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STUDIES ON SULFHYDRYL GROUPS OF *ASPERGILLUS NIGER* AMINE OXIDASE

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Summary

Amino acid analysis of the amine oxidase of *Aspergillus niger* (monoamine : O_2 oxidoreductase (deaminating), EC 1.4.3.4) showed a composition similar to that of bovine plasma enzyme. One molecule of enzyme contained 25 Cys residues. It was shown that 9 to 11 residues of Cys were titrated to be SH groups.

The amine oxidase reaction was markedly inhibited by metal ions (Cu^{2+}, Hg^{2+}, Ag^{+}). The enzyme was inactivated with SH reagents (phenyl mercuric acetate, Cl-HgBzO$^-$) and the extent of this inactivation was dependent on the time of incubation with SH reagents. Also, the Cl-HgBzO$^-$-inactivated enzyme was reactivated with cysteine and this reactivation was biphasic with the time of incubation.

The Cl-HgBzO$^-$-inactivated amine oxidase was compared with the native enzyme in their reactivity with phenylhydrazine and their spectral properties. The results showed that the Cl-HgBzO$^-$-inactivated enzyme had lower reactivity with phenylhydrazine than the native enzyme and had higher absorbance values than the native enzyme around 400 nm wavelengths.

Introduction

It was reported that the amine oxidase of *Aspergillus niger* (monoamine : O_2 oxidoreductase (deaminating), EC 1.4.3.4) was dissociated into 3

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Abbreviations: Cl-HgBzO$^-$, *p*-chloromercuribenzoate; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid).

subunits in the presence of 6 M guanidine hydrochloride containing 0.1 M mercaptoethanol [1]. It was suggested that these subunits were covalently bonded with S-S bridges [1]. On the other hand, the bovine plasma amine oxidase was shown to contain 2 SH groups per molecule of enzyme, but these groups were not essential for the activity of enzyme [2]. It will be intersting to see whether or not the *Aspergillus* amine oxidase contains SH groups.

This paper deals with the SH content in *Aspergillus* amine oxidase, the effect of SH reagents on the amine oxidase reaction, and the results of amino acid and carbohydrate analyses. The Cl-HgBzO$^-$-inactivated enzyme was compared with the native one in their reactivities with phenylhydrazine and in their spectral properties.

Materials and Methods

The amine oxidase of *Aspergillus niger* was prepared by the method described previously [1]. The concentration of the enzyme was estimated spectrophotometrically by using the molar extinction coefficient for protein of $2.98 \cdot 10^5\ M^{-1} \cdot cm^{-1}$ at 280 nm [3]. This value was obtained by using $E_{1\%} = 11.8$ [4] and a molecular weight of 252 000 [5]. Cl-HgBzO$^-$ was purified by the method of Boyer and its concentration was determined spectrophotometrically [6]. Other reagents used were of the highest grade of purity and were used without further purification.

Assay of amine oxidase

The activity of amine oxidase was estimated by measuring the oxygen uptake using a Clark oxygen electrode with *n*-butylamine as substrate [3]. The activity of amine oxidase was also measured by the absorbance change at 250 nm with benzylamine as substrate [7]. All the assays were performed in 0.06 M phosphate buffer, pH 7.5 at 25° C. When Pb^{2+}, Ca^{2+} and Ag^+ were used as inhibitor, the buffer was 0.06 M pyrophosphate/sulfuric acid, pH 7.5.

Amino acid analysis and carbohydrate content

The crystalline amine oxidase (50 mg) was dialyzed two times for 24 h against 1 liter of 0.01 M phosphate buffer, pH 7.5. The enzyme solution thus obtained was further dialyzed three times for 12 h against the deionized water. It was then lyophilized and placed over silica gel in the desiccator under the reduced pressure. The lyophilized enzyme was used for amino acid and carbohydrate analyses. A part of the lyophilized enzyme was hydrolyzed with 6 M HCl at 110° C for 32 h, then the amino acid contents were determined by using a Beckman amino acid analyzer. The tryptophan content was determined spectrophotometrically [8]. Cysteine and/or cystine was determined as cysteic acid after performic acid oxidation [9]. The carbohydrate content was determined by the phenol/sulfuric acid method using D-mannose as standard [10].

Determination of SH content of amine oxidase

The SH groups of the enzyme were quantitated by DTNB [11]. A given concentration of amine oxidase was incubated with a sufficient amount of DTNB in 0.08 M Tris buffer, pH 8.0 containing 0.5% sodium dodecyl sulphate

for a given time. The reduction in the concentration of DTNB was followed by the absorbance increment at 412 nm. The SH equivalent was calculated from the maximum absorbance by using an extinction coefficient for 3-carboxy-4-nitrothiophenol anion of $1.36 \cdot 10^4$ $M^{-1} \cdot cm^{-1}$ [11].

Spectrometry

Spectrophotometric measurements were done with a Shimadzu spectrophotometer, model MPS-50 at room temperature. Absorbance at the fixed wavelength was measured with Hitachi-Perkin-Elmer spectrophotometer model 139.

Results and Discussion

Carbohydrate content and amino acid composition

Carbohydrate content of *Aspergillus* amine oxidase was determined by the phenol/sulfuric acid method using D-mannose as standard [10]. The lyophilized enzyme of 0.88 mg and 1.63 mg was found to contain 4.12 μg and 7.58 μg carbohydrate, respectively. These values correspond to 0.47% of carbohydrate content in the molecule of enzyme. The content of carbohydrate in the bovine plasma amine oxidase was reported to be 4.6% [12]. This is ten times as much carbohydrate as the *Aspergillus* enzyme.

Table I shows the amino acid composition of the *Aspergillus niger* amine oxidase estimated on a molecular weight of 252 000 [5]. Similarly, in amine oxidase of bovine plasma [12], high contents of Asx, Glx and Val, and low contents of Cys, Met and Trp were observed. It will be worth mentioning the

TABLE I

AMINO ACID COMPOSITION OF *ASPERGILLUS* AMINE OXIDASE

Amino acid	Number of amino acid residues per 252 000 g of protein
Asx	255
Thr	94
Ser	102
Glx	219
Pro	152
Gly	141
Ala	127
Val	171
Met	31
Ile	99
Leu	136
Tyr	55
Phe	95
Lys	89
His	63
Arg	127
Ammonia	(225)
Trp	24
Total half cystine	25
Total	2005

low yields of residue weights. This may be due to the presence of carbohydrate, 3 gatoms of copper [13] and 2 molecules of pyridoxal 5′-phosphate in the enzyme [3]. As this enzyme was reported to be dissociated into 3 subunits [1] and the relation among these subunits is not clear, the amino acid composition in Table I should be treated as tentative.

Effect of metal ions on the activity of amine oxidase

A 0.1 ml aliquot of a solution of amine oxidase was added to the buffer (2.5 ml, 0.06 M phosphate at pH 7.5) containing a given concentration of metal ions and 1 mM *n*-butylamine. Then the oxygen uptake was measured to estimate the activity of enzyme. As Table II shows, Cu^{2+}, Hg^{2+} and Ag^{+} were found to be strong inhibitors among the metal ions studied.

Effect of SH reagents on the activity of amine oxidase

To see if the inhibition by metal ions was caused by a blocking of SH groups of enzyme, the effect of SH reagents on the activity of the enzyme was studied. A solution of amine oxidase was incubated in the medium containing a given concentration of SH reagents. Then, the activity of enzyme was estimated by measuring oxygen uptake. As Table III shows, the enzyme was inactivated by the low concentrations of phenyl mercuric acetate and Cl-$HgBzO^{-}$ among the reagents studied. However, other reagents such as monoiodoacetic acid, *N*-ethylmaleimide, mercaptoethanol and arsenite did not inactivate the enzyme. These results were further confirmed by the incubation experiments.

A solution of enzyme was incubated with a given concentration of SH reagents. Then, a part of the incubation mixture was taken at a given time to estimate its enzyme activity. As Fig. 1 shows, the activity of enzyme was rapidly lost with phenyl mercuric acetate and Cl-$HgBzO^{-}$ in the 10 min incubation. However, monoiodoacetic acid, *N*-ethylmaleimide, arsenite and mercaptoethanol had no effect on the enzyme activity at 10 min after incubation. Since this enzyme was inactivated by phenyl mercuric acetate and Cl-$HgBzO^{-}$, it is

TABLE II

EFFECTS OF METAL IONS ON THE AMINE OXIDASE REACTION

Activity was measured in the medium containing a given concentration of metal ions and expressed as the percentage to the activity without metal ions. The concentration of enzyme was 0.33 μM and the buffer was 0.06 M phosphate or 0.06 M pyrophophate/sulfuric acid(*), pH 7.5. Temperature was 25° C.

Metal ions	Concn. (M)	Activity (% of control)
Fe^{3+}	10^{-3}	84
Ni^{2+}	10^{-3}	100
Zn^{2+}	10^{-3}	100
Pb^{2+}*	10^{-3}	70
Ca^{2+}*	$1.5 \cdot 10^{-3}$	53
Cu^{2+}	10^{-5}	79
	$6.7 \cdot 10^{-4}$	27
Hg^{2+}	10^{-3}	0
Mg^{2+}	$1.33 \cdot 10^{-3}$	75
Ag^{+}*	$3.3 \cdot 10^{-4}$	0

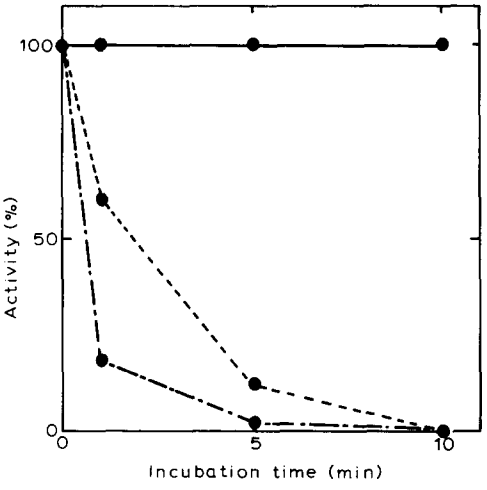


Fig. 1. The effect of time on the incubation of amine oxidase with SH reagents. Aliquots of 0.5 ml enzyme (10 μM) in 0.06 M phosphate buffer, pH 7.0, were mixed with 0.5 ml SH reagents. The mixture was incubated at 25°C for a given time, then aliquots of 0.2 ml incubation mixture were taken and added to 2.8 ml assay medium. The enzyme activity was estimated by measuring oxygen uptake. The concentrations of SH reagents in the incubation mixture were 1 mM for *N*-ethylmaleimide, monoiodeacetic acid, and arsenite (●———●), 84 μM for Cl-HgBzO$^-$ (●- - - - -●) and 80 μM for phenyl mercuric acetate (●— · — · ●). The control experiment (●———●) was carried out without SH reagents.

suggested that the inhibition by Cu^{2+}, Hg^{2+} and Ag^+ was caused by blocking SH groups of enzyme.

It was reported that the reaction of the bovine plasma amine oxidase was inhibited at the high concentration of Cl-HgBzO$^-$ [2]. The inhibition was postulated to be due to π-bonding between the benzene ring of Cl-HgBzO$^-$ and aromatic residues in the enzyme [2]. This possibility seems to be unlikely for the *Aspergillus* enzyme, since the enzyme was not inactivated by benzoic acid (Table III). Rather, the inactivation by phenyl mercuric acetate and Cl-HgBzO$^-$ was caused by blocking some of the SH groups of enzyme.

TABLE III

EFFECTS OF SH REAGENTS ON THE ACTIVITY OF AMINE OXIDASE

A 0.2 ml aliquot of SH reagents was mixed with 0.2 ml of 10 μM amine oxidase in 0.06 M phosphate buffer, pH 7.0. The mixture was incubated for 30 min at 25°C. Then, a 0.2 ml aliquot of incubation mixture was taken and added to 2.8 ml assay medium to estimate the residual activity of enzyme by measuring the oxygen uptake. The concentrations of SH reagents in the incubation mixture are shown. In the assay medium, the buffer was 0.06 M pyrophosphate/hydrochloric acid, pH 7.5 and 25°C. The concentration of *n*-butylamine was 1 mM.

Reagents	Concn. (M)	Activity (% of control)
Arsenite	10^{-3}	100
Mercaptoethanol	$4.4 \cdot 10^{-3}$	100
N-Ethylmaleimide	10^{-3}	89
Monoiodeacetic acid	10^{-3}	89
Phenyl mercuric acetate	$8.0 \cdot 10^{-5}$	0
Cl-HgBzO$^-$	$8.4 \cdot 10^{-5}$	0
Benzoic acid	$2.5 \cdot 10^{-2}$	100

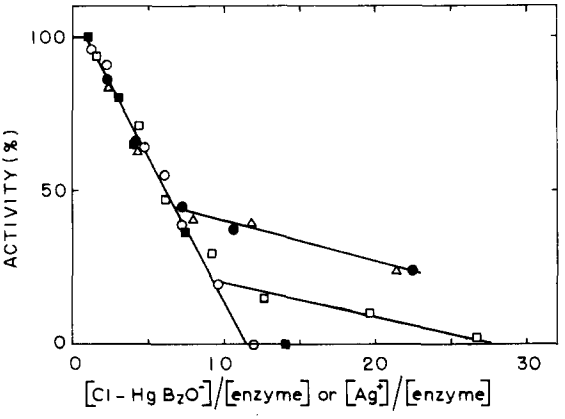


Fig. 2. Titration of amine oxidase with Ag^+ and Cl-HgBzO$^-$. Aliquots of 0.2 ml enzyme (8.94 μM) in 0.06 M phosphate buffer, pH 7.0, were mixed with given concentrations of 0.2 ml Ag^+ or Cl-HgBzO$^-$. After incubation for a given time, a 0.1 ml aliquot of incubation mixture was taken and added to 2.9 ml assay medium to estimate the enzyme activity. The changes in activity were plotted against the molar ratios of reagents to the enzyme. The activity of enzyme was measured at 30 (●), 60 (□) and 120 min (■) after the incubation with a given concentration of Cl-HgBzO$^-$ and at 30 min (○) after incubation with a given concentration of Ag^+. The enzyme (8.94 μM) containing 3.3 mM *n*-butylamine in 0.2 ml of 0.06 M phosphate buffer, pH 7.0, was mixed with 0.2 ml of a given concentration of Cl-HgBzO$^-$, and the mixture was incubated for 30 min. Then the residual activity was measured as described above (△).

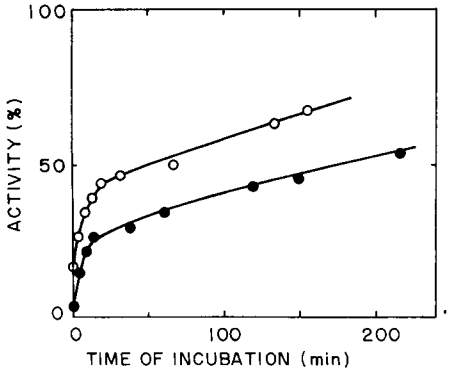
Titration of amine oxidase with Ag^+ and Cl-HgBzO$^-$

A solution of the amine oxidase was incubated with a given concentration of Ag^+ or Cl-HgBzO$^-$ at pH 7.0 and 25° C. After incubation for a given time, the residual activity was measured. As Fig. 2 shows, the activity of amine oxidase was linearly decreased with an increasing concentration of Ag^+ and completely lost with 11 g ions of Ag^+ per mol of enzyme. When the enzyme was titrated with Cl-HgBzO$^-$, the activity was decreased on the same line. However, the activity was not completely lost with 11 mol of Cl-HgBzO$^-$ per mol of enzyme after 30 min incubation, but completely lost after 2 h incubation (Fig. 2). The results in Fig. 2 suggest that at least 11 mol of SH groups are present in one molecule of enzyme.

It was shown that the prosthetic groups of the enzyme are pyridoxal 5′-phosphate form in the absence of substrate, amines, but the pyridoxamine 5′-phosphate form in the presence of substrate [3]. As Fig. 2 shows, the loss of the enzyme activity with Cl-HgBzO$^-$ was the same fashion in spite of the presence or the absence of substrate, *n*-butylamine. Therefore, this result clearly shows that the activity loss of enzyme with Cl-HgBzO$^-$ is independent of the state of the prosthetic groups of enzyme. This will mean that the SH groups of this enzyme are not involved in the main path of this enzyme reaction [3].

Determination of the total SH groups of amine oxidase

The total SH residues per mol of enzyme were determined by using DTNB. A solution of 2.7 μM amine oxidase (3 ml) was incubated for 30 min with 145 μM DTNB in 0.08 M Tris buffer, pH 8.0 containing 0.5% sodium dodecyl sulphate. The absorbance at 412 nm increased slowly and became almost constant after 10 min incubation. From this constant absorbance



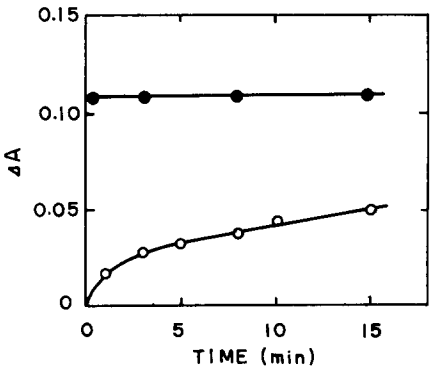


Fig. 3. Reactivation of Cl-HgBzO$^-$-inactivated enzyme with cysteine. Amine oxidase (1.62 μM) was incubated with 11.7 (○) or 23.2 (●) molar excess of Cl-HgBzO$^-$ for 1.5 h at pH 7.0 and 25°C. The volume of the incubation mixture was 2.4 ml. Then, 0.1 ml aliquots of cysteine were added to the incubation mixture to the final concentration of 4.0 mM. 0.2 ml aliquots of incubation mixture were taken at a given time after incubation with cysteine and assayed by using 2.5 mM benzylamine as substrate (see methods). The volume of the assay medium was 3 ml. The activity (percentage to the control) was plotted against the time of incubation of enzyme with cysteine. Without Cl-HgBzO$^-$ treatment, the enzyme activity was not changed during the experiments.

Fig. 4. Reactivity of Cl-HgBzO$^-$-inactivated amine oxidase with phenylhydrazine. The amine oxidase (1.62 μM) was incubated 34.5 molar excess of Cl-HgBzO$^-$ for 2 h at pH 7.0 and 25°C. Then, a solution of phenylhydrazine (0.1 ml) was added to the final concentration of 3.8 mM. The absorbance increments at 442.5 nm (○———○) were plotted against the time of incubation with phenylhydrazine. The control experiment (●———●) was performed by using the native enzyme.

(0.335), the concentration of SH groups was calculated to be 24.6 μM in the 2.7 μM amine oxidase (3 ml). This corresponds to 9.0 mol of SH groups per mol of enzyme.

Combining this with the data in Fig. 2, it will be concluded that the amine oxidase of *Aspergillus niger* contains 9 to 11 mol of SH groups. Since the content of Cys is 25 residues per molecule of enzyme (Table I), the enzyme can be estimated to contain 8 S-S bridges per molecule.

Reactivation of Cl-HgBzO$^-$-inactivated amine oxidase

A solution of 1.62 μM amine oxidase was incubated with a given concentration of Cl-HgBzO$^-$ in 0.06 M phosphate buffer (pH 7.0) at 25°C for 1.5 h, then a solution of cysteine was added at the final concentration of 4.0 mM. A part of the incubation mixture was taken to estimate the activity of enzyme at a given time. As Fig. 3 shows, about 25% of the initial activity was recovered in a few minutes, but the rest of the activity was recovered very slowly. This results indicate that the reactivation is composed of at least two steps. Therefore, it is probable that there are at least two states of SH groups in the enzyme molecule. Since the Cl-HgBzO$^-$-inactivated enzyme was reactivated with cysteine, it is reasonable to assume that the inactivation by Cl-HgBzO$^-$ was due to the mercaptidation of some of the SH groups of enzyme.

Reactivity of Cl-HgBzO$^-$-inactivated amine oxidase with phenylhydrazine

Aspergillus amine oxidase contains two pyridoxal 5'-phosphates per molecule of enzyme [3] and these groups are essential for the enzyme activity

[3,14,15]. Therefore, it will be interesting to see whether or not the reactivity of the Cl-HgBzO$^-$-inactivated enzyme with phenylhydrazine is different from the native one.

A solution of 1.62 μM amine oxidase (2.5 ml) was incubated with a 34.5 molar excess of Cl-HgBzO$^-$ in 0.06 M phosphate buffer, pH 7.0, for 2 h, then a solution of phenylhydrazine (0.1 ml) was added to a final concentration of 3.8 mM. Fig. 4 shows the time course of absorbance change at 442.5 nm [3]. The reaction of the native enzyme with phenylhydrazine was too rapid to detect, but that of the Cl-HgBzO$^-$-inactivated enzyme was very slow. This result suggests that the Cl-HgBzO$^-$ reacts with SH groups of enzyme and modifies the structure of enzyme to decrease the reactivity of phenylhydrazine with the prosthetic groups of the enzyme, pyridoxal 5'-phosphate.

Difference absorbance spectrum between the Cl-HgBzO$^-$-inactivated and the native amine oxidase

To obtain further information about the structural change of the enzyme by the Cl-HgBzO$^-$ treatment, the spectral property of the Cl-HgBzO$^-$-inactivated enzyme was compared with that of the native one. A solution of amine oxidase (2.5 ml) was incubated with a given concentration of Cl-HgBzO$^-$ (14 or 17.7 equivalents of the concentration of enzyme) in 0.06 M phosphate buffer, pH 7.0 at 24°C for 2 h. The spectrum of the Cl-HgBzO$^-$-inactivated enzyme was measured by Shimadzu spectrometer in the absorbance range 0 to 0.200. The spectrum of the native enzyme was also measured. Then the difference absorbance spectrum between the Cl-HgBzO$^-$-inactivated and the native enzymes was obtained by calculation. The difference spectrum was obtained at two different concentrations of enzyme. Fig. 5 shows the difference spectrum obtained. The

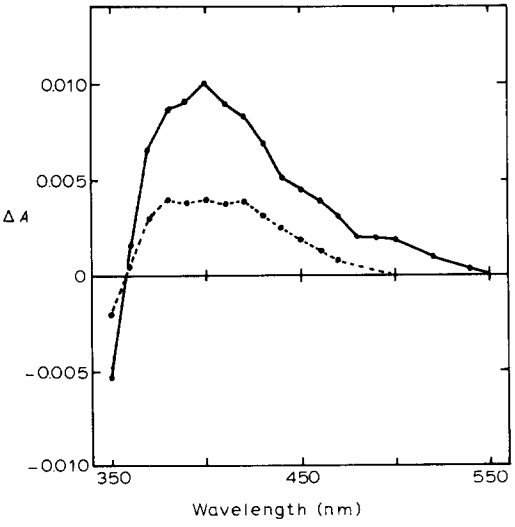


Fig. 5. Difference absorbance spectrum between the Cl-HgBzO$^-$-inactivated and the native amine oxidases. The amine oxidase was incubated with 14 (●———●) or 17.7 (●- - - - -●) molar excess of Cl-HgBzO$^-$ for 2 h at pH 7.0 and 25°C. Then, the absorbance spectrum of the Cl-HgBzO$^-$-inactivated enzyme and the native enzyme was measured. The absorbance difference between these enzymes was plotted against the wavelength. The concentration of the enzyme was 3.18 μM (●- - - - -●) and 6.5 μM (●———●). The absorbances at 400 nm were 0.050 for (●———●) and 0.020 for (●- - - - -●).

absorbance increment of the Cl-HgBzO$^-$-inactivated enzyme at 400 nm was calculated to be 20% of the absorbance of the native enzyme in each of the enzyme concentrations. There may be several possibilities to explain these large spectral changes shown in Fig. 5.

One possibility is that the mercaptide formed by Cl-HgBzO$^-$ caused the increment of absorbance around 400 nm wavelengths. Other possibilities are as follows. Though the mercaptide formed by the Cl-HgBzO$^-$ treatment of enzyme does not contribute to the spectral changes, the mercaptidation of SH groups caused alterations in Cu^{2+}-ligand chelation, alterations in pyridoxal 5′-phosphate binding, or alterations in the pyridoxal 5′-phosphate environment. The large spectral change observed might be due to some of these alterations, since the *Aspergillus* amine oxidase contains 2 molecules of pyridoxal 5′-phosphate [3] and 3 g atoms of copper [13] per one molecule of enzyme, and these prosthetic groups can be presumed to contribute absorbances around these wavelengths. However, further works will be required to elucidate which possibility contributes to the spectral changes observed and to find out the mechanism to explain the inactivation of enzyme with SH reagents.

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