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BACILLUS CEREUS β-LACTAMASE

REACTION WITH *N*-BROMOSUCCINIMIDE AND THE PROPERTIES OF THE PRODUCT

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Summary

The effect of *N*-bromosuccinimide on the enzymatic activity and the conformation of a *Bacillus cereus* β-lactamase (penicillin amido-β-lactamase EC 3.5.2.6) was studied. Incubation with 10 μM *N*-bromosuccinimide caused over 95% decrease of the enzymatic activity within 15 min. Spectrophotometric titration with *N*-bromosuccinimide showed that the reaction proceeded in two steps.

The half-inactivated enzyme was prepared by the reaction with *N*-bromosuccinimide and its properties examined. Amino acid analysis showed that the half-inactivated enzyme contained one residue of tryptophan less while other amino acid contents were similar. Neither the molecular weight nor the mobility in disc electrophoresis was changed. However, the affinity to a cephalexin-CH-Sepharose column was increased, and the K_m value for cloxacillin was one-third that of the native enzyme, although that for benzylpenicillin was similar. These results indicate that a tryptophan residue sensitive to *N*-bromosuccinimide is essential for the maintenance of the rigid conformation and that its oxidation alters the enzyme in a manner such that a substrate with a bulky group in its side chain can form an enzyme-substrate complex more easily.

In the native enzyme, the value of $(f_a)_{eff}$ (Lehrer, S.S. (1971) Biochemistry 10, 3254—3263), did not vary significantly in the absence or the presence of cloxacillin. In contrast, in the half-inactivated enzyme the presence of cloxacillin affected the conformation such that over two thirds of the tryptophyl fluorescence were accessible for quenching by KI, although about half was accessible in the absence of cloxacillin.

Introduction

β-Lactamases of *Bacillus cereus* were isolated in crystalline forms by Pollock and co-workers [1,2] and Kuwabara [3]. The amino acid sequences of some β-lactamases from Gram-positive bacteria [4,5], and a preliminary crystallographic analysis of an *Escherichia coli* β-lactamase were reported [6].

Dupue et al. [7] described the presence of a histidine residue in the active site of *Staphylococcus aureus* 147 β-lactamase and Meadway [4] and Csànyi et al. [8] reported the presence of a tyrosine residue in that of β-lactamases of *Bacillus cereus* and *Bacillus licheniformis*. As reported in previous papers [9,10], we identified a histidine residue in the substrate-binding site of a *B. cereus* β-lactamase and an *E. coli* enzyme using benzylpenicillin isocyanate and carbenicillin isocyanate as affinity labeling reagents.

β-Lactamases are interesting enzymes. Many enzymes with a variety of substrate specificities have been isolated from a number of bacteria. One species of bacterium produces many enzymes with different properties such as substrate specificity, reactivity to chemical reagents and to the antibodies. Small changes of the substrate structure greatly affect the kinetics of the reaction of the enzyme with the substrate. Finally, many substrates have been synthesized for specific purposes. Such a variety of properties suggest possible differences in their substrate-induced conformational changes [11] and in the amino acid residues involved in attracting and repulsing the substrate.

To explore such properties, we attempted to study the effect of chemical reagents on the enzymatic activity of a *B. cereus* β-lactamase and to determine how such chemical modification affects the conformation and the kinetic properties of the enzyme. The present paper describes the results of the chemical modification of a *B. cereus* β-lactamase with *N*-bromosuccinimide and the properties of the half-inactivated enzyme.

Materials and Methods

Chemicals

Iodoacetic acid, bromoacetone, tetranitromethane and 0.05 M iodine solution were purchased from Wako Pure Chemicals, acetylimidazole and 2-hydroxy-5-nitrobenzyl bromide from Seikagaku Kogyo. Diethylpyrocarbonate and β-lactamase of *B. cereus* were obtained from Calbiochem. This β-lactamase was disc-electrophoretically pure and did not contain cysteine and zinc. This suggests that this enzyme belongs to a group of β-lactamase I of Kuwabara [3]. Benzylpenicillin and ciclacillin (6-(1-aminocyclohexane carboxamido) penicillanic acid) were products of Takeda Pharmaceutical Co., ampicillin was a product of Meiji Seiko Co. Carbenicillin was obtained from Taito Pfizer Co., cloxacillin from Fujisawa Pharmaceutical Co., methicillin from Banyu Pharmaceutical Co., and cephalexin and cephaloglycin were obtained from Shionogi Pharmaceutical Co. Myoglobin, trypsin, α-chymotrypsinogen and pepsin used as external standards in the disc electrophoresis were obtained from Schwarz and Mann. *N*-bromosuccinimide was purchased from Wako Pure Chemicals and was recrystallized from water. CH-Sepharose 4B was obtained from Pharmacia Fine Chemicals. 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide was a product

of the Protein Research Foundation, Osaka, Japan. Dimethyl(2-hydroxy-5-nitrobenzyl)sulfonium bromide was prepared by the method of Horton and Tucker [12].

Reaction of B. cereus β-lactamase with N-bromosuccinimide

The enzyme solution used for the preparation of the half-inactivated enzyme was prepared by adding 1.2 ml of the aqueous enzyme solution to 1.2 ml of 0.2 M acetate buffer, pH 5.0. The concentration of the enzyme was calculated from the optical density at 278 nm, assuming that the $E^{1\%}_{1\,cm}$ is 10.0 [13] and the molecular weight is 29 000, which had been determined by sodium dodecyl sulfate-polyacrylamide disc electrophoresis. The initial concentration of the enzyme was found to be $3.6 \cdot 10^{-5}$ M from the optical densities at 278 nm of the sample solution and of 0.1 M acetate buffer of pH 5.0. Ten-μl portions of 0.01 M *N*-bromosuccinimide solution were added to each solution in cuvettes stirred magnetically with small stirring bars. At each time the absorbance at 278 nm and the enzymatic activity of an aliquot were determined using benzylpenicillin as a substrate. When a total of 50 μl of *N*-bromosuccinimide solution had been added, the reaction was stopped by dialysing the reaction mixture overnight against four changes of 2.5 l of water, followed by lyophilization.

Coupling of cephalexin to CH-Sepharose 4B

To a suspension of 5 g of CH-Sepharose 4B in 29 ml of water, solutions of 226 mg (0.65 mmol) of cephalexin in 20 ml of water and 350 μl of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide in 15 ml of water, which had been adjusted to pH 5.0, were added. The whole reaction mixture was gently stirred for 1 h at room temperature maintaining the pH at 5.0 by adding 1 M HCl and then for 44 h at 4°C. The product was washed extensively with 0.1 M phosphate buffer containing 1 M NaCl, pH 8.0; 0.1 M acetate buffer containing 1 M NaCl, pH 3.8; and water. The cephalexin content was estimated as 6.4 μmol/ml of Sepharose by iodometric titration.

Disc electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel disc electrophoresis was performed at room temperature according to the procedure of Weber and Osborn [14].

Enzyme assay

β-Lactamase activity was determined iodometrically at pH 7.0 using a slight modification of the method of Perret [15]. The enzyme was incubated at 30°C for 30 min in a total volume of 1 ml of 0.1 M phosphate buffer with 6 μmol of benzylpenicillin. Then, the enzyme was inactivated by heating at 100°C for 2 min, cooled in an ice bath and 5 ml of 0.005 M iodine solution in water were added. After 10 min, the remaining iodine was titrated with 0.005 M sodium thiosulfate solution. The microiodometric assay of Novick [16] was used for the kinetic measurement of the enzyme reaction.

Fluorescence spectrophotometry

Fluorescence measurements were performed with Hitachi MPF-4 spectrophotofluorometer. An excitation slit of 4 nm and an emission slit of 10 nm were used for all experiments. Emission spectra were obtained by means of an excitation wavelength of 290 nm. Measurements of fluorescence as a function of KI concentration were performed at an excitation wavelength of 290 nm. Monitoring was done at the peak near 340 nm except the case of the half-inactivated enzyme and the presence of cloxacillin, where fluorescence was monitored at the peak near 350 nm. Data were obtained at 35 ± 2°C in 0.01 M Tris · HCl buffer of pH 6.9 and were uncorrected.

Results

Effects of various chemical reagents on the enzymatic activity of Bacillus cereus β-lactamase

When this β-lactamase was incubated with 1 mM of iodoacetic acid, diethylpyrocarbonate, bromoacetone, acetylimidazole or tetranitromethane, only a slight decrease in the enzymatic activity was observed. On the other hand, when the enzyme was incubated with *N*-bromosuccinimide (0.1 mM) or 2-hydroxy-5-nitrobenzyl bromide (1 mM) for 30 min to 1 h at room temperature, marked decrease was observed. In the case of *N*-bromosuccinimide, over 99% of the enzymatic activity was lost within 30 min (Table I).

Effects of concentration of N-bromosuccinimide on the inactivation

Incubation of the enzyme with various concentrations of *N*-bromosuccinimide produced a rapid inactivation (Table II). At 10 μM, over 95% of the

TABLE I

EFFECTS OF VARIOUS INHIBITORS ON THE ENZYMATIC ACTIVITY OF A *BACILLUS CEREUS* β-LACTAMASE

An enzyme solution (approx. $1.7 \cdot 10^{-7}$ M) was incubated, in a total volume of 250 μl, with various inhibitors at the indicated concentration for the indicated time at room temperature in a dark room. Diethylpyrocarbonate, bromoacetone, acetylimidazole, tetranitromethane and 2-hydroxy-5-nitrobenzyl bromide were added in 2 μl of acetone solution. Iodoacetic acid and *N*-bromosuccinimide were added in 25 μl of 0.1 M phosphate buffer. In the control, the same volume of acetone or the buffer was added to the incubation mixture. The enzymatic activity in an aliquot was measured. The remaining activity in the table was expressed as the percentage of the remaining activity in an aliquot of that of the control.

Reagents	Concentration (mM)	Time (min)	Buffer	pH	Remaining activity (%)
Iodoacetic acid	1	60	0.1 M phosphate	7.0	62
	0.1	60	0.1 M phosphate	7.0	89
Diethylpyrocarbonate	1	60	0.1 M acetate	4.0	85
Bromoacetone	1	60	0.1 M phosphate	6.5	74
Acetylimidazole	1	60	0.01 M Tris · HCl	7.4	100
Tetranitromethane	1	60	0.1 M phosphate	7.0	78
N-Bromosuccinimide	0.1	30	0.1 M phosphate	7.0	0.7
2-Hydroxy-5-nitrobenzyl bromide	1	60	0.1 M acetate	4.0	33
	0.1	60	0.1 M acetate	4.0	47

TABLE II

EFFECTS OF THE CONCENTRATIONS OF *N*-BROMOSUCCINIMIDE ON THE ENZYMATIC ACTIVITY OF A *BACILLUS CEREUS* β-LACTAMASE

An enzyme solution (approx. $1.7 \cdot 10^{-7}$ M) was incubated, in a total volume of 250 μl, with various concentrations of *N*-bromosuccinimide at room temperature for the indicated time in 0.1 M phosphate buffer of pH 7.0. *N*-bromosuccinimide was added in 2 μl of the buffer solution.

Concentration (μM)	(*N*-bromosuccinimide)/(enzyme) (mol/mol)	Time (min)	Remaining activity (%)
10	60	15	3.7
		30	2.8
5	30	15	19
		30	15
1	6	15	82
		30	67

enzymatic activity disappeared within 15 min, and at 1 μM, 33% of the activity was lost after 30 min incubation.

Spectrophotometric titration

Effects of *N*-bromosuccinimide on the enzymatic activity and the optical density at 278 nm of the β-lactamase were examined at pH 5.0. Addition of increasing amounts of *N*-bromosuccinimide resulted in gradual decrease of the absorbance and the enzymatic activity up to a 50% decrease of the latter (Fig. 1). At the point of 50% decrease in activity the absorbance at 278 nm was 77% of the initial absorbance, which suggested the destruction of about 1.5 residues of tryptophan. On further addition of *N*-bromosuccinimide, that is, addition of 7 and 11 molar excess, both the absorbance and the enzymatic

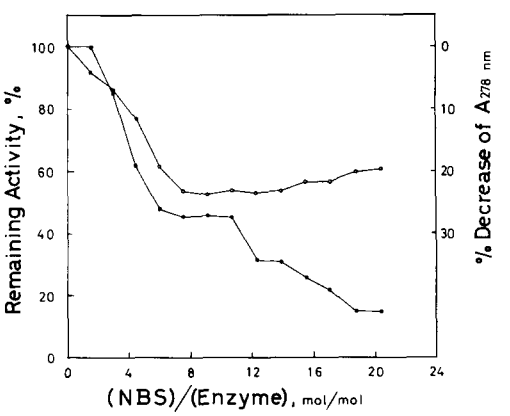

Fig. 1. Effects of *N*-bromosuccinimide on the enzymatic activity and the optical absorption of the β-lactamase. The enzyme solution was prepared as follows: 0.75 ml of the stock enzyme solution, 0.25 ml of water, 1.0 ml of 0.2 M acetate buffer of pH 5.0 and 0.3 ml of 0.1 M acetate buffer of pH 5.0 were pipetted into a 1.0 cm cuvette. The initial concentration of the enzyme was $2.9 \cdot 10^{-6}$ M. 10-μl portions of 0.001 M solution of *N*-bromosuccinimide were added to each solution in the cuvette stirred magnetically with a small stirring bar. At each time the optical density at 278 nm (○) and the remaining enzymatic activity (●) in a 10 μl aliquot were measured.

activity did not change, and on further addition of *N*-bromosuccinimide, the absorbance increased and the enzymatic activity gradually decreased.

Effects of dimethyl (2-hydroxy-5-nitrobenzyl) sulfonium bromide

The enzyme ($4.5 \cdot 10^{-6}$ M) in 1.5 ml of 0.1 M acetate buffer, pH 4.0, was treated with dimethyl (2-hydroxy-5-nitrobenzyl) sulfonium bromide (0.45 mM) for 3 h at room temperature in the dark. The enzymatic activity in an aliquot (3 μl) was determined as described. The solution was passed through a column of Sephadex G-25, which had been equilibrated with 0.1 M acetic acid, and eluted with 0.1 M acetic acid. The protein-containing fractions were collected, lyophilized, dissolved in 1 M NaOH and the absorbance at 410 nm was measured [17]. The 2-hydroxy-5-nitrobenzyl group bound to the enzyme was determined to be 0.25 mol/mol of the enzyme, and no loss of enzymatic activity was observed.

Isolation of the half-inactivated enzyme

When the enzyme ($3.6 \cdot 10^{-5}$ M) was treated with increasing amounts of *N*-bromosuccinimide, the absorbance at 278 nm and the enzymatic activity decreased gradually in the same manner as those in the spectrophotometric titration. On the addition of 5.8 molar excess of *N*-bromosuccinimide, a faint turbidity was observed and the reaction was stopped by dialysing the reaction mixture and lyophilization. The enzymatic activity at this point was 60% of the initial activity. Amino acid analysis performed by the methods of Matsubara and Sasaki [18], Scoffone et al. [19] and Edelhoch [20] showed that the half-inactivated enzyme contained 1.10 residues less tryptophan while other amino acid contents including those of tyrosine (9.4 and 9.3 residues for the native and the half-inactivated enzymes, respectively), and histidine (3.9 and 3.2 residues) were similar.

Properties of the half-inactivated enzyme

Disc electrophoresis. The molecular weights of the native and the half-inactivated enzymes were determined by sodium dodecyl sulfate-polyacrylamide disc electrophoresis using myoglobin (mol. wt 17 200), trypsin (23 300), α-chymotrypsinogen (25 700) and pepsin (35 000) as external standards. No change in molecular weight was observed after the oxidation of a tryptophan residue in the enzyme. This shows that no cleavage occurred during oxidation with *N*-bromosuccinimide. In addition, when disc electrophoresis was performed at pH 8.9 and in 7.5% acrylamide gel for 3 h, no difference was observed between the mobilities of the native and the half-inactivated enzymes.

Affinity chromatography. The behavior of the half-inactivated enzyme toward an affinity column was compared with that of the native enzyme. The native enzyme was eluted from the column immediately after the buffer was changed to 0.05 M phosphate buffer containing 1 M NaCl (Fig. 2). In contrast, the half-inactivated enzyme was eluted shortly after the native enzyme.

Comparison of the hydrolysis rates and the K_m values of penicillins

The initial hydrolysis rates of benzylpenicillin, ampicillin, carbenicillin, ciclacillin, cloxacillin and methicillin by the half-inactivated enzyme were de-

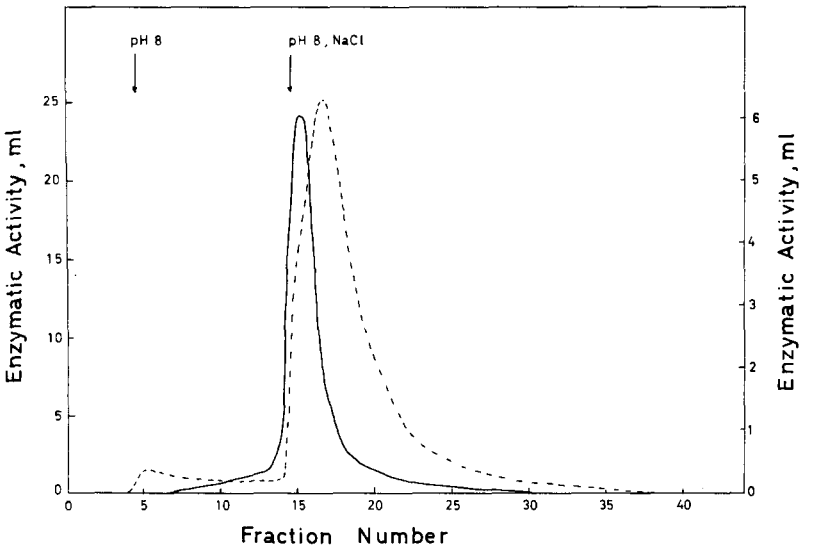

Fig. 2. Affinity column chromatography of the native and the half-inactivated enzymes. A 1 ml column of cephalexin-CH-Sepharose 4B was equilibrated in a small capillary tube with 0.1 M acetate buffer, pH 4.0, at 4°C. An enzyme solution in 1 ml of 0.1 M acetate buffer was passed through the column and washed with 6 ml of the same buffer and 20 ml of 0.1 M phosphate buffer of pH 8.0 and then with 0.05 M phosphate buffer containing 1 M NaCl, pH 8.0, at a speed of about 10 ml/h. Two-ml portions of fractions were collected and the enzymatic activities in aliquots were determined iodometrically [15]. The enzymatic activity in the figure is expressed as the consumption of 0.005 M iodine solution in 100 μl of the fraction. The left side scale is for the native enzyme (solid line) and the right side scale is for the half-inactivated enzyme (broken line).

TABLE III

COMPARISON OF THE RELATIVE HYDROLYSIS RATE OF PENICILLINS BY THE NATIVE AND THE HALF-INACTIVATED ENZYMES

The rate of hydrolysis of penicillins was measured microiodometrically [16] by incubating penicillins (0.1 mM) and the enzyme at pH 7.0 and 25°C. The decrease of absorbance at 620 nm was recorded every 30 s for 5 min by the Hitachi 124 recording spectrophotometer. The initial hydrolysis rate of penicillins by the native enzyme and that by half-inactivated enzyme were measured, and the ratio was calculated. The relative hydrolysis rate of penicillins is expressed taking the ratio in benzylpenicillin as 100.

Penicillins	$V_{\text{native enzyme}}$ ($\Delta A_{620\,nm}$/min)	$V_{\text{modified enzyme}}$ ($\Delta A_{620\,nm}$/min)	$\frac{V_{\text{native enzyme}}}{V_{\text{modified enzyme}}}$	Relative hydrolysis rate of penicillins (benzylpenicillin = 100)
Benzylpenicillin	0.0830	0.0963	0.862	100
Ampicillin	0.0640	0.0714	0.896	104
Carbenicillin	0.0228	0.0273	0.835	97
Ciclacillin	0.0540	0.0614	0.879	102
Cloxacillin	0.0151	0.0125	1.21	140
Methicillin	0.0777	0.0898	0.865	100

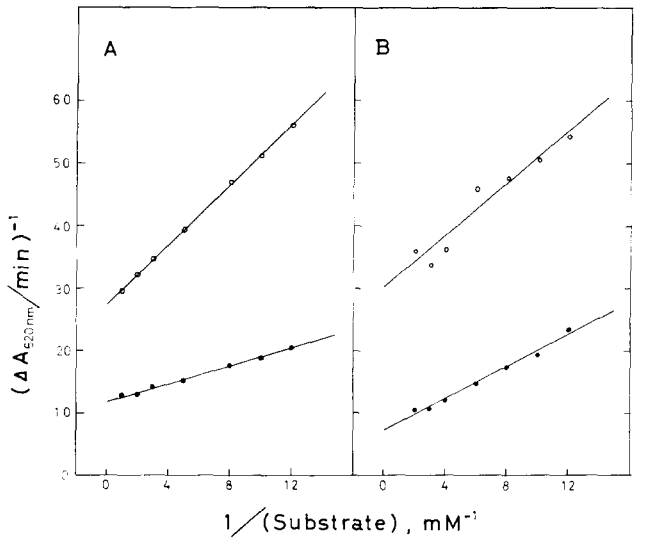

Fig. 3. The double reciprocal plots of the initial velosity (ΔA_{620nm}/min) against the concentration of benzylpenicillin and of cloxacillin. (A), the hydrolysis of benzylpenicillin by the native (●) and the half-inactivated (○) enzymes; (B), the hydrolysis of cloxacillin by the native (●) and the half-inactivated (○) enzymes.

termined and compared with those by the native enzyme by the spectrophotometric method. From the results shown in Table III, it is clear that the hydrolysis rates of penicillins were not changed by the modification of a tryptophan residue of the enzyme except the case of cloxacillin. The half-inactivated enzyme hydrolyzed cloxacillin about 1.4 times less rapidly than the native enzyme. Then, affinities of benzylpenicillin and cloxacillin to these enzymes were measured by the spectrophotometric method. The double-reciprocal plots of v, the velocity of the hydrolysis, against the concentration of benzylpenicillin (Fig. 3A) were linear and gave values of K_m for the native enzyme of $6 \cdot 10^{-5}$ M and for the half-inactivated enzyme of $9 \cdot 10^{-5}$ M. This shows that the affinities of these enzymes to benzylpenicillin were almost the same. However, although the double-reciprocal plots were linear in the case of cloxacillin, the K_m value calculated from the ordinate intercept and the slope using the least-squares method (Fig. 3B) for the half-inactivated enzyme ($6.4 \cdot 10^{-5}$ M) was about 3 times less than that for the native enzyme ($1.8 \cdot 10^{-4}$ M).

Fluorescence spectrophotometry

Native enzyme. The fluorescence emission spectrum of the native enzyme showed a maximum at 338 nm and did not change its maximum wavelength when excitation was carried out at 280, 285, 290 and 295 nm. When cloxacillin was added at a concentration of 0.5 mM, the wavelength of the maximum did not change but the fluorescence intensity was decreased by about 10%, as shown in Fig. 4A. This indicates that a substrate quenches the tryptophyl fluorescence and that a substrate changes the conformation or blocks the tryptophan residue such that some tryptophan residues can not emit their fluorescence.

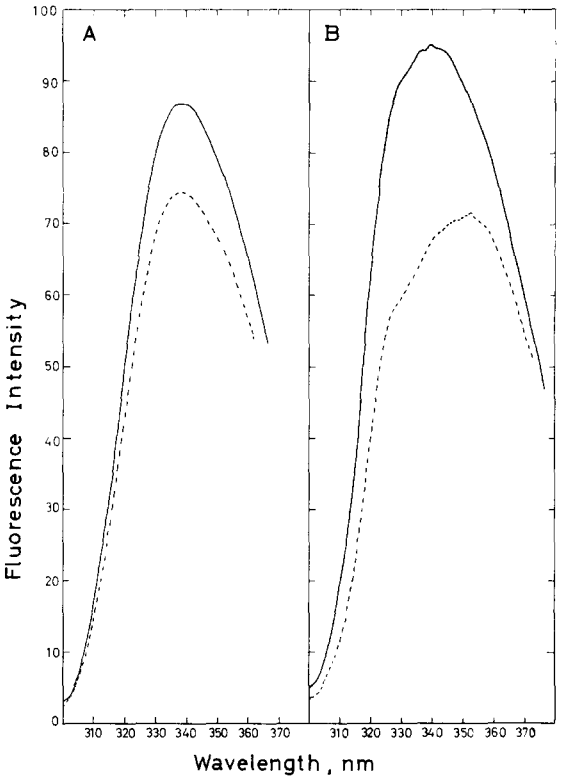

Fig. 4. Fluorescence spectra of the native (A) and the half-inactivated (B) enzymes in the absence (solid line) and the presence (broken line) of cloxacillin. The buffer is 0.01 M Tris · HCl, pH 6.9, and the protein concentration is approx. $5 \cdot 10^{-7}$ M. $\lambda_{excitation}$: 290 nm.

In order to learn more about the quenching mechanism of the enzyme, quenching of fluorescence emission of the enzyme in the presence and the absence of cloxacillin was studied by adding increasing amounts of KI. In the absence or the presence (0.5 mM) of cloxacillin, KI quenched the fluorescence emission of the enzyme and the modified Stern-Volmer plot gave a straight line (Fig. 5A). From the intercept, values of $(f_a)_{eff}$, effective fractional maximum accessible fluorescence [21], were calculated to be 0.53 and 0.59 for those in the absence and the presence of cloxacillin, respectively. Thus, about half of the tryptophyl fluorescence is accessible both in the absence and the presence of cloxacillin for quenching by KI at pH 6.9. Quenching by KI does not shift the wavelength of the maximum emission.

Half-inactivated enzyme. The fluorescence emission spectrum of the half-inactivated enzyme showed a maximum at 340 nm. When cloxacillin was present in the protein solution, the maximum wavelength was shifted to a longer wavelength (352 nm) and at the same time the fluorescence intensity was decreased by about 27% (Fig. 4B). This indicates that cloxacillin affects the conformation of the half-inactivated enzyme. The effects of KI on the quenching of the fluorescence emission were examined in the absence and the presence of cloxacillin. In the absence of cloxacillin, KI effectively quenched the fluorescence emission of the half-inactivated enzyme, and the modified Stern-Volmer

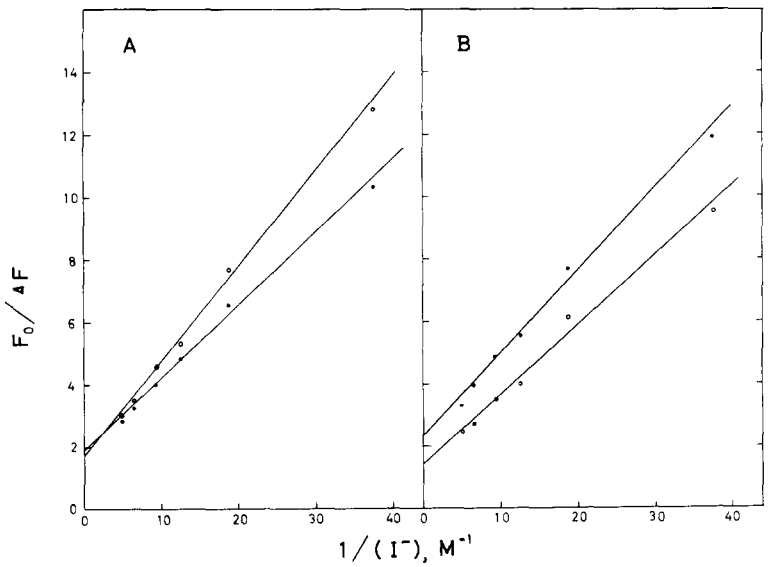

Fig. 5. Modified Stern-Volmer plot of the quenching of the native and the half-inactivated enzymes' fluorescences by KI. To a protein solution (approx. $5 \cdot 10^{-7}$ M in 0.01 M Tris · HCl, pH 6.9) in the absence and the presence of $5 \cdot 10^{-4}$ M cloxacillin, increasing amounts of 3 M KI in 0.1 mM sodium thiosulfate solution were added. A control cuvette was obtained by adding increasing amounts of 3 M NaCl in 0.1 mM sodium thiosulfate. F_0 is the fluorescence in the NaCl-containing control and ΔF is the difference in fluorescence of sample and control cuvettes. The iodide concentration was calculated using the proper dilution factor. (A), the native enzyme in the absence (●) and the presence (○) of cloxacillin; (B), the half-inactivated enzyme in the absence (●) and the presence (○) of cloxacillin.

plot gave a straight line (Fig. 5B). In contrast to the native enzyme, the fluorescence maximum was shifted to a shorter wavelength by the addition of KI. This effect was not observed when NaCl was added to the enzyme solution. When cloxacillin was present at 0.5 mM in the enzyme solution, a slight increase of the quenching by KI was observed, as shown by the decreased slope of the modified Stern-Volmer plot. From the intercept, values of $(f_a)_{eff}$ were calculated to be 0.44 and 0.72 for those in the absence and the presence of cloxacillin, respectively. Thus, about half of the tryptophyl fluorescence is accessible in the absence of cloxacillin for quenching by KI, and the presence of cloxacillin is suggested to change the conformation of the half-inactivated enzyme in a manner such that over two thirds of the tryptophyl fluorescence are accessible for quenching by KI.

Discussion

Effects of chemical reagents

Some essential amino acid residues have been reported in the active site of β-lactamases. As for a histidine residue, Dupue et al. [7] reported that this amino acid is involved in the active site of the *S. aureus* 147 enzyme. We proposed in previous papers [9,10] that penicillin isocyanates, affinity labeling reagents, inactivated a *B. cereus* and an *E. coli* enzyme through the reaction with an essential histidine residue first and then its migration to the adjacent

amino group to form a stable, inactivated urea derivative of the enzyme. On the other hand, tyrosine was also reported to be essential for the activity of the β-lactamases of *B. cereus* and *B. licheniformis* [4,8]. In contrast to these reports, diethylpyrocarbonate, bromoacetone and iodoacetic acid, so-called histidine-specific reagents [22], and acetylimidazole and tetranitromethane, so-called tyrosine-specific reagents [22], did not inactivate a *B. cereus* enzyme (Table I). In a previous paper [23], we reported that a tyrosine residue was not essential for the activity of an *E. coli* enzyme, because 0.5 mM of tetranitromethane did not inactivate this enzyme. In a study of the isolation of an active site peptide, Scott [24] reported that about 1.2 mM tetranitromethane inactivated an *E. coli* enzyme by only 36% and that the activity loss was not increased even at a higher concentration and by a prolonged incubation. He also used iodoacetic acid at a concentration of 0.25 M to inactivate the enzyme and to isolate an active site peptide containing an essential histidine residue.

On the other hand, *N*-bromosuccinimide [25] and 2-hydroxy-5-nitrobenzyl bromide [26], so-called tryptophan-specific reagents, did inactivate the enzyme (Table I). The inhibitory activity of *N*-bromosuccinimide is comparable to or even stronger than that of iodine. During studies on the effects of pH on inactivation by *N*-bromosuccinimide, the same inactivation rate was observed in the pH range 5.0—7.0, and a slight decrease of the inactivation effect was observed at pH 8.0.

Spectrophotometric titration

Studies on the effects of *N*-bromosuccinimide on the enzyme at pH 5.0 showed that the process followed two or three chemical steps. The first was the oxidation of a tryptophan residue which resulted in a gradual decrease of the absorbance at 278 nm and a concomitant decrease of the enzymatic activity up to about 50% (Fig. 1). The enzyme in this condition appears inert to the further attack of *N*-bromosuccinimide as far as the optical density and the enzymatic activity are concerned. However, during this process, the conformation is supposed to be transformed to a different state than that of the native enzyme. Addition of a little more molar excess of *N*-bromosuccinimide to this state of enzyme resulted in an increase in the absorbance due to a modification of a tyrosine residue, which corresponded to a gradual decrease in enzymatic activity. From these facts it can be concluded that a tryptophan residue susceptible to *N*-bromosuccinimide is not essential for the enzymatic activity but is necessary for the maintenance of the rigid conformation of the fully active enzyme. On the other hand, a tyrosine residue is an essential amino acid for enzymatic activity but in the native state it is not accessible to attacks of *N*-bromosuccinimide and other chemical reagents. This residue must be buried inside the enzyme molecule. Csànyi et al. [27] and Mile et al. [28] showed in their study on the iodine sensitivity of a *B. cereus* enzyme that the inactivation of the enzyme by iodine took place in two steps: "pH-sensitive" reaction and "pH-resistant" reaction. It might be suspected that the first step in the reaction with *N*-bromosuccinimide corresponds to the "pH-sensitive" reaction with iodine and the second step, modification of a tyrosine residue, corresponds to the "pH-resistant" reaction with iodine. Csànyi et al. [8] claimed that oxidation of a *B. cereus* enzyme with NaIO, KIO_3, K_2CO_4, $KMnO_4$ and H_2O_2 gave

no notable change in enzymatic activity and induced a conformational change to a more sensitive state for iodine at pH 6. However, data in the present studies indicate that oxidation of a tryptophan residue gives rise to the decrease of the enzymatic activity and also a conformational change.

Properties of the half-inactivated enzyme

Oxidation of a tryptophan residue resulted in no change of the molecular weight and the mobility in disc electrophoresis. This indicates that no cleavage and no change in the charge occurred by the reaction. The effect of iodine on the enzymatic activity of the half-inactivated enzyme as compared with that of the native enzyme was studied. When the enzymes were incubated with 5 μM of iodine at room temperature for 10 to 20 min, no difference was observed in the decrease of the enzymatic activity. However, the affinity to substrates was greatly affected. This is supported by the following facts. First, the elution pattern of the modified enzyme from an affinity column showed that the modified enzyme was attached a little stronger to the column (Fig. 2). Second, although the K_m value for benzylpenicillin was not affected, that for cloxacillin was about 3 times less than that of the native enzyme (Fig. 3A and 3B). This indicates that the conformation of the enzyme was transformed to a state that binds to cloxacillin, having a bulky group in its side chain, more strongly attached than the native enzyme.

Fluorescence spectrophotometry

In the present study, no contribution of tyrosine residue was observed in the fluorescence emission spectrum of the native enzyme [29]. The complex formation of an enzyme with a ligand often causes quenching of tryptophyl fluorescence emission [30—32]. This is also the case in the present study. In fact, the presence of a substrate caused a 10% decrease of the intensity of the fluorescence emission (Fig. 4A). This is due to the changes in charge distribution near a tryptophan residue at or near the binding site of the substrate and due to the conformational change and blocking of a tryptophan residue. This effect was more marked in the case of the half-inactivated enzyme. Here the intensity decrease reached 27% through the binding of a substrate (Fig. 4B).

In contrast to the native enzyme, where no shift of the maximum wavelength was observed by the addition of cloxacillin, the half-inactivated enzyme showed a shift of the maximum to a longer wavelength. This indicates that while in the native enzyme cloxacillin induces the changes in charge distribution and conformation but the polarity at or near the tryptophan residue does not vary significantly, in the modified enzyme cloxacillin transforms the conformation to a state that the tryptophan residue is in a more polar environment. Polar solvents are known to shift tryptophan fluorescence emission to a longer wavelength [33,34]. This conclusion is supported by the results from solute perturbation studies by KI [21,35]. In the native state, values of $(f_a)_{eff}$, effective fractional maximum accessible fluorescence, do not vary significantly in the absence and the presence of cloxacillin, and about half of the tryptophyl fluorescence is accessible for quenching by KI. In contrast, in half-inactivated enzyme the presence of cloxacillin has influence over the conformation in such a way that over two thirds of the tryptophyl fluorescence are accessible for

quenching by KI, although about half is accessible in the absence of cloxacillin. From these facts, it is suggested that while the native enzyme is in a rigid state, the half-inactivated enzyme is in a flexible state. If we may be allowed to speculate, the substrate specificity of β-lactamases might be controlled by the rigid conformation which is maintained by some hydrophobic residues such as tryptophan and cysteine [23] rather than the direct attraction and repulsion by the electrostatic force.

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