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# Cross-Linking Preserves Conformational Changes Induced in Penicillinase by its Substrates

Yoel KLEMES and Nathan CITRI
*Institute of Microbiology, Hebrew University–Hadassah Medical School, Jerusalem, Israel*

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Exopenicillinase of *Bacillus cereus* 569/H was cross-linked with toluene 2,4-di-isocyanate in the presence of cephalothin, cloxacillin or no substrate. The derivatives show significant differences in susceptibility to inactivation by heat, urea, iodination or proteolysis. Such differences can be predicted from the contrasting effects of these substrates on the conformation of the enzyme.

It has been frequently observed that the binding of substrate is accompanied by a conformational change in the active site of the enzyme (Koshland, 1958; Citri, 1973). The change (conformative response) is fully reversible, i.e. the native conformation is restored after the release of the product or after the dissociation of the enzyme–substrate complex. The question before us was whether we can prevent the reversal by cross-linking the enzyme in the substrate-induced conformation. Penicillinase secreted by *Bacillus cereus* ($\beta$-lactamase I, EC 3.5.2.6) provides a convenient model, since it shows two distinct kinds of conformative response (Citri & Zyk, 1965) and a perceptible lag before reverting to the native conformation (Citri *et al.*, 1976). In the presence of S-type ligands, represented here by cephalothin, the enzyme assumes a compact stable conformation. By contrast, A-type ligands, such as cloxacillin used here, induce in the enzyme a significant degree of unfolding and, consequently, lability. We show that both conformational states can be recognized in derivatives cross-linked in the presence of the respective substrates.

The choice of a bifunctional reagent (Wold, 1972) for arresting the conformative response in penicillinase was limited by the following considerations. First, the reagent must be effective under the conditions (pH, temperature and ionic strength) that favour the formation of the enzyme–substrate complex. Secondly, the reagent must not lead to blocking of the active site by covalently bonding the substrate or product of the reaction. Thirdly, the reagent must act quickly in view of the fact that the conformative responses studied are induced by hydrolysable substrates. Of the several reagents tested (cf. Klemes & Citri, 1979), we found that the most suitable was toluene 2,4-di-isocyanate, which, at neutral pH or above, binds to the $\varepsilon$-amino group of lysine residues in a protein and forms a stable ureido bond (Schick & Singer, 1961).

The cross-linked derivatives of penicillinase, prepared as described in Fig. 1 legend, were isolated by chromatography on a Sepharose 6B column. The cross-linking was associated with loss of activity, which was dependent on the structure of the ligand. This is illustrated by the typical results presented in Table 1. Titration with 2,4,6-trinitrobenzenesulphonic acid (Habeeb, 1966) revealed that five out of 19 lysine residues were substituted both in the absence and in the presence of an active-site ligand. [The determination could not be confirmed by amino acid analysis, since ureido bonds between toluene 2,4-di-isocyanate and the protein tend to break in the acid hydrolysis (Wold, 1972; Shick & Singer, 1961).] Further incubation with toluene 2,4-di-isocyanate did not alter the proportion of substituted lysine residues or the characteristics of the derivatives. The involvement of other amino acid residues in the cross-linking has not been ruled out.

The effect of the substitution as such, i.e. without the accompanying cross-linking, was examined

Table 1. *Specific activity of derivatives cross-linked in the presence and absence of ligands*

Experimental details are given in the text. One unit of penicillinase hydrolyses 1 $\mu$mol of benzylpenicillin in 60 min at 30°C.

| Ligand present during cross-linking | Specific activity (units/$\mu$g of protein) |
|---|---|
| Cloxacillin | 62 |
| Cephalothin | 121 |
| None | 136 |
| None (no cross-linking) | 260 |



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with the aid of *p*-tolyl isocyanate. This monofunctional analogue of toluene 2,4-di-isocyanate causes considerable inactivation (up to 80%) of the native enzyme, but has little effect on the derivatives cross-linked with toluene 2,4-di-isocyanate. This is consistent with both reagents sharing common bonding sites. It also indicates that substitution of the five accessible lysine residues will cause departure from the active conformation: the loss of activity is prevented by bridge formation between the substituted sites.

Cross-linking generally increases the thermostability of enzymes (Zaborsky, 1974), including penicillinase (Klemes & Citri, 1979), presumably by preventing thermal disruption of the native structure. This has now been confirmed for penicillinase cross-linked with diverse bifunctional reagents, including toluene 2,4-di-isocyanate. Significantly modification with the corresponding monofunctional reagent, *p*-tolyl isocyanate, lowers the thermostability of the enzyme (Fig. 1). The other results included in Fig. 1 show the difference in thermostability of derivatives cross-linked in the presence of cephalothin and cloxacillin. Cephalothin is known to protect penicillinase against thermal inactivation (Citri & Zyk, 1965). Nevertheless cross-linking in its presence yields a derivative that is only slightly more stable than cross-linked native penicillinase. This is as expected if the cephalothin-induced conformation is not very different from that of the native enzyme. The previously observed protective effect of this substrate is thus analogous to cross-linking in that it prevents disruption of a catalytically active conformation. This is in sharp contrast with the effect of cloxacillin, a substrate previously shown to labilize penicillinase to heat (Citri & Zyk, 1965). Stabilization, by cross-linking, of the cloxacillin-induced conformation yields a paradoxical derivative. It is a stable derivative in the sense that it retains the imprint of cloxacillin indefinitely. [In the absence of cross-links, the enzyme will revert to the native conformation (Citri *et al.*, 1976).] Its stability to heat is, however, considerably lower than that of the non-cross-linked (native) enzyme.

These observations are further confirmed in Fig. 2, where we compare the rates of inactivation of the native enzyme and its derivative in 8 M-urea. In this conformation-disrupting medium, the difference between the cloxacillin-induced derivative and the other enzyme preparations is particularly striking. This is mainly because cross-linking by itself, or in the presence of cephalothin, appears to have less effect on the stability of penicillinase in urea (Fig. 2). It is possible that here is a clue to the paradoxical properties of the cloxacillin derivative. Denaturation by urea is known to be, on the whole, more readily reversible than thermal inactivation (Nozaki & Tanford, 1963). Indeed, there is evidence from past

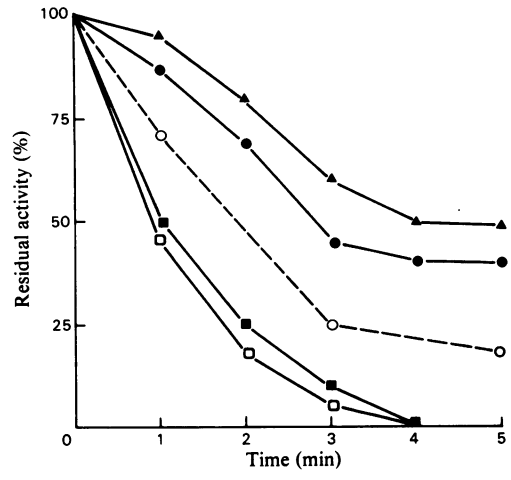


Fig. 1. *Thermostability of penicillinase and its derivatives*
Penicillinase was isolated from culture supernatant of *B. cereus* strain 569/H (Citri *et al.*, 1960) and cross-linked by toluene 2,4-di-isocyanate. Cross-linking in the presence of substrates was started by adding toluene 2,4-di-isocyanate 6 min after the substrate. The reagent (0.1 ml) was added to 1.0 mg of the enzyme dissolved in 30 ml of 30 mM-potassium phosphate buffer, pH 7.8. After 45 min of stirring in a pH-stat at 4°C at pH 7.8 the reaction was terminated by addition of $(NH_4)_2CO_3$ (final concentration 0.25%). The supernatant obtained after centrifugation at 12000 *g* for 20 min was dialysed extensively against 0.25% $(NH_4)_2CO_3$ and three changes of 3 mM-potassium phosphate buffer, pH 7, at 4°C, concentrated *in vacuo* to 2.0 ml, and dialysed as before. *p*-Tolyl isocyanate (0.01 ml) was stirred into precooled mixtures containing 6.0 ml of 30 M-potassium phosphate buffer, pH 7.3, and 200 $\mu$g of enzyme protein. Stirring was stopped after 30 min at 0°C, and the residual activity was assayed iodometrically (Citri, 1964). Samples of each preparation (125 units in 0.2 ml of 0.5% gelatin) were added to preheated 80 mm × 15 mm test tubes containing 0.2 ml of 0.1 M-potassium phosphate buffer, pH 7.3. After exposure to 56°C for the indicated time intervals, the test tubes were transferred to an ice bath for 60 s, and the residual activity was assayed iodometrically (Citri, 1964). Enzyme preparations: ○, native; □, modified by *p*-tolyl isocyanate; ▲, ■ and ●, cross-linked by toluene 2,4-di-isocyanate with cephalothin (20 mM) (▲), cloxacillin (50 mM) (■) or no substrate present (●).

work on penicillinase that the catalytically active conformation is lost in urea but restored by the assay substrate (benzylpenicillin) (Garber & Citri, 1962). Hence cross-linking, which stabilizes a catalytically favourable conformation, clearly prevents irreversible inactivation, but will make little difference when extensive re-activation is possible,

as after exposure to urea. On the other hand, molecules stabilized in the cloxacillin-induced state and subsequently exposed to urea are less likely to revert to an active conformation because of the cross-links.

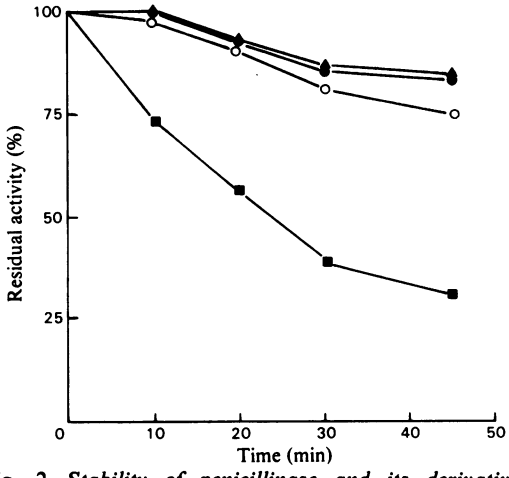


Fig. 2. *Stability of penicillinase and its derivatives in 8 M-urea*

Samples of the enzyme preparations (60 units in 0.1 ml of 0.1% gelatin) were added to 0.9 ml of 0.1 M-potassium phosphate buffer, pH 7.0, containing 8 M-urea. After incubation at 30°C, 0.1 ml samples were removed at the indicated times and assayed by the spectrophotometric method (Samuni, 1975). The derivatives were cross-linked as described in Fig. 1 with cephalothin (20 mM) (▲), cloxacillin (50 mM) (■) or no substrate (●) present. O, Native enzyme.

We next asked whether derivatives cross-linked in the presence of the two types of substrates differ in their response to substrates subsequently presented. A very sensitive test of conformative response is based on the observation that A-type substrates cause exposure of a tyrosine residue that is essential for catalytic activity (Citri, 1973; Citri & Zyk, 1965; Csanyi *et al.*, 1971). Thus iodination in the presence of methicillin, an A-type penicillin, causes rapid loss of activity.

In a typical experiment samples of the enzyme preparations (180 units in 0.1 ml of 0.5% gelatin) were added to 0.9 ml of 50 mM-potassium phosphate buffer, pH 7.3, containing 3.5 mM-$I_2$/KI (Citri & Zyk, 1965) and 10 $\mu$g of methicillin. After 5 min at 0°C the residual activity was assayed as previously described (Citri & Zyk, 1965) and expressed as a percentage of the initial activity. The residual activity of the native (non-cross-linked) preparation was 38%. By this criterion maximal stability (82%) was obtained by cross-linking in the presence of cephalothin, whereas the derivative cross-linked in the presence of cloxacillin was least stable (51%). Cross-linking in the absence of substrate yielded a derivative of intermediate stability (72%). These results are consistent with the assumption that the derivatives retain an 'imprint' that is characteristic of the substrate present during the cross-linking procedure. That imprint is not evident in the absence of methicillin, when all cross-linked derivatives are highly resistant to iodination ($t_{\frac{1}{2}} >$ 60 min). The half-life of the native enzyme under these conditions is 18 min, presumably reflecting the fluctuations of the essential tyrosine residue in

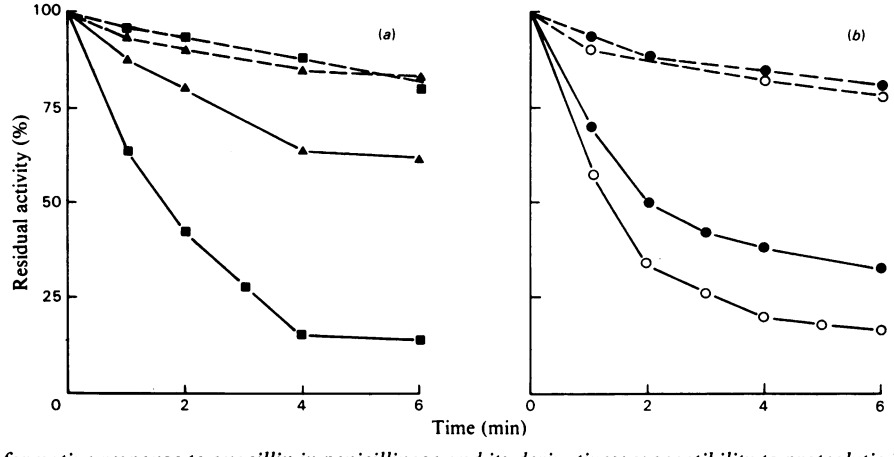


Fig. 3. *Conformative response to oxacillin in penicillinase and its derivatives: susceptibility to proteolytic inactivation*

Samples of the enzyme preparations (125 units in 0.1 ml of 0.1 M-potassium phosphate buffer, pH 8.0) were incubated at 37°C with 100 $\mu$g of Pronase, dissolved in 0.3 ml of 10 mM-$CaCl_2$, in the presence (——) or absence (– – – –) of oxacillin (100 $\mu$g). The colorimetric assay (Imsande, 1965) was used to determine the residual activity at the indicated time intervals (*a*) Enzyme cross-linked in the presence of cloxacillin (50 mM) (■) or cephalothin (20 mM) (▲). (*b*) Controls: native enzyme (O) or cross-linked enzyme (●), with no ligand present.

the non-cross-linked molecule. Such fluctuations appear to be suppressed in the derivatives, as indicated by the stabilizing effect of the cross-linking. However, the precise position of the essential tyrosine residue is clearly different in the three derivatives. That residue is most readily exposed by methicillin in the derivative bearing the imprint of an A-type ligand. It is possible that a sizeable proportion of the enzyme cross-linked in the presence of cloxacillin was 'frozen' with the tyrosine residue exposed, but such molecules are unlikely to retain catalytic activity (Klemes & Citri, 1979). That may explain the fact that we have never encountered a stable iodine-sensitive form of this enzyme and also the low specific activity of preparations cross-linked in the presence of cloxacillin (see Table 1). Finally, the need to introduce methicillin for bringing out differences between the derivatives confirms that our preparations were free of contamination by ligands used in the process of cross-linking.

Another criterion for conformative response to A-type penicillins is based on an earlier observation (Citri, 1973; Citri & Zyk, 1965) that these substrates induce susceptibility to proteolytic inactivation. Typical results obtained with oxacillin, an A-type penicillin, and Pronase are shown in Fig. 3. In the absence of oxacillin, the rates of inactivation by Pronase are slow and virtually identical for the native enzyme and its derivatives. In the presence of oxacillin the rates of inactivation clearly depend on the nature of the derivative. (The requirement for an A-type ligand is thus analogous to that described above, and so is our interpretation.) As expected, the cephalothin-induced derivative is much more stable than that induced by cloxacillin, which is actually more labile than the native enzyme. As before, cross-linking in the absence of substrates yielded a derivative of intermediate stability.

In conclusion, we have demonstrated that substrate-induced changes, which are normally reversible, can be stabilized by cross-linking of the enzyme molecule. The derivatives thus obtained retained catalytic activity and the specific imprint of the conformation-modifying substrate. A more extensive study of such derivatives should improve our understanding of the role of substrate-induced changes in the function of enzymes. In a more general sense, our results illustrated the possibility of designing enzyme derivatives with predictable properties by an appropriate choice of conformation-modifying site-specific ligands.

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