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AFFINITY LABELING OF AN *ESCHERICHIA COLI* β-LACTAMASE

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SUMMARY

1. A β-lactamase from *Escherichia coli* was inactivated by a substrate derivative, benzylpenicillin isocyanate.

2. The inactivation process followed pseudo-first-order kinetics with K and k_3 values of $4 \cdot 10^{-5}$ M and $9 \cdot 10^{-2}$ min^{-1}, respectively.

3. The inactivation rate was reduced by the presence of benzylpenicillin or methicillin as would be expected for a specific reaction with a group in the active site of the enzyme.

4. Benzylpenicillin isocyanate was hydrolyzed by the enzyme with a Michaelis constant identical with the K value described above.

5. The inactivated enzyme was not reactivated by dialysis.

6. It is concluded that the inhibition by benzylpenicillin isocyanate was specific and irreversible through its binding to the catalytic site of the enzyme.

INTRODUCTION

A β-lactamase such as penicillinase (penicillin amido-β-lactamhydrolase, EC 3.5.2.6) has been known since Abraham and Chain[1] reported that an extract of *Escherichia coli* destroyed the antibacterial activity of benzylpenicillin. Thereafter, many such enzymes were isolated both from Gram-negative and Gram-positive bacteria[2]. The β-lactamases of Gram-negative bacteria were classified into a number of types according to their substrate specificity and to their sensitivity to *p*-chloromercuribenzoate and cloxacillin[3–5]. As reported in a previous paper[6], we isolated, from *E. coli*, a new type of β-lactamase whose synthesis was mediated by an R factor derived from a *Proteus* strain. The enzymes of Gram-positive bacteria were isolated from *Bacillus cereus* in the crystalline form by Pollock and co-workers[7,8] and Kuwabara[9]. The amino acid sequences of some β-lactamases from Gram-positive bacteria have been reported[10,11].

There have been extensive studies on β-lactamases from the point of view of enzyme induction, resistance to penicillins, and transfer of genetic elements. With respect to the active site of the enzyme, Dupue *et al.*[12] claimed that a histidine residue

is involved in the active site of *Staphylococcus aureus* 147 β-lactamase and Meadway[10] and Csányi *et al.*[13] reported that a tyrosine residue is necessary for the activity of the β-lactamases of *B. cereus* and *B. licheniformis*. Affinity labeling, initiated by Singer and his colleagues[14,15] in the study of the antibody combining site, is an important tool in exploring the structural features of the catalytic site of enzymes. Thus, derivatives of benzylpenicillin which is a substrate of all β-lactamases should be useful for affinity labeling and active site analysis. In this report, the inactivation of an *E. coli* β-lactamase, β-lactamase$_{75}$, by a substrate derivative, benzylpenicillin isocyanate, is described.

MATERIALS AND METHODS

Syntheses of reagents

Benzylpenicillin isocyanate. A suspension of 435 mg (1.0 mmole) of benzylpenicillin triethylammonium salt in 3 ml of methylene chloride was cooled to −10 °C and 100 μl (1.0 mmole) of ethylchloroformate was added in one portion. The resulting mixture was stirred at −10 °C for 1.5 h, and the solution became clear. Then, a solution of 65 mg (1.0 mmole) of NaN_3 in 1 ml of water was added in 50-μl portions during a period of 30 min. The stirring was continued for a further 10 min at −5 to −10 °C. The reaction mixture was diluted with 2.5 ml of ice-cold water, and extracted with three portions of 2.5 ml of ice-cold methylene chloride. The extracts were combined, washed with 2.5 ml of ice-cold 1% $NaHCO_3$ and with three portions of 2.5 ml of ice-cold water, dried over $MgSO_4$, and evaporated to dryness at room temperature under reduced pressure, yielding 260 mg of oily substance (73%). It was characterized by its infrared spectrum: azide, 2145 cm^{-1}; β-lactam, 1785 cm^{-1}; and aromatic ring, 770 and 695 cm^{-1}. When the benzylpenicillin azide thus obtained was dried under reduced pressure for 3 days at room temperature, it was quantitatively converted to the corresponding isocyanate. Its infrared spectrum showed an intense isocyanate band at 2270 cm^{-1}; β-lactam, 1785 cm^{-1}; and aromatic ring, 770 and 700 cm^{-1}. Analysis: Calculated for $C_{16}H_{17}N_3O_3S$: C, 57.99; H, 5.17; N, 12.68; S, 9.68. Found: C, 58.34; H, 5.61; N, 12.60; S, 9.46.

6-Phthalimidopenicillanic acid isocyanate. This substance was prepared according to the method of Perron *et al.*[16]. Its infrared spectrum showed an intense isocyanate band at 2260 cm^{-1}; phthalimide, 1803 and 1723 cm^{-1}; β-lactam, 1780 cm^{-1}; and aromatic ring, 790 and 720 cm^{-1}. Analysis: Calculated for $C_{16}H_{13}N_3O_4S$: C, 55.97; H, 3.82; N, 12.24; S, 9.34. Found: C, 55.84; H, 3.82; N, 12.07; S, 9.39.

β-Lactamase

β-Lactamase$_{75}$ was prepared as described previously[6].

Chemicals

Crystalline bovine serum albumin and dithiothreitol were purchased from the Nutritional Biochemicals Co., and the 0.05 M iodine solution was obtained from the Takeda Pharmaceutical Co. All inorganic salts and other organic reagents were of analytical reagent grade.

Enzyme assays

Method A. The reaction mixture (total volume 1.0 ml) contained 6 mM benzylpenicillin and 0.1% bovine serum albumin in 0.1 M sodium phosphate buffer (pH 7.0). A suitable aliquot of enzyme in the above solution was incubated for 30 min at 30 °C, heated for 1 min in a boiling water bath, and cooled in an ice bath. The velocity of the reaction was linear for at least 30 min in these experimental conditions. A 5-ml portion of 0.05 M iodine solution was added, and the remaining iodine was determined by measurement of the absorbance at 520 nm with the Carl Zeiss PMQ-II spectrophotometer.

Method B. For the determination of K_m values, the microiodometric assay of Novick[17] was used. The reaction was carried out in 0.1 M sodium phosphate buffer (pH 7.4) at 30 °C. The decrease in absorbance at 620 nm was recorded by the Hitachi 124 spectrophotometer.

Affinity labeling of β-lactamase$_{75}$

Affinity labeling of β-lactamase$_{75}$ was carried out at 30 °C in a reaction mixture composed of the enzyme (approx. $1.5 \cdot 10^{-7}$ M), and benzylpenicillin isocyanate which was added in 5 μl of acetone solution to a total volume of 0.5 ml of 0.1 M sodium phosphate buffer (pH 7.4) containing 1 mM dithiothreitol. Aliquots of 50 μl were removed and assayed for enzymatic activity by Method A.

Because β-lactamase$_{75}$ at high dilution is relatively unstable, and the conditions employed in these studies resulted in activity loss in the control samples varying from 0 to 20% (usually about 10%), the enzymatic activity loss was calculated as a percentage of the control activity under identical conditions.

RESULTS

Reaction of various isocyanates with β-lactamase$_{75}$

Table I summarizes the effect of various isocyanates on the activity of β-lactamase$_{75}$. An isocyanate derivative of benzylpenicillin, benzylpenicillin isocyanate, inactivated the enzymatic activity both at pH 7.4 and 5.9. 6-Phthalimidopenicillanic acid isocyanate, a derivative of a poor substrate of the enzyme, also had inhibitory activity. Both benzylpenicillin isocyanate and 6-phthalimidopenicillanic acid isocyanate have a penicillin nucleus. However, *n*-butylisocyanate and phenylisocyanate did not inhibit the enzymatic activity even at 10 times higher concentration. Thus it is clear that inactivation is related to the structure of the isocyanate derivative and not just to its chemical reactivity.

Effect of concentration of benzylpenicillin isocyanate on the inactivation rate

Incubation of β-lactamase$_{75}$ with various concentrations of benzylpenicillin isocyanate produced a rapid inactivation of its enzymatic activity which followed pseudo-first-order kinetics (Fig. 1). When both the inactivation and the catalytic reaction take place concomitantly, the mechanism will follow Eqn 1.

$$E + \mathrm{BPI} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E \cdot \mathrm{BPI} \xrightarrow{k_3} E\text{–BPI} \qquad (1)$$
$$\downarrow k_2$$
$$E + \mathrm{BPI}'$$

TABLE I

EFFECT OF VARIOUS ISOCYANATES ON THE ACTIVITY OF β-LACTAMASE$_{75}$

β-Lactamase$_{75}$ (approx. $1.5 \cdot 10^{-7}$ M) was incubated, in a total volume of 0.5 ml, with each isocyanate at the indicated concentration in 0.1 M sodium phosphate buffer containing 1 mM of dithiothreitol, pH 7.4 or pH 5.9 at 30 °C for the indicated time. The isocyanate was added in 5 μl of acetone solution. The activity was measured by Method A. In the control, 5 μl of acetone was added to the incubation mixture instead of isocyanate solution.

Reagents	*Concn (μM)*	*pH*	*Time of reaction (min)*	*Activity remaining (%)*
Benzylpenicillin isocyanate	20	7.4	15	56
	10	5.9	30	47
6-Phthalimidopenicillanic acid isocyanate	100	7.4	15	40
	20	7.4	15	55
	10	5.9	30	74
n-Butylisocyanate	100	7.4	15	90
	200	7.4	15	92
	10	5.9	30	81
Phenylisocyanate	100	7.4	15	87
Control		7.4	15	93
		5.9	30	89

Here, E–BPI is the irreversibly-inactivated enzyme and BPI′ is the hydrolyzed product of benzylpenicillin isocyanate. In the absence of a substrate or a competitive

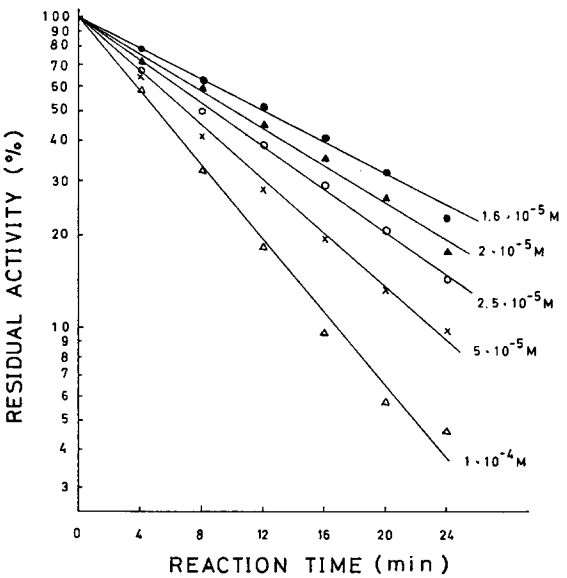

Fig. 1. Rate of inactivation of β-lactamase$_{75}$ by various concentrations of benzylpenicillin isocyanate at pH 7.4 and 30 °C. The reaction mixture contained, in 0.5 ml, β-lactamase$_{75}$ (approx. $1.5 \cdot 10^{-7}$ M), 0.1 M sodium phosphate buffer (pH 7.4), 1 mM dithiothreitol, and benzylpenicillin isocyanate. The concentration of benzylpenicillin isocyanate present is indicated on the lines. At various times, aliquots (50 μl) were removed and the remaining enzymatic activity was assayed by Method A.

inhibitor, and with [BPI] $\gg E_0$, where E_0 is the total enzyme concentration and K is $(k_{-1} + k_2 + k_3)/k_1$,

$$\frac{1}{k_{obsd}} = \frac{1}{k_3} + \frac{K}{k_3} \cdot \frac{1}{[BPI]} \tag{2}$$

will be followed[18]. The rate constants in Fig. 1 (k_{obsd}) were plotted in double-reciprocal fashion against varied concentrations of benzylpenicillin isocyanate (Fig. 2). The fact that a straight line plot did not pass through the origin but intercepted the positive y axis indicates that a reversible complex ($E \cdot$BPI) is formed before irreversible inactivation occurs. From data plotted in this fashion, K and k_3 were calculated to be $4 \cdot 10^{-5}$ M and $9 \cdot 10^{-2}$ min^{-1}, respectively.

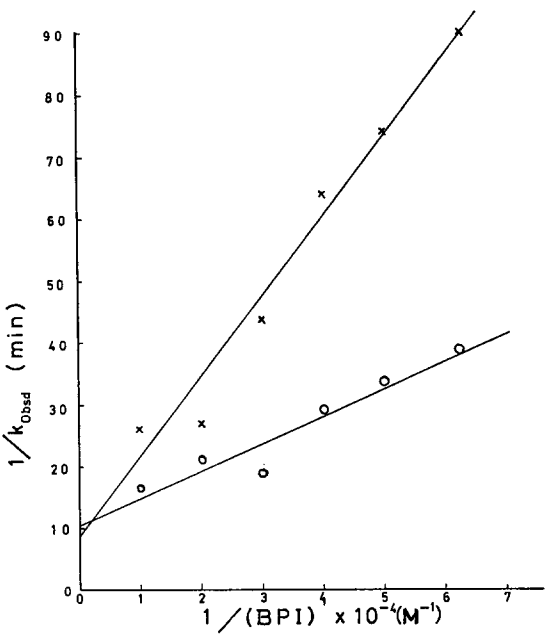

Fig. 2. Dependence of pseudo-first-order constant of the inactivation on the concentration of benzylpenicillin isocyanate (BPI). The reaction was carried out in the absence (○) or in the presence (×) of $2.73 \cdot 10^{-3}$ M benzylpenicillin at pH 7.4 and 30 °C.

Effect of substrate and competitive inhibitor on inactivation of β-lactamase$_{75}$ by benzylpenicillin isocyanate

When benzylpenicillin isocyanate reacts with the same site as the substrate, the presence of a substrate (A) or a competitive inhibitor (A), methicillin, must reduce the inactivation rate of the enzyme in the fashion described below[18].

$$E + A \overset{K_A}{\rightleftharpoons} E \cdot A \tag{3}$$

$$\frac{1}{k_{obsd}} = \frac{1}{k_3} + \frac{K}{k_3}\left(1 + \frac{[A]}{K_A}\right)\frac{1}{[BPI]} \tag{4}$$

Here, K_A is the dissociation constant of $E \cdot A$. Actually, the presence of $2.73 \cdot 10^{-3}$ M benzylpenicillin in the reaction mixture reduced the inactivation rate of the enzyme

(Fig. 3, *cf.* with the results in Fig. 1). A double-reciprocal plot of the rate constants (k_{obsd}) thus obtained against varied concentrations of benzylpenicillin isocyanate (Fig. 2) intercepted the y axis at the same point as that without benzylpenicillin, but the slope was much greater. From the slope, the K_A value was calculated to be $1 \cdot 10^{-3}$ M. This value was coincident within the experimental error with the K_m value ($7 \cdot 10^{-4}$ M) obtained from the hydrolysis rate of benzylpenicillin in a separate experiment. The same value should be obtained when the reaction is carried out in the presence of a reversible competitive inhibitor instead of a substrate. In fact, the

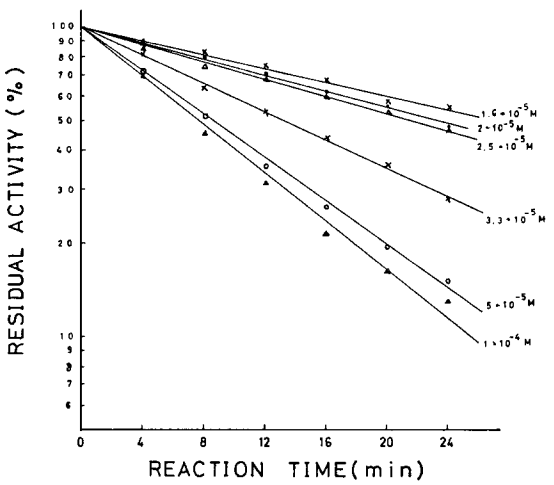

Fig. 3. Rate of inactivation of β-lactamase$_{75}$ by various concentrations of benzylpenicillin isocyanate in the presence of $2.73 \cdot 10^{-3}$ M benzylpenicillin at pH 7.4 and 30 °C. The reaction was carried out in the same way as in Fig. 1.

presence of $3 \cdot 10^{-4}$ M methicillin in the reaction mixture greatly reduced the inactivation rate of the enzyme (data no shown). Again, the intercept at the y axis was the same as that without methicillin. Furthermore, evidence for inactivation by modification of an active-site residue is the protection afforded by various concentrations of methicillin on the inactivation of β-lactamase$_{75}$ by $5 \cdot 10^{-5}$ M benzylpenicillin isocyanate (Fig. 4). Scrutton and Utter[19] have derived Eqn 6 to describe a system represented by Eqns 1, 3 and 5.

$$E \cdot A + \text{BPI} \xrightarrow{k_4} E \cdot A \cdot \text{BPI} \tag{5}$$

$$\frac{v_i}{v_0} = \frac{k_4}{k_3} + K_A \cdot \left(\frac{1 - v_i/v_0}{[A]} \right) \tag{6}$$

In Eqn 6, v_0 and v_i are the pseudo-first-order rate constants for inactivation in the absence and presence of methicillin, respectively. It is assumed in this equation that the concentrations of benzylpenicillin isocyanate and methicillin are much larger than that of the enzyme and that the rate of reversible dissociation of the methicillin–enzyme complex is greater than that of inactivation. A plot of the data of Fig. 4 according to this equation indicates that the relationship between v_i/v_0 and $(1 - v_i/v_0)/[A]$

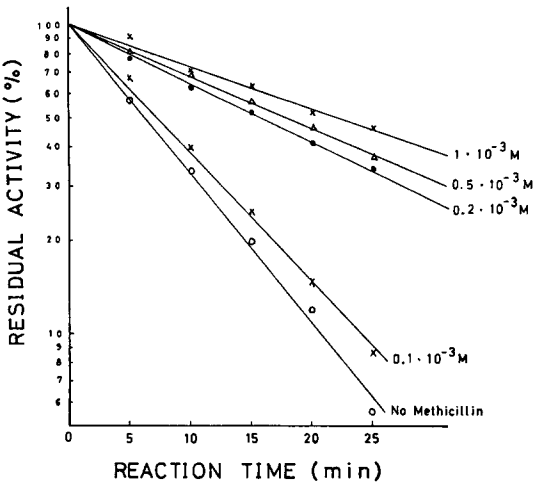

Fig. 4. Rate of inactivation of β-lactamase$_{75}$ by benzylpenicillin isocyanate in the presence of various concentrations of methicillin at pH 7.4 and 30 °C. The reaction as carried out in the same way as in Fig. 1.

was linear and that the intercept on the y axis was approximately zero. Thus, it is evident that the reaction represented by Eqn 5 does not contribute significantly to the inactivation process.

Kinetics of β-lactamase$_{75}$-catalyzed hydrolysis of benzylpenicillin isocyanate

Benzylpenicillin isocyanate has a β-lactam ring which could be hydrolyzed by the enzyme. When benzylpenicillin isocyanate binds to the catalytic site and hydrolysis and inactivation proceed simultaneously, these reactions should take place through the same reversible complex, $E \cdot$BPI, as represented in Eqn 1. For the hydrolysis, one obtains rate Eqn 7:

$$v = \frac{V}{K/[\text{BPI}] + 1} \tag{7}$$

Here, K, the concentration of benzylpenicillin isocyanate giving a half-maximum rate, is $(k_{-1} + k_2 + k_3)/k_1$, v is the initial velocity of hydrolysis, and V is the maximum velocity of the hydrolysis reaction.

Although the rate was over 100 times less than that of benzylpenicillin, the hydrolysis of benzylpenicillin isocyanate occurred with concomitant inactivation (Fig. 5A). The double-reciprocal plot of v against the concentration of benzylpenicillin isocyanate (Fig. 5B) was linear and gave a value for K of $7 \cdot 10^{-5}$ M. The fact that this value was coincident within the experimental error with K ($4 \cdot 10^{-5}$ M) determined in the inactivation reaction described above supports the idea that both of these processes proceed through a common intermediary enzyme–substrate complex, and that benzylpenicillin isocyanate attacks the catalytic site of the enzyme.

DISCUSSION

The work reported in this paper was initiated to get an active-site-directed

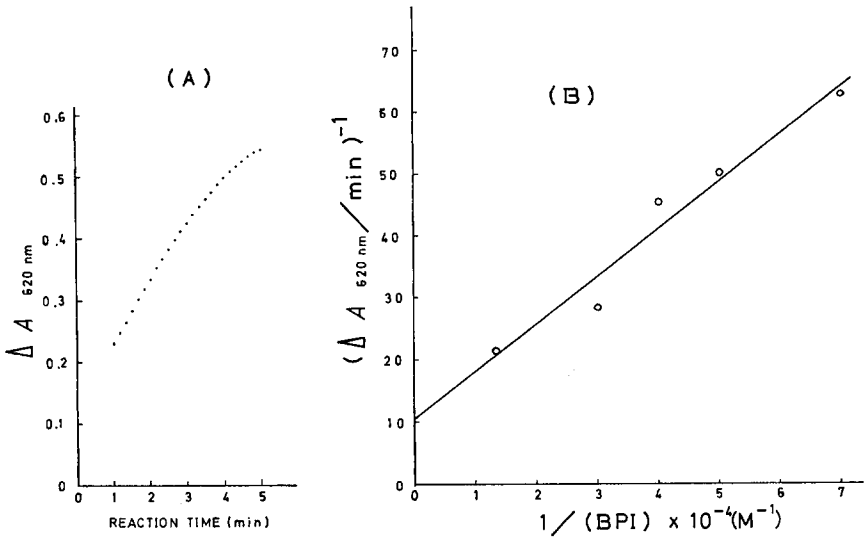

Fig. 5. Hydrolysis of benzylpenicillin isocyanate (BPI) by β-lactamase$_{75}$. (A) The rate of hydrolysis was determined by the microiodometric assay of Novick[17]. The decrease of absorbance at 620 nm was recorded at pH 7.4 and 30 °C every 10 s by the Hitachi 124 spectrophotometer. The concentration of benzylpenicillin isocyanate shown in the figure was $1 \cdot 10^{-4}$ M. (B) The double-reciprocal plot of the initial velocity ($\Delta A_{620\,nm}$/min) *vs* the concentration of benzylpenicillin isocyanate.

irreversible inhibitor or an affinity labeling reagent. Benzylpenicillin is a substrate for all β-lactamases. A derivative which will attack the catalytic site specifically can be applied to other β-lactamases as well. The results presented above show that inhibition by benzylpenicillin isocyanate occurs with a group within the active site of β-lactamase$_{75}$ and that this group is essential to the catalytic activity of the enzyme. This conclusion is supported by the following: First, although *n*-butylisocyanate and phenylisocyanate did not show the inactivation even at a high concentration, benzylpenicillin isocyanate and phthalimidopenicillanic acid isocyanate, both having a β-lactam ring, showed strong inhibition of the enzyme. Thus the structure of the isocyanate is important for inactivation. Second, the fact that a substrate, benzylpenicillin, and a competitive inhibitor, methicillin, have a Michaelis constant and an inhibition constant for inhibition of β-lactamase-catalyzed hydrolysis of benzylpenicillin identical with those for protection of the enzyme from inactivation by benzylpenicillin isocyanate shows that the reaction is occurring with a group in the active site of the enzyme. Third, the fact that the relationship between v_i/v_0 and $(1-v_i/v_0)/[A]$ is linear and that the intercept on the y axis is approximately zero supports the idea that benzylpenicillin isocyanate and the substrate and the competitive inhibitor bind to the same site in the enzyme. Finally, benzylpenicillin isocyanate is hydrolyzed by β-lactamase[75] with a Michaelis constant identical with K obtained from Eqn 2. The inactivated enzyme did not recover its enzymatic activity by dialyzing overnight against two changes of 1000 times volume of the buffer. From these results, it is concluded that benzylpenicillin isocyanate inactivates the enzyme specifically and irreversibly through binding to the catalytic site of the enzyme.

Recently, Patil and Day[20] described the irreversible inactivation of penicillinase from *Bacillus cereus* 569/H using "diazotization products" of 6-aminopenicillanic acid and ampicillin. They claimed that a carboxyl group is related to the inactivation. However, in the case of the β-lactamase described in this paper, preliminary experiments using "diazotization products" from many penicillins and cephalosporins including 6-aminopenicillanic acid and ampicillin and nitrous acid produced by passing sodium nitrite through a Dowex 50 column (H^+), showed no inactivation of the enzymatic activity. Thus, a carboxyl group could not be shown to be involved in the active site of this enzyme. As reported previously[6], β-lactamase$_{75}$ has a cysteinyl residue which might be involved in the enzymatic activity. The fact that the enzyme was inactivated by isocyanate derivatives in the presence of a large excess of dithiothreitol suggests that the active site region does not include the cysteinyl residue, and that inactivation by *p*-chloromercuribenzoate described before[6] is related to a change in conformation of the enzyme.

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