ISOLATION OF A COVALENT INTERMEDIATE IN β-LACTAMASE I CATALYSIS

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1. Introduction

Resistance of bacteria to penicillins and cephalosporins is often due to the production of a β-lactamase (penicillinase), which results in the degradation of these clinically important antibiotics. Relatively little is known regarding the catalytic mechanism by which β-lactam ring hydrolysis occurs. Although studies with inhibitors [1–8] and poor cephalosporin and penicillin substrates [9–11] suggest the intermediacy of an acyl-enzyme, the existence of such an intermediate in the catalysis of good (specific) substrates must still be considered speculative. In fact the lack of definitive evidence for an acyl-enzyme intermediate in penicillin catalysis has been held to support a general-base-type mechanism [12].

Studies on the site acylated by penicillin G and a specific substrate in 2 bacilliary carboxypeptidases [13,14] have shown striking homology with the sites of several β-lactamases acylated by a variety of inactivators [7,15,16]. Here, we show that a covalent intermediate, consistent with an acyl-enzyme, is formed in the reaction of β-lactamase I (from *B. cereus* 569/H) with the specific substrate dansyl-penicillin and can be trapped at low pH.

2. Materials and methods

β-lactamase I was prepared and assayed by standard procedures [17]. Dansyl-penicillin was synthesized as follows: A solution of 4.5 g 6-aminopenicillanic acid in 300 ml 3% sodium bicarbonate/300 ml acetone was mixed with 4.5 g dansyl-chloride in 40 ml acetone. The mixture was stirred at room temperature for 2 h, filtered and the acetone removed under vacuum. The aqueous residue was extracted twice with ether and the latter discarded. The aqueous phase was further extracted with 2 × 100 ml butyl-acetate at pH 2. The aqueous phase was then titrated with bicarbonate to pH 7.8 and lyophillized. The product, the potassium salt of dansyl-penicillin was homogeneous by several TLC systems.

The low-pH intermediate trapping experiment was carried out as follows: 0.1 ml enzyme (35 mg/ml) in pH 5.5 buffer, was added to 0.9 ml solution of dansyl-penicillin in pH 2.0 aqueous buffer (either HCl/KCl or HCl/HAc) at 4°C, to give a substrate/enzyme ratio of 100:1. Five volumes of acetone were immediately added to precipitate the protein; after centrifugation and washing twice with acetone, the pellet was taken up in 5 M Gu · HCl. The stoichiometry of the labelled enzyme was determined from standard calibration curves using dansyl-penicillin or its hydrolysis product (excitation 350 nm, emission 550 nm) as a function of pH and solvent. Linear log–log plots of fluorescence emission intensity *vs* concentrations were obtained over 3–4 orders of magnitude of concentration. The stoichiometry of the labelled protein was usually determined at pH 4.3 in the presence of 5 M Gu · HCl since the sensitivity was much higher than at pH 2.

Peptide fragmentation was performed by digesting the dansyl-penicillin–β-lactamase I (2 mg) with pepsin (200 μg) for 1 h at pH 2.0, 25°C.

3. Results and discussion

Dansyl-penicillin was the substrate of choice for this investigation on the basis of its fluorescence properties, which provide a sensitive means of detecting its presence, and its catalytic properties, which show it to be as good a substrate as benzyl-penicillin (penicillin G). The catalytic parameters were determined as follows: $k_{cat} = 2 \times 10^3\ s^{-1}$, $K_m = 200\ \mu M$, and $k_{cat}/K_m = 1 \times 10^7\ M^{-1}\ s^{-1}$ (pH 7.0, 25°C).

The approach chosen to demonstrate the presence

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of the putative acyl-enzyme was as follows:

(1) To build up a significant concentration of the intermediate;
(2) To rapidly denature it;
(3) To subject the denatured (hence trapped) acyl-enzyme to gel filtration under conditions where coelution of the fluorescence label with the enzyme would signify a covalent linkage;
(4) To use protease fragmentation and enzyme digestion to locate the site, or sites, of covalent attachment.

Three different methods to trap the putative acyl-enzyme were investigated. Two utilized very low temperatures, in conjunction with cryosolvents, to accumulate the intermediate, followed by rapid denaturation [18]. The third method, reported herein, made use of the fact that the enzyme is rapidly inactivated by acid, and yet the anticipated rate of deacylation at low pH should be quite slow (assuming deacylation is dependent on a $pK > 4$).

The inactivation of β-lactamase I at pH 2.0 was monitored by the intrinsic fluorescence emission of the enzyme at 4°C, where the half-life was found to be 30 s. Since formation of the putative acyl-enzyme would be a very facile process as it involves opening of the strained β-lactam ring, it is reasonable to assume that deacylation would be slower and hence probably rate-limiting. If this is so, one can calculate that at pH 2, 4°C the rate of deacylation will be comparable to, or somewhat faster than, that of inactivation. Therefore, if the enzyme were added to a solution of substrate at pH 2.0 a competition would be established between denaturation and acrylation; similarly any acyl-enzyme formed would also be subject to a competition between denaturation and deacylation.

When β-lactamase I at pH 5 was added to a solution of dansyl-penicillin at pH 2, 4°C, the apparent rate of inactivation, as measured by changes in the intrinsic fluorescence of the enzyme, was unchanged. No turnover was observed under these experimental conditions. The enzyme–substrate complex was precipitated by the addition of acetone and centrifuged. The pellet was dissolved in 5 M Gu · HCl. From measurement of the fluorescence emission, and knowledge of the enzyme concentration, a 1:0.9 ± 0.1 stoichiometry of enzyme to substrate was calculated. The dissolved pellet was subjected to gel filtration on Sephadex G-25 using 5 M Gu · HCl as eluant. The fluorescent label co-chromatographed with the enzyme with no significant change in the stoichiometry of substrate to enzyme. In control experiments in which either the substrate was added to the enzyme, already incubated at pH 2.0 (and hence inactive), or the enzyme was pre-inactivated with 6-β-bromopenicillanic acid, <0.02 mol bound substrate/mol enzyme were found in the pellet, indicating that the dansyl-penicillin binds to the active site and that non-specific interactions are minimal. Thus, we conclude that the intermediate trapped by this low-pH procedure involves covalent bonding between enzyme and substrate, with the substrate bound in the active-site.

Dialysis of the dansyl-penicillin-labelled enzyme against 5 M Gu · HCl solutions at various pH-values confirmed the acid-stability of the labelled derivative, but indicated lability in neutral or alkaline solutions (fig.1). In [14] a similar acid stability for the penicilloyl moiety bound to *B. stearothermophilis* carboxypeptidase was described, and its susceptibility to base-catalyzed hydrolysis interpreted to indicate an ester linkage [14]. If an aliquot of the dansyl-penicillin–enzyme derivative at pH 2.0 was added to pH 7.0 buffer at 25°C the loss of the label could be followed by changes in its fluorescence spectrum. Over 65% of the label was lost with a first-order rate constant of 9×10^{-3} s $^{-1}$. Presumably this rate is that of the renaturation of the acyl-enzyme at pH 7.0. Analysis of the product by TLC indicated it co-chromatographed with authentic dansyl-penicillinoic acid.

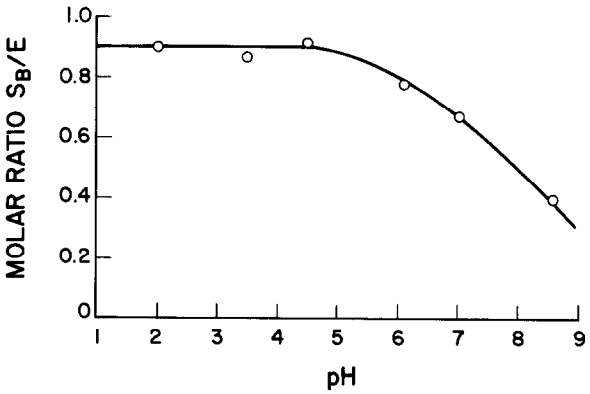

Fig.1. pH-Stability of the dansyl-penicillin–β-lactamase complex. Labelled enzyme was dialyzed against 5 M Gu · HCl at the pH values shown for 1 h at 4°C. The molar ratios of enzyme-bound dansyl-penicillin are corrected for the effect of pH and denaturant on the fluorescence intensity. S_B is the bound substrate. The decreased ratios of S_B/E at higher pH reflect the lability of the denatured acyl-enzyme under these conditions, since the fluorescence of the covalently bound dansyl-penicillin is much greater than that of dansyl-penicilloic acid (see text).

The dansyl-penicillin–β-lactamase I complex was found to be sufficiently stable at pH 2 in the absence of additional denaturant to permit digestion of the labelled enzyme with pepsin. The resulting peptide fragments were initially subjected to gel filtration on Sephadex G-25. The rationale behind this method was that the anticipated dansyl-penicillin peptide would be rather hydrophobic and thus retarded by the non-polar adsorption properties of Sephadex G-25. Comparison of the absorbance and fluorescence of the eluant revealed 4 peaks, the last 2 both having fluorescence emission from the dansyl group. The last peak to elute was shown to contain dansyl-penicillinoic acid. The preceding fluorescent fraction presumably contained the desired dansyl-penicillin–peptide and other peptides with hydrophobic side-chains. The desired fraction was subjected to high-voltage paper electrophoresis at pH 1.4 which revealed a single fluorescent spot, which was then eluted. The preliminary results of subsequent studies on this fluorescent peptide, using a variety of purification techniques, end-group analysis, and carboxy- and amino-peptidase sequencing, are consistent with attachment of the dansyl-penicillin to Ser-44.

An interesting aspect of the dansyl-penicilloyl–enzyme intermediate is the dependence of its fluorescence on environmental factors. For example, in 5 M Gu · HCl, in which the native enzyme is denatured [19], the excitation and emission maxima of the acyl-enzyme at pH 2 are at 300 and 565 nm, respectively. In the absence of the guanidine, at pH 2, the fluorescence intensity is 30-fold greater with the excitation and emission maxima at 350 and 500 nm, respectively (not shown). We conclude that the dansyl-penicillinoyl–enzyme complex at pH 2 in the absence of Gu · HCl is not fully denatured, but retains at least some structure in the vicinity of the substrate binding site. However, since the intermediate does not deacylate significantly under these conditions the complex is not fully native either. This observation indicates that not only is the dimethylamino-group of the fluorophor unprotonated in the latter instance, but that the binding site of the dansyl group on the enzyme is highly hydrophobic [20,21]. Raising the pH to 7 results in a rapid loss of a portion of this emission maximum at 500 nm and a concomitant red shift to 565 nm, with a much lower fluorescence emission intensity, characteristic of free dansyl-penicilloic acid in aqueous solution.

These results provide the first definite evidence that β-lactamase catalysis of specific penicillin substrates proceeds via a covalent intermediate. Moreover, the preliminary elucidation of the site of attachment of the substrate is consistent with the postulated relationship between the β-lactamases and the penicillin-sensitive enzymes of the bacterial cell wall.

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