Vol. 82, No. 3, 1978 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
June 14, 1978 Pages 951-956

REVERSIBLE INHIBITION OF PENICILLINASE BY QUINACILLIN: EVALUATION OF MECHANISMS INVOLVING TWO CONFORMATIONAL STATES OF THE ENZYME

by

Richard Virden, Adrian F. Bristow*and Roger H. Pain

Biochemistry Department, The University,

Newcastle upon Tyne, NE1 7RU, U.K.

Received April 14,1978

SUMMARY

Staphylococcal penicillinase (EC 3.5.2.6) is shown to undergo partial, fully reversible inactivation of benzylpenicillinase activity on incubation with the substrate quinacillin, the hydrolysis of which follows a corresponding biphasic time-course. The kinetics fit a scheme involving slow isomerization of the enzyme between conformational states that differ in K_m and V_{max} for quinacillin. The possibility that inactivation is related to formation of a previously observed covalent enzyme-quinacillin conjugate is ruled out because the kinetics of its formation differ from those of inactivation. This implies that the conjugate arises from a state of the enzyme substrate complex present during the normal catalytic cycle. The multiplicity of binding sites found suggests that a reactive catalytic intermediate substitutes several amino-acid side chains during denaturation of the enzyme-quinacillin mixture, thus providing an explanation of earlier results.

Quinacillin and certain other semisynthetic penicillins induce slow inactivation of staphylococcal penicillinase. Although inactivation was complete and irreversible in most of his experiments, Dyke (1) suggested that it was associated with reversible conformational isomerization. In the present work, using purified enzyme, inactivation has been shown to be partial and fully reversible. An alternative explanation of reversible inactivation is raised by the recent observation that quinacillin was bound covalently in almost equimolar proportions after denaturation of a mixture of enzyme and

* Present address: Biochemistry Laboratory, School of Biological Sciences, University of Sussex, Falmer, Brighton, BN19QG, U.K.

0006-291X/78/0823-0951$01.00/0

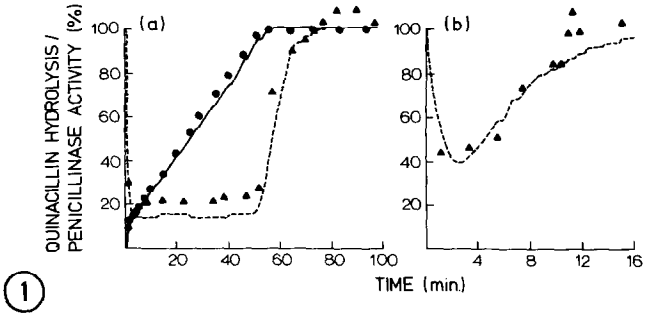
and quinacillin (2). No such complex was obtained after completion of quinacillin hydrolysis. Inactivation could thus reflect reversible, covalent attachment of quinacillin close enough to the active site to cause loss of activity by steric hindrance. The kinetics of covalent modification and the nature of the covalent product have therefore been investigated.

MATERIALS AND METHODS

Penicillinase from Staphylococcus aureus PC1 was purified and assayed for benzylpenicillinase activity as previously described (2-4) except that 0.05% gelatin was included in the assay mixture (5). Hydrolysis of quinacillin was measured by the fall in optical rotation at 400 nm (6). Quinacillin was incubated with penicillinase at 25°C in phosphate-EDTA buffer, pH 7.0, containing 39mM KH_2PO_4/61mM Na_2HPO_4/1mM Na_2 EDTA. Digestion of the enzyme-quinacillin complex with chymotrypsin and purification of peptides by chromatography on Sephadex G-25 and on sulphopropyl-Sephadex, and by high voltage paper electrophoresis were as previously described (2). Digestion with trypsin and pepsin and the strategy of peptide purification were as described by Ambler (7). Quinacillin attached to peptides was detected by the absorbance at 326 nm (8) in samples eluted from columns or from paper. On paper, quinacillin (>50pmol) was detected by contact printing on to Ilford DR 4.5L document paper using u.v. light.

RESULTS AND DISCUSSION

Effect of Quinacillin on benzylpenicillinase activity: Fig.1a shows the effect on the activity of the enzyme of incubating it with a saturating concentration of quinacillin. At various times after mixing, samples were diluted into an excess of benzylpenicillin solution such that the final quinacillin concentration was less than that at which competitive inhibition of benzylpenicillinase can be detected (5-10mM). The benzylpenicillinase activity of each partly inactivated sample remained at a constant value for 1-2 min after dilution from quinacillin; thereafter significant reactivation was observed. The variation in benzylpenicillinase activity as a function of time of exposure to quinacillin correlated with the changes in rate of hydrolysis of quinacillin. Both processes reached similar limiting values relative to their initial values (approx. 20%); increasing the quinacillin concentration to 14mM led to no



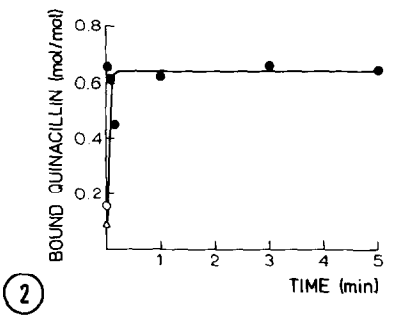


Fig.1 The effect of incubating penicillinase (144 μg/ml) with quinacillin on the benzylpenicillinase activity of the enzyme. Quinacillin (a) 5.4 mM, (b) 0.5 mM. ●, Extent of quinacillin hydrolysis (points taken from a continuous trace of optical rotation). ▲, Benzylpenicillinase activity of samples diluted 500-fold into 1.4 mM benzylpenicillin in the pH-stat cell. Hatched lines were calculated using the scheme described in the text, setting $k_1 = 10^7 M^{-1}.s^{-1}$, $k_1 = 3 \times 10^3 s^{-1}$, $kcat = 2.4 s^{-1}$, $k_3 = 2.5 \times 10^{-2} s^{-1}$, $k_{-3} = 3.8 \times 10^{-3} s^{-1}$, $k_2 = k_{-2} = k'_1 = k'_{-1} = k'_{cat} = 0$.
——— Extent of quinacillin hydrolysis, – – – – concentration of active enzyme.

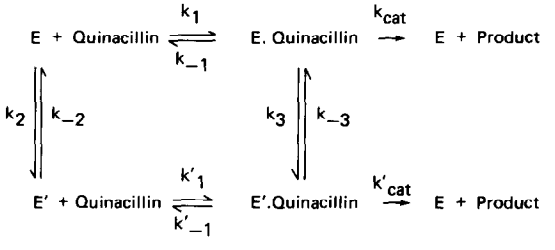
Fig.2 Enzyme-quinacillin conjugate obtained by quenching reaction mixtures with formic acid. ●, An equal volume of formic acid was added to a reaction mixture containing enzyme (1.2 mg/ml) and 43 mM quinacillin. In controls, the final concentration of each component was identical to those in other quenched mixtures, i.e. 50% (v/v) formic acid; enzyme, 0.6 mg/ml; 21.5 mM quinacillin. These mixtures were made up in the following way: ○, enzyme added to quinacillin in formic acid; △, quinacillin added to enzyme in formic acid.

greater inactivation. With lower, non-saturating concentrations of quinacillin less than maximal inactivation was observed (Fig.2). In all cases benzylpenicillinase activity returned to its initial value after quinacillin had been completely hydrolysed.

Analysis (9) of the initial and secondary rates of hydrolysis as a function of quinacillin concentration (0.5-4.3mM) gave K_m values for the fast and slow phases of the reaction of 0.31 ± 0.10 and 0.09 ± 0.03mM respectively. The respective values of V_{max} were 12.0 ± 0.6 and 1.55 ± 0.05 $\mu mol.min^{-1}.mg^{-1}$. Within experimental error both K_m and V_{max} fell by the same factor in the transition from the fast to slow phases of the reaction.

The main features of these experimental results are as follows: (i) quinacillin hydrolysis follows a biphasic time-course; (ii) the time-course of the fall in benzylpenicillinase activity parallels the transition between the fast and slow phases of quinacillin hydrolysis; (iii) the inactivation of benzylpenicillinase is fully reversed after the quinacillin is fully hydrolysed; (iv) only partial loss of activity is observed with saturating concentrations of quinacillin; (v) the rates of inactivation ($t_{\frac{1}{2}} \approx 30s$) and reactivation ($t_{\frac{1}{2}} \approx 200s$) on dilution into benzylpenicillin are slow compared with the turnover of quinacillin in the slow phase of hydrolysis ($t_{\frac{1}{2}} = 2s$); (vi) in addition, the degree of maximal inactivation varies when a different β-lactam compound is substituted for quinacillin (Table 1).

Biphasic kinetics of hydrolysis suggest modelling by a scheme (I) that postulates an active state (E) and an inactive or less active state (E') of penicillinase.



(I)

The observed kinetics of quinacillin hydrolysis (Fig.1a) were fitted by choosing reasonable values for k_1 and k_1', constraining others to fit the experimental values of K_m and V_{max}, and adjusting the remainder to obtain a reasonable fit to the time-course of hydrolysis (10). The constants used to generate the curve in Fig.1 illustrate a simple, special case of the scheme in which isomerization may occur only between alternative states of the enzyme-substrate complex, one of which is completely inactive. This scheme demands that values of k_3 and k_{-3} be small, consistent with an appreciable conformational change.

Table 1. Inactivation of penicillinase by β-lactam antibiotics. Samples were taken at about 6 min. intervals from a mixture of enzyme and β-lactam compound.

Compound	Concn. (mM)	Enzyme concn. (μg/ml)	Maximum extent of inactivation (per cent)	Extent of reactivation (per cent)
Quinacillin	5	144	80	107
"	14	52	71	97
Cloxacillin	11	108	82	101
"	40	1400	91	76
Pyrazocillin	6	88	92	95 - 100
Cephaloridine	1	69	86	90

The predicted concentration of remaining active enzyme gives a reasonable fit to the observed level of benzylpenicillinase activity (Fig.1a). Further, the same rate constants provide a good prediction of the time course of fall and recovery of activity during incubation with a lower concentration of quinacillin (Fig.1b). It would be equally possible to model the kinetic data by assuming that E' represented a partially active state or states. In each case, however, the maximum extent of inactivation is predicted to depend on the position of the equilibrium between active enzyme and the form(s) of the enzyme represented by E'. This equilibrium would plausibly vary with the nature of the β-lactam compound, again in accord with experiment.

Significance of the quinacillin-penicillinase complex. Fig.2 shows that 0.7 mol of quinacillin per mol of enzyme were rapidly bound ($t_{\frac{1}{2}} < 10s$) after incubation of enzyme and quinacillin followed by quenching with 50% formic acid. This binding is covalent (2). The complex is formed too rapidly for it to be responsible for quinacillin-induced inactivation ($t_{\frac{1}{2}} \approx 30s$).

The attachment of quinacillin to the enzyme was further investigated by isolating peptides after digestion with either chymotrypsin, trypsin

or pepsin. In each case, quinacillin was recovered in low yield and attached to many peptides. Thus, material thought to consist of one or two peptides (2) was found to include at least eight quinacillin-containing components when a larger sample (containing 220 nmol of quinacillin chromophore) was fractionated by high voltage paper electrophoresis at pH 6.5. The largest recovery of quinacillin in a single peptide was 6% of the total quinacillin originally bound to the enzyme.

The rapid kinetics of formation of the quinacillin-enzyme complex favour its being derived from a catalytic intermediate. The heterogeneity of the binding location suggests that this is an activated form of quinacillin which reacts with a number of groups on the enzyme during denaturation.

The fact that the partial inactivation is fully reversible with a number of β-lactam-containing compounds and that the associated kinetics can be modelled by a scheme involving a conformational change of enzyme in the enzyme-substrate complex provides quantitative support for the conformational hypothesis as the basis of the modification of penicillinase activity.

We thank Gail Burgham and Bob Nicholson for technical assistance, The Smith, Kline and French Foundation for a Radiometer Autoburette and the Science Research Council for financial support. Quinacillin, cloxacillin and cephaloridine were generously given by The Boots Company Ltd, Beecham Pharmaceuticals, and Glaxo Research Ltd, respectively.

REFERENCES

1. Dyke, K. G. H. (1967) Biochem. J., 103, 641-646.
2. Virden, R., Bristow, A. F. and Pain, R. H. (1975) Biochem. J. 149, 397-401.
3. Robson, B. and Pain, R. H. (1976) Biochem. J., 155, 325-330
4. Bristow, A. F. and Virden, R. (1978) Biochem. J. 169, 381-388.
5. Al Shamaony, L. (1972) M.Sc. Thesis, University of Newcastle upon Tyne.
6. Kersey, R. C., Iriye, T., Meehav, B. M. and Kasper, M. A. (1955) Antibiotics Ann. 47-50.
7. Ambler, R. P. (1975) Biochem. J. 151, 197-218.
8. Richards, H. C., Housley, J. R. and Spooner, D. F. (1963) Nature, (London) 199, 354-356.
9. Wilkinson, G. N. (1961) Biochem. J. 80, 324-332.
10. Wright, K. (1970). BIT 10, 217-227.