Interaction of the pBR 322-coded RTEM α -lactamase with substrates

Evidence for specific conformational transitions

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(Received 26 October 1981/Accepted 13 November 1981)

The rate of inactivation of RTEM-1 β -lactamase by Pronase is accelerated by class A ('resistant') penicillins. Other substrates (class S penicillins and cephalosporins) protect against the inactivation. Cefoxitin, a semi-synthetic cephamycin, induces a more extensive, hysteretic response. In its presence the enzyme is inactivated by trypsin as well as by Pronase.

The structure of the *N*-acyl substituent ('side-chain') of a penicillin determines the rate of hydrolysis of the β -lactam bond by β -lactamase (EC 3.5.2.6); side-chains of class S promote, while those of class A retard, the catalytic reaction. These contrasting effects have been correlated with differences in conformational states induced by the two classes of substrates in several β -lactamases (Citri *et al.*, 1976; Pain & Virden, 1979; Kiener *et al.*, 1980). Thus β -lactamase I of *Bacillus cereus*, which has been studied in some detail, has been shown to acquire a generally labile, partly unfolded and catalytically inefficient conformation in the course of its interaction with class A substrates. By contrast, substrates of class S induce, without a perceptible lag, a stable and efficient conformation (Citri & Zyk, 1965). The most obvious consequence is the difference in the kinetics, which are linear with all S substrates but biphasic (initially decelerating) with all A substrates (Citri *et al.*, 1976). However, biphasic kinetics have not been observed with RTEM β -lactamase in its reaction with class A penicillins. In the absence of any other evidence of substrate-induced changes in the conformation of that enzyme, it has been assumed that conformational adaptation has no role in its interaction with substrates (Fisher *et al.*, 1978, 1980; Frère, 1981).

We have now examined the effect of substrates on the β -lactamase coded by the *amp^r* of plasmid pBR 322 and expressed in a K-12 strain (C-600) of *Escherichia coli*.

Methods

The enzyme, classified as RTEM-1 β -lactamase (Datta & Kontomichalou, 1965; Ambler & Scott, 1978; Sutcliffe, 1978) was purified as described (Melling & Scott, 1972). We found it to be

cold-sensitive and to require 1–2 h at 30°C before regaining full activity after cold storage (N. Citri & N. Zyk, unpublished work). The reactivated enzyme was kept at 30°C throughout our experiments. The substrates were all from commercial sources, except for Cephamycin C which was a gift from Merck Sharp and Dohme (Rahway, NJ, U.S.A.). Pronase (type V) was obtained from Sigma and trypsin (twice crystallized) from Worthington.

Results and discussion

Our initial observations confirmed that this enzyme tends to retain a stable, native conformation and does not respond to the presence of substrates in a readily observable manner. We found, however, that differential proteolytic inactivation (Citri, 1973) detects in this enzyme substrate-induced changes which are remarkably consistent with those seen in β -lactamases secreted by Gram-positive organisms.

RTEM β -lactamase is very resistant to trypsin, but moderately susceptible to inactivation by Pronase. The rate of inactivation by Pronase, which follows first-order kinetics (results not shown), was strongly accelerated by A penicillins (Table 1), whereas S-penicillins (Table 2) protected the enzyme against proteolysis. The unsubstituted penam nucleus had no effect (Table 1). The protection by cephalosporins (Tables 1 and 2) is also consistent with the previously reported stabilization of bacillary β -lactamases by these substrates (Citri & Zyk, 1965). The competitive interaction of a labilizing and a stabilizing substrate (Table 1) is as expected if either must bind to the active site to alter the conformation of the enzyme.

The only compounds, outside the class of A penicillins, which we found to induce susceptibility

Table 1. *Rate of inactivation of RTEM β -lactamase by Pronase: effect of substrates*

Substrate (0.5 mM) was present in the treatment solution (final volume 100 μ l) containing 50 μ g of Pronase, CaCl₂ (10 mM), Tris buffer, pH 7.5 (20 mM) and 10 units (Pollock & Torriani, 1953) of the RTEM enzyme, added last. The incubation (at 30°C) was terminated by dilution into the assay solution. Half-life is based on survival of activity assayed, at appropriate intervals, by either the spectrophotometric or timed iodometric procedures.

Substrate	Half-life of β -lactamase (min)
None	7-9*†
6-Aminopenicillanic acid	7-9*
A penicillins	
Oxacillin	0.9†
Cloxacillin	0.8*†
Dicloxacillin	0.8†
Flucloxacillin	0.7†
Methicillin	1.0†
Cephalosporins	
Cephaloridine	>60 (see Table 2)
Cephalexin	37†
Cephalosporin C	>60*
Cephalothin	>60*†
Cephalothin (1.0 mM)	
+ Cloxacillin (0.25 mM)	3.6†
+ Cloxacillin (0.5 mM)	2.7†
Cefoxitin	3.0*†

* Assayed spectrophotometrically with 8 mM-cephaloridine in 10 mM-Tris buffer, pH 7.5, at 324 nm (ϵ 305 M $\cdot$ cm⁻¹). (Total volume 3.0 ml.)

† Assayed (with benzylpenicillin) by the time iodometric procedure (Citri, 1964) adapted for this enzyme by replacing gelatin with starch (2%), and lowering the iodine (to 1.0 mM), in the assay solution (total volume 1.2 ml). The modified assay is most precise ($\pm$ 5%) in the range of 0.4 to 4.0 units (Pollock & Torriani, 1953) of β -lactamase.

Table 2. *Progress of the β -lactamase reaction in the presence of Pronase*

Protection by rapidly hydrolysed substrates (S penicillins, cephaloridine) was evident from the course of the catalytic reaction in the presence of Pronase (500 μ g/ml). A colorimetric assay (Imsande, 1965) was used because absorption by Pronase at that concentration precluded direct spectrophotometry. The rapid decline in the rate of hydrolysis of oxacillin (an A penicillin) under these conditions confirmed the validity of this procedure.

Substrate (8 mM)	Half-life of β -lactamase (min)
Cephaloridine	>60
S penicillins	
Benzylpenicillin	>60
Phenoxymethylpenicillin	>60
Ampicillin	>60
A penicillin (oxacillin)	<1.0

Table 3. *Tryptic inactivation is induced by cefoxitin and prevented by cloxacillin*

Treatment was as in Table 1 except for Pronase, which was replaced with 10 μ g of trypsin. Residual activity was measured spectrophotometrically (as in Table 1) after 10 min at 30°C.

Treatment		Residual activity
[Cefoxitin] (mM)	[Cloxacillin] (mM)	
0	0	100
0.05	0	35
0.10	0	18
0.45-2.0	0	4
0.45	0.45	68

to proteolytic inactivation were members of the cephamycin family, namely cephamycin C (a naturally occurring 7-methoxy cephalosporin) and cefoxitin, a semisynthetic cephamycin. Since the latter is more available, we used it in subsequent studies. In spite of the apparent similarity, cefoxitin differs from class A substrates in its effect. It induces a conformation which is sensitive to tryptic (Table 3), as well as Pronatic (Table 1), inactivation. The difference between the effect of A penicillins and cefoxitin on the conformation is best illustrated in Table 3, where cloxacillin, an A penicillin which induces a Pronase-sensitive conformation, protects the enzyme against trypsin by displacing cefoxitin from the active site.

Furthermore, cefoxitin at concentrations below its K_m (0.69 mM) (Fisher *et al.*, 1980) attains near-saturation in converting the enzyme to the trypsin-sensitive state (Table 3; N. Citri & N. Zyk, unpublished results). This implies that the extensive conformational change, induced by cefoxitin, persists after the dissociation of the RTEM lactamase-cefoxitin complex. In other words, the response of the enzyme to this substrate appears to be hysteretic, like that of β -lactamase I to A penicillins (Citri *et al.*, 1976). An obvious alternative, namely the persistence of the complex itself, is suggested by the compelling evidence, presented by the group of Knowles (Fisher *et al.*, 1980), that an acyl-enzyme intermediate is formed which is slow to dissociate. While we do not dispute the conclusion that cefoxitin can acylate the enzyme, it is not at all clear that a significant fraction of the enzyme was acylated in our experiments which required a shorter exposure to substantially (approx. 200 times) lower concentrations of cefoxitin (cf. Table 3 and Fisher *et al.*, 1980). Also note the presence of CaCl₂ in the tryptic, as well as pronatic, treatment; calcium ions, and several other divalent cations, have been found to prevent termination of the hysteretic response in

β -lactamases (N. Citri & N. Zyk, unpublished results).

The structural basis of the conformation-distorting effect of A penicillins has been recently elucidated; the side-chains of this class of substrates, unlike those of other penicillins, have been shown to have a rigid internal structure (Blanpain *et al.*, 1980). That A-type side chains contribute nevertheless to a tighter binding of the substrate provides impressive evidence for the conformational adaptability of the enzyme. However, in the course of adapting, the enzyme acquires a partly unfolded conformation (Citri *et al.*, 1976; Citri, 1973). It is probably significant that this pattern of conformational response, derived from studies of Gram-positive β -lactamases, can now be extended, *mutatis mutandis*, to the RTEM enzyme. The details of the interaction show differences which are consistent with the existence of a disulphide bridge in RTEM β -lactamase (Ambler & Scott, 1978) and the absence of covalent cross-links in the other enzymes. Thus the RTEM enzyme, which is generally more stable, is not perceptibly destabilized (e.g. to heat or urea) by A substrates; also, it does not display the hysteretic behaviour, and the consequent biphasic kinetics, in catalysing the hydrolysis of such penicillins. In that sense, the RTEM enzyme resembles cross-linked derivatives of β -lactamase I (Klemes & Citri, 1979, 1980), and the membrane-bound γ -penicillinase (Citri & Kalkstein, 1967) of *B. cereus*. Conformational changes in such constrained enzyme molecules are more restricted and may be difficult to detect. However, as pointed out elsewhere (Citri, 1973), proteolytic inactivation provides us with a very sensitive criterion, since a proteinase with broad specificity can be viewed as a general probe for slight unfolding in a tightly packed enzyme molecule. The more extensive and persistent response to cefoxitin is likely to be due to the 7-methoxy substituent, absent from the other sub-

strates and presumably responsible for the unusual resistance of cephamycins to enzymic hydrolysis. Its pivotal position suggests a different and possibly more exacting challenge to the conformational adaptability of β -lactamase.

References

- Ambler, R. P. & Scott, G. K. (1978) *Proc. Natl. Acad. Sci. U.S.A.* **75**, 3732–3736
- Blanpain, P. C., Nagy, J. B., Laurent, G. H. & Durant, F. V. (1980) *J. Med. Chem.* **23**, 1283–1292
- Citri, N. (1973) *Adv. Enzymol. Relat. Areas Mol. Biol.* **37**, 397–648
- Citri, N. (1964) *Methods* **10**, 221–232
- Citri, N. & Kalkstein, A. (1967) *Arch. Biochem. Biophys.* **121**, 720–728
- Citri, N. & Zyk, N. (1965) *Biochim. Biophys. Acta* **99**, 427–441
- Citri, N., Samuni, A. & Zyk, N. (1976) *Proc. Natl. Acad. Sci. U.S.A.* **73**, 1048–1052
- Datta, N. & Kontomichalou, P. (1965) *Nature (London)* **208**, 239–241
- Fisher, J., Charnas, R. L. & Knowles, J. R. (1978) *Biochemistry* **17**, 2180–2184
- Fisher, J., Belasco, J. G., Khosla, S. & Knowles, J. R. (1980) *Biochemistry* **19**, 2895–2901
- Frère, J.-M. (1981) *Biochem. Pharmacol.* **30**, 549–552
- Imsande, J. (1965) *J. Bacteriol.* **89**, 1322–1327
- Kiener, P. A., Knott-Hunziker, V., Petursson, S. & Waley, S. G. (1980) *Eur. J. Biochem.* **109**, 575–580
- Klemes, Y. & Citri, N. (1979) *Biochim. Biophys. Acta* **567**, 401–409
- Klemes, Y. & Citri, N. (1980) *Biochem. J.* **187**, 529–532
- Melling, J. & Scott, G. K. (1972) *Biochem. J.* **130**, 55–62
- Pain, R. H. & Virden, R. (1979) in *Beta-Lactamases* (Hamilton-Miller, J. M. T. & Smith, J. T., eds.), pp. 141–180, Academic Press, London
- Pollock, M. R. & Torriani, A. M. (1953) *C. R. Acad. Hebd. Séances Sci.* **237**, 276–278
- Sutcliffe, J. G. (1978) *Proc. Natl. Acad. Sci. U.S.A.* **75**, 3737–3741