Identification of an essential glutamic acid residue in β -lactamase II from *Bacillus cereus*

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β -Lactamase II from *Bacillus cereus* was readily inactivated by incubation at pH 4.75 with a water-soluble carbodi-imide plus a suitable nucleophile. In the early stages of the reaction, 1 equivalent of nucleophile was incorporated/equivalent of enzyme, whereas during the later stages a second equivalent of nucleophile was also incorporated. This latter process correlated with the blocking of the enzyme's single thiol group. Enzyme inactivated in the presence of the coloured nucleophile *N*-(2,4-dinitrophenyl)ethylenediamine was fragmented by pepsin digestion, and coloured peptides were isolated by gel filtration and h.p.l.c. Two major peptides, representing 52% of the incorporated label, were isolated and sequenced. Both peptides contained the incorporated label on glutamic acid-37, and it is concluded that this latter residue represents a catalytically essential carboxylic residue in β -lactamase II.

INTRODUCTION

Among the penicillin-hydrolysing enzymes, β -lactamase II of *Bacillus cereus* stands out as being rather unusual. This enzyme, which hydrolyses both penicillins and cephalosporins, is a zinc-metalloenzyme (Sabath & Abraham, 1966) that is not susceptible to those reagents that inactivate other β -lactamases by reacting with a serine residue. β -Lactamase II is readily inactivated by chelating agents and, especially in the absence of free Zn^{2+} , by agents modifying the single thiol residue, a moiety that seems involved in metal co-ordination in β -lactamase II (Sabath & Abraham, 1966; Davies & Abraham, 1974; Baldwin *et al.*, 1980; Bicknell & Waley, 1985a,b). No other inactivators of this enzyme have been reported. As part of an examination of the active site of β -lactamase II, the possibility of the involvement of carboxylic residues in the catalytic activity was studied. For this purpose the combination of a water-soluble carbodi-imide and a nucleophile was used to treat the enzyme. Such reagents are commonly used for the modification of protein carboxy groups (Hoare & Koshland, 1967; Carraway & Koshland, 1972).

MATERIALS AND METHODS

Materials

β -Lactamase II was purified from the culture supernatant of *B. cereus* 569/H/9 as described by Davies *et al.* (1974). EDC [1-ethyl-3-(3-dimethylaminopropyl)carbodi-imide] was obtained from Sigma Chemical Co., St. Louis, MO, U.S.A., and *N*-(2,4-dinitrophenyl)ethylenediamine hydrochloride (DNP-ethylenediamine) from Calbiochem A.G., Lucerne, Switzerland.

Determination of enzyme activity

Enzyme activity was measured at 30 °C and pH 6.0 by continuous titration as described by Davies *et al.* (1974).

Inactivation conditions

Unless otherwise stated, enzyme (1–2 mg/ml) was incubated in 0.15 M-NaCl/10 mM-ZnCl₂/5 mM-DNP-ethylenediamine. The reaction mixture was maintained at pH 4.75 and 22–24 °C in a pH-stat. When the extent of incorporation of nucleophile into the enzyme was to be measured, the enzyme was incubated with EDC and DNP-ethylenediamine for 45 min and then dialysed at 4 °C against three changes of 0.15 M-NaCl/0.1 mM-ZnCl₂/10 mM-sodium acetate buffer, pH 6.0. Nucleophile concentration was measured at 360 nm by using $\epsilon = 15000 \text{ M}^{-1} \cdot \text{cm}^{-1}$ (Huc *et al.*, 1975). Enzyme concentrations were measured by A_{280} by using $\epsilon = 26000 \text{ M}^{-1} \cdot \text{cm}^{-1}$ (Davies, 1973). The A_{280} of solutions of enzyme containing incorporated nucleophile was corrected for the A_{280} of the latter according to the equation:

$$A_{280}(\text{enzyme}) = A_{280}(\text{complex}) - \frac{A_{360}(\text{complex})}{3}$$

where $A_{280}(\text{enzyme})$ is the A_{280} from the protein, and $A_{280}(\text{complex})$ and $A_{360}(\text{complex})$ refer to the absorbances of the modified enzyme.

For the preparation of labelled peptides, 25 mg of β -lactamase II was inactivated to 30% residual activity with EDC and DNP-ethylenediamine and dialysed against 0.15 M-NaCl/0.1 mM-ZnCl₂/10 mM-sodium acetate buffer, pH 6.0, and then against 50 mM-NH₄HCO₃/0.1 mM-ZnCl₂, and finally freeze-dried. The residue was divided into two, and each half was taken up in 0.5 ml of 5% (v/v) formic acid, and to this was added 0.1 ml of 1% (w/v) pepsin in 10% (v/v) acetic acid. The mixture was agitated at 37 °C for 6 h and then subjected to gel filtration.

Thiol assays were carried out as described by Ellman (1959) on enzyme samples denatured by heating very briefly at 100 °C in 1 mM-EDTA/0.1% (w/v) SDS/0.1 M-potassium phosphate buffer, pH 7.6.

Abbreviations used: EDC, 1-(3-dimethylaminopropyl)-3-ethylcarbodi-imide; DNP, 2,4-dinitrophenyl.

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H.p.l.c.

Reverse-phase h.p.l.c. was performed with a Lichrosorb RP (5 μ m particle size) column (4.6 mm $\times$ 250 mm). The elution medium was 0.1% trifluoroacetic acid (solvent A) and acetonitrile/propanol/0.1% trifluoroacetic acid (4:2:1, by vol.) (solvent B). A linear gradient of solvent B in solvent A was developed over 60 min.

Peptide analysis

For amino acid analyses, performic acid-oxidized samples were hydrolysed in 6 M-HCl at 110°C for 18 h. Amino acid composition was determined by using a Biochrom 4101 analyser (LKB, Bromma, Sweden). *N*-Terminal peptide residues were identified by the dansyl (5-dimethylaminonaphthalene-1-sulphonyl) procedure of Bruton & Hartley (1970).

Automatic liquid-phase sequencing of peptides was performed in a Beckman 890C sequencer, and the amino acid residues were identified by h.p.l.c. (Campbell *et al.*, 1981).

RESULTS

Inactivation of β -lactamase II by carbodi-imide plus nucleophile

Incubation of β -lactamase II under mild conditions of temperature and reagent concentrations caused a rapid loss of catalytic activity (Fig. 1). The rate of inactivation and the maximum extent of reaction increased with increasing concentrations of the carbodi-imide. Inactivation was demonstrated with the nucleophiles serine methyl ester and DNP-ethylenediamine, and was not reversed upon exhaustive dialysis of the treated enzyme against 0.15 M-NaCl/0.1 mM-ZnCl₂/10 mM-sodium acetate buffer, pH 6.0. β -Lactamase II was incubated with EDC plus DNP-ethylenediamine, and the extent of incorporation of the nucleophile into the enzyme together with the extent of enzyme inactivation were measured. It can be seen (Fig. 2) that for extents of inactivation less than 50% the loss of enzyme activity correlates with the incorporation of 1 mol of nucleophile/mol of enzyme. However, for near-total inactivation, the amount of nucleophile incorporated approaches 2 mol/mol of enzyme. Carbodi-imides generally react equally readily with thiol and carboxy groups (Carraway & Koshland, 1972), and so the possibility that thiol-group modification might be occurring under the present reaction conditions was examined. The data in Fig. 3 indicate thiol blocking, but only after considerable enzyme inactivation has already taken place. Thus at 80% inactivation the extent of thiol blocking is only about 0.2 mol/mol of enzyme. Assuming that 1 mol of DNP-ethylenediamine is incorporated/mol of thiol group blocked, the data in Fig. 2 may be corrected for this additional incorporation of label. So doing yields the data also shown in Fig. 2, where an excellent linear relationship between the loss of catalytic activity and DNP-group incorporation can be seen. Total inactivation now corresponds to the incorporation of about 1.1 mol of DNP group/mol of enzyme.

To check for the possible incorporation of nucleophile on to tyrosine residues, the modified enzyme was dialysed against 0.2 M-hydroxylamine for 24 h at room temperature. Since no change in A_{360} occurred (results not shown), it was assumed that no modification of tyrosine residues had occurred (Carraway & Koshland, 1972).

Fig. 1. Inactivation of β -lactamase II by a carboxy-group reagent

Enzyme (40 μ M) in 0.15 M-NaCl/10 mM-ZnCl₂ was incubated at pH 4.5 at 21 °C in a pH-stat with 5 mM-DNP-ethylenediamine and 6 mM-(●) or 61 mM-EDC (■), or with 20 mM-serine methyl ester and 20 mM-EDC (▲). Samples were removed from the reaction mixture after different times of incubation and assayed for enzyme activity.

Fig. 2. Stoichiometry of inactivation of β -lactamase II by carbodi-imide plus nucleophile

Enzyme was incubated in 3 ml portions with DNP-ethylenediamine plus various amounts of EDC (1.5–25 mM). The residual enzyme activity and extent of incorporation of nucleophile into the enzyme were measured (●). Further details are given in the Materials and methods section. The data were also corrected for incorporation of label into the cysteine residue by using data shown in Fig. 3 (□).

Separation and identification of labelled peptide

Peptides from a peptic digest of modified β -lactamase II (see the Materials and methods section) were fractionated by gel filtration (Fig. 4). The major band of yellow material was strongly retarded, having an elution volume slightly greater than the total column volume. Column fractions containing the DNP-labelled material

Fig. 3. Thiol-group modification during inactivation of β -lactamase II

Enzyme was incubated with DNP-ethylenediamine and EDC as described in the legend to Fig. 2, and the inactivated enzyme was then analysed for residual enzyme activity and thiol-group content. Further details are given in the Materials and methods section.

were pooled as shown in Fig. 4, concentrated by freeze-drying and further purified by h.p.l.c. The major peak of labelled material from gel filtration (peak B) yielded two major components of labelled material (Fig. 5). The amino acid compositions, *N*-terminal residues and amino acid sequences of these two components (I and II) were determined and are shown in Table 1 and Fig. 6. It was concluded that peptides I and II were identical except that peptide I was longer by a leucine residue at the *C*-terminus. By comparing the amino acid sequences of peptides I and II with the amino acid compositions, the unknown yellow residue, Xaa, at position three in the sequence must be glutamic acid to which the yellow-coloured nucleophile has been bound.

Analysis by h.p.l.c. of the other labelled fractions from Fig. 4 indicated that pools A-1 and A-2 contained several labelled components all present in low amounts. Pool C comprised mainly peptides I and II, as found in pool B. About 51% of the labelled material in pool C was in the form of peptides I and II. At least four other labelled peptides were also present (results not shown). By measuring the areas under the peaks in all the chromatograms, it was estimated that peptides I and II accounted for 52% of all the label in the peptic digest of modified enzyme.

DISCUSSION

The ready inactivation of β -lactamase II (*B. cereus*) by a water-soluble carbodi-imide in the presence of nucleophile suggests that the enzyme may contain one or more catalytically essential carboxylic residues. Extrapolation of the initial linear portion of a plot of fractional enzyme activity against nucleophile incorporation into the enzyme suggests that the inactivation arises from the modification of a single essential residue. However, for more extensive inactivation (over 50%), this plot becomes

Fig. 4. Separation of peptic peptides of modified β -lactamase II in gel filtration

A peptic digest of 25 mg of modified enzyme (0.9 ml) was applied to a column (0.45 cm $\times$ 140 cm) of Sephadex G-25 and eluted with 10% (v/v) acetic acid. Fractions (approx. 1 ml) were collected and the A_{280} (—) and A_{380} (----) were measured. Peak fractions containing the nucleophile label were pooled as indicated (A and B).

markedly non-linear, a feature that Rakitzis (1984) suggests is consistent with the involvement of many modifiable groups per active site. Fortunately, in the present case, extensive inactivation was associated with thiol-group modification, so that when this process was taken into consideration (see Figs. 2 and 3) the above-mentioned plot becomes linear throughout with total inactivation corresponding to the incorporation of approx. 1 mol of nucleophile/mol of enzyme. Thiol-group blocking was observed only at extents of inactivation greater than 40% and hence cannot be the primary mechanism of inactivation. Modification of the catalytically essential carboxylic residue apparently exposes the thiol group. Possibly the blocking of an active-site carboxylic residue results in the loss of the active-site Zn^{2+} , thereby exposing the metal-ion-liganding cysteine residue. Further evidence for the selective nature of the enzyme modification process comes from the fact that some 52% of the total incorporated nucleophile label was found to reside in two peptides isolated from the pepsin digest of the modified enzyme. Closer inspection of these two peptides showed that they were identical except that one was longer than the other by a single leucine residue. By comparing the sequences of these two peptides with that of the total protein, known thanks to the work of

Fig. 5. H.p.l.c. of pool B of nucleophile-labelled material from gel filtration of pepsin digest of modified β -lactamase II

Pool B of labelled peptides (see Fig. 4) was further fractionated by h.p.l.c. The column was eluted with a linear gradient of 30–60% (v/v) acetonitrile/0.1% trifluoroacetic acid/propanol (4:2:1, by vol.) in 0.1% trifluoroacetic acid. Fractions (approx. 0.75 ml) were collected and the A_{230} (—) and A_{360} (-----) were measured. Fractions were pooled (I and II) as indicated. Further details are given in the Materials and methods section.

R. P. Ambler on the enzyme, and of M. Hussain and J. O. Lampen on the gene coding for the enzyme (R. P. Ambler, personal communication, and Ambler *et al.*, 1985), the peptides can be identified as Asn-35–Gly-43 and Asn-35–Leu-44. Both peptides contained the incorporated label on Glu-37, and this residue can therefore be regarded as being catalytically essential. Since the modification of this residue seems to render the thiol group more reactive, the inactivation process might involve an active-site distortion whereby the catalytically essential Zn^{2+} dissociates from the enzyme.

An outline mechanism for β -lactamase II action may be proposed. This mechanism is based on the amino acid sequence, the work reported in the present paper, earlier

Table 1. Composition of labelled β -lactamase II peptides

Component	Composition (mol/mol of peptide)	
	Peptide I	Peptide II
Aspartic acid	1.6 (2)	1.7 (2)
Serine	1.0 (1)	1.2 (1)
Glutamic acid	1.3 (1)	1.4 (1)
Proline	1.1 (1)	0.7 (1)
Glycine	2.2 (2)	1.9 (2)
Alanine	1.2 (1)	0.9 (1)
Valine	1.1 (1)	1.2 (1)
Leucine	0.7 (1)	1.4 (1)*
DNP	1.1 (1)	1.2 (1)
No. of amino acid residues	10	9*
<i>N</i> -Terminus	Asparagine (or aspartic acid)	(Leucine)*, asparagine (or aspartic acid)

* Sample used for amino acid analysis gave results suggesting leucine and asparagine (or aspartic acid) as *N*-terminal residues. A second sample contained no *N*-terminal leucine. From this and from the sequencing data, it was concluded that the sample of peptide II used above contained free leucine.

Labelled peptide I	Asn-Gly-Xaa-Ala-Val-Pro-Ser-Asn-Gly-Leu
Labelled peptide II	Asn-Gly-Xaa-Ala-Val-Pro-Ser-Asn-Gly

Fig. 6 Sequences of labelled β -lactamase II peptides

The unidentified residue, Xaa, was strongly yellow-coloured.

spectroscopic work (Davies & Abraham, 1974; Baldwin *et al.* 1980; Hill *et al.*, 1980) and analogy with other Zn^{2+} -containing hydrolases (Vallee & Galdes, 1984), particularly thermolysin (Hangauer *et al.*, 1984) and carbonic anhydrase (Kannan *et al.*, 1977). A water molecule bound to the catalytic Zn^{2+} attacks the β -lactam carbonyl group, with glutamate-37 acting as a general

Scheme 1. Hypothetical outline mechanism for β -lactamase II action

base deprotonating the water molecule. Subsequently the proton withdrawn from the water molecule may be donated to the nitrogen atom of the β -lactam ring (Scheme 1). The advantage of proposing an explicit hypothesis is that it draws attention to some of the features needing experimental test. These features include the role of glutamate-37 and of Zn^{2+} -bound water. Further progress on the mechanism of β -lactamase II would be greatly assisted by knowledge of the three-dimensional structure of the enzyme.

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