-dithiobis(nitrobenzoic acid); PMI, N-(1-pyrene)maleimide; dansyl, 2,5-dimethylaminonaphthalene-1-sulfonfonyl; TPCK, 1-tosylamide-2-phenylethyl chloromethyl ketone.
in the void volume of the column were combined and used for absorption and fluorescence spectral measurements. The amount of PMI bound was determined using an extinction coefficient of $4 \times 10^4$ M$^{-1}$cm$^{-1}$ at 345 nm for the pyrene chromophore (10).

**Trypsin Digestion of PMI/Aminotransferase**—Solutions of enzyme (200 mg in 1 M potassium phosphate (pH 7), were allowed to react with N-(1-pyrene)maleimide, molar mixing ratio, 1 mol of protein/10 mol of PMI, for 8 min at 25 °C. The reaction was stopped by addition of β-mercaptoethanol (1 mm), and the reaction mixture was exhaustively dialyzed against 0.1 M NH$_4$HCO$_3$ at 4 °C. The modified protein (18 mg) was heated at 90 °C for 5 min and then cooled to 37 °C. TPCK/trypsin (0.1 mg) was added to the denatured protein and incubated at 37 °C for 2 h. The resulting peptide solution was dried under vacuum and redissolved in 500 μl of NH$_4$HCO$_3$ (100 mM).

The peptide solution was applied to a column of Sephadex G-25 (100 x 1 cm), eluted with 100 mM NH$_4$HCO$_3$ and the fractions monitored by fluorescence measurements (emission 386 nm, excitation 345 nm).

Fractions containing pyrene-labeled peptides were combined, dried under vacuum, dissolved in acetic acid (3 n), and subjected to "finger-print" analysis.

Electrophoresis in the first dimension was conducted at 600 V using a polyacrylamide gel (10% acrylamide/bisacrylamide, 29:1; pH 8.6). The gel was cast in a 12 cm x 12 cm x 0.15 cm box, and the sample was applied to the top of the gel as a 15% stock solution in 0.01 M Tris-HCl (pH 8.0) and 0.1 M sodium dodecyl sulfate (SDS). The gel was run for 2 h at 25 °C.

The amino acid composition was determined in a Jeol-6-AH autoanalyzer using the expansion scale of 0.15 absorbance as full scale.

**Determination of Free Cysteine Residues**—The inactivation reactions were carried out in 2-ml reaction values containing 0.7 mg of enzyme/ml and the inactivating reagent (iodosobenzoate) at an initial concentration of 100 μM in 0.1 M phosphate buffer (pH 7). The reaction was stopped after 20 min incubation at 25 °C, and the reacted sulfhydryl reagent was removed by chromatography at 4 °C with ultraviolet light, was eluted with 6 N HCl to final concentration of 5 M, and titrated with DTNB. The absorption and emission spectra of the modified enzyme were recorded.

**RESULTS**

**Inactivation by N-(1-Pyrene)maleimide**—The conjugation reaction of PMI with protein’s sulfhydryl groups is relatively fast and can be monitored by the increase in fluorescence intensity of pyrene chromophores (12). The fluorescence enhancement is due to a formation of a succinimide derivative.

The reaction of 4-aminobutyrate aminotransferase (1 μM) with various concentrations of PMI was monitored at 386 nm, upon excitation at 345 nm in 0.1 M phosphate buffer (pH 7.4) at 25 °C.

The time course for a typical reaction between the enzyme (1 μM) and PMI (10 μM) is given in Fig. 1, where it may be seen that a maximum increase in fluorescence at 386 nm is reached after 10 min incubation at 25 °C.

The kinetics of the reaction investigated at several concentra-
4-Aminobutyrate Aminotransferase Reactivity of SH Groups

**Fig. 2.** Bottom, emission spectra of 4-aminobutyrate aminotransferase reacted with N-(1-pyrene)maleimide recorded after completion of the reaction (1) and after dialysis against 0.1 M phosphate buffer (pH 7) (2). Top, absorption spectra of 4-aminobutyrate aminotransferase reacted with N-(1-pyrene)maleimide (mixing molar ratio 1:10) in the absence (1) and presence (2) of 10 mM \( \alpha \)-ketoglutarate. Absorption spectra were recorded after the samples were applied to a Sephadex G-25 column (1 x 20 cm) to remove excess reagent.

To this end, the enzyme reacted with PMI was digested with trypsin and subjected to fingerprint analysis as described under “Experimental Procedures.” The fingerprint pattern of the trypsin-digested enzyme showed the presence of many peptide zones, only one of which exhibited the strong fluorescence characteristics of pyrene chromophores.

After gel filtration through a Sephadex-G-25 column (1 x 100 cm), the peptide fractions displaying fluorescence at 386 nm (excitation 345 nm) were pooled, concentrated, and analyzed by fingerprint techniques. As shown in Fig. 3, only one fluorescent spot was detected when viewed under UV light.

The fluorescent peptide, eluted from the cellulose plate, was hydrolyzed in 6 M HCl for 20 h at 110 °C. Amino acid analysis of the hydrolyzed peptide revealed the presence of glycine (3), aspartic (1), glutamic (1), threonine (1), leucine (1), and serine (1). Aromatic amino acids, i.e., tryptophan, tyrosine, phenylalanine, and the amino acid cysteine were not detected in the hydrolyzed samples.

The NH₂-terminal residue of the fluorescent peptide, determined by dansylation (13) and identified by three-dimensional chromatography on micropolyamide sheets (14), was found to be aspartate.

The finding that only one fluorescent peptide labeled with pyrene was detected after fingerprint analysis of trypsin-digested PMI/aminotransferase, together with the observation that one NH₂-terminal group was identified, lends strong support to the contention that N-(1-pyrene)maleimide blocks specific sulfhydryl groups of the enzyme.

**Cross-linking of the Subunits—**Like N-(1-pyrene)maleimide, maleimide and N′-N′-o-phenylenedimaleimide block SH groups of 4-aminobutyrate aminotransferase and irreversibly inhibit catalytic activity. In marked contrast to these reagents, the reaction of 4-aminobutyrate aminotransferase with iodosobenzoate could be easily reversed by addition of either \( \beta \)-mercaptoethanol or dithiothreitol.

Iodosobenzoate was chosen for these studies because it is well established (15) that it reacts with vicinal SH groups in proteins to form disulfide bonds according to Scheme 1.

![Scheme 1](image)

**The time course of inactivation of 1-aminobutyrate aminotransferase (7 \( \mu \)M) by iodosobenzoate (100 \( \mu \)M) at pH 7 is given in Fig. 4. At the end of the reaction, the mixture was chromatographed on Sephadex G-25 at 4 °C to remove any unreacted iodosobenzoate, and the extent of inactivation, the concentration of free thiol groups and the protein concentration in the chromatographed solutions were determined as described under “Experimental Procedures.” As can be seen in Table I, this reaction resulted in 85% inactivation of the enzyme and covalent modification of 1.9 SH residues/dimer.

If intersubunit cross-linking has taken place in the iodosobenzoate-inhibited enzyme, then the oligomeric species produced would be of molecular weight higher than 50,000; and they would be detected by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Indeed, the electrophoresis results clearly show the presence of oligomeric species which can be assigned to dimers of 4-aminobutyrate aminotransferase (Fig. 5). When the iodosobenzoate-inhibited enzyme was incubated with either \( \beta \)-mercaptoethanol or dithiothreitol prior to electrophoresis this oligomeric species was not present (Fig. 5). Moreover, aminotransferase dissociated by guanidinium HCl, then incubated with iodosobenzoate did not yield cross-linked species, suggesting that the intact protein dimer is necessary...
butyrate aminotransferase (7 pM) at pH reduced glutathione (1 mM) to the inhibited enzyme (5).

The method chosen for demonstration of cross-linked subunits would be unsuitable for detection of intramolecular cross-linked monomers, since modified monomers would be expected to display the same electrophoretic mobility as monomeric species of 4-aminobutyrate aminotransferase on sodium dodecyl sulfate-polyacrylamide gel electrophoresis. However, the predicted stoichiometry for the reaction of iodosobenzoate with sulphydryl groups in proteins is consistent with the titration results (Scheme 1). Accordingly, 1.9 sulphydryl groups in the modified aminotransferase failed to react with DTNB in the presence of 5 M guanidinium HCl (Table I) because iodosobenzoate has promoted the formation of the disulfide bond.

The enzyme treated with iodosobenzoate exhibited 15% of the normal catalytic activity, but the addition of 100-fold molar excess of dithiothreitol with respect to protein rapidly restored normal catalytic activity (Fig. 4). The rate of restoration of enzymatic activity with either β-mercaptoethanol or dithiothreitol, is a very efficient process as revealed by the results included in Fig. 4; in striking contrast, reduced glutathione failed to restore full catalytic activity. Hence, the degree of restoration of normal enzymatic activity decreases significantly with increasing thiol size, suggesting that there are steric restrictions in the immediate vicinity of the modified cysteinyl residues of the protein which cause larger thiol compounds to be at least partially excluded.

**FIG. 4.** Reaction of iodosobenzoate (100 pM) with 4-aminobutyrate aminotransferase (7 μM) at pH 7, 25 °C. Enzymatic activity changes in the absence (1) and presence (2) of 10 mM α-ketoglutarate. Restoration of enzymatic activity when 2-mercaptoethanol (3) and (4) at a concentration of 1 mM were added to the inhibited enzyme. Restoration of catalytic activity upon addition of reduced glutathione (1 mM) to the inhibited enzyme (5).

**FIG. 5.** Sodium dodecyl sulfate-polyacrylamide gel electrophoresis patterns of native 4-aminobutyrate aminotransferase (A), enzyme reacted with iodosobenzoate (mixing molar ratio 1:14) and applied to a Sephadex G-25 column to remove excess iodosobenzoate (B), and sample B reacted with 0.1 mM 2-mercaptoethanol (C). Molecular weights standards were phosphorylase b (93,000), bovine serum albumin (69,000), and ovalbumin (45,000). Prior to electrophoresis, samples A and C exhibited full enzymatic activity, sample B displayed 23% of the native catalytic activity.

**TABLE I**

<table>
<thead>
<tr>
<th>Reaction mixture</th>
<th>Activity</th>
<th>SH groups reacted %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Enzyme (1 μM) + PMI (10 μM)</td>
<td>10</td>
<td>2</td>
</tr>
<tr>
<td>Enzyme (1 μM) + α-ketoglutarate (10 mM) + PMI (10 μM)</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>1 Enzyme (1 μM) + α-ketoglutarate (10 mM) + iodosobenzoate (100 μM)</td>
<td>15</td>
<td>1.9</td>
</tr>
<tr>
<td>Enzyme (7 μM) + α-ketoglutarate (10 mM) + iodosobenzoate (100 μM)</td>
<td>100</td>
<td>0</td>
</tr>
</tbody>
</table>

**DISCUSSION**

The results presented indicate that the fluorescence probe, N-(1-pyrene)maleimide, blocks two sulphydryl groups in 4-aminobutyrate aminotransferase and abrogates catalytic activity.

The modified enzyme, containing 2-pyrene molecules/dimer, exhibits several emission peaks in the 375–440 nm region characteristic of pyrene monomer fluorescence. Emission peaks centered at 386 and 405 nm have been reported for pyrene maleimide modified bovine serum albumin and for the pyrene maleimide adducts of cysteine and cysteamine (12).

Experimental evidence from both chemical analysis and nuclear magnetic resonance studies of the cysteine-pyrene adducts support the hypothesis that the appearance of two emission peaks at 386 and 405 nm is the result of intramolecular aminolysis of the succinimido ring in the adducts. Although intramolecular aminolysis as described for other maleimide adducts, i.e. N-ethylmaleimide, N-4-dimethylamino-3,5-dinitrophenylmaleimide (16, 17), may have occurred in the reaction of N-(1-pyrene)maleimide with the aminotransferase, the intramolecular nucleophilic attack on the succinimido ring of the adduct by primary amino groups appears to be a fast process, since the emission spectrum of N-(1-pyrene)maleimide conjugated aminotransferase recorded 12 min after completion of the reaction was identical to the emission spectrum obtained after separation of noncovalently bound pyrene (Fig. 2).

Excimers of pyrene derivatives typically exhibit a broad emission band at 480 nm, unlike the monomer fluorescence which is characterized by several sharp peaks at shorter wavelengths (18).

Quantum mechanical calculations (19), on the other hand, have shown that two pyrene molecules must be oriented parallel to each other with an interplanar distance of 3.6 Å for excimer formation; and it has been demonstrated that N-(1-pyrene)maleimide-conjugated tropomyosin displays a strong emission band centered at 480 nm (20). Surprisingly, we were unable to detect any emission band indicative of excimer fluorescence in pyrene-conjugated 4-aminobutyrate aminotransferase.

Since fingerprint analysis of PMI/aminotransferase revealed the presence of only one pyrene-labeled peptide, it seems reasonable to suggest that the absence of excimer...
emission in the undigested enzyme is due to unfavorable orientation of the interacting pyrene rings.

The reaction of 4-aminobutyrate aminotransferase with another reagent, \( N'N' \)-o-phenylenedimaleimide, also resulted in irreversible loss of catalytic activity, but in this instance cross-linking of the subunits to form oligomeric structures was detected by electrophoresis techniques (results not shown).

Further evidence for involvement of sulfhydryl groups in the cross-linking process was derived from studies of the reaction between iodosobenzoate and the sulfhydryl groups of the aminotransferase. The reaction of approximately 1.9 SH residues/dimer with iodosobenzoate resulted in enzyme inactivation together with the formation of oligomeric species of \( M_r = 100,000 \).

The cross-linked subunits are dissociated by addition of \( \beta \)-mercaptoethanol and dithiothreitol which also restore the catalytic activity of iodosobenzoate-inhibited enzyme. Altogether, these observations are consistent with the concept that inactivation of 4-aminobutyrate aminotransferase by iodosobenzoate proceeds through disulfide bond formation between vicinal cysteinyl residues in the dimeric protein. An important corollary of these studies is related to the position of those critical SH residues in the spatial structure of the dimeric enzyme. They are able to participate in the formation of a disulfide bond because they are in close proximity, within 2 Å of each other, and they are situated on opposite sides of the enzyme dimer at the subunit interfaces.

The other interesting aspect of the inactivation of 4-aminobutyrate aminotransferase by pyrene maleimide and iodosobenzoate is the protection afforded by the substrate \( \alpha \)-ketoglutarate. In both cases, it was demonstrated that \( \alpha \)-ketoglutarate exerts its protective effect by preventing the reaction of SH groups with the attacking reagent.

The fact that the ligand (\( \alpha \)-ketoglutarate) that protects two sulfhydryl groups against modification by \( N'-(1\text{-pyrene}) \) maleimide is the same ligand that protects against inactivation by iodosobenzoate, and the fact that modification by either reagent results in approximately 90% inactivation of the enzyme, suggest that these two reagents modify the same set of sulfhydryl residues.

The results of our studies raise some questions about the role of the reactive sulfhydryl groups in enzyme function. Two distinct possibilities emerge. The sulfhydryl groups are essential for catalysis, i.e. they participate in some catalytic events.

Alternatively, blocking of the reactive sulfhydryl groups triggers a conformational change which affects the catalytic site domain.

The first interpretation seems to be consistent with the protective effect exerted by \( \alpha \)-ketoglutarate, but binding \( \alpha \)-ketoglutarate can also result in stabilization of protein conformations which are no longer accessible to attacking sulfhydryl reagents. In this connection, it should be noted that the other substrate of the enzyme, i.e. 4-aminobutyrate, failed to exert any protective effect against the bulky sulfhydryl reagents used in our experiments. Since 4-aminobutyrate converts pyridoazal-5-P into pyridoxamine-5-P, it appears that there is a correlation between events occurring at the catalytic site and structural fluctuations at the level of the subunit interfaces where the reactive sulfhydryl groups are located.

REFERENCES