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A thiono-β-lactam substrate for the β-lactamase II of *Bacillus cereus*

Evidence for direct interaction between the essential metal ion and substrate

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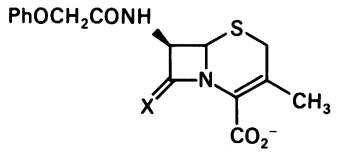
An 8-thionocephalosporin was shown to be a substrate of the β-lactamase II of *Bacillus cereus*, a zinc metalloenzyme. Although it is a poorer substrate, as judged by the $k_{cat.}/K_m$ parameter, than the corresponding 8-oxocephalosporin, the discrimination against sulphur decreased when the bivalent metal ion in the enzyme active site was varied in the order Mn^{2+} (the manganese enzyme catalysed the hydrolysis of the oxo compound but not that of the thiono compound), Zn^{2+}, Co^{2+} and Cd^{2+}. This result is taken as evidence for kinetically significant direct contact between the active-site metal ion of β-lactamase II and the β-lactam carbonyl heteroatom. No evidence was obtained, however, for accumulation of an intermediate with such co-ordination present.

INTRODUCTION

β-Lactamase II of *Bacillus cereus* is unusual among β-lactamases in that it is a zinc metalloenzyme [1]. Chemical-modification experiments [2,3], spectral studies [3–6], differential tritium-exchange experiments [7] and, more recently, a 0.35 nm-resolution X-ray-crystallographic structure [8] have shown that the protein provides four ligands, three histidine imidazole groups and one cysteine thiolate, to the essential active-site metal ion. Elegant pre-steady-state and cryoenzymological studies by Bicknell, Waley and co-workers [9, 10] have identified three enzyme–substrate complexes involved in turnover, and indicated the metal ion co-ordination geometry in these complexes. On the basis of these results and those from other chemical-modification experiments [11], detailed chemical mechanisms of action of this enzyme have been proposed [9–10]. An important but unresolved issue in these mechanistic speculations is whether the substrate comes in direct contact with the metal ion in a kinetically significant intermediate complex. This question, of course, has also long dogged mechanistic thought with respect to the prototypical metalloamidase carboxypeptidase A [12,13].

One approach to the answer of the above-mentioned question that has apparently yielded an unambiguous, or at least an unchallenged, conclusion comes from employment of thioamides and metalloenzymes containing active-site metal ions of distinctly different thiophilicity. Although thiopeptides are generally poorer substrates, of carboxypeptidase A for example, than are the analogous peptides [14–17], they are much less poor, again relative to the peptides, when the active-site Zn^{2+} ion is replaced by a more thiophilic metal ion such as Cd^{2+} [14, 17]. Conversely, a metal ion less thiophilic than Zn^{2+}, such as Mn^{2+}, yields a relatively poorer thioamidase [17]. Since β-lactamase II of *B. cereus* is known to yield active enzymes when Zn^{2+} is replaced with a variety of metal ions [3], it appeared that the thioamide probe might be useful in the case of this enzyme. We report here the results of such experiments with a thionocephalosporin substrate and its oxo analogue, (Ib) and (Ia) respectively. The results do support kinetically important metal ion–substrate contact.



(Ia) X = O
(Ib) X = S

EXPERIMENTAL

Materials

The β-lactamase II of *B. cereus* was separated from β-lactamase I (the mixture was purchased from the P.H.L.S. Centre for Applied Microbiology and Research, Porton Down, Wilts., U.K.) by the method of Davies *et al.* [18]. 7-(Phenoxyacetamido)-3′-desacetoxycephalosporanic acid (Ia) and 7-phenoxyacetamido-8-thiono-3′-desacetoxycephalosporanic acid (Ib) were prepared as previously described [19]. $ZnSO_4,7H_2O$ was Baker Analysed quality, and $Co(NO_3)_2,6H_2O$, $Cd(NO_3),4H_2O$ and $MnSO_4,4H_2O$ were Puratronic grade from Johnson Matthey Chemicals.

The apoenzyme of the β-lactamase II was prepared by the method of Bicknell *et al.* [20] with a little modification. The holoenzyme (3 ml) was dialysed at 4 °C against two 500 ml portions of 20 mM-sodium succinate buffer, pH 6.0, containing 20 mM-EDTA, the first for 11 h and

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the second for 6 days. The resulting apoenzyme solution was then dialysed at 4 °C twice, for 24 h each time, against 500 ml of 20 mM-sodium succinate buffer, pH 6.0, containing sufficient NaCl to give an ionic strength of 1.0. All glassware, including cuvettes, in contact with these solutions was washed successively with 35 % (v/v) HNO_3, deionized water, aqueous EDTA at pH 8.0 and then with large volumes of deionized water. The apoenzyme prepared as described above had no detectable β-lactamase activity (less than 0.1 % of the original) before metal ion addition.

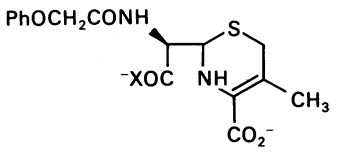
Analytical and kinetic methods

Absorption spectra and spectrophotometric reaction rates were obtained by means of a Cary 219 spectrophotometer. β-Lactamase concentrations were determined spectrophotometrically with the assumption of an absorption coefficient of 22000 $M^{-1} \cdot cm^{-1}$ at 280 nm [10]. Spectrophotometric steady-state rate determinations were generally initiated by addition of the apoenzyme to a thermostatically controlled cuvette containing the metal ion and substrate in buffer, although the order of addition did not affect the measured rates. In the case of Co^{2+}, however, the metal ion was added last in order to avoid a slow spontaneous loss of activity of the cobalt enzyme. All of the reported experiments were performed at 30 °C in 0.02 M-Hepes buffer containing sufficient NaCl to achieve an ionic strength of 1.0. Final metal ion concentrations were 75 μM for Zn^{2+} and 1 mM for Co^{2+}, Cd^{2+} and Mn^{2+}. These concentrations are sufficient essentially to bring about saturation of the catalytic metal-ion-binding site [6,18]. The wavelengths chosen for monitoring the enzyme-catalysed reactions were 265 nm ($\Delta\epsilon = 4500\ M^{-1} \cdot cm^{-1}$) and 307 nm ($\Delta\epsilon = 10600\ M^{-1} \cdot cm^{-1}$) for compounds (Ia) and (Ib) respectively. Steady-state kinetic parameters were obtained from the initial-velocity measurements by the method of Wilkinson [21].

Stopped-flow experiments with the use of a Durrum D-110 spectrophotometer were performed with the cobalt β-lactamase II and compound (Ib) in order to search for a cobalt–sulphur charge-transfer absorption. Measurements were made at 343 nm and 370 nm with solutions whose concentrations after mixing were 40 μM in enzyme, 0.5 mM in substrate and 1 mM in Co^{2+} ion.

RESULTS AND DISCUSSION

Native (Zn^{2+}-containing) *B. cereus* β-lactamase II catalyses the hydrolysis of both compounds (Ia) and (Ib) to compounds (IIa) and (IIb) respectively. The former product is characterized by loss of the intact cephem chromophore and its ^1H-n.m.r. spectrum [19,22]. Formation of the latter product is accompanied by loss of the thionocephem chromophore (Fig. 1) and the appearance of a ^1H-n.m.r. spectrum very similar to that



(IIa) X = O
(IIb) X = S

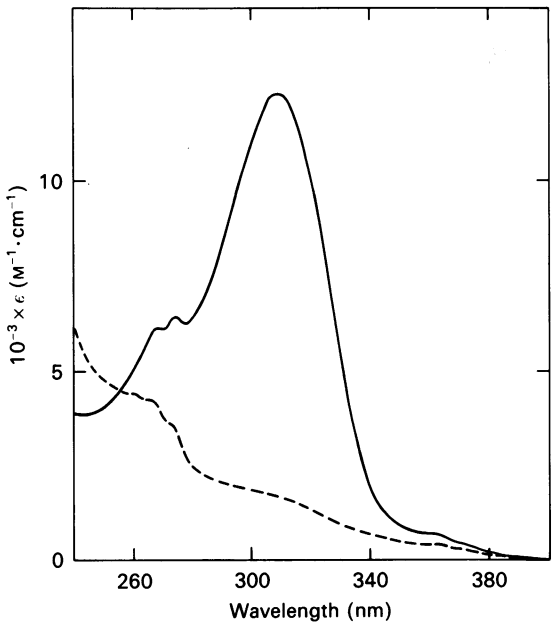


Fig. 1. Absorption spectrum of compound (Ib) before (——) and after (----) hydrolysis catalysed by the zinc β-lactamase II

of compound (IIa) [19,22]; the product spectra are not identical, however, and thus the product of hydrolysis of compound (Ib) is not compound (IIa). The cobalt, cadmium and manganese enzymes appear to catalyse the same reactions, as judged by the absorption-spectral changes [except for the manganese enzyme and compound (IIb), as described below].

The spectrophotometrically determined steady-state parameters for the hydrolysis of compounds (Ia) and (Ib) catalysed by β-lactamase II containing Zn^{2+}, Co^{2+}, Cd^{2+} or Mn^{2+} at its active site are shown in Table 1. In the case of the manganese enzyme, but not with the others, the reaction with compound (Ia) consists of two phases. This phenomenon has been previously observed with β-lactamase II, in particular during the manganese-β-lactamase II-catalysed hydrolysis of benzylpenicillin, and has been interpreted in terms of a branched mechanism [9] (see below). The values for the manganese enzyme reported in Table 1 refer to the initial faster phase.

Comparison of the effectiveness of the various metal ions shows, for both substrates, perhaps surprisingly, that the cobalt and cadmium enzymes are superior ($k_{cat.}/K_m$) to the native zinc enzyme. The difference, with the cobalt enzyme at least, is not usually so great, but a comparably large difference has been previously noted in the kinetics of hydrolysis of cephalosporins without C-3′ leaving groups [23]. The manganese enzyme also appears to be more effective than the zinc enzyme in catalysing hydrolysis of compound (Ia) (in its initial phase at least), but did not appear to affect compound (Ib), a point amplified below.

The thiono compound (Ib) is markedly inferior ($k_{cat.}/K_m$) to the oxo compound (Ia) as a substrate of the native zinc enzyme. This is in accord with all previous experience with thiono substrates and zinc metalloamidases [14–17, 24,25], and has been variously ascribed to the greater length of the C–S bond than C–O or to the greater

Table 1. Steady-state kinetic data for the hydrolysis of cephalosporins (Ia) and (Ib) in the presence of β-lactamase II containing various metal ions

Metal ion	Compound (Ia) (X = O)			Compound (Ib) (X = S)			$\frac{(k_{cat.}/K_m)_{(Ib)}}{(k_{cat.}/K_m)_{(Ia)}}$
	$k_{cat.}$ (s^{-1})	K_m (mM)	$k_{cat.}/K_m$ ($M^{-1} \cdot s^{-1}$)	$k_{cat.}$ (s^{-1})	K_m (mM)	$k_{cat.}/K_m$ ($M^{-1} \cdot s^{-1}$)	
Mn^{2+}	33±10	1.7±0.9	1.94×10^4	0	–	0	0
Zn^{2+}	2.7±0.2	0.74±0.16	3.69×10^3	0.40±0.02	1.8±0.2	2.15×10^2	0.06
Co^{2+}	126±1	0.57±0.11	2.18×10^5	4.4±0.3	0.10±0.03	4.48×10^4	0.20
Cd^{2+}	30±4	0.48±0.17	6.36×10^4	6.3±0.6	0.22±0.05	2.80×10^4	0.44

resistance of the thionamide group to torsion. This trend is seen with the other metal ions also. The most important feature of Table 1, however, and, indeed, the main point of the present paper, is the relative effectiveness of compound (Ib) with respect to (Ia) as a function of metal ion. The ratio $(k_{cat.}/K_m)_{(Ib)}/(k_{cat.}/K_m)_{(Ia)}$ increases, from zero with Mn^{2+}, to 0.44 with Cd^{2+} (Table 1). Thus the preference of the metalloenzyme for the thiono substrate with respect to the oxo compound increases in this order. Setting aside the question of Co^{2+} for a moment, the order $Mn^{2+} < Zn^{2+} < Cd^{2+}$ is also the order of increasing thiophilicity of these metal ions [17,26,27], and the correlation between this and the order of catalytic preference for compound (Ib) is striking and can be interpreted [14,17] as evidence for kinetically significant direct contact between the active-site metal ion of β-lactamase II and the β-lactam carbonyl heteroatom.

The position of Co^{2+} is unexpected, but not completely surprising. Most estimates of thiophilicity [17,26,27] place Co^{2+} between Mn^{2+} and Zn^{2+} rather than between Zn^{2+} and Cd^{2+} where it is seen in Table 1, but this is to some extent ligand-dependent, and in fact Bond *et al.* [17] find Co^{2+} between Zn^{2+} and Cd^{2+} in the order of thiono substrate preference of carboxypeptidase A. One significant difference between Co^{2+} and the other metal ions in Table 1 is the availability of ligand field-stabilization energy to Co^{2+}, i.e. Co^{2+} should have stronger directional preferences in the arrangement of its ligands and these preferences should be strong in the field of a sulphur ligand. Manganese (d^5), zinc (d^{10}) and cadmium (d^{10}) have spherically distributed *d* electron density and thus no stabilization of specific geometries. The 'anomalous' position of Co^{2+} in the order of selectivity for compounds (Ib) and (Ia) then may reflect the importance of a specific geometry, or perhaps of rigidity, at a kinetically significant stage of catalysis.

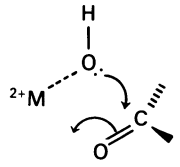
Waley and co-workers [9, 10] have looked carefully at the details of β-lactamase II catalysis using fast kinetics, cryoenzymological and spectroscopic techniques. They have concentrated principally on the cobalt β-lactamase II/benzylpenicillin system and propose eqn. (1) to explain their data:

$$\begin{array}{l} E+S \rightleftharpoons ES^* \rightleftharpoons ES^1 \xrightarrow{k_2} E+P \\ \qquad\qquad\quad \Big\downarrow k_3 \\ \qquad\qquad\quad ES^2 \xrightarrow{k_4} E+P \end{array} \qquad (1)$$

Acid quenching experiments suggested that none of the three ES complexes detected involved covalent interactions between the enzyme and substrate, and that all were enzyme–substrate (rather than enzyme–product) complexes (or could rapidly revert to substrate). Spectroscopic evidence suggested that the Co^{2+} in the free enzyme and ES^2 was five-co-ordinate whereas ES^1 was four-co-ordinate. They proposed that the fifth ligand in the free enzyme was a water molecule, which was displaced (but not replaced) by the substrate, perhaps forming a tetrahedral intermediate with it, in ES^1. The breakdown of ES^1 was then largely rate-determining. The present result therefore indicates that a transition state and perhaps also a non-accumulating intermediate, subsequent to ES^1 and involved in the slow step designated by the rate constant k_2, has the substrate more directly linked to the metal ion, through the β-lactam carbonyl heteroatom, than in ES^1 (assuming that the same series of intermediates are traversed by the current substrates; the cephalosporin nitrocefin appears to do so, it might be noted [9]). The five-co-ordinate branch intermediate ES^2 may derive from the putative subsequent intermediate rather than from ES^1.

Direct evidence for co-ordination of the sulphur atom of compound (Ib) to the metal ion in cobalt β-lactamase II should be, in principle, obtained from absorption spectroscopy. The free enzyme and ES^1 display typical cobalt–sulphur charge-transfer absorption bands at 348 nm and 332 nm respectively [9] that are due to the presence of the cysteine ligand. One would expect that co-ordination of a second sulphur atom would lead to a band of enhanced intensity [6]. Stopped-flow studies of the reaction between the cobalt enzyme and compound (Ib) showed no evidence for such enhanced absorption between 340 and 370 nm. It could be that the band had moved to lower wavelength, where it would be obscured by substrate absorption, although the probable five-co-ordinate nature of the metal ion complex would suggest that this explanation is not likely. It seems more likely that no such intermediate accumulates, which would be in accord with the picture arrived at in the previous paragraph.

Finally, with respect to the actual mechanism of the β-lactam hydrolysis, it should be noted that the currently favoured mechanism for metalloamidases [28–31], nucleophilic attack of metal-ion-bound hydroxide (or base-catalysed water) concerted or partly concerted with carbonyl co-ordination to the metal ion (III), does not as easily fit into the above scheme of events as one would like. It may be, as Waley and co-workers have most recently proposed [10], that ES^1 represents an enzyme-bound tetrahedral intermediate, but experimental evidence for the accumulation of such species, even metal-ion-bound, is not strong, and the question of their



(III)

stability should be pursued further. This scenario would also seem to require transient six-co-ordination of the metal ion unless a protein ligand is also transiently displaced, since β-lactamase II has four such ligands rather than the three seen in carboxypeptidase A. On the other hand, if ES^1 contains substrate that has not yet undergone nucleophilic attack, then it would seem that the more classical scenario of general base-catalysed water attack on the metal-ion-co-ordinated substrate [9,11] would seem more likely, since re-co-ordination of water before nucleophilic attack would seem too great an assault on the principles of parsimony.

We conclude that the data support a mechanism where direct co-ordination of the β-lactam carbonyl heteroatom to the metal ion is kinetically significant. Excluded therefore are mechanisms where the sole role of the metal ion is activation of water or of the substrate by co-ordination to the β-lactam nitrogen atom. The latter mechanism has been proposed to occur in the metal-ion-catalysed hydrolysis of penicillins [32,33].

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