BACILLUS CEREUS 569/H β -LACTAMASE I: STRUCTURE-FUNCTION RELATIONSHIP

by

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Various functional groups of β -lactamase I were modified using appropriate group specific reagents. Modification of five of the ten arginine residues and approximately fifteen of twenty-one lysine residues present in the protein could readily be achieved by reaction with diacetyl trimer and trinitrobenzene sulfonic acid respectively, without any adverse effect on catalytic activity. Reaction with N-bromosuccinimide revealed that two of the three tryptophan residues could be oxidized without impairment of enzymatic activity. Treatment with tetranitromethane resulted in the modification of two of the seven tyrosine residues of the protein with approximately 30 % diminution in activity. Reaction of the enzyme with a water soluble carbodiimide resulted in a rapid inactivation, complete loss of activity being accompanied by the modification of five of the carboxylic functions. Conversion of all the four histidine residues of the enzyme to the corresponding N-carbethoxy derivatives had no effect on the catalytic function. All these observations suggest that chemical modification of the enzyme in the presence of substrate or its analog is essential for the identification and/or localization of functional groups participating in the catalytic mechanism.

Abbreviations: DCC = 1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide; DEP = diethylpyrocarbonate; DT = trimer of 2,3-butane dione; NBS = N-bromosuccinimide; TNBS = trinitrobenzene sulfonic acid; TNM = tetranitromethane.

1. INTRODUCTION

Beta-lactamases, enzymes capable of hydrolyzing penicillins and cephalosporins, have attracted considerable attention in recent years in view of their inherent role in microbial resistance to β -lactam antibiotics (15). Beta-lactamase I, specific to the cleavage of the lactam ring in penicillins, has been isolated from both gram-positive and gram-negative microorganisms. Available information indicates considerable homology in the primary structures of β -lactamases derived from different microbial sources (2). Despite the successes in the isolation, purification and elucidation of the primary structure of these proteins, very little is known about the nature of the functional groups as well as the mechanism involved in the reaction catalyzed by these enzymes. Modification of a tyrosine residue in the enzyme derived from *Escherichia coli* (33), *Bacillus licheniformis* (1), *Staphylococcus aureus* (3), and *Bacillus cereus* (5), has been found to result in partial impairment of the catalytic function. Conversion of the four histidine residues of β -lactamase of *B. cereus* 569/H to the corresponding N-carbethoxy derivative has been accomplished with no effect on enzymatic activity (12, 9). Reaction of the enzyme with carbodiimides and other carboxyl specific reagents has been found to effect inactivation (37). Hitherto, no efforts have been directed towards the assessment of the role played by either the amino or the guanidino functions present in the enzyme. The current study concerns a systematic and comprehensive approach towards identification of functional groups that participate in the catalytic function of β -lactamase I. The observations recorded in this study emphasize the vital need for conducting the chemical modification of the enzyme in the presence of its substrate or analog to establish the identity of functional groups participating in the catalytic mechanism. The identity of the C-terminal amino acid residue, which could not be detected in earlier studies (35) of β -lactamase I from *B. cereus* 569/H, has been established using carboxypeptidase Y.

2. EXPERIMENTAL

2.1. Materials

Spore suspensions of *B. cereus* 569/H, a constitutive β -lactamase producing strain were

kindly provided by Dr. DAVID SHAPIRO of the Weizmann Institute of Science. Carboxypeptidase Y was a laboratory preparation. Tetranitromethane (TNM), diethyl pyrocarbonate (DEP), 2,4,6-trinitrobenzene sulfonate (TNBS), and 1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide were purchased from Aldrich Chemical Co., Milwaukee, Wisc. Ampicillin and methicillin were kindly provided by Bristol Laboratories, Syracuse, N. Y., and benzylpenicillin was a gift from Eli Lilly Co., Indianapolis.

2.2. Isolation and purification of β -lactamase I

Colonies highly active in β -lactamase production were selected (19), and subsequently used in the isolation of β -lactamase I, according to the procedure of MILLER et al. (27). The crude enzyme was further purified by chromatography on DEAE-cellulose (21) and Sephadex G-100.

2.3. Measurement of activity

Beta-lactamase I activity was determined by the spectrophotometric procedure with benzylpenicillin as substrate (36). In some experiments, activity measurements were also performed using N-(2-furyl) acryloyl penicillin as substrate (10).

2.4. General methods

The nitrogen content of β -lactamase preparation was determined by the procedure of LANG (24).

SDS-polyacrylamide gel electrophoresis was used to assess the homogeneity (38) and the molecular weight of β -lactamase I.

Amino acid analysis: Protein samples (1–2 mg) were hydrolyzed with 2.0 ml of 6 N HCl in sealed tubes under vacuum at 105 °C for 36 h. The amino acid analysis was performed by ion-exchange chromatography (34) with the aid of a Beckman model 120B amino acid analyzer.

2.5. Chemical modification of functional groups of β -lactamase I

Modification of the various functional groups of β -lactamase I was accomplished by treatment of the enzyme with group specific reagents.

Details of the procedures adopted for each of the functional groups investigated are presented below.

Modification of guanidino groups was accomplished using DT (13). Changes in enzymatic activity during the reaction were monitored using aliquots taken at desired intervals and assaying with benzylpenicillin (1 mM in phosphate buffer, pH 7.0) as substrate. In studies designed to assess the influence of light on DT induced modifications, the control and DT-treated samples, following an initial 12 h incubation in the dark, were irradiated for 5 min using a Black-Lit U.V. light source (13).

Tyrosine residues of the protein were modified by reaction with TNM (16). The progress of the reaction was monitored by following the changes in enzymatic activity (with 1 mM-benzylpenicillin in acetate buffer, pH 5.5, as substrate) using aliquots drawn at appropriate intervals.

The conversion of histidine residues of the enzyme to the corresponding N-carbethoxy derivatives was accomplished by treatment with DEP according to the procedure of FERENCZ et al. (12). The number of histidine residues modified was determined from $\Delta A_{240\text{nm}}$ using the ϵ_m value of $3.2 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$ for N-carbethoxy imidazole.

Modification of tryptophan residues with NBS was accomplished according to the procedure of PATCHORNIK et al. (29).

Trinitrophenylation of the ϵ -NH₂ groups of lysine residues was accomplished by reaction with TNBS (28). In these experiments, β -lactamase solutions were extensively dialyzed against water to remove all traces of free amino acids and other small molecular weight components. Similar modification of lysine residues of β -lactamase was also performed in the presence of methicillin (2 mM) in the reaction mixture. The presence of methicillin or the penicilloic acid of methicillin (a product of β -lactamase catalyzed hydrolysis) was found to have no effect on the rate of hydrolysis of TNBS in the absence of the enzyme.

The modification of the carboxyl groups of β -lactamase I was achieved by reaction with a water soluble carbodiimide (100 mM) in the presence of taurine (500 mM). The pH of the reaction was maintained at 4.75 by the addition

of appropriate amounts of HCl. At the indicated times, samples were removed for the spectrophotometric determination of residual enzymatic activity, and the remainder of the mixture treated with 4.0 ml acetate buffer (pH 4.0) in order to terminate further reaction. This mixture was dialyzed extensively against water, then against hydroxylamine (500 mM) at pH 7.0 for 5 h. After removal of hydroxylamine by dialysis against water, the material was acid hydrolyzed and subjected to amino acid analysis in order to assess the amount of taurine incorporated. A reagent blank, subjected to identical operational procedures but excluding the enzyme, served as a control in the estimation of free residual taurine in the dialyzed preparation. The amount of taurine incorporated into β -lactamase samples by this procedure provided a reliable and quantitative assessment of the extent of carboxyl modification by DCC.

2.6. C-terminal analysis

A sample of lyophilized β -lactamase I (extensively dialyzed against water prior to freeze-drying) was dissolved in 2.0 ml of acetate buffer (200 mM), pH 5.5, and treated with formamide to achieve a final concentration of 55.2 μM and 6.0 M with respect to enzyme and denaturant, respectively. The solution was heated to 60 °C for 10 min and was subsequently cooled to room temperature and allowed to stand for 15 min. Following removal of a 100 μl aliquot (which served as control), the remainder of the solution was treated with 100 μl of a carboxypeptidase Y solution, to achieve a final exopeptidase concentration of 0.5 μM . The reaction was allowed to proceed at room temperature for 10 h. Aliquots (250 μl , corresponding to 10 nmoles of β -lactamase) were taken at regular intervals and used in the determination of enzymatic activity and of amino acid released.

Two methods (A & B) were employed in the assessment of enzymatic activity by the spectrophotometric procedure using N-(2-furyl) acryloyl penicillin (1 mM in 100 mM-acetate buffer, pH 5.5) as substrate. In method A, β -lactamase activity was determined, either prior to or following heating to 60 °C and carboxypeptidase Y treatment, on aliquots that had been appropri-

ately diluted in acetate buffer (pH 5.5) and allowed to stand for 2 min prior to use in the assay. In method B, such dilution of aliquots and subsequent standing was omitted, and activity measurements were performed by directly introducing 2 μ l aliquots of reaction mixture to assay medium containing formamide (6M). Method A allows for the estimation of activity potentially recoverable due to refolding of the denatured enzyme upon reduction of the concentration of denaturant. In method B, such reactivation is minimized, thereby giving an estimation of the degree of enzyme denaturation.

The assessment of the free amino acids released was accomplished as follows: each aliquot was added to 1.5 g Dowex-50 X-8 (100–200 mesh H⁺ form) in 3.0 ml water (pH 2–3). After stirring for 30 min at room temperature, the resin was packed into a small column (10 $\times$ 0.5 cm) and eluted with two volumes of water. This fraction was discarded. Amino acids (released from the C-terminus of β -lactamase by carboxypeptidase Y treatment) were then eluted from the resin with 10 ml ammonium hydroxide (5 N). The eluted material was taken to dryness under reduced pressure and transferred to vials with small volumes of water. The various samples were lyophilized, redissolved in 75 μ l HCl (10 mM) and analyzed for amino acid content on a Durrum amino acid analyzer.

3. RESULTS

3.1. Characterization of the enzyme

SDS-polyacrylamide gel electrophoresis of the enzyme preparation chromatographed twice on Sephadex G 100 gel revealed the presence of a single component representing more than 95 % of the protein sample applied. The molecular weight of the purified enzyme preparation, estimated from SDS-polyacrylamide electrophoresis, is 27,500, which agrees well with previously published data (25). In addition, the specific activity of the enzyme (0.35 moles benzylpenicillin hydrolyzed/mg protein/h at 30 °C, pH 7.0) is similar to that reported by PATIL and DAY (30). However, wide variations exist in the amino acid content of β -lactamase I isolated in different laboratories (6, 22, 25); thus, in this case, amino acid composition appears to be an unsatisfactory method for determining purity.

3.2. Chemical modification

3.2.1. Arginine

Treatment of the enzyme with a 100-fold molar excess of DT for 24 h in the dark resulted in modification of three of the ten arginine residues present in the protein (Table I). Very little change in catalytic activity was noted under these conditions. Irradiation of DT treated samples with UV light had no observable effect upon enzymatic activity. Prolonged incubation of the enzyme with DT in the absence of light resulted in the modification of two additional arginine residues with no effect on enzymatic activity. Thus, it would appear that only half of the ten residues present in the native protein are susceptible to reaction with DT.

3.2.2. Tyrosine

Incubation of the enzyme with a 10 fold molar excess of TNM resulted in approximately 30 % loss in activity with concomitant nitration of two of the seven tyrosine residues present in the protein (Table II). At this concentration of TNM, extending the incubation period beyond 2 h failed to produce further inactivation of the enzyme. Treatment with a 100 fold molar excess of the reagent resulted in 50 % loss of enzymatic activity as well as the modification of an additional tyrosine residue. Conversion of the nitro groups in the modified enzyme to amino groups by reduction with dithionite (18) did not result in the restoration of a fully active enzyme. Thus, the partial loss in activity accompanying nitration appears to be due to steric effects rather than due to change in pK_a of phenolic functions.

3.2.3. Histidine

Reaction of β -lactamase with DEP, at pH 6.0, resulted in the modification of approximately 3.8 to 4.0 histidine residues present in the protein. Despite such modification of all the histidine residues, enzymatic activity remained unaffected. These data are in agreement with those recorded earlier (12).

3.2.4. Tryptophan

Oxidation of the indole side chains of tryptophan residues was accomplished by treatment of

Table I**Amino acid analysis of DT treated β -lactamase I^a.**

Amino Acid	Residues/Mole Control	Protein ^b DT Treated	Amino Acid	Residues/Mole Control	Protein DT Treated
Lys	(21)	21.0	$\frac{1}{2}$ Cys	(0)	0
His	3.8 (4)	3.8	Ala	23.9 (24)	23.6
Arg	9.9 (10)	6.8	Val	15.2 (15)	13.5
Asx	28.0 (28)	28.3	Met	2.0 (2)	2.8
Thr	18.6 (19)	18.9	Ile	16.5 (17)	17.0
Ser	13.2 (13)	13.7	Leu	16.1 (16)	16.9
Glx	21.6 (22)	21.9	Tyr	7.4 (7)	6.2
Pro	7.0 (7)	6.9	Phe	5.9 (6)	6.0
Gly	17.1 (17)	16.5	Trp	2.9 (3)	

^a Two millilitres of β -lactamase solution (37.4 μ M) in 200 mM-phosphate buffer, pH 6.0 was treated with DT (19.1 mg), to achieve a final concentration of 3.7 mM with respect to the reagent and allowed to react at 30 °C for 24 h. An identical solution of the enzyme, untreated with DT, served as control. After completion of the reaction, the samples were dialyzed extensively against distilled water and lyophilized prior to the assessment of their amino acid content.

^b Based upon 21 lysine residues/mole of protein.

Values in parentheses denote the probable number of residues in the protein. Tryptophan content was estimated by titration with N-bromosuccinimide (13).

Table II**Effect of TNM on enzymatic activity and tyrosine content of β -lactamase I^a**

Time of Reaction (min)	$\frac{[\text{TNM}]}{[\text{ENZ}]}$			
	10		100	
	Activity (% control)	Tyrosine ^b Residues/Mole	Activity (% control)	Tyrosine Residues/Mole
0	100	7.00	100	7.00
5	—	—	61.6	5.3
20	95.3	—	—	—
30	—	—	48.6	—
45	89.8	—	—	—
60	—	—	46.2	4.3
90	83.8	—	—	—
120	73.4	5.2	—	—

^a One millilitre of β -lactamase (44 μ M) in 100 mM-Tris buffer, pH 8.5 was treated with aliquots of an ethanolic solution of TNM (41 mM) to achieve either a 10 or 100 fold molar excess of the reagent with respect to the protein. An identical sample of enzyme treated with an equivalent amount of ethanol served as control. Upon achievement of approximately 30% inactivation in TNM treated samples, further reaction was terminated by the addition of 50 μ l of mercaptoethanol (1.5 M). Following extensive dialysis against water, the protein solutions were lyophilized prior to the assessment of their tyrosine content. Activity measurements were performed spectrophotometrically in 50 mM-acetate buffer, pH 5.5, with benzylpenicillin (1 mM) as substrate. Activity of control (untreated with TNM) remained unaffected over a period of 2 h. Degree of inhibition was estimated with reference to control.

^b Values based on 6.0 moles of phenylalanine per mole of protein.

Figure 1. β -Lactamase I activity as a function of the number of tryptophan residues modified by N-bromosuccinimide.

A solution of β -lactamase I (21.3 μ M) in acetate buffer (100 mM), at pH 4.0, was treated stepwise with aliquots (10 μ l) of N-bromosuccinimide solution (5 mM) and the number of tryptophan residues modified was estimated (29). The activity determinations were performed after each addition of an aliquot of N-bromosuccinimide.

Figure 2. Relationship between the number of lysine residues modified and enzymatic activity upon treatment with trinitrobenzenesulfonate.

Reaction conditions employed were: borate buffer (500 mM), pH 8.25; enzyme concentration 2.15 μ M; trinitrobenzenesulfonate concentration, 350 μ M; temperature, 25 °C. Enzyme activity was assessed spectrophotometrically at various times using benzylpenicillin (1 mM) at pH 7.0. The reaction was conducted in the absence and presence of methicillin (2 mM). The number of lysine residues modified was calculated on the basis of the observed increase in $A_{367\text{nm}}$ accompanying trinitrophenylation of the amino acid, using an ϵ_M value of $1.1 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$. No methicillin (▼); methicillin (2 mM) (▽); activity data in the absence (○) and presence (●) of methicillin.

Figure 3. Inactivation of β -lactamase I by a water soluble carbodiimide.

An aqueous solution of enzyme (51.4 μ M) containing taurine (500 mM) was treated with solid carbodiimide to achieve a final concentration of 100 mM with respect to the reagent. The pH of the reaction was maintained at 4.75 by the addition of appropriate amounts of HCl. At the intervals shown, activity was determined spectrophotometrically using benzylpenicillin as substrate at pH 7.0; temperature 25 °C.

the enzyme with NBS. Results illustrated in Figure 1 show that approximately 2 tryptophan residues can be modified with no apparent alteration in catalytic function. The loss of activity accompanying the modification of the third tryptophan residue could well be due to gross conformational changes in the protein in view of the appearance of turbidity in the latter stages of the reaction.

3.2.5. Lysine

Reaction of β -lactamase I with TNBS resulted in the facile modification of 12 of the 21 lysine residues in the protein without any effect on catalytic activity (Figure 2). Inclusion of methicillin, a substrate known to induce discrete conformational changes in the protein (7, 32), in the reaction medium resulted in an initial rapid rate of modification. After 50 min the rate reverted to that normally observed in the absence of methicillin. The initial rapid increase in $A_{367\text{nm}}$ in the presence of methicillin corresponded to the modification of 3–4 additional lysine residues over that normally noted in the absence of this substrate (Figure 2). It would appear that methicillin induced conformational changes in the enzyme are such as to expose additional lysine residues, rendering them susceptible to rapid reaction with TNBS.

3.2.6. Dicarboxylic amino acids

Previous studies had demonstrated rapid inactivation of β -lactamase I in the presence of a water soluble carbodiimide (37). In the current investigation, taurine was used to convert the reactive O-acylisourea intermediate (formed during the reaction of DCC with carboxyls) to a stable amide derivative. An estimation of the amount of taurine incorporated provided a means of assessing the number of carboxyl groups modified. As shown in Figure 3, reaction of the enzyme with DCC in the presence of taurine resulted in a rapid loss of activity, 50 % inactivation being noted within 5 min. Complete inactivation was found within 1 h of the initiation of the reaction. Based on the amount of taurine incorporated into the enzyme, complete inactivation of the enzyme was found to be accompanied by the modification of 4 to 5

carboxyl functions of the protein. Analysis of the partially inactivated enzyme (approximately 50 % loss of activity) revealed the modification of two to three carboxyl groups of the protein. Kinetic analyses of the partially inactivated enzyme showed its K_m value (47 μM) for benzylpenicillin to be very similar to that found in the case of unmodified enzyme.

3.3. C-terminal amino acid

Earlier attempts using enzymatic and chemical methods have failed to reveal any C-terminal residue in β -lactamase I (35, 37). The broad specificity of carboxypeptidase Y as well as its resourcefulness as an agent in the elucidation of the amino acid sequence of the C-terminal region of proteins (17, 26) prompted us to reinvestigate the nature of the C-terminal residue of β -lactamase I. Initial experiments were directed towards finding conditions that would selectively denature β -lactamase without appreciably affecting the function of carboxypeptidase Y. Media containing high concentrations of urea or guanidine hydrochloride were found to effect denaturation of both proteins and hence could not be used. When enzymatic activities were determined in the presence of formamide (6 M), carboxypeptidase Y was found to retain 33 % of its original activity, while β -lactamase activity appeared to be unaffected. However, β -lactamase solutions, heated to 60 °C for 10 min in the presence of formamide (6 M), were found to be devoid of activity when assayed (following cooling to room temperature) in medium containing formamide (6 M). Such heat inactivated preparations of β -lactamase reacquired more than 50 % of their original activity provided they were incubated in buffer (formamide free) solutions for two minutes prior to their assay in medium devoid of denaturant. These results are consistent with earlier reports which have shown that partial reactivation of denatured β -lactamase occurs subsequent to the removal of denaturing agents or conditions (8). Thus, this procedure not only rendered possible the treatment of partially denatured (inactive) β -lactamase with active carboxypeptidase Y but also permitted a means for estimating the degree of β -lactamase activity recoverable following such treatment.

Table III

Amino acid release from denatured β -lactamase I upon carboxypeptidase Y treatment.

Time of Reaction (h)	Activity ² (% Control)	Moles Released/Mole Protein ¹				
		Leu	Ile	Ala	Val	Pro
0	—	0	0	0	0	0
0.5	100	0.62	0.02	0.03	0.02	0.02
1	100	0.63	0.01	0.17	0.09	0.03
2	51.8	0.83	0.20	0.16	0.13	0.01
2.75	50.3	0.55	0.18	0.12	0.14	0.01
3.75	60.4	—	—	—	—	—
4.75	33.3	1.08	0.60	0.45	0.41	0.07
5.75	23.3	1.04	0.75	0.58	0.49	0.15
6.75	20.9	1.61	1.26	1.07	0.85	0.31
10	6.4	1.78	1.73	1.37	1.02	0.43

¹ Values normalized by subtracting values at $t = 0$. The amounts of other amino acids released were negligible, corresponding to less than 0.1 mole per mole of protein.

² Measured spectrophotometrically at pH 5.5, in the presence and absence of formamide (6 M) with N-(2-furyl) acryloyl penicillin (1 mM) as substrate.

Treatment of partially denatured β -lactamase (by the method described above) with carboxypeptidase Y resulted in a gradual decrease in the recoverable enzymatic activity (Table III). Analyses for the released amino acids revealed a rapid appearance of leucine in amounts corresponding to one mole per mole of β -lactamase. Upon prolonged incubation release of Ile, Val, Ala and another Leu was observed in amounts corresponding to one mole of residue per mole of protein. The sequence of the release of these amino acids was not determined. However, the rapid and stoichiometric release of leucine suggests that it occupies the C-terminal position.

4. DISCUSSION

Elucidation of the chemical basis for catalytic function of β -lactamase is vital to the development of appropriate antagonists that can help overcome β -lactam resistance noted with β -lactamase producing microorganisms. The extensive homology in the primary structures of penicillinases from different microbial origin (2) would suggest that all these enzymes probably share a common catalytic mechanism in the hydrolysis of penicillins. Consequently, identification of functional groups essential for catalytic activity in one of these enzymes is of considerable

importance. The availability of *B. cereus* 569/H β -lactamase I in large amounts as well as the relative ease of its purification, renders this enzyme an attractive choice for studies designed to elucidate the structure-function relationship.

A systematic study of the role played by various functional groups of the enzyme revealed that nearly 5 of the ten arginine residues and of 12 to 15 of the twenty-one lysine residues present in the protein can be altered without impairment of catalytic function. Likewise, all four histidine residues of the enzyme can be modified with no effect on catalytic activity. The observations suggest that these susceptible residues are neither involved in the catalytic mechanism nor in the maintenance of the protein in a conformation essential for catalysis. A substantial percentage of the protein's cationic residues could be modified without affecting activity, suggesting that electrostatic interactions may not play a critical role in maintaining active enzyme conformations.

Two of the three tryptophan residues could be readily oxidized with no apparent effect on enzymatic activity. The loss of activity accompanying the oxidation of the third tryptophan residue would appear to be due to side reactions leading to gross conformational changes in the enzyme. Thus, tryptophan residues do not

appear to play a major role in either the binding of the substrate or the preservation of conformation(s) essential for catalytic function. This view is further supported by the observation that β -lactamase I fails to undergo photodynamic inactivation in the presence of DT, a compound known to induce rapid photo-destruction of tryptophan residues (13).

Previous reports have shown that partial inactivation of β -lactamases (from a variety of sources) accompanies treatment with tyrosine modifying reagents. In at least one case (33) a tyrosine residue has been implicated in the mechanism of β -lactamase (from *E. coli*) catalysis. We have found that modifications of 2–3 tyrosine residues in the enzyme for *B. cereus* result in only a modest degree of inactivation. These results suggest that either tyrosine residues are not involved in the catalytic mechanism of β -lactamase I or that a critical phenolic group becomes exposed only under conditions which induce the enzyme to adapt to specific conformation(s).

Rapid inactivation of the enzyme by DCC (37) and other carboxyl modifying agents have indicated the presence of carboxyl function(s) in the active site of the β -lactamase I. However, these studies were qualitative in that no information concerning the actual number of carboxyl groups undergoing modification was provided. Current investigations have revealed that the rapid and complete inactivation of the enzyme by DCC is accompanied by the modification of approximately five carboxyl groups of the protein. Furthermore, approximately 50% loss of activity was found to be associated with the modification of two to three carboxyl functions. The correspondence noted between the extent of inactivation and the number of modified carboxyls would imply that a large number of such groups are equally susceptible to the action of DCC, although only one or two of them may actually be involved in the catalytic mechanism (9, 30). Thus, the partially inactive enzyme (with 50% loss of activity) may simply be a mixture of fully active, unmodified enzyme and totally inactive protein with nearly five of its carboxyls modified. However, the K_m value (47 μM) of the partially inactivated enzyme for benzylpenicillin was almost identical with the value (54 μM) obtained with the unmodified enzyme. These

observations provide indirect evidence for the view that partially inactivated enzyme is probably a mixture of fully active and inactive species. A change in K_m value for the substrate would have been expected for the alternative situation of homogeneity of the enzyme molecules, each with 2 to 3 of its carboxyls modified.

A survey of the results of modification of various functional groups indicates that none of those investigated in the current study, with the probable exception of carboxyl functions, may be involved in the catalytic mechanism of the enzyme. The results of carboxyl modification are also ambiguous since the reaction does not appear to be specific to those residues present in the active site of the enzyme. The large number of carboxyl groups modified raises the possibility of the inactivation occurring as a consequence of gross conformational changes in the protein. Thus, the results of the current studies point to the futility of the conventional approach of chemical modification by group specific reagents for the identification and/or localization of functional groups essential for the function of β -lactamase. These observations may simply indicate that the active site does not pre-exist in the enzyme but that its formation is induced by the presence of substrate. Thus, β -lactamase I may serve as an excellent model for substrate induced conformational changes (20) leading to the formation of a functional catalytic site. This idea derives substantial support from the recently demonstrated conformational responses of the enzyme of A and S type penicillins (7, 32). The observation that the number and reactivity of TNBS susceptible lysine residues in the enzyme are altered in the presence of substrate (Figure 2) is also consistent with this concept. Thus, chemical modification of β -lactamase I in the presence of substrate or its analogues appears to be vital for the identification of chemical groups involved in the catalytic process. The rapid and specific inactivation of β -lactamase I with active-site orientated reagents, such as ampicillin-nitrite (9, 30) and clavulanic acid (4, 11, 14, 23) reinforces this conclusion.

Earlier attempts using carboxypeptidase A and B and the hydrazinolysis procedure had failed to detect any amino acid at the C-terminus of β -lactamase I. In the current study, treatment of partially denatured enzyme with carboxypepti-

dase Y resulted in the rapid release of nearly one mole of leucine per mole of protein. Further treatment resulted in the liberation of additional amino acids (Ile, Val, Ala, and Leu). While the sequence of release of these residues was not established, their highly non-polar nature suggests that the C-terminal region of the protein may occupy a hydrophobic location in the native enzyme. In addition, the release of the C-terminal leucine from the protein prevents the recovery of enzymatic activity upon removal of denaturing conditions, presumably due to the inability of the modified protein to refold and renature under the normal conditions of the assay.

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REFERENCES

1. AMBLER, R. P. & R. J. MEADWAY: Chemical structure of bacterial penicillinases. *Nature* 222, 24–26 (1969)
2. AMBLER, R. P. & G. K. SCOTT: Partial amino acid sequence of penicillinase coded by *Escherichia coli* plasmid RGK. *Proc. Nat. Acad. Sci. (USA)* 75, 3732–3736 (1978)
3. BRISTOW, A. & R. VIRDEN: Preferential nitration with tetranitromethane of a specific tyrosine residue in penicillinase from *Staphylococcus aureus* DCI. *Biochem. J.* 169, 381–388 (1978)
4. CHARNAS, R. L., J. FISHER & J. R. KNOWLES: Chemical studies of the inactivation of *Escherichia coli* RTEM β -lactamase by clavulanic acid. *Biochemistry* 17, 2185–2189 (1978)
5. CITRI, N. & N. GARBER: The effect of urea on the activity of penicillinase. *Biochim. Biophys. Acta* 30, 664–665 (1958)
6. CITRI, N. & M. R. POLLOCK: The biochemistry and function of β -lactamase. *Adv. Enzymology* 28, 237–323 (1966)
7. CITRI, N., A. SAMUNI & N. ZUK: Acquisition of substrate-specific parameters during the catalytic reaction of penicillinase. *Proc. Nat. Acad. Sci. (USA)* 73, 1948–1952 (1976)
8. DAVIES, R. B., E. P. ABRAHAM & D. G. DALGLEISH: Conformation changes in the extracellular β -lactamase I from *Bacillus Cereus* 569/H/9. *Biochem. J.* 143, 137–141 (1974)
9. DURKIN, J. P., G. I. DMITRIENKO & T. VISWANATHA: Reversibility of the ampicillin and nitrite induced inactivation of β -lactamase I. *Can. J. Biochem.* 55, 453–457 (1977)
10. DURKIN, J. P., G. I. DMITRIENKO & T. VISWANATHA: N-(2-furyl) acryloyl penicillin: A novel compound for the spectrophotometric assay of β -lactamase I. *J. Antibiotics* 30, 883–885 (1977)
11. DURKIN, J. P. & T. VISWANATHA: Clavulanic acid inhibitor of β -lactamase I from *B. cereus* 569/H. *J. Antibiotics* 31, 1162–1169 (1978)
12. FERENCZ, I., I. MILE & V. CSANY: The role of histidine side chains in the catalytic activity of *B. cereus* exopenicillinase. *Acta. Biochim. Biophys. Acad.* 6, 5–7 (1971)
13. FLISS, H., N. M. TOZER & T. VISWANATHA: The reaction of chymotrypsin with 2,3-butanedione trimer. *Can. J. Biochem.* 53, 275–283 (1975)
14. FISHER, J., R. L. CHARNAS & J. R. KNOWLES: Kinetics studies on the inactivation of *Escherichia coli* RTM β -lactamase by clavulanic acid. *Biochemistry* 17, 2180–2184 (1978)
15. FISHER, J. & J. R. KNOWLES: Bacterial resistance to β -lactams: The β -lactamases. *Ann. Rep. Medicinal Chemistry* 13, 239–248 (1978)
16. GLAZER, A. N., R. J. DELANGE & D. S. SIGMAN: In *Laboratory Techniques, in Biochemistry and Molecular Biology* (Work T. S. & Work E., eds.) Vol. 4, Pt. 1, North-Holland, Amsterdam (1975)
17. HAYASHI, R. S., S. MOORE & W. H. STEIN: Carboxypeptidase from yeast. Large scale preparation and the application to COOH-terminal analysis of peptides and proteins. *J. Biol. Chem.* 248, 2296–2302 (1973)
18. KENNER, R. A. & H. NEURATH: Activity and fluorescent derivatives of aminotyrosyl trypsin and trypsinogen. *Biochemistry* 10, 551–557 (1971)
19. KOGUT, M., M. E. POLLOCK & E. J. TRIDGELL: Purification of penicillin-induced penicillinase of *Bacillus cereus* NRRL 569: A comparison of its properties with those of a similarly purified penicillinase produced spontaneously by a constitutive mutant strain. *Biochem. J.* 62, 391–401 (1956)
20. KOSHLAND, D. E.: Application of a theory of enzyme specificity to protein synthesis. *Proc. Nat. Acad. Sci. (USA)* 44, 98–104 (1958)
21. KUWABARA, S.: Purification and properties of two extracellular β -lactamases from *Bacillus cereus* 569/H. *Biochem. J.* 118, 457–465 (1970)
22. KUWABARA, S., E. D. ADAMS & E. P. ABRAHAM:

- Composition of β -lactamase I and β -lactamase II from *Bacillus cereus* 569/H. *Biochem. J.* 118, 475–480 (1970)
23. LABIA, R. & J. FEDUZZI: Cinetique de l'inhibition de beta-lactamases par l'acide clavulanique. *Biochim. Biophys. Acta* 526, 572–579 (1978)
 24. LANG, C. A.: Simple microdetermination of Kjeldahl nitrogen in biological materials. *Anal. Chem.* 30, 1692–1694 (1958)
 25. LLOYD, P. H. & A. R. PEACOCK: Sedimentation-equilibrium studies of the heterogeneity of two β -lactamases. *Biochem. J.* 118, 467–474 (1970)
 26. MARTIN, B., I. SVENDSEN & M. OTTESEN: Use of carboxypeptidase Y for carboxy-terminal sequence determination in proteins. *Carlsberg Res. Commun.* 42, 99–102 (1977)
 27. MILLER, G., G. BACH & Z. MARKUS: Production of *Bacillus cereus* exo-penicillinase on a pilot plant scale. *Biotechnol. Bioeng.* 7, 517–528 (1965)
 28. OKUYAMA, T. & K. SATAKE: On the preparation and properties of 2,4,6-trinitrophenyl-amino acids and peptides. *J. Biochem. (Tokyo)* 47, 454–466 (1960)
 29. PATCHORNIK, A., W. B. LAWSON, E. GROSS & B. WITKOP: The use of N-bromosuccinimide and N-bromoacetamide for the selective cleavage of C-tryptophyl peptide bonds in model peptides and glucagon. *J. Am. Chem. Soc.* 82, 5923–5927 (1960)
 30. PATIL, G. U. & R. A. DAY: Involvement of a carboxyl group in the active site of *Bacillus cereus* 569/H penicillinase (β -lactamase I). *Biochim. Biophys. Acta* 293, 490–496 (1973)
 31. POLLOCK, M. R.: In the enzymes (Boyer, P. D., Lardy, H., & Myrback, K., eds.) Vol. 4, ed. 2, p. 269 (1960), Academic Press, New York.
 32. SAMUNI, A. & N. CITRI: Diphasic kinetics induced by modified substrates of penicillinase. *Biochim. Biophys. Res. Commun.* 62, 7–11 (1975)
 33. SCOTT, G. K.: Structure and mechanism of β -lactamase from *Escherichia coli*. *Biochem. Soc. Trans.* 1, 159–162 (1973)
 34. SPACKMAN, D. H., W. H. STEIN & S. MOORE: Automatic recording apparatus for use in the chromatography of amino acids. *Anal. Chem.* 30, 1190–1206 (1958)
 35. THATCHER, D. R.: The partial amino acid sequence of the extracellular β -lactamase I of *Bacillus cereus* 569/H. *Biochem. J.* 147, 313–326 (1975)
 36. WALEY, S. G.: A spectrophotometric assay of β -lactamase action on penicillins. *Biochem. J.* 139, 789–790 (1974)
 37. WALEY, S. G.: The pH-dependence and group modification of β -lactamase I. *Biochem. J.* 149, 547–551 (1975)
 38. WEBER, K. & M. OSBORN: The reliability of molecular weight determinations by dodecyl sulfate-polyacrylamide gel electrophoresis. *J. Biol. Chem.* 244, 4406–4412 (1969)