Use of electrospray mass spectrometry to directly observe an acyl enzyme intermediate in β-lactamase catalysis

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Electrospray mass spectrometry was used to directly observe intact acyl enzyme complexes formed between a class C β-lactamase (from *Enterobacter cloacae* P99) and four poor substrates/inhibitors. In each case the molecular weight difference between the unreacted and the reacted β-lactamase was consistent with the formation of an acyl enzyme.

β-Lactamase; Electrospray mass spectrometry; Inhibition; Acyl enzyme complex

1. INTRODUCTION

β-Lactamases are bacterial enzymes that by catalysing the hydrolysis of the lactam bond of the β-lactam type antibiotics, play an important role in the antibiotic resistance of pathogens. Mechanistically they are either metallo-enzymes or serine enzymes [1]; despite the wealth of evidence demonstrating the formation of a covalent bond between substrate and β-lactamase, the only direct spectroscopic evidence for a covalent ester linkage, *during catalysis* remains a Fourier transform infra-red study [2].

The serine enzymes are subdivided, on the basis of their amino acid sequences into classes A or C. Class C β-lactamases, which resemble each other closely [3], have the favourable property that, for several substrates both k_{cat} and K_m are low; this permits the direct observation of an intermediate. Indeed such a substrate was used in the first isolation of an acyl enzyme from a class C β-lactamase, but this was only achieved after denaturation of the enzyme [4]. The goal of detecting such an intermediate during catalytic turnover has now been attained, and is the subject of this paper. One of the intermediates observed here is analogous to the one characterised crystallographically with the class C β-lactamase from *Citrobacter freundii* [5].

The newly developed technique of electrospray mass spectrometry provides a rapid method for the determination of the molecular weight of proteins, and has found recent successful application in the analysis of heterogeneous mixtures of proteins [8,9]. In this paper we describe our attempts to characterise the acyl-enzyme intermediate in the catalytic hydrolysis of the β-lactam antibiotics by mass spectrometry.

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2. EXPERIMENTAL

2.1. *Materials*

Carbenicillin, oxacillin, flucloxacillin, penicillin G, and ampicillin, were purchased from Sigma Chemicals. Aztreonam was a gift of the Bristol-Meyers Squibb Pharmaceutical Institute.

β-Lactamase from *Enterobacter cloacae* was purchased from PHLS Centre for Applied Microbiology (Division of Porton Down Research Biochemicals Biotechnology, Porton Down, Salisbury, Wilts SP4 0JG).

2.2. *Methods*

Electrospray mass spectra were measured on a VG BIO Q triple quadruple atmospheric pressure mass spectrometer equipped with an electrospray interfere (VG Biotech, Tudor Road, Altrincham, Cheshire).

Samples (10 μl) were injected into the electrospray source via a loop injector (Rheodyne 5717) as a solution, typically 25–50 pmol/μl, in water/methanol (1 : 1) containing 1% acetic acid at a flow rate of 4 μl per minute (Applied Biosystems model 140A dual syringe pump). Sample solutions were prepared immediately prior to analysis by mixing 1 : 1 molar proportions of solutions of P99 β-lactamase and β-lactam in Mili-Q water at pH 7. Aliquots were taken after 5 and 10 min, mixed with an equal volume of methanol containing 2% (v/v) acetic acid (resulting solution pH 3.5) and immediately analysed by electrospray mass spectrometry. The mass spectrometer was scanned over the mass range 600–1400 Da. The instrument was calibrated with myoglobin (20 pmol/μl, M_w 16 950.5 Da).

3. RESULTS AND DISCUSSION

Initially we examined electrophoretically pure P99 β-lactamase by electrospray mass spectrometry (Fig. 1a). Analysis of the resultant data revealed a single species with a molecular mass of 39 203.36 Da. ($\delta_D = 7$), which is in good agreement with the value predicted 39 206.41 Da from the gene sequence [3].

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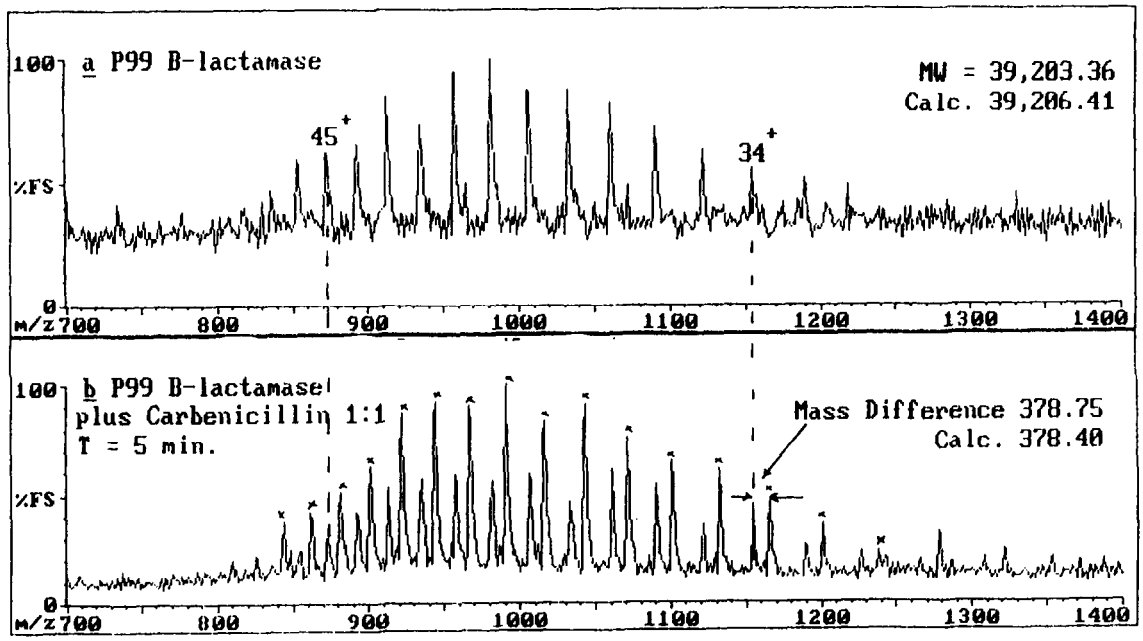

Fig. 1. (a) Electrospray mass spectrum of P99 β-lactamase. (b) Electrospray mass spectrum of P99 β-lactamase plus carbenicillin ($\times$ indicates peaks assigned as acylated enzyme).

The acyl-enzyme from the P99 β-lactamase has been previously isolated by reaction with cloxacillin followed by the addition of 1 vol. of acetic acid [10]. We speculated that it may be possible to directly observe an acyl-enzyme intermediate by electrospray mass spectrometry, of the P99 β-lactamase, for which kinetic parameters for both good and poor substrates have been reported [11,12]. Thus, initially an aqueous solution of the P99 β-lactamase was treated with aztreonam, followed by acid quenching (to pH 3.5), and examined by mass spectrometry. A new set of peaks was observed with measured mass shift of $437.6 \pm$ Da (calculated = 435.43) consistent with the fact that $>90\%$ of the protein had been acylated (Table I, entry 1). Similarly we were able to obtain mass shifts consistent with the formation of acyl enzyme complexes, using oxacillin (Table I, entry 2) and flucloxacillin (Table I, entry 3).

In the case of carbenicillin, under similar conditions, we observed ca. 70% formation of the complex after 5 min (Fig. 1b). After 10 min the level of acylated material had decreased to ca. 20% and after 15 min only the molecular mass corresponding to the native protein was observed, consistent with kinetic data [11] that indicate carbenicillin is a poor substrate. The apparent difference in stabilities of the acyl-enzyme complexes formed between the P99 β-lactamase and carbenicillin or oxacillin might not have been anticipated on the basis of previous kinetic studies [11]. These prior studies were, however, carried out at pH 8.2, whereas our electrospray analyses were made at pH 3.5. Hence, it is conceivable that the relative stabilities of the complexes is altered at the lower pH, especially since carbenicillin contains an acidic side chain whereas that of oxacillin is neutral.

Table I

Molecular mass shifts observed for acylated P99 β-lactamases

Entry	β-Lactam	Measured mass shift	Theoretical mass shift
1	Aztreonam	+437.65	435.43
2	Oxacillin	+401.49	401.44
3	Flucloxacillin	+455.39	453.87
4	Carbenicillin	+378.66	378.40

In contrast, in the case of the good substrates penicillin G and ampicillin [11] we were unable to detect any acyl enzyme intermediates, under the conditions we were using in this study, even in the presence of a large excess of substrate (not all the substrate was hydrolysed before quenching).

In summary, this mass spectrometric study provides further evidence for the formation of an acyl-enzyme intermediate during β-lactam hydrolysis by β-lactamases and illustrates the analytical power of electrospray mass spectroscopy, in searching for possible enzyme bound intermediates.

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