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CATALYTIC AND CONFORMATIONAL PROPERTIES OF CROSS-LINKED DERIVATIVES OF PENICILLINASE

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Summary

Exopenicillinase (penicillin amido-β-lactamhydrolase, EC 3.5.2.6) has been isolated from the culture supernatant of *Bacillus cereus*, strain 569/H. Cross-linking of the enzyme by covalent attachment of bifunctional reagents to non-essential amino acid residues yielded catalytically active derivatives with altered properties. The reagents used include cyanuric chloride, hexamethylene diisocyanate, diethyl malonimidate and glutaraldehyde at several concentrations. All derivaties showed a marked increase in thermostability consistent with a general stabilization of the native conformation of the enzyme. Further consequences of cross-linking were studied in some detail in the glutaraldehyde derivatives.

The native enzyme responds to the presence of certain substrates (A-type penicillins) by a characteristic change in conformation and in reaction kinetics. The effects of such substrates can be virtually eliminated by extensive cross-linking. Similarly, the antigenic identity of the native enzyme appeared to be lost in the glutaraldehyde derivative, although it was not altered by an analogous modification with a monofunctional reagent. Surprisingly, the Michaelis constants of the enzyme were not affected by cross-linking. The results are discussed in terms of the effect of constraint of conformational flexibility on the behavior of the enzyme.

Introduction

The conformational adaptability of enzymes [1] implies flexibility of the tertiary structure of the enzyme molecule. The role of flexibility in the function of an enzyme can be deduced from studies comparing the properties of the native molecule and those of less flexible derivatives. In this report we show

that cross-linking with bifunctional reagents provides a useful tool for reducing the conformational flexibility of an enzyme. The system described here consists of the exopenicillinase (penicillin amido-β-lactamhydrolase, EC 3.5.2.6) secreted by *Bacillus cereus*, strain 569/H, and of substrates representing A-type penicillins. These substrates have been previously shown to induce striking changes in the conformation [2,3] and the kinetic behavior [4] of penicillinase.

The remarkable flexibility of penicillinase is most likely related to the fact that the tertiary structure of the native exoenzyme is not stabilized by covalent links [5]. As shown below, introduction of cross-links does indeed suppress the conformational response of penicillinase to its substrates.

The study of such constrained derivatives allowed us to identify several properties of the enzyme which are strongly dependent on its conformational flexibility.

Materials and Methods

Benzylpenicillin was purchased from Rafa Laboratories (Israel), and 6-aminopenicillanic acid from Aldrich Chemical Co. (WI, U.S.A.); methicillin, oxacillin and cloxacillin were purchased from Beecham Research Laboratories (Brentford, U.K.). Glutaraldehyde (70%) was the product of Lodd Laboratories (Israel), and cyanuric chloride was obtained from Merck (Darmstadt). Hexamethylene diisocyanate was purchased from Pierce Chemical Co. (IL, U.S.A.), and *n*-butyraldehyde from British Drug Houses (Poole, U.K.). All other reagents were of analytical grade.

Bacterial growth and purification of the enzyme were as described previously [6].

Antisera to penicillinase of *B. cereus* 569/H were prepared in rabbit according to the method of Citri and Strejan [7]. Pooled antisera were used.

Penicillinase activity was determined by iodometric [8], spectrophotometric [9] or colorimetric [10] methods. A unit of activity is defined as the amount of enzyme which hydrolyzes 1 μmol benzylpenicillin/h at pH 7.0 at 30° C [11].

For cross-linking with glutaraldehyde 1200 μg/ml penicillinase in 0.05 M phosphate buffer (pH 7.5) was stirred with glutaraldehyde (0.1% or as indicated) at room temperature. The reaction was terminated after 18 h, either by dialysis against 0.05 M sodium glycine for 6 h at 4° C or by the addition of $NaBH_4$ to a final concentration of 0.05 M and incubation for an additional 20 min at 4° C. The enzyme derivative was separated from low molecular weight material by dialysis against 0.003 M phosphate buffer (pH 7.0) at 4° C.

Cyanuric chloride (dissolved at 1 mg/ml in acetone) was added slowly to the enzyme (60 μg/ml in 0.05 M phosphate buffer, pH 7.5) to a final concentration of 25 μg/ml. After incubation of 15 min at 4° C the mixture (total volume, 60 ml) was dialyzed against 0.003 M phosphate buffer (pH 7.0) at 4° C.

The treatment with hexamethylene diisocyanate was based on the procedure of Snyder et al. [12].

Cross-linking with diethyl malonimidate was carried out as described by Dutton et al. [13] in 0.05 M Na_2HPO_4 adjusted to pH 8.5 with 1 M NaOH. After 60 min at room temperature, the preparation was dialysed for 18 h at 4° C against 0.03 M phosphate buffer, pH 7.0. The insoluble residue was

removed by centrifugation and the preparation stored at −20°C in 10% glycerol.

For modification with *n*-butyraldehyde 2 mg/ml penicillinase in 0.05 M phosphate buffer (pH 7.5) was stirred with the reagent (final concentration 10%) for 30 min at room temperature. The reagent was removed by dialysis as described for the glutaraldehyde derivative. In the treatment, the enzyme lost 50% of its original activity. The dialyzed derivative was centrifuged and stored at −20° C in 10% glycerol.

Protein content of the derivatives was determined according to Lowry et al. [14]. Amino acid analysis was carried out on samples containing 250 μg protein in a Beckman model 120 B Amino Acid Analyzer.

Results

Cross-linking protects penicillinase against thermal activation

The introduction of intramolecular bridges was expected to stabilize the native conformation of penicillinase and thus to increase its resistance to thermal denaturation. We found that stabilization is indeed observed when the enzyme is cross-linked with bifunctional reagents which spare its catalytic activity. Several examples of cross-linking leading to increased thermostability are listed in Table I. The effect of cross-linking was further analyzed with glutaraldehyde as the bifunctional reagent. Glutaraldehyde is known to be bound mainly to lysine residues in proteins but other interactions have not been ruled out [15]. We determined the number of free lysin residues and found 9.2 residues in the derivative cross-linked with 0.10% glutaraldehyde as compared to 19 residues in the native enzyme. The number of arginine, histidine and other amino acid residues was unchanged. In order to ascertain that glutaraldehyde imparts thermostability by cross-linking, we prepared a derivative with *n*-butaraldehyde, a monofunctional reagent otherwise analogous to glutaraldehyde [16]. As seen in Fig. 1, the butyraldehyde derivative shows no increased thermostability and is, in fact, less stable than the untreated enzyme. We also show that treatment with the monofunctional reagent prevents stabilization by glutaraldehyde and hence serves as the appropriate non-cross-

TABLE I

MODIFICATION OF PENICILLINASE BY CROSS-LINKING REAGENTS

Thermostability is expressed as residual activity (percent of initial) after 3 min exposure to 56°C at pH 7.3 as described in legend to Fig. 1. Conformative response is expressed as loss of activity (percent of initial) as a result of iodination (5 min at 0°C) in the presence of methicillin (10 μg/ml). For other details see legend to Fig. 2.

Cross-linking reagent	Specific activity (units/μg protein)	Thermostability	Conformative response
Cyanuric chloride	52	50	48
Hexamethylene diisocyanate	57	72	49
Dimethyl malonimidate	48	59	5
Glutaraldehyde 0.02%	208	79	42
Glutaraldehyde 0.10%	52	93	15
None	310	25	93

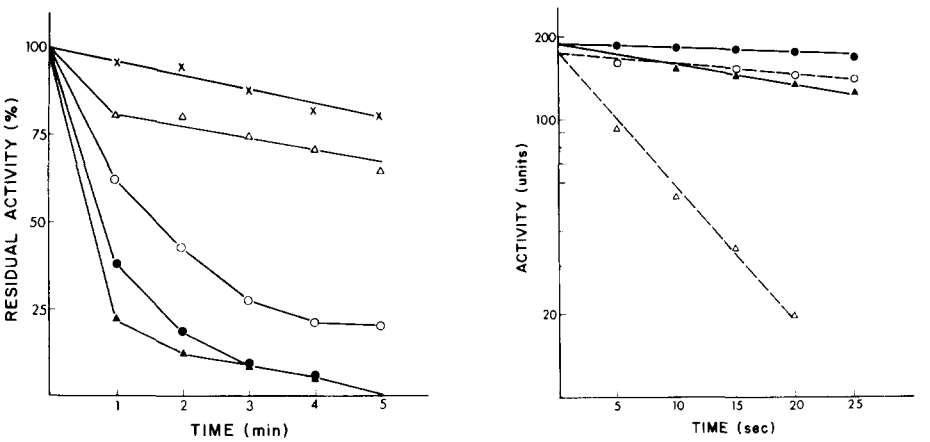

Fig. 1. Thermostability of native and modified penicillinase. Samples of the native enzyme or its derivatives (180 units in 0.2 ml 0.5% gelatin) were added to 0.2 ml 0.1 M phosphate buffer (pH 7.3) and immersed in a 56°C water-bath for the indicated time intervals. After cooling for 1 min at 0°C, the residual activity was assayed by the iodometric method [8] and expressed as percent of the initial activity. ○, native enzyme; △, treated with 0.02% glutaraldehyde; ✕, treated with 0.1% glutaraldehyde; ●, treated with 2.5% *n*-butyraldehyde; ▲, treated with 2.5% *n*-butyraldehyde and subsequently with 0.1% glutaraldehyde.

Fig. 2. Cross-linking prevents cloxacillin-induced inactivation. Samples of cross-linked or native enzyme (280 units in 0.1 ml 0.5% gelatin) were exposed at 0°C to 0.01 M I_2 in 0.5 ml 0.04 M phosphate buffer (pH 7.3). The residual activity at the indicated times was determined as previously described [8]. ———, 0.1% glutaraldehyde cross-linked derivative treated in the absence (●) or presence (▲) of 300 μg/ml cloxacillin; - - - - - -, native enzyme treated in the absence (○) or presence (△) of 100 μg/ml cloxacillin.

linking control. We can thus conclude that the stabilization is entirely due to cross-linking.

We further compared the kinetics of thermal inactivation of a 'lightly' and a 'heavily' cross-linked derivative, prepared with 0.01% and 0.10% glutaraldehyde, respectively. As seen in Fig. 1, the heavily cross-linked derivative is homogeneous in terms of thermostability. By contrast, the kinetics of inactivation of the lightly cross-linked preparation shows a break, implying that about 20% of its activity is associated with molecules of lower stability. However, the bulk of that preparation displays thermostability which is identical with that of the heavily cross-linked derivative.

Cross-linking restricts the conformative response

The conformational change induced in an enzyme by its substrate ('conformative resonse') which has been extensively studied in penicillinase [2,3] depends by definition on the flexibility of the active site. Hence, it was of interest to examine the effect of cross-linking of the enzyme on the conformative response. The simple test shown in Fig. 2 is based on earlier evidence [2—4] that cloxacillin and all other so-called A-type penicillins induce a reversible deformation in the active site of penicillinase. One of the results is that a tyrosine residue essential for catalytic activity becomes exposed [17]. Iodination of the exposed tyrosine causes inactivation of penicillinase. The extremely rapid progress of cloxacillin-induced inactivation of the native

enzyme is not seen in the cross-linked derivative (Fig. 2). It is, however, important to note that the conformative response is not entirely eliminated by cross-linking. The slight, but significant response of the derivative to cloxacillin supports the view (see Discussion) that some degree of flexibility is essential for catalytic activity. Similar results were obtained with another A-type penicillin (methicillin) and other cross-linked derivatives of penicillinase (Table I).

Cross-linking suppresses the biphasic kinetics of penicillinase

It is well-known [4] that the rate of hydrolysis of A-type penicillins decelerates with time until a steady, low rate of the catalytic reaction is attained. The biphasic kinetics observed at saturating substrate concentrations have been shown to reflect the conformational transition induced by these substrates [3] and thus to depend on the flexibility of the active site of the enzyme. The phenomenon is illustrated in Fig. 3 where the substrate is one of the A-type penicillins (oxacillin). Cross-linking of the enzyme with glutaraldehyde was found to suppress the biphasic character of the catalytic reaction, presumably by restricting the flexibility of the active site. Kinetics obtained with derivatives cross-linked to a varying degree (Fig. 3) show that the deviation from a linear course decreases as the extent of cross-linking is increased. Apart from this obvious conclusion, several further points emerge from the results in Fig. 3. First, the kinetic transition is not entirely eliminated even when the cross-linking yields a uniformly stabilized derivative (cf. Fig. 1). This adds further support for the view that some degree of flexibility must be preserved for the enzye to retain catalytic activity. Next, it will be noted that

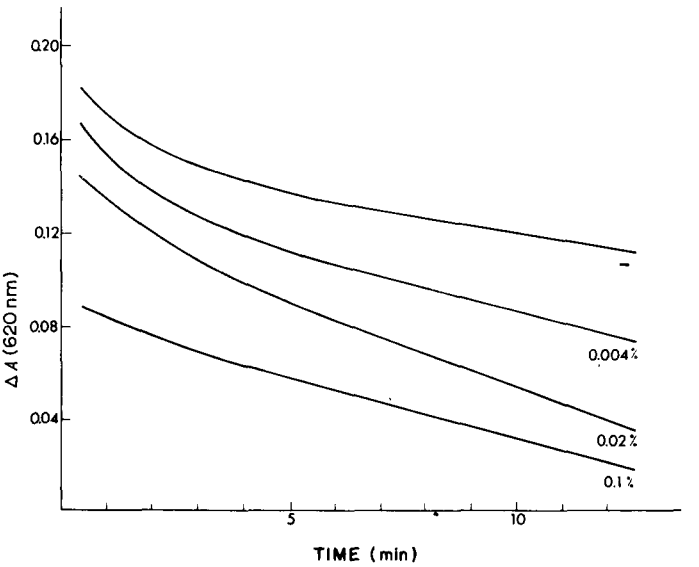

Fig. 3. Suppression of biphasic kinetics of hydrolysis of oxacillin. Samples of native or cross-linked penicillinase (120 units in 0.1 ml 0.5% gelatin) were assayed by the colorimetric method [10] with 7 mM oxacillin as substrate. The cross-linked derivatives were prepared with 0.004%, 0.02% or 0.1% glutaraldehyde.

TABLE II

SUPPRESSION OF BIPHASIC KINETICS BY CROSS-LINKING OF THE ENZYME

Catalytic rates in the first (I) and second (II) phase of the biphasic kinetics were determined by the spectrophotometric assay [9]. Expressed as percent of the rate of hydrolysis of the unsubstituted 6-aminopenicillanic acid. Benzylpenicillin is an S-type penicillin and the rate of its hydrolysis is constant. Cross-linked derivatives were prepared with 0.1% glutaraldehyde.

Substrate	Free enzyme			Cross-linked derivative		
	I	II	I/II	I	II	I/II
Methicillin	37	23	1.6	49	48	1.0
Oxacillin	100	21	4.8	75	47	1.6
Cloxacillin	30	6.4	4.7	30	13	2.3
Benzylpenicillin	—	714	—	—	750	—

the rate of the catalytic reaction in the second, linear phase is actually increased by cross-linking. This is clearly seen in the partly cross-linked derivatives, and it is fully consistent with earlier evidence [4] that the deceleration of hydrolysis of A-type penicillins can be effectively prevented by restricting the flexibility of the enzyme (see Discussion). Note also the kinetic behavior of the maximally cross-linked derivative, prepared with 0.10% glutaraldehyde. The rate of catalysis by that derivative, which is initially low (cf. Table I), is nevertheless effectively maintained throughout the reaction.

Similar results have been obtained with other A-type penicillins (methicellin and cloxacillin). By contrast, the course of hydrolysis of S-type penicillins (e.g. benzylpenicillin) which is linear in the first place [4] was not modified by cross-linking (Table II).

Michaelis constants of native and cross-linked preparations

We found no indication that the K_m values of penicillinase for either A-type or S-type substrates are significantly altered by cross-linking. Typical results are shown in Table III, where we compared several such constants derived for the native enzyme and for a 'heavily' cross-linked derivative. The spectrophotometric method was used [9] and the values obtained for unmodified penicillinase are in agreement with the published data based on other assay

TABLE III

MICHAELIS CONSTANTS OF NATIVE AND CROSS-LINKED ENZYME

For details of procedure see Ref. 9. Cross-linked derivatives were prepared with 0.1% glutaraldehyde.

Substrate	Native enzyme K_m (mM)	Cross-linked derivative K_m (mM)
6-Aminopenicillanic acid	1.5	1.45
Benzylpenicillin	0.06	0.07
Methicillin *	0.5	0.6
Oxacillin *	0.24	0.24

* Derived from the second phase of the biphasic kinetics as described in Ref. 9.

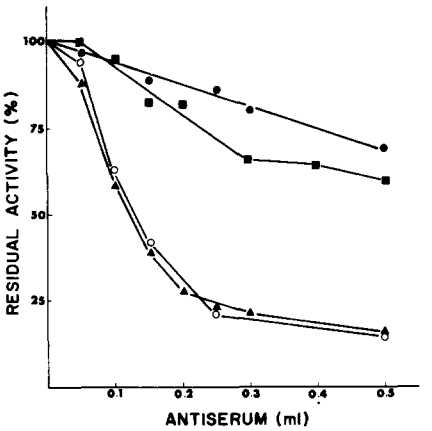

Fig. 4. Neutralization of activity of penicillinase and its derivatives by antibodies to the native enzyme. Samples of native or modified enzyme (180 units in 0.1 ml 0.5% gelatin) were incubated with the indicated amounts of pooled antisera. The final volume (0.5 ml) was made up with normal rabbit serum. The activity was assayed iodometrically [8] and expressed as percent of the activity recorded in 0.5 ml serum. ●, 0.1% glutaraldehyde derivative; ■, 2.5% cyanuric chloride derivative; △, 2.5% *n*-butyraldehyde derivative; ○, native enzyme.

procedures [4]. The K_m values for the A-type substrates, namely methicillin and oxacillin, pertain to the second phase of the biphasic reaction.

Antigenic properties of the modified enzyme

Antibodies prepared against the native enzyme may be used to detect structural differences between the native and the chemically modified enzyme. We used an activity neutralization test to detect such differences (Fig. 4). The results indicate that antibodies which neutralize the activity of native penicillinase have little effect on the cross-linked derivatives of the enzyme. Such antibodies do react with *n*-butyraldehyde-treated enzyme, and neutralize its activity to the same extent as that of the native enzyme. These observations suggest that the flexibility of the enzyme molecule may play a role in enzyme-antibody interactions.

Discussion

The main purpose of this work was to examine the role of conformational flexibility in the function of the enzyme. We must, however, consider other possible consequences of the treatment which yields a cross-linked derivative. First we need to distinguish the effect of substitution of the participant amino acid residues from that of the cross-link formation. By using the monofunctional reagent, *n*-butyraldehyde, as a control in the study of the effect of glutaraldehyde we have been able to make that distinction. We have shown that *n*-butyraldehyde prevents cross-linking by glutaraldehyde and hence substitutes the lysine residues which would otherwise bind the bifunctional reagent. None of the changes observed with glutaraldehyde were seen in the active derivative obtained with the monofunctional reagent. We may thus conclude that the

altered properties are due to cross-link formation rather than to any other modifications introduced by glutaraldehyde.

Next we need to consider the effect of cross-linking on the general stability, as opposed to its effect on the conformational flexibility, of the enzyme. Stabilization of the tertiary structure of an enzyme is an obvious and well-known result of introducing covalent links into the molecule. This is particularly true of enzymes such as penicillinase which contain no disulfide bridges. As shown above, all bifunctional reagents tested did indeed improve the thermostability of penicillinase.

Relatively light cross-linking confers nearly maximal stabilization (Fig. 1) whereas more extensive cross-linking leads to progressive inactivation (Table I), possibly by increasingly constraining the flexibility of the enzyme molecule. The implication is that a certain degree of conformational flexibility is essential for either binding of the substrate or catalyzing its hydrolysis or both.

The constraint of flexibility imposed by glutaraldehyde cross-links was striking indeed, as seen in Fig. 2, but it was not sufficient to eliminate the conformative response to cloxacillin. This was further confirmed with methicillin and a series of cross-linked penicillinase derivatives. The effect of cross-linking on the kinetics of hydrolysis of A-type penicillins is similarly consistent with the postulated role of conformational flexibility in the catalytic reaction. The biphasic kinetics which reflect the conformational adjustment of the enzyme to the structure of the substrate [4] are suppressed as the extent of cross-linking is increased (Fig. 3). There is even a hint (Fig. 3 and Table II) that in some cases the kinetic response may be entirely eliminated without total loss of activity. It is of course conceivable that the residual flexibility which may be essential for activity is still preserved in such cross-linked derivatives.

This view is further supported by the unexpected observation (Table III) that the K_m values for a series of substrates were found to be unchanged in the cross-linked derivatives. It must be remembered, however, that cross-linking causes partial inactivation of the enzyme. We suggest, therefore, that molecules which retain activity are cross-linked outside the active site; conversely, cross-links which directly involve the active site cause total loss of activity rather than increase in K_m. This interpretation is consistent with our previous observations on penicillinase [18].

Our final comment refers to the observations presented in Fig. 4. The characteristic 'neutralization curve', normally seen when native penicillinase is incubated with increasing amounts of homologous antibodies [7] was largely eliminated when the enzyme was cross-linked with either cyanuric chloride or glutaraldehyde. By contrast, an analogous modification without cross-linking had no effect whatsoever on the enzyme-antibody interaction. This may seem surprising since the surface structure of the enzyme molecule must have been similarly altered by the monofunctional and the bifunctional reagent. A plausible explanation of these results may emerge from considering once more the role of flexibility in enzyme function. Earlier studies in this laboratory have provided evidence [6,18] that antibodies inhibit penicillinase activity indirectly, rather than by blocking the active site of the enzyme. In fact, it has been postulated that the bulky antibody molecule suppresses the catalytic activity by restricting the conformational flexibility of the native enzyme. No

such effect is expected when the antibody binds to a derivative of penicillinase which retains a catalytically active conformation firmly stabilized by cross-links.

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