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REACTIVITY OF ONE THIOL GROUP IN THE DIMERIC PROTEIN, 4-AMINOBUTYRATE AMINOTRANSFERASE

UDOUDO MOSES and JORGE E. CHURCHICH

Department of Biochemistry, University of Tennessee, Knoxville, TN 37916 (U.S.A.)

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Summary

The enzyme 4-aminobutyrate aminotransferase (4-aminobutyrate:2-oxoglutarate aminotransferase, EC 2.6.1.19) from pig brain is inactivated by incubation with 5-5′-dithiobis-2-nitrobenzoic acid at pH 7.4. The reaction of one SH group per dimer brings about 95% loss of aminotransferase activity. The reaction with 5-5′-dithiobis-2-nitrobenzoic acid under pseudo-first-order conditions proceeds at a slow rate ($K_{obs} = 0.05$ min^{-1}) and the substrate 4-aminobutyrate has no effect on the rate of inactivation. The inactivation of the enzyme cannot be related to dissociation of the cofactor (pyridoxal-*P* and pyridoxamine-*P*) from the catalytic site. One thiol group of the enzyme reacts with *N*-iodoacetylaminoethyl-5-naphthylamine-1-sulfonic acid at pH 7.4. The reaction of approximately one SH group per dimer causes 95% loss of aminotransferase activity. The absorption and fluorescence properties of the enzyme tagged with the chromophore were used to gain information on the microenvironment of the reactive SH group critically connected with catalytic activity. The fluorescent properties, emission maximum (470 nm), fluorescence lifetime (19 ns) and degree of exposure to the collisional quencher acrylamide ($K_q = 1.8 \cdot 10^8$ M$^{-1} \cdot$ s^{-1}) indicate that the chromophore is shielded by the protein matrix.

Introduction

The enzyme 4-aminobutyrate aminotransferase (4-aminobutyrate:2-oxoglutarate aminotransferase, EC 2.6.1.19) has been purified and characterized in

Abbreviation: AEDANS, *N*-iodoacetylaminoethyl-5-naphthylamine-1-sulfonic acid.

several laboratories [1—4]. The mechanism of action of this enzyme is well established and there is strong evidence supporting the contention that the enzyme from pig brain is a dimeric protein made up of two subunits of identical molecular weights [4—5]. The cofactor pyridoxal-5-*P* is tightly bound to the catalytic site, and recent studies have demonstrated that the irreversible inhibition by some substrate analogues is due to chemical modification of the cofactor, followed by binding of the adduct to amino acid residues of the catalytic site [6].

However, no information is available about the amino acid residues which are critically connected with the enzymatic activity. The aims of the present investigation are 2-fold. Firstly, to study the reactivity of the SH groups of the enzyme, and secondly, to investigate the effect of irreversible blocking of the SH groups on the affinity of the protein for the cofactors pyridoxal and pyridoxamine-5-*P*.

Experimental Procedures

Purification of enzymes. 4-Aminobutyrate aminotransferase from pig brain purified according to a procedure previously described [5], except that pyridoxal-5-*P* was omitted from all the purification steps. This preparations has a specific activity of 20 units/mg at 37°C and it migrates as a single protein and activity band on polyacrylamide gel electrophoresis. The enzyme was resolved into apoprotein and free cofactor by a method already described [5]. Succinic semialdehyde dehydrogenase from pig brain was purified according to the procedure of Blaner and Churchich [7]. This preparation has a specific activity of 10 units/mg at 37°C. One unit of enzymatic activity is defined as that amount of enzyme which produces 1 μmol NADH/min at 37°C.

Enzymatic assays. Two methods were used in the assay of 4-aminobutyrate aminotransferase activity.

Method I: This enzyme assay procedure is base on fluorimetric measurements of the condensation product of cyclohexane-1-3-dione with succinic semialdehyde [5].

The reagent was a solution containing 0.25 g of cyclohexane-1-3-dione, 10 g ammonium acetate, 5 ml glacial acetic acid in 100 ml of water. The substrate solution contained 20 mM 4-aminobutyrate and 5-mM 2-oxoglutarate adjusted to pH 8.4 with NaOH. The 5 ml substrate solution was incubated with the enzyme at 37°C. 0.5 ml aliquots were withdrawn from the incubation mixture at several time intervals (2 min), mixed with 0.5 ml of the reagent solution, heated for 15 min in a water bath at 60°C, diluted to 3 ml by addition of water, and the fluorescence intensity recorded at 460 nm (excitation 365 nm). A standard curve of succinic semialdehyde reacted wity cyclohexane-1-3-dione was run in parallel. The fluorimetric method permits the detection of concentrations of succinic semialdehyde lower than 0.1 μM.

Method II: 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. Enzyme assays were performed in 0.1 M sodium pyrophosphate (pH 8.4) containing 5 mM NAD^+, 20 mM 4-aminobutyrate and 5 mM 2-oxo-

glutarate. The progress of the reaction was followed by monitoring changes in absorbance at 340 nm. The coupled assay system effectively measures the rate of transamination when the concentration of succinic semialdehyde dehydrogenase is at least 5-fold greater than the concentration of aminotransferase.

Titration of sulfhydryl groups. Prior to the titration experiments designed to determine the number of reactive thiol groups, the protein dissolved in 0.1 M potassium phosphate (pH 7.4) containing 0.1 mM β-mercaptoethanol and 0.1 mM EDTA was run through a column of Sephadex G-25 (30 × 1 cm) equilibrated with 0.1 M potassium phosphate (pH 7.4). The latter procedure removed all traces of β-mercaptoethanol from the proteins and the holoenzyme retained full catalytic activity.

The number of reactive SH groups was determined by reaction with 5-5′-dithiobis-2-nitrobenzoic acid using the procedure of Ellman [8] and by reaction with *p*-hydroxymercuribenzoic acid according to the procedure of Boyer [9]. Protein concentration was determined by the method of Lowry et al. [10].

Labeling of 4-aminobutyrate aminotransferase with N-iodoacetylaminoethyl-5-naphthylamine-1-sulfonic acid. To obtain the dye-protein conjugate, 5 μM 4-aminobutyrate aminotransferase was allowed to react with a 100-fold molar excess of *N*-iodoacetylaminoethyl-5-naphthylamine-1-sulfonic acid at pH 7.4, 4°C. The reaction was allowed to proceed for 12 h at 4°C. Excess of free reagent was removed by dialysis against 0.1 M phosphate buffer (pH 7.4), followed by gel filtration through Sephadex G-25, equilibrated with the same buffer. The degree of labeling of the enzyme was determined spectrophotometrically using an excitation coefficient of $6.2 \cdot 10^3\ M^{-1} \cdot cm^{-1}$ at 340 nm. The labeled enzyme, containing 1 mol of chromophore per mol of enzyme is inactive.

Spectroscopic methods. Fluorescence spectra were recorded in a precision spectrofluorimeter equipped with two Bausch and Lomb monochromators. The slits of the monochromators were set to give a band width of 3 nm. Spectrophotometric measurements were carried out in a Cary model 15 spectrophotometer. Polarization of fluorescence measurements were performed in an instrument designed in our laboratory [11]. Fluorescence lifetime measurements were made using the monophoton technique on an Ortec model 9200 nanosecond fluorimeter. A free-running falsh lamp operating in air at 1 atm pressure was used as exciting light source. The excitation wavelength (maximum transmission at 365 nm) was selected by a Corning glass filter (C-S-7-83) and the mission was filtered through a C-S-3-72 Corning glass filter. Analysis of the data was performed with a computer program based on the least-squares method of Ware et al. [12].

Materials. Pig brains were obtained from East Tennessee Packing Co. The brains were placed in ice as quickly as possible after slaugher, and preparation of enzymes was begun with 1 h.

Succinic semialdehyde, NAD^+, pyridoxal-5-*P* and pyridoxamine-5-*P*, 4-aminobutyrate and 2-oxoglutarate were purchased from Sigma Chemical Company. Carboxymethyl-Sephadex C-50, DEAE-Sephadex and Sephadex G-25 were purchased from Pharmacia. Cyclohexane 1-3-dione obtained from Aldrich Chemical Company was crystallized twice before being used in the fluorimetric experiments.

Results

The enzyme 4-aminobutyrate aminotransferase is easily inactivated by 5-5′-dithiobis-2-nitrobenzoic acid and *p*-hydroxymercuribenzoic acid which are specific reagents for thiol groups in proteins.

To determine the number of thiol groups critically connected with catalytic activity, the enzyme was titrated in 0.1 M potassium phosphate (pH 7.4) with increasing concentrations of 5-5′-dithiobis-2-nitrobenzoic acid. The extent of the reaction was determined by measuring the increase in absorbance at 412 nm after addition of a few mole equivalents of reagent per mol of enzyme and incubation of the reaction mixture for 12 h at 4°C. The addition of 4 and 6 mol equiv. of reagent causes the release of less than 2 mol of 2-nitro-5-mercaptobenzoate after an incubation time of 12 h (Table I). Under this set of experimental conditions, the reacted enzyme loses 95% of the aminotransferase activity. A similar effect on the catalytic activity was observed after preincubation of the enzyme with 2 mol equiv. of *p*-hydroxymercuribenzoic acid as shown by the results of Table I.

The kinetics of the reaction of the aminotransferase with 5-5′-dithiobis-2-nitrobenzoic acid were monitored at 25°C under pseudo-first-oder conditions. The time course of the reaction of the 2.5 μM enzyme with the reagent is given in Fig. 1, where it may be seen that the reaction of approx. 1 thiol group per mol of enzyme has taken place within 60 min of incubation at 25°C.

20-μl aliquots withdrawn at different time intervals from the incubation mixture containing 2.5 μM enzyme and 75 μM reagent were assayed for enzymatic activity using the amino acid substrate 4-aminobutyrate. As shown by the results included in Fig. 1, 95% loss of the aminotransferase activity has taken place after the reaction of 1 thiol group per mol of enzyme (dimer). The loss of catalytic activity is not prevented by the addition of the substrates (10 mM) 4-aminobutyrate plus (1 mM) succinic semialdehyde prior to the reaction of the thiol group (Fig. 1). Hence, the slow reactivity of the thiol group critically connected with enzymatic activity, suggests that this amino acid residue is positioned on a region of the polypeptide backbone which is

TABLE I

REACTIVITY OF THE THIOL RESIDUES 4-AMINOBUTYRATE AMINOTRANSFERASE (3 μM) AT pH 7.4

All samples were preincubated at 4° C for 12 h except for 5-5′-dithiobis-2-nitrobenzoic acid (20 μM) + 5 M guaridinum HCl which was preincubated at 25° C for 10 min.

Sample	mol SH/mol enzyme	Enzyme activity (%)
5-5′-dithiobis-2-nitrobenzoic acid (12 μM)	1.2	5
5-5′-dithiobis-2-nitrobenzoic acid (18 μM)	1.2	4
5-5′-dithiobis-2-nitrobenzoic acid (20 μM) + 5M guanidinum HCl	5	0
p-hydroxymercutibenzoic acid (6 μM)	1.2	4

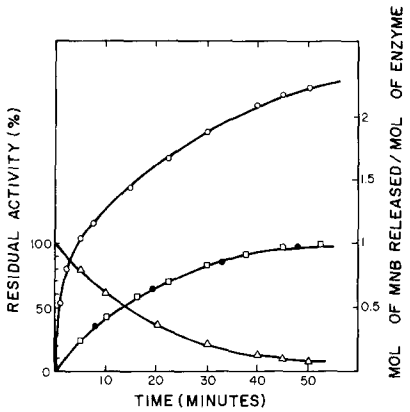
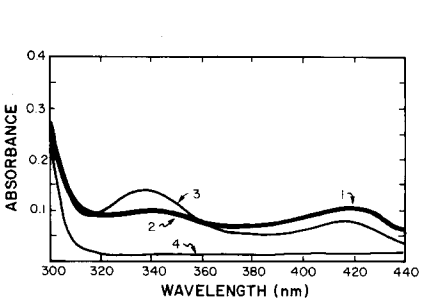


Fig. 1. Time course of reaction of 2.5 μM 4-aminobutyrate aminotransferase in the presence (•) and absence (□) of 10 mM 4-aminobutyrate + 1 mM succinic semialdehyde with 75 μM 5-5′-dithiobis-2-nitrobenzoic acid at pH 7.4, 25° C. Time course of the reaction of the 2.5 μM apoprotein with 75 μM reagent (○). The pseudo-first-order rate constant for the holotransaminase reaction is $K_1 = 0.05$ min^{-1}, whereas the pseudo-first-order rate constants for the reaction of the apoprotein are $K_1 = 0.453$ min^{-1}, $K_2 = 0.04$ min^{-1}. The loss of aminotransferase activity as a function of incubation time with 5-5′-dithiobis-2-nitrobenzoic acid (△) is given in the figure. Method I was used in the enzymatic assays. MNB, 2-nitro-5-mercaptobenzoate.

unaffected by the presence of the substrates. In view of the lack of protection afforded by 4-aminobutyrate, we decided to investigate whether the chemical modification of the enzyme results in dissociation of the cofactor from the catalytic site. To this end, the absorption and fluorescence properties of the holoenzyme were studied after inactivation with *p*-hydroxymercuribenzoic acid.

The absorption spectra of the cofactor pyridoxal-5-*P* covalently bound to the protein is shown in Fig. 2. Two absorption bands, centered at around 330 and 415 nm, are detected at pH 7.4. Although the addition of 2 mol equiv. of *p*-hydroxymercuribenzoic acid per mol of enzyme brings about 95% loss of the catalytic activity, there is no effect on the intensity of the absorption bands covering the spectral range 300—450 nm as shown by the results of Fig. 2. It should be noted that pyridoxal-5-*P* dissociated from the enzyme displays a maximum of absorption at 388 nm. Therefore, the simplest interpretation of this result is that pyridoxal-*P* remains covalently attached to the amino acid residues of the catalytic site despite irreversible blocking of one of the thiol groups of the protein.

This finding prompted us to investigate whether the microenvironment surrounding the cofactor pyridoxamine-*P* is perturbed by addition of 2 mol equiv. of *p*-hydroxymercuribenzoic acid per mol of enzyme. Conversion of the pyridoxal-*P* form of the enzyme (aldimine) into the pyridoxamine-*P* form (ketimine) was attained by the addition of the substrate (10 mM) 4-aminobutyrate to (10 μM) 4-aminobutyrate aminotransferase at pH 7.4, and the emission spectra of the enzyme mixture was recorded over the spectral range 350—460 nm (Fig. 3). Under this set of experimental conditions, the pyridoxamine-*P* molecules bound to the enzyme (electrostatic interaction) are characterized by a fluorescence yield at least 10-fold lower than the corresponding



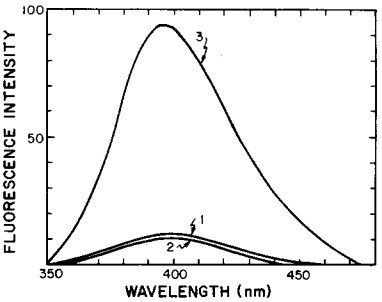


Fig. 2. Absorption spectra of 5 μM 4-aminobutyrate aminotransferase (1), 5 μM 4-aminobutyrate aminotransferase reacted with *p*-hydroxymercuribenzoic acid (2), 5 μM 4-aminobutyrate aminotransferase + 10 mM 4-aminobutyrate (3), and apoprotein (4) in 0.1 M phosphate (pH 7.4).

Fig. 3. Fluorescence spectra of pyridoxamine-5-*P* released from 4-aminobutyrate aminotransferase (3) at pH 5.5 after incubation with 0.5 M H_2KPO_4 for 60 min at 25° C. Fluorescence spectra of the pyridoxamine-5-*P* form of the enzyme before (1) and after (2) incubation with *p*-hydroxymercuribenzoic acid at pH 7.4. Excitation wavelength 330 nm. The fluorescence yield of free pyridoxamine-5-*P* is independent of pH over the range 5—7.4.

fluorescence yield of pyridoxamine-5-*P* dissociated from the enzyme at pH 5.5, after incubation with 0.5 M KH_2PO_4 (Fig. 3).

The release of pyridoxamine-*P* from the catalytic site is paralleled by a substantial increase in the quantum yield as shown by the results included in Fig. 3. If pyridoxamine-5-*P* is dissociated from the enzyme after blocking the reactive thiol group, then one should observe an increase in the fluorescence yield upon excitation at 330 nm. If, on the other hand, the cofactor remains bound to the enzyme after loss of the catalytic activity, the fluorescence yield should remain constant.

The results of these measurements clearly indicate that the intensity of the emission band due to pyridoxamine-5-*P* remains unaltered after irreversible inactivation of the enzyme.

Thus, the spectroscopic results indicate that the loss of catalytic activity induced by *p*-hydroxymercuribenzoic acid cannot be ascribed to dissociation of the cofactor from the enzyme. They also lend strong support to the concept that the thiol group connected with enzymatic activity does not overlap with the cofactor pyridoxamine-5-*P*.

Binding of fluorescent probes

The effect of fluorescence probes on the catalytic activity of 4-aminobutyrate aminotransferase was studied by using fluorescence compounds which react with the chemically accessible thiol group of the enzyme. Several fluorescent probes were tested, but *N*-iodoacetylaminoethyl-5-naphthylamine-1-sulfonic acid was chosen for these studies because it is sensitive to small perturbations of the polarity of the surrounding microenvironment.

The labeling outlined in Experimental Procedures yielded a tagged enzyme containing approx. 1 mol of chromophore per mole of enzyme. The modified enzyme, which is enzymatically inactive, exhibits an emission band centered at

TABLE II

Sample	Emission maximum (nm)	Fluorescence lifetime (ns)	Quenching rate constant (K_q) ($M^{-1} \cdot s^{-1}$)
Cysteine-AEDANS	500	11.0	$9 \cdot 10^8$
E-AEDANS *	470	19.0	$1.3 \cdot 10^8$
E-AEDANS + 0.04 M acrylamide	470	16.5 (19)	
E-AEDANS + 0.1 M acrylamide	470	15.2 (19)	
E-AEDANS + 0.12 M acrylamide	470	12.7 (19)	

* Enzyme labeled with *N*-iodoacetylaminoethyl-5-naphthylamine-1-sulfonic acid

around 470 nm upon excitation at 360 nm. The maximum of emission is blue shifted by 30 nm when compared to cysteine-AEDANS (Table II). Upon attachment to the thiol group of 4-aminobutyrate aminotransferase, the fluorescence of the probe decays in a monoexponential manner with a lifetime of 19.0 ns (Table II).

Although it is difficult to ascertain whether the thiol group reacted with *N*-iodoacetylaminoethyl-5-naphthylamine-1-sulfonic acid is the same that undergoes exchange reaction with 5-5′-dithiobis-2-nitrobenzoic acid, it is worth noting that the enzyme labeled with the fluorescent probe does not react with 5-5′-dithiobis-2-nitrobenzoic acid at pH 7.4.

In an effort to gain more information on the degree of accessibility of the thiol group critically connected with enzymatic activity, we decided to measure the effect of solute perturbation on the fluorescence emitted by the fluorescent probe covalently bound to the protein. The effect of acrylamide on the spectroscopic properties of the conjugated protein was examined at pH 7.4 using steady and nanosecond fluorescence techniques. As shown by the results included in Table II, the fluorescence emitted by the conjugated enzyme was was progressively decreased as the acrylamide concentration varied from 0 to 0.2 M.

The perturbation exerted by acrylamide is also reflected in the fluorescence decay measurements. The fluorescence decay of the conjugated protein in the presence of acrylamide is no longer monoexponential and exhibits fluorescence decay components shorter than 19 ns, which is the fluorescence lifetime of the conjugated enzyme in the absence of quencher (Table II).

Thus, the interaction of acrylamide with the probe bound to the thiol group of the protein can be detected by either fluorescence intensity or fluorescence lifetime measurements. In view of these results, it seems reasonable to assume that a collisional process is taking place between the excited state of the chromophore and the quencher. Therefore, the magnitude of the quenching rate constant (K_q) obtained from measurements of the dynamic quenching constant (K) and the fluorescence lifetime (τ_0) (Equation 1 and 2) is related to the degree of exposure of the emitting molecule to the collisional quencher.

$$K = K_q \cdot \tau_0 \qquad (1$$

From the results included in Table II, it is immediately apparent that the fluorescent probe bound to the thiol group of the enzyme is less accessible to the perturbing action of acrylamide than the probe bound to cysteine.

The simplest interpretation of these results is that the fluorescent group covalently attached to the enzyme is shielded by the protein matrix.

Discussion

The experiments designed to study the number of thiol groups critically connected with enzyme activity have shown that the reaction of one thiol group per dimer leads to irreversible inactivation of 4-aminobutyrate aminotransferase. The presence of the substrate 4-aminobutyrate in the reaction mixture does not afford any protection against the reactivity of the thiol group towards 5-5′-dithiobis-2-nitrobenzoic acid. Similar results were obtained when the reagent *p*-hydroxymercuribenzoic acid was used to determine the number of thiol groups implicated in catalysis. There are two possible ways of explaining the effect of the thiol reagents on the catalytic function of the enzyme. The first one is that blocking of the thiol group results in dissociation of the cofactor from the catalytic site of the enzyme. The second is that the reaction of the thiol group triggers a conformational change which affects the structure of the catalytic sites. The first possibility was investigated by examining the spectroscopic properties of the enzyme before and after reaction with *p*-hydroxymercuribenzoic acid. The results of those measurements clearly demonstrated that the loss of aminotransferase activity cannot be ascribed to dissociation of the cofactor from the enzyme.

Therefore, the second possibility, i.e., binding of the reagent to the critical thiol group of the enzyme causes a conformational change which influences the catalytic function, seems more plausible.

In this connection, it should be noted that the blocking of only one thiol group by any of the bulky reagents tested results in loss of the aminotransferase activity. This unusual stoichiometry of binding is significant, since the enzyme is made up of two subunits of identical molecular weight.

In an effort to understand the effect of the thiol reagent on the enzyme conformation, the spectroscopic properties of the fluorescent ligand, *N*-iodoacetylaminoethyl-5-naphthylamine-1-sulfonic acid, were examined by different methods. The fluorescent probe covalently bound to the reactive thiol group of the enzyme interacts with the protein matrix as revealed by a blue shift in the emission, an increase in the fluorescence lifetime and by the degree of exposure to the collisional quencher acrylamide. The apparent value of the rotational correlation time obtained by linear extrapolation of the Perrin's plot is low (40 ns) in comparison with the value predicted for a spherical protein of 100 000 molecular weight, and suggests that the rotational unit is smaller than the dimeric protein, as would be the case if some form of internal flexibility were present.

However, it remains to be demonstrated whether the increase in flexibility of the inactivated enzyme is due to perturbation of the monomer-monomer interface by insertion of a bulky reagent.

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