Reaction of Cystathionase with the Fluorescent Probe Bis(dansyl)cystine*

KYUNG-JA OH AND JORGE E. CHURCHICH
From the Department of Biochemistry, The University of Tennessee, Knoxville, Tennessee 37916

SUMMARY

The enzyme cystathionase from rat liver is inactivated by incubation with 5,5'-dithiobis(2-nitrobenzoic acid) at pH 7.4. The reaction of four -SH groups per mole of enzyme brings about 95% loss of the homoserine deaminase activity. Kinetically, the reactive -SH groups can be classified into two classes. The substrate L-homoserine or the competitive inhibitor L-alanine has no effect on the rate of inactivation.

The thiol groups of the enzyme undergo an exchange reaction with the dye bis(5-dimethylaminonaphthalene sulfonyl)-L-cystine (bis(dansyl)cystine). The reaction of approximately two -SH groups with the dansylated reagent causes 40% loss of the homoserine deaminase activity. The absorption and fluorescence properties of the dansylated enzyme were used to gain information about the microenvironment surrounding the reactive -SH groups. The fluorescence properties—emission maximum (515 nm), relative fluorescence yield (20), and fluorescence decay time (18 ns)—of the dye bound to the enzyme are identical to the fluorescence properties of bis(dansyl)cystine in a nonpolar mixture containing dioxane:water (95:5). On the basis of these results, it is proposed that the reactive -SH groups which react with bis(dansyl)cystine are located in a nonpolar environment. The inactivation of the enzyme by bis(dansyl)cystine cannot be related to dissociation of the cofactor pyridoxal-5-P from the catalytic site.

In our laboratory we have been investigating the functional role played by the -SH groups during enzyme activation. The present paper is concerned with the reaction of the -SH groups with the reagents DTNB and bis(dansyl)cystine. It is shown that the reaction of four -SH groups with DTNB brings about complete inactivation of the enzyme, while the reaction of two -SH groups with the fluorescent reagent bis(dansyl)cystine causes 40% loss of the homoserine deaminase activity. The absorption and fluorescence properties of the dansyl moiety covalently bound to the enzyme were used to gain information about the microenvironment surrounding the reactive -SH groups. On the basis of absorption and fluorescence results, it is proposed that the loss of catalytic activity cannot be correlated with the dissociation of the cofactor. It is suggested that the reactive -SH groups do not participate in the binding of pyridoxal-5-P to the catalytic site of the enzyme.

EXPERIMENTAL PROCEDURE

Methods

Fluorescence and Absorption Spectroscopy—Fluorescence measurements were performed in a fluorometer built in our laboratory. Calibration of the exciting source (Xenon lamp, 150 watt) and detector system (EM1 62658 photomultiplier) was carried out as described in a previous publication (3). Absorption spectra were recorded in a Cary model 10 spectrophotometer. Fluorescence lifetime measurements were made using the monophoton technique on an ORTEC 9200 nanosecond fluorometer. Time-base calibration of the multichannel analyzer was performed both directly, using a standard delay line, and indirectly, using a 1 X 10^{-4} M solution of quinine sulfate in 0.01 n NH_{4}SO_{4}, whose lifetime is 19.5 ns.

Purification of Cystathionase—The enzyme cystathionase from rat liver was purified by the procedure of Matsuo and Greenberg (4). The last step of the purification of the enzyme included stepwise elution from a hydroxyapatite column with phosphate buffer (pH 7.5) as described by Mushahwar and Koeppe (5). The pyridoxal-5-P content of cystathionase was 1 mole of pyridoxal-5-P per 45,000 g of enzyme as determined by the method of Wada and Snell (6). Enzymatic activity was assayed by measuring α-ketobutyric acid production with L-homoserine as substrate (7). Protein concentration was determined by the method of Lowry et al. (8).

Reduction and Carboxymethylation—Reduced and S-carboxymethylated protein was prepared following the procedure of Crestfield et al. (9) with some modifications. The protein was dissolved in 0.1 M Tris-HCl at pH 8.6 containing 10 M urea and 0.1% EDTA, and the solution was thoroughly deoxygenated with nitrogen. Reducing conditions were obtained by adding 100-fold molar excess of 2-mercaptoethanol. After 4 hours at 25°C, a neutral solution of iodoacetate was added under nitrogen. The amount of iodoacetate added was a 1.2-fold excess.

* This work was supported by Grant GB 33305 from the National Science Foundation.

1 The abbreviations used are: DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); NMB, 2-nitro-5-mercaptobenzoate; bis(dansyl)cystine, bis(5-dimethylaminonaphthalene sulfonyl)-L-cystine.

(Received for publication, January 18, 1974)

From the Department of Biochemistry, The University of Tennessee, Knoxville, Tennessee 37916
molar excess over the reducing agent. The carboxymethylation reaction was allowed to proceed in the dark at room temperature for 15 min. The reduced carboxymethylated protein was run through Sephadex G-25 (2.5 X 40 cm) equilibrated with 0.2 N acetic acid. S-Carboxymethylation of cystathionase in 10 mM urea was carried out under similar experimental conditions, except that the reduction with 2-mercaptoethanol was omitted.

**Ammonium Acrylate Analysis—**Samples of carboxymethylated and reduced carboxymethylated cystathionase in 0.2 N acetic acid were lyophilized and then dissolved in 6 M HCl. The hydrolysis tubes were flushed with nitrogen, evacuated, and maintained at 110° for 24, 48, and 72 hours. The analyses were performed on a Beckman model 116 automatic amino acid analyzer using resin types PA-35 and UR-30.

**Titration of Thiol Groups—**The number of reactive —SH groups were determined by reaction with DTNB using the procedure of Ellman (10). The experiments with native cystathionase were carried out at 25° in 0.1 M phosphate buffer (pH 7.4). Titration of the thiol groups of the denatured enzyme were conducted in 6 M guanidine HCl at 25°. The molar extinction coefficient of 13,600 at 412 nm for the NMB anion was employed for all calculations. The reaction of the thiol groups of cystathionase with the fluorescent reagent bis(dansyl)cysteine was conducted in 0.1 M phosphate buffer (pH 7.4). The molar ratio of bis(dansyl)cysteine to protein was 100. The mixture was incubated at 4° in the dark for 24 hours, dialyzed for 3 hours at 4° against several changes of 0.1 M phosphate buffer (pH 7.4) and finally passed through a small column of Sephadex G-25 which had been equilibrated with the same buffer.

The latter procedure removed all traces of free dye from the conjugate. The degree of labeling was determined from the absorption spectrum using a molar absorption for the bis(dansyl)cysteine moiety of 4300 to 330 nm.

**Materials**

Frozen, perfused rat livers were purchased from Pel-Freez Biologicals, Inc., Rogers, Ark. Protamine sulfate (salmine) was obtained from Calbiochem. 5,5'-Dithiobis(2-nitrobenzoic acid) was purchased from the Aldrich Chemical Co. Ultrapure guanidine HCl (16,000 g) was purchased from Schwarz-Mann. Iodoacetate acid for carboxymethylation, obtained from Sigma, was twice recrystallized from petroleum ether. Pyridoxal-5-P, L-homoserine, Trizma base, and EDTA were obtained from Sigma.

The amino acid calibration mixture was obtained from the Aldrich Chemical Co. Ultrapure guanidine HCl and urea were purchased from Schwarz-Mann. Iodoacetate acid for carboxymethylation, obtained from Sigma, was twice recrystallized from petroleum ether. Pyridoxal-5-P, L-homoserine, Trizma base, and EDTA were obtained from Sigma.

**RESULTS**

**Determination of S-Carboxymethylcysteine—**The amino acid analysis of the enzyme cystathionase has been published by Loiselet and Chatagner (11). Except for the cysteine and cystine content of the enzyme, there is no significant difference between our results and those reported by the above mentioned authors.

Analysis of the cystine content of carboxymethylated and reduced carboxymethylated cystathionase after acid hydrolysis for 24, 48, and 72 hours are presented in Table I. The samples of enzyme carboxymethylated in the presence of 10 mM urea yields a S-carboxymethylcysteine content of 17 groups per mole of enzyme (160,000 g).

This value derived from amino acid analysis does not differ significantly from the value obtained by spectrophotometric titration with DTNB in the presence of 6 M guanidine HCl (10 —SH cysteine residues per 160,000 g). However, the sample of cystathionase which was reduced and carboxymethylated in 10 mM urea exhibits 25 S-carboxymethylcysteine residues per mole of enzyme. Since this value was obtained under reducing conditions, it seems reasonable to suggest that the 8 additional S-carboxymethylcysteine residues are derived from the splitting of four S-S bonds.

**Reaction with DTNB—**The enzyme cystathionase from rat liver is inactivated by incubation with DTNB at 25°, whereas the enzyme is stable under these conditions in the absence of reagent. To determine the number of —SH groups critically connected with enzyme activity, the enzyme was titrated with increasing concentrations of DTNB. The extent of the reaction was determined by measuring the increase in absorbance at 412 nm after addition of a few moles eq of DTNB per mole of enzyme (molecular weight, 160,000).

The time course of the reaction of the enzyme with 4 moles eq of DTNB is shown in Fig. 1, where it may be seen that the addition of either 4 or 6 moles eq of DTNB causes the release of less than 4 moles of NMB after an incubation time of 60 min. at 25°. Under this set of experimental conditions—temperature, 25°; pH 7.4; incubation time, 60 min—the enzyme loses 96% of the homoserine deaminase activity.

Experiments designed to correlate changes in enzymatic activity with the number of —SH groups titrated, revealed that the enzyme lost 95% of the original homoserine deaminase activity after the reaction of approximately four —SH groups per mole of enzyme (Fig. 2). This amount of reactive —SH groups represents a fraction (25%) of the total —SH content of the protein as determined by amino acid analysis and by spectrophotometric titrations with DTNB in the presence of 6 M guanidine HCl (Table I). Thus, the results of the titration experiments indicate that the inhibitory action of DTNB is attributable to the reaction of this ligand with approximately four —SH groups.

**TABLE I**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Hydrolysis time</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>24 hrs</td>
</tr>
<tr>
<td>S-Carboxymethylcysteine content</td>
<td></td>
</tr>
<tr>
<td>cystathionase</td>
<td></td>
</tr>
<tr>
<td></td>
<td>18</td>
</tr>
<tr>
<td>Reduced S-carboxymethylated cysta-</td>
<td>25.0</td>
</tr>
<tr>
<td>thionase</td>
<td></td>
</tr>
</tbody>
</table>

**Fig. 1.** Time course of reaction of cystathionase (5 X 10⁻⁴ M) in 0.1 M phosphate buffer (pH 7.4) with 4 (●), 5 (△), and 6 (□) mole eq of DTNB at 25°. The three samples of enzyme lost 95% of the homoserine deaminase activity after 60 min incubation with DTNB.
FIG. 2. Titration of -SH groups of cystathionase (3 X 10^{-4} M) with DTNB in 0.1 M phosphate buffer (pH 7.4) at 25°. The reaction was followed by measuring the increase in absorbance at 412 nm for 1 hour at 25°. After 1-hour incubation, aliquots withdrawn from the incubation mixture were tested for homoserine deaminase activity.

The kinetics of the reaction of the -SH groups of cystathionase with DTNB was also examined at increasing molar ratios of ligand to enzyme at 25°. When the reaction was followed in the presence of a large excess of DTNB under pseudo-first order conditions, it was found that the profile of the reactivity curve is biphasic, exhibiting an initial rapid increase in absorbance followed by a slower increase in the absorbance at 412 nm. Fig. 3 illustrates this pattern. The pseudo-first order rate constant for the first reaction is calculated to be $K_1 = 8.0 \text{ min}^{-1}$, whereas that for the second reaction is $K_2 = 1.04 \text{ min}^{-1}$. These data can be best described by the model that assumes the reaction of DTNB with two classes of -SH groups critically connected with enzymatic activity: 1.8 -SH groups which react with a pseudo-first order rate constant of 8 min⁻¹, and 2.2 -SH groups, which exhibit a pseudo-first order rate constant of 1.04 min⁻¹.

The reactivity of the two classes of -SH groups is not altered by the addition of either the substrate L-homoserine or the competitive inhibitor L-alanine to the enzyme prior to the addition of DTNB. When the substrate L-homoserine is included in the reaction mixture, the reactivity curve still follows a biphasic pattern (Fig. 3).

The same type of biphasic kinetics is observed when the enzyme preincubated with L-alanine is allowed to react with DTNB (Fig. 3). In view of these results, it is evident that the substrate does not modify the reactivity of the two classes of -SH groups critically connected with the homoserine deaminase activity of the enzyme.

Reaction with Bis(dansyl)cystine—The thiol groups of the enzyme cystathionase also undergo a disulfide exchange reaction with the dye bis(dansyl)cystine; the degree of labeling of the protein after the exchange reaction can be easily determined by spectrophotometric methods.

The labeling procedure outlined in "Experimental Procedure" yielded a bis(dansyl)cystine-enzyme complex containing approximately 2 or less moles of dansyl fluorophores per mole of enzyme. Under the set of experimental conditions chosen, namely reaction with the dye for 24 hours at 4° in a phosphate buffer of pH 7.4, the labeled enzyme, which lost 40% of the original homoserine deaminase activity, exhibits two absorption bands covering the spectral range from 310 to 500 nm (Fig. 4). The absorption band centered at around 330 nm is due to the dansyl fluorophore, while the absorption band with maxima at around 410 nm is due to the cofactor pyridoxal-5-P. From these results, it should be noted that the intensity of the absorption band of the cofactor in the modified enzyme, which lost 40% of the enzymatic activity, is comparable in magnitude to the intensity of the absorption band of the cofactor in the native enzyme. This observation is interpreted to mean that the loss of homoserine deaminase activity caused by reaction of thiol groups with bis(dansyl)cystine cannot be ascribed to the dissociation of the cofactor from the catalytic site of the enzyme.

FIG. 3. Graphical analysis of reaction of cystathionase (6 X 10^{-4} M) with 100-fold molar excess of DTNB in the presence of L-homoserine (10^{-4} M) (○), in the presence of L-alanine (10^{-4} M) (△), and in the absence of substrate (●). The pseudo-first order rate constants are $K_1 = 8 \text{ min}^{-1}$ and $K_2 = 1 \text{ min}^{-1}$ for the fast and slow steps of the biphasic reaction. The samples incubated in the presence or absence of L-homoserine lost 95% of the homoserine deaminase activity after 3-min incubation with DTNB at 25°. The reaction of bis(dansyl)cystine-cystathionase (2 X 10^{-4} M) with 100-fold molar excess of DTNB (■) at pH 7.4 is also shown in the figure.

FIG. 4. Absorption spectra of samples of cystathionase (1) and bis(dansyl)cystine-cystathionase (2) after chromatography through Sephadex G-25. The degree of labeling of the modified enzyme is 1.6 bis(dansyl)cystine residues per mole of enzyme. This sample lost 40% of the homoserine deaminase activity when compared to the native enzyme.
Attempts to increase the degree of labeling of the enzyme at pH 7.4 by either increasing the concentration of bis(dansyl)-cystine or by prolonging the incubation time of the reaction mixture from 24 to 36 hours at 4°C were unsuccessful. However, incorporation of more than 2 moles of bis(dansyl)-cystine per mole of enzyme was achieved at alkaline pH (pH 8.6), but at this pH the native enzyme is less stable. The fluorescence properties of the dansylated enzyme were examined at neutral pH and compared to the properties of free bis(dansyl)-cystine in solvents of varying dielectric constants. As shown in Fig. 5, the labeled enzyme exhibits an emission band centered at 515 nm, which is shifted by 20 nm when compared to free bis(dansyl)-cystine in the same buffer. The quantum yield of the bound fluorophore is 20 times larger than the corresponding value of bis(dansyl)-cystine in the same buffer, but it is identical to the fluorescence yield of bis(dansyl)-cystine in the solvent mixture containing 95% dioxane-5% water (Table II).

The fluorescence properties of dansyl-cystine and dansyl-cysteine are quite similar to several dansyl-amino acids in the sense that they all show a blue shift in their emission spectra and an appreciable increase in fluorescence quantum yield on going from water to less polar solvents, such as ethanol and dioxane (12, 13).

Upon attachment to the enzyme, the fluorescence of dansyl-cystine decays in an exponential manner with a fluorescence lifetime of 18 ns (Fig. 6). This fluorescence decay time value is considerably larger than the decay value of bis(dansyl)-cystine in water (τ = 3.5 ns) but identical to the fluorescence lifetime of the dye in the mixture dioxane-water (95:5) (Fig. 6).

All these fluorometric results lend strong support to the hypothesis that the microenvironment surrounding the dansyl groups in the protein is less polar than water. Therefore, it is possible to postulate that the two —SH groups that undergo an exchange reaction with bis(dansyl)-cystine are located in a nonpolar environment.

Although it is difficult to ascertain whether the thiol groups which reacted with bis(dansyl)-cystine are the same that undergo a rapid exchange reaction with DTNB, it is worth noting that the dansylated enzyme is completely inactivated by the subsequent incorporation of 2 moles of DTNB per mole of enzyme. Furthermore, the reaction of the dansylated enzyme with DTNB does not follow a biphasic kinetic pattern as indicated in Fig. 3.

**DISCUSSION**

The experiments presented have shown that the enzyme cystathionase contains 17 —SH groups and 4 disulfide bridges per mole of enzyme. Titration of four —SH groups with DTNB causes 95% inactivation of the homoserine deaminase activity; while the presence of either the substrate L-homoserine or the inhibitor L-alanine has no effect on the rate of the reaction between the thiol groups of the enzyme and the ligand DTNB.

Loiselet and Chatagner (11) have reported that the enzyme subjected to performic acid oxidation followed by acid hydrolysis contains approximately 80 residues of cysteic acid per mole of enzyme. Subsequently, Démé et al. (2) proposed that 60 of the cysteic acid residues result from the oxidation of 30 S—S bonds. Although there is no significant difference between our determination of —SH content and the value reported by Démé et al. (2), it is evident that our value for the disulfide content is considerably smaller than the value of 30 S—S residues per mole of enzyme as reported by Loiselet and Chatagner (11).

The reactive —SH groups of cystathionase also undergo an exchange reaction with the fluorescent dye bis(dansyl)-cystine.

**FIG. 5.** Emission spectra of bis(dansyl)-cystine-cystathionase (1) and bis(dansyl)-cystine (2) in 0.1 M phosphate buffer (pH 7.4) at a sensitivity of 1 and 20, respectively. Both samples have the same absorbance (0.1) at the exciting wavelength of 330 nm.

**TABLE II**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Fluorescence maximum</th>
<th>Relative fluorescence yield</th>
<th>Fluorescence lifetime</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bis(dansyl)-cystine in water (pH 7.4)</td>
<td>535</td>
<td>1</td>
<td>3.5</td>
</tr>
<tr>
<td>Bis(dansyl)-cystine in dioxane-water (95:5, v:v)</td>
<td>515</td>
<td>20</td>
<td>18</td>
</tr>
<tr>
<td>Bis(dansyl)-cystine-cystathionase (pH 7.4)</td>
<td>515</td>
<td>20</td>
<td>18</td>
</tr>
<tr>
<td>Bis(dansyl)-cystine-epocystathionase (pH 7.4)</td>
<td>515</td>
<td>20</td>
<td>18</td>
</tr>
</tbody>
</table>

**FIG. 6.** Fluorescence decay times of bis(dansyl)-cystine-cystathionase (1) and bis(dansyl)-cystine in the solvent mixture; dioxane-water (95:5) (O); excitation at 330 nm. The emission band was isolated using a C.S.3.71 Corning glass filter.

In analogy to the reaction with DTNB, the reaction of less than two —SH groups with bis(dansyl)-cystine brings about 40% decrease in the homoserine deaminase activity.

Since the dansyl fluorophores exhibit fluorescence properties which are influenced by the polarity of the medium (13), it was thought to be of interest to correlate these fluorescence properties with the microenvironment surrounding the dansyl fluorophores which are covalently bound to the thiol groups.

An analysis of the fluorescence properties of the dansylated
enzyme, fluorescence lifetime, emission spectra, and fluorescence yield reveals that all these properties are identical with those exhibited by the dye bis(dansyl)cystine in the nonpolar mixture, dioxane-water (95:5, v/v). Judging from these results, it seems reasonable to propose that the reactive —SH groups of cystathionase critically connected with catalytic activity are surrounded by an environment of very low polarity.

There are two possible ways of explaining the effect of bis(dansyl)cystine on the catalytic function of cystathionase. The first one is that the reaction of the thiol groups with bis(dansyl)cystine results in the dissociation of the cofactor pyridoxal-5-P from the catalytic site of the enzyme. The second is that the reaction of the thiol groups triggers a conformational change which affects the catalytic site. As result of this conformational change, the protein cannot facilitate the transfer reaction between substrate and coenzyme by bringing them into a suitable spatial juxtaposition.

The first possibility was investigated by examining the absorption spectra of the cofactor in the native and dansylated enzymes. After loss of 40% of the homoserine deaminase activity, there is very little effect on the intensity of the absorption band due to pyridoxal-5-P bound to the catalytic site (Fig. 4). Furthermore, the addition of pyridoxal-5-P to the dansylated enzyme failed to restore the enzymatic activity. These two independent lines of experimental evidence lend support to the contention that the loss of homoserine deaminase activity cannot be ascribed to dissociation of the cofactor from the catalytic site. The second possibility, namely, the binding of bis(dansyl)cystine to a nonpolar area of the protein causes conformational changes which influence the catalytic function, seems more plausible.

The fluorescence properties of the dansyl groups covalently bound to the enzyme can be used to deduce proximity relationships between the thiol groups which reacted with bis(dansyl)cystine and the cofactor pyridoxal-5-P. A comparison of the absorption and fluorescence spectra (Figs. 4 and 5) reveals that the absorption spectrum of the cofactor pyridoxal-5-P overlaps the emission band of the dansyl moiety bound to the thiol group. If the cofactor and the dansyl moiety are in close contact, then one should expect to observe a dramatic quenching effect on the fluorescence emitted by the dansyl moiety due to either short or long range energy transfer mechanisms (13, 14). This effect is not observed, since the fluorescence emitted by the dansyl moiety as well as its fluorescence decay time remain essentially unaffected after resolution of the enzyme as indicated in Table II. Thus it appears that the inactivation of the enzyme cystathionase by the fluorescent dye bis(dansyl)cystine cannot be attributed to the reaction of thiol groups which are near the cofactor binding site.

Acknowledgment—We thank Professor Roger E. Koppel, Oklahoma State University, for communicating his method of purification of cystathionase prior to publication of his paper with Isa K. Mushahwar.

REFERENCES
10. Ellman, G. L. (1959) Arch. Biochem. Biophys. 82, 70
Reaction of Cystathionase with the Fluorescent Probe Bis(dansyl)cystine
Kyung-Ja Oh and Jorge E. Churchich


Access the most updated version of this article at http://www.jbc.org/content/249/15/4737

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/249/15/4737.full.html#ref-list-1