Biochimica et Biophysica Acta, 293 (1973) 490–496

BBA 66809

INVOLVEMENT OF A CARBOXYL GROUP IN THE ACTIVE SITE OF *BACILLUS CEREUS* 569/H PENICILLINASE (β-LACTAMASE I)

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(Received August 1st, 1972)

SUMMARY

1. Penicillinase (penicillin amido-β-lactamhydrolase, EC 3.5.2.6) *Bacillus cereus* 569/H, was concentrated by means of the humidifier technique and low temperature evaporation to obtain the maximum yield of enzyme activity.

2. The enzyme was irreversibly inactivated by the products of diazotization of 6-aminopenicillanic acid and ampicillin.

3. The inactivation was found to be pH dependent.

4. The inactivated enzyme shows a new CD band which was eliminated by hydroxylamine treatment.

5. The inactivated enzyme was acid hydrolyzed under standard conditions. The acid hydrolyzates show approximately 1 mole of penicillamine/penicillinase.

INTRODUCTION

Penicillinases (penicillin amido-β-lactamhydrolase, EC 3.5.2.6) from Gram-positive and Gram-negative bacteria have been isolated and purified and in a few cases have been crystallized[1,2]. The amino acid composition of several penicillinases, the N-terminal analysis and peptide maps[1] of a few penicillinases have been reported[3]. The complete amino acid sequence of penicillinase from *Staphylococcus aureus* PC 1 and the partial sequence of penicillinases from *Bacillus licheniformis* 749/C have been reported by Ambler and Meadway[4]. The effect of the side chains on the interaction of penicillin with staphylococcal and bacillary penicillinases has been investigated by Depue *et al.*[5]. Considering the pH *vs* activity curve with benzyl penicillin the authors suggest the participation of histidine in the active site. The inactivation of penicillinase from *Bacillus cereus* 569/H by "diazotization products" of 6-aminopenicillanic acid and ampicillin had been reported by Day and Patil[6], and Patil and Day[7]. Similar results had been reported earlier by Day *et al.*[8]. Here we report the studies on the nature of this inactivation presenting evidence from which it may be deduced that an ester bond is formed between the inactivating agent and peni-

cillinase. The inactivating agents are unstable; attempts to isolate them have not been successful. The inactivation has been observed only when the diazotization of 6-aminopenicillanic acid or ampicillin is carried out in the presence of penicillinase.

MATERIALS AND METHODS

Spores of *Bacillus cereus* 569/H were provided by Dr Pollock, University of Edinburgh. 6-Aminopenicillanic acid (in part) and 7-amino-cephalosporanic acid were gifts from Eli Lilly and Co., and the ampicillin from Bristol Laboratories, Inc. Penicillin G was obtained from Nutritional Biochemical Corporation. The remainder of the 6-aminopenicillanic acid used in this work was purchased from Calbiochem. Penicillamine was obtained from Sigma Chemical Corporation.

The enzyme was prepared essentially by the method of Kogut *et al.*[2] or by the methods of Falter and Day[9] and of Madaiah[10]. Each time approximately 25 l of crude enzyme were concentrated by means of a humidifier (a commercial Kenmore portable humidifier Model 758,72910 Sears and Roebuck Company) to about 1.5 l. The crude mixture was centrifuged for 15 min at 12 000 × *g*, dialyzed in a double layered dialysis sack for 24 h against 0.1 M phosphate buffer (pH 7.0). It was further concentrated to approximately 300 ml by flash evaporator at 35–38 °C under reduced pressure, taking care to avoid bubbling or foaming. Several combined batches of the concentrated enzyme solution were then fractionated by $(NH_4)_2SO_4$ (ref. 10). All preparations of the enzyme were checked for purity and homogeneity by chromatography and by specific activity measurements. Some preparations were also checked for homogeneity by Tiselius electrophoresis and sedimentation velocity experiments. Assays were done by the method of Perret[11].

For preparation of enzyme for amino acid analyses, CD studies, and mapping $1 \cdot 10^6$ to $10 \cdot 10^6$ units of enzyme were treated with ampicillin or 6-aminopenicillanic acid (10 mg/ml) added as solid to the enzyme solution containing 10 mg/ml $NaNO_2$, pH 5 in 1 M sodium acetate buffer. The reaction was allowed to proceed at room temperature for approximately 0.5 h beyond the dissolution of the solid penicillin. The treated enzyme was then dialyzed against water (4–10 times 1000 vol.) in a double layered dialysis sack for approximately 24 h. In some preparations the treated enzyme was desalted by gel filtration through columns of Sephadex G-10. For inactivation studies alone about $1 \cdot 10^3$ units were used.

CD spectra were obtained on a Cary 60 Spectropolarimeter with a CD attachment.

Amino acid analyses were carried out essentially by the automated single column methods of Piez and Morris[12]. Penicillinase samples were hydrolyzed with 6 M HCl at 110 °C for 24 h in evacuated tubes.

Hydroxylamine treatment of the inactivated enzyme was done at room temperature for 24 h in a 1 M solution of NH_2OH (pH 7.5). For tryptic digestion the inactivated enzyme was lyophilized to dryness. The solid was treated with 80% (v/v) ethanol for a few hours. Alcohol-denatured enzyme was suspended in 2 ml of 0.1 M NH_4HCO_3 (pH 8.4) and to that trypsin (EC 3.4.4.4) (2% of the weight of the penicillinase contained in 0.1 ml, $1 \cdot 10^{-3}$ M HCl) was added. The mixture was then incubated for 6–8 h at 37 °C.

Peptide mapping was done on Whatman 3 MM chromatography paper (23 cm × 56.5 cm). The solvent system *n*-butanol–acetic acid–water (4:1:5, by vol.) was used for descending chromatography.

Penicillin derivatives were detected by the method of Helberg[15]. For pH dependence inactivation studies 1 M sodium acetate buffers of different pH were used when the enzyme was treated with 6-aminopenicillanic acid and nitrite.

TABLE I

CONCENTRATION OF CRUDE PENICILLINASE PRODUCED FROM *Bacillus cereus* 569/H

Stage of concentration	*Total activity (units)*	*Yield (%)*
1. Supernate of culture	$4.5 \cdot 10^7$	100
2. Humidifier	$3.8 \cdot 10^7$	84.4
3. Dialysis, then flash evaporation	$3.42 \cdot 10^7$	76
4. After complete precipitation in saturated $(NH_4)_2SO_4$ and dissolving the precipitate in 0.1 M phosphate and dialysis	$2.8 \cdot 10^7$	62.2
5. Fractional precipitation with $(NH_4)_2SO_4$	$2.25 \cdot 10^7$	50*

* Kogut *et al.*[2] at the end of their concentration work report the yield to be 20.2%; Madaiah reported the yield to be 23.4% by the procedure of Kogut *et al.*[2].

RESULTS

As shown in Table I, the recovery of the enzyme activity is up to 50% when crude penicillinase is concentrated by the humidifier followed by the low-temperature evaporation of water. The better yields of enzyme activity could possibly be due to the fact that the crude enzyme solution is concentrated before the fractional precipitation with $(NH_4)_2SO_4$. The concentrated crude enzyme when further purified by gel filtration through columns of Sephadex G-100, Biogel P-60 and DEAE-Sephadex gave a symmetrical single peak in the ultraviolet absorption ($A_{280\ nm}$) elution profile. The enzyme activity peak coincided with the ultraviolet absorption elution profile. Some of the preparations which were checked for homogeneity by Tiselius electrophoresis and sedimentation velocity experiments gave results which were in agreement with the results of Madaiah and Day[13].

Treatment of penicillinase with combinations (1) of 6-aminopenicillanic acid and nitrite and (2) ampicillin and nitrite lead to inactivation of the activity (Table II). It should also be noted that 7-aminocephalosporanic acid, an analogue of 6-aminopenicillanic acid, does not generate an inhibitor of penicillinase in the presence of nitrite. Neither nitrite, nor nitrite and any amino acid (D or L), nor nitrite and any "nonamino" penicillin led to any detectable inhibition of the β-lactamase I activity of the enzyme. An elution profile of the acid hydrolyzate of penicillinase not treated with penicillin gave analyses consistent with those observed by Madaiah and Day[13]. The hydrolyzate of the nitrite-treated enzyme showed no significant differences from the control profile. The hydrolyzates of the 6-aminopenicillanic acid–nitrite-treated enzyme and the ampicillin–nitrite-treated enzyme showed one new peak in the elution profile in the Val–Met region. This new peak corresponded to the elution time found for penicillamine. Approximately 1 mole of penicillamine was found per mole

TABLE II

INACTIVATION STUDIES WITH *Bacillus* CEREUS 569/H PENICILLINASE

The enzyme was treated with the penicillin and $NaNO_2$ or nitrite alone in 0.1 M sodium acetate (pH 5) buffer until the penicillin was completely dissolved.

Addition	*β-Lactam (mg/ml)*	*Activity remaining (%)*
None	—	100
Nitrite	—	96
Ampicillin + nitrite	2.15	5
Nitrite	—	96
6-Amino penicillanic acid + nitrite	9.5	12
Nitrite	—	96
Penicillin G + nitrite	20.9	95
Nitrite	—	96
7-Aminocephalosporanic acid + nitrite	13	110

TABLE III

MOLES OF PENICILLAMINE PER MOLE OF INACTIVATED PENICILLINASE IN THE ACID HYDROLYZATES

Penicillin	*Percent inactivation**	*Moles penicillamine per mole penicillinase***	
		16 Leu/mole	*19 Leu/mole*
6-Amino penicillanic acid		1.061	0.893
6-Amino penicillanic acid	60–80	0.800	0.674
6-Amino penicillanic acid		0.662	0.557
Ampicillin		1.89	1.59
Ampicillin	80–90	1.87	1.57
Ampicillin		1.39	1.17

* Percentage inactivation in larger batches ($>10^6$ units) was less than that shown for smaller batches (10^{-3} units) reported in Table II.

** Leucine in 24-h hydrolyzates was used as the internal standard utilizing the data of Madaiah and Day[13] and of Kuwabara *et al.*[14] which report 16 and 19 leucine/mole of 569/H enzyme, respectively. Each value reported is that of separate preparation of the inactivated enzyme. The values were obtained by transposing the elution profile in the Val–Met region to non-log paper and analyzing with a Dupont Curve resolver (Model 310). It was assumed that the time of elution of each peak was about 8 min which is the observed elution times for >95% of most components.

of enzyme (Table III). The results were obtained with exhaustively dialyzed (4–10 times 1000 vol.) and in some cases column desalted samples. The values for 6-aminopenicillanic acid–nitrite–treated enzyme fall in the range 0.01 × % inactivation/mole of enzyme; values for the ampicillin–nitrite-treated enzyme appear to be somewhat higher. Other components are apparently present in the latter case.

A new CD band was found in both 6-aminopenicillanic acid–nitrite- and ampicillin–nitrite-treated penicillinase samples (see Fig. 1). The CD spectrum of the enzyme (A) is modified by the ampicillin-nitrite treatment (B) and was restored (C) by treatment with hydroxylamine. The difference between spectra A and B indicates a relatively large residue ellipticity. The protein concentration for (C) < (B) < (A) the dilution and/or loss of the sample occurred as it was carried through the successive manipulations. The hydroxylamine-treated enzyme showed no recovery of activity.

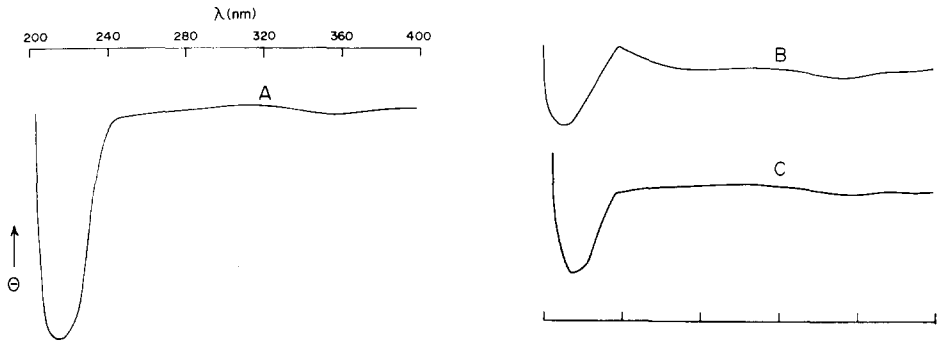

Fig. 1. Circular dichroic (CD) spectra. The CD spectra of penicillinase (A), of penicillinase treated with ampicillin–nitrite (B), and of the enzyme treated sequentially with (1) ampicillin–nitrite mixture (sample for the spectrum shown in B) and (2) hydroxylamine (C). The latter was prepared as described in Materials and Methods.

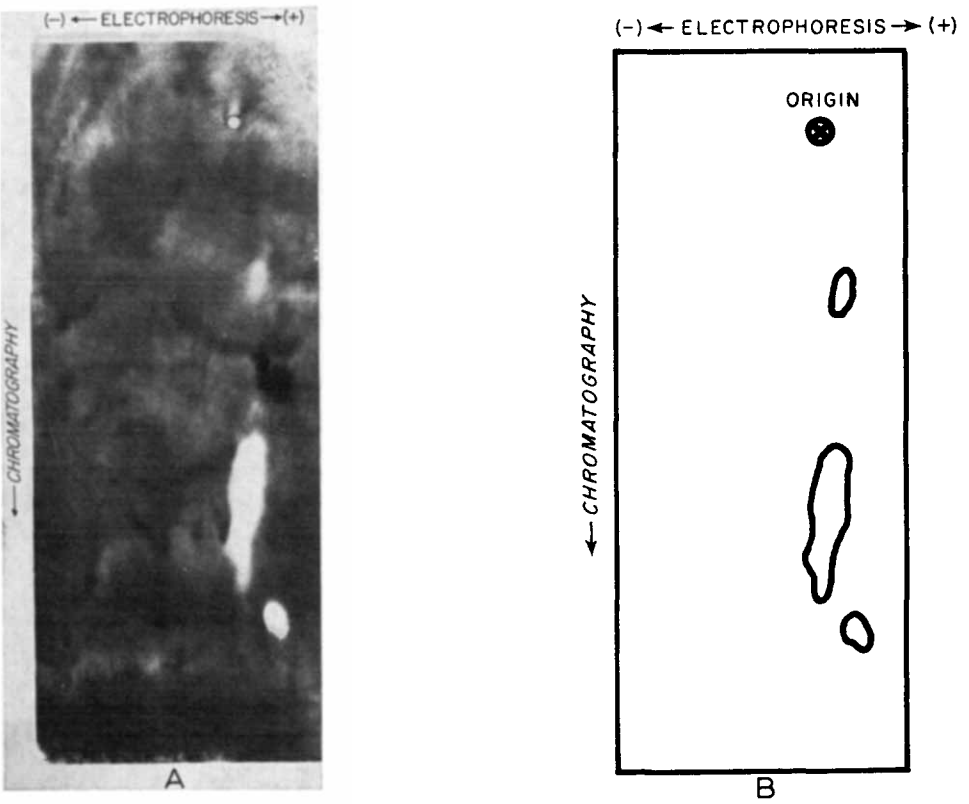

Fig. 2. Azide–KI_3 positive zones on tryptide map of ampicillin–nitrite treated penicillinase. The map was produced and the zones detected as described in Materials and Methods. Part A is a photograph of the map and part B is a tracing of A. The spot with highest R_F appears immediately and is relatively more intense than two other spots. The spot with intermediate R_F appears in about 1 h. The spot with lowest R_F appears in 24 to 48 h and is very faint.

The tryptic digest of the ampicillin–nitrite treated enzyme after electrophoresis using pyridine–acetic acid–water buffer (100:4:900, v/v/v) at pH 6.5 was chromatographed by descending chromatography. The chromatogram was then treated with azide–KI_3 reagent[15] to detect the modified tryptide. This treatment gave two spots. The one with the higher R_F value appears quickly and is relatively more "intense" than the other more slowly appearing spot (Fig. 2).

The inactivation is pH dependent, proceeding more rapidly with decreasing pH down to about pH 5 (Fig. 3).

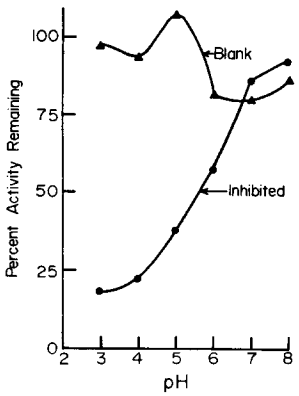

Fig. 3. The pH dependence of the inactivation of penicillinase. The enzyme was treated with the 6-aminopenicillanic acid and nitrite under conditions described in Materials and Methods.

DISCUSSION

The results are consistent with the formation of a diazo product which in turn functions as an alkylating agent. In a study of the products of di- and deazotization of 6-aminopenicillanic acid[16], various products were identified which were consistent with the formation of 6-diazopenicillanic acid or the corresponding diazonium salt although its isolation was not reported. No comparable studies have been done with ampicillin, but by analogy with numerous other reactions studied a diazo intermediate is probable and we postulate its presence.

No modified residues were found in the acid hydrolyzates of the 6-aminopenicillanic acid–nitrite inactivated enzyme, but the presence of approximately 1 mole of penicillamine/mole of penicillinase indicates the binding of a penicillin derivative to the enzyme. Penicillins and some related compounds are readily hydrolyzed to penicillamine and other products (*cf.* ref. 17); furthermore, penicillamine itself is stable to the conditions used in carrying out the acid hydrolysis of penicillinase.

The ampicillin–nitrite treated enzyme, on the other hand, apparently gave more than 1 residue of penicillamine/mole of penicillinase. This value may be in error because of the presence of at least two additional components in the valine to methionine region of the elution profile as indicated by analysis of the data on a curve resolver.

The hydroxylamine reversal of the CD effect, occasioned by treating the enzyme with ampicillin–nitrite, is consistent with the binding of a penicillin derivative by an ester linkage. Chemically, of course, the hydroxylamine treatment should give the hydroxamate, not the carboxylate; although the CD effect is reversed (Fig. 1, Curve C) presumably through removal of a large moiety, it is replaced by a small residue, –NHOH, which cannot be optically active nor can it be expected to contribute significantly to an extrinsic Cotton effect. The hydroxylamine-treated, modified enzyme showed no measurable recovery of activity. Similarly the pH dependence of the binding is that expected for alkylation of a carboxyl group. The rate of formation of the reactive intermediate may be pH dependent and could influence the pH profile of the inactivation. It has been noted that a carboxylate in pepsin (EC 3.4.4.1) is esterified by diazo substrate analogues[18–20]; this is accompanied by loss of enzymatic activity. The esterification of the pepsin presumably occurs by a normal mechanism.

The mapping experiment indicates that the penicillin-related moiety is covalently bound to one or two sites on the enzyme.

In conclusion, we have presented evidence that some derivatives of certain penicillins alkylate a carboxylate in the active site of penicillinase.

ACKNOWLEDGMENTS

This work was supported by a grant of the U.S.P.H.S. (AI-06375). R.A.D. is a Career Development Grant Awardee (U.S.P.H.S. 5 K04 07654). We gratefully acknowledge support for part of this work provided through the courtesy of Prof. H. Rudney.

REFERENCES

1 POLLOCK, M. R., TORRIANI, A. M. and Tridgell, E. J. (1956) *Biochem. J.*, 62, 387
2 Kogut, M., Pollock, M. R. and Tridgell, E. J. (1956) *Biochem. J.* 62, 391
3 Citri, N. and Pollock, M. R. (1966) in F. F. Nord, *Adv. Enzymol.* 28, 237
4 Ambler, R. P. and Meadway, R. J. (1969) *Nature* 222, 24
5 DePue, R. H., Moat, A. G. and Bondi, A. (1964) *Arch. Biochem. Biophys.* 107, 374
6 Day, R. A. and Patil, G. V. (1967) *Bacteriol. Proc.* 18
7 Patil, G. V. and Day, R. A. (1971) *IUPAC, Macromolecular Preprint* Vol. II, 1088
8 Day, R. A., Berstein, A., Murthy, L. and Spicer, N. (1966) *Bacteriol. Proc.* 88
9 Falter, H. and Day, R. A. (1967) *Arch. Biochem. Biophys.* 120, 231
10 Madaiah, M. (1970) Ph. D. Dissertation, University of Cincinnati
11 Perret, C. J. (1954) *Nature* 174, 1012
12 Piez, K. A. and Morris, L. (1960) *Anal. Biochem.* 1, 187
13 Madaiah, M. and Day, R. A. (1971) *Biochim. Biophys. Acta* 236, 191
14 Kuwabara, S., Adams E. P. and Abraham, E. P. (1970) *Biochem. J.* 118, 475
15 Helberg, H. (1968) *J. Assoc. Official Anal. Chem.* 51, 552
16 Stoodley, R. J. (1967) *Tetrahedron Lett.* 941
17 Peck, R. L. and Folkers, K. (1949) in *Penicillin*, (Clarke, H. T. *et al.*, eds) p. 67, Princeton University Press, Princeton, N. J.
18 Delpierre, G. R. and Fruton, J. S. (1965) *Proc. Natl. Acad. Sci. U.S.* 54, 1161
19 Rajagopalan, T. G., Stein, W. H. and Moore, S. (1966) *J. Biol. Chem.* 241, 4295
20 Fry, K. T., Kim, O. K., Spona, J. and Hamilton, G. A. (1970) *Biochemistry* 9, 4624