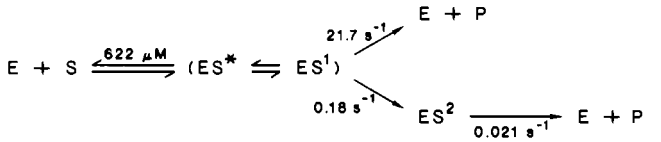
Changes in the Coordination Geometry of the Active-Site Metal during Catalysis of Benzylpenicillin Hydrolysis by *Bacillus cereus* β-Lactamase II†

Roy Bicknell,‡ Andreas Schäffer,‡ Stephen G. Waley,§ and David S. Auld*,‡

Center for Biochemical and Biophysical Sciences and Medicine and Department of Pathology, Harvard Medical School, Boston, Massachusetts 02115, and Sir William Dunn School of Pathology, University of Oxford, Oxford, U.K.

Received June 12, 1986; Revised Manuscript Received August 13, 1986

ABSTRACT: Rapid-scanning stopped-flow spectroscopy (425–700 nm) has been used to study spectral changes in cobalt(II)-substituted *Bacillus cereus* β-lactamase II during the binding and hydrolysis of benzylpenicillin. The experiments were carried out in aqueous solution over a temperature range of 3–20 °C. Three metallointermediates have been characterized by their visible absorption spectra. Two of them have visible absorption spectra identical with the intermediates ES^1 and ES^2 previously observed at subzero temperatures in a mixed aqueous/organic solvent [Bicknell, R., & Waley, S. G. (1985) *Biochemistry 24*, 6876–6887]. In addition, the branched kinetic pathway observed with the zinc(II) and cobalt(II) β-lactamase II at subzero temperatures has been shown to occur with the cobalt(II)-substituted enzyme in aqueous solution at above-zero temperatures; thus, at pH 6.0 and 3 °C, the rate and equilibrium constants are readily determined for the reaction scheme:



A third transient intermediate (called ES*) was found to precede ES^1 in the pre-steady-state time period. The identity of the intermediates formed in aqueous solution with those previously observed in the cryostudy confirms that the mechanism is not changed either by the presence of an organic cosolvent or by subzero temperatures. Further characterization of ES^1 and the steady-state intermediate ES^2 at subzero temperatures, where their lifetime may be extended for up to several hours, has involved circular and magnetic circular dichroic studies. The magnetic circular dichroic spectra identify changes in the coordination sphere of the active-site metal during catalysis. Thus, on the basis of cobalt(II) complex ion models, the metal coordination sphere of resting enzyme and ES^2 is fivelike, while that of ES^1 is fourlike.

The replacement of zinc(II) by the spectroscopic probe cobalt(II) in zinc metalloenzymes has enabled extensive characterization of enzyme–inhibitor complexes [see, for example, Vallee & Holmquist (1980)]. These studies have served as a point of reference to those in which cobalt enzyme intermediates have been characterized through a combination of rapid-scanning and rapid-mixing techniques at subzero temperatures (Geoghegan et al., 1983a; Auld et al., 1984, 1986b).

Two structurally unrelated β-lactamases are now known to be zinc metalloenzymes, namely, *Bacillus cereus* β-lactamase II (Sabath & Abraham, 1966) and the L1 β-lactamase of *Pseudomonas maltophilia* (Bicknell et al., 1985). Low-temperature studies of β-lactamase II have shown that in fluid media containing an organic cosolvent, the enzyme followed a branched kinetic pathway and permitted cryospectrokinetic characterization of two metallointermediates during the catalysis of benzylpenicillin by the cobalt(II)-substituted enzyme (Bicknell & Waley, 1985a,b). It was further shown by low-temperature acid quenching that the β-lactam ring remained intact in the pre-steady-state intermediate ES^1.

This paper describes a study of cobalt(II)-substituted β-lactamase II catalysis in aqueous solution over the temperature range 3–20 °C. It is shown that the mechanistic details of catalysis elucidated at subzero temperatures in aqueous/organic media also pertain to catalysis in aqueous solution at above-zero temperatures. Cryostabilization of the metallointermediates has permitted their further characterization by circular and magnetic circular dichroism, including elucidation of the overall metal coordination number of the metallointermediates ES^1 and ES^2.

Materials and Methods

β-Lactamase II from *Bacillus cereus* 569/H/9 was prepared as described previously (Davies et al., 1974; Baldwin et al., 1980a). Cobalt(II) substitution of β-lactamase II was carried out according to Bicknell et al. (1983). As previously reported (Bicknell et al., 1983), the apoenzyme showed less than 0.1% of the activity of the zinc enzyme. The residual activity probably arises from adventitious metal contamination of the assay. β-Lactamase II concentrations were determined on the basis of $\epsilon_M(280\text{ nm}) = 2.2 \times 10^4\ M^{-1}\ cm^{-1}$. The following buffers were used, all in 0.2 M NaCl; 0.2 M 2-(*N*-

† Supported in part by the Medical Research Council and by Grant-in-Aid GM-24967 from the National Institutes of Health, Department of Health and Human Services, to Harvard Medical School. This paper is a contribution from the Oxford Enzyme Group. R.B. and A.S. received postdoctoral fellowships from SERC/NATO and the "Deutsche Forschungsgemeinschaft", respectively.

* Correspondence should be addressed to this author at the Center for Biochemical and Biophysical Sciences and Medicine, Harvard Medical School.

‡ Harvard Medical School.

§ University of Oxford.

0006-2960/86/0425-7208$01.50/0

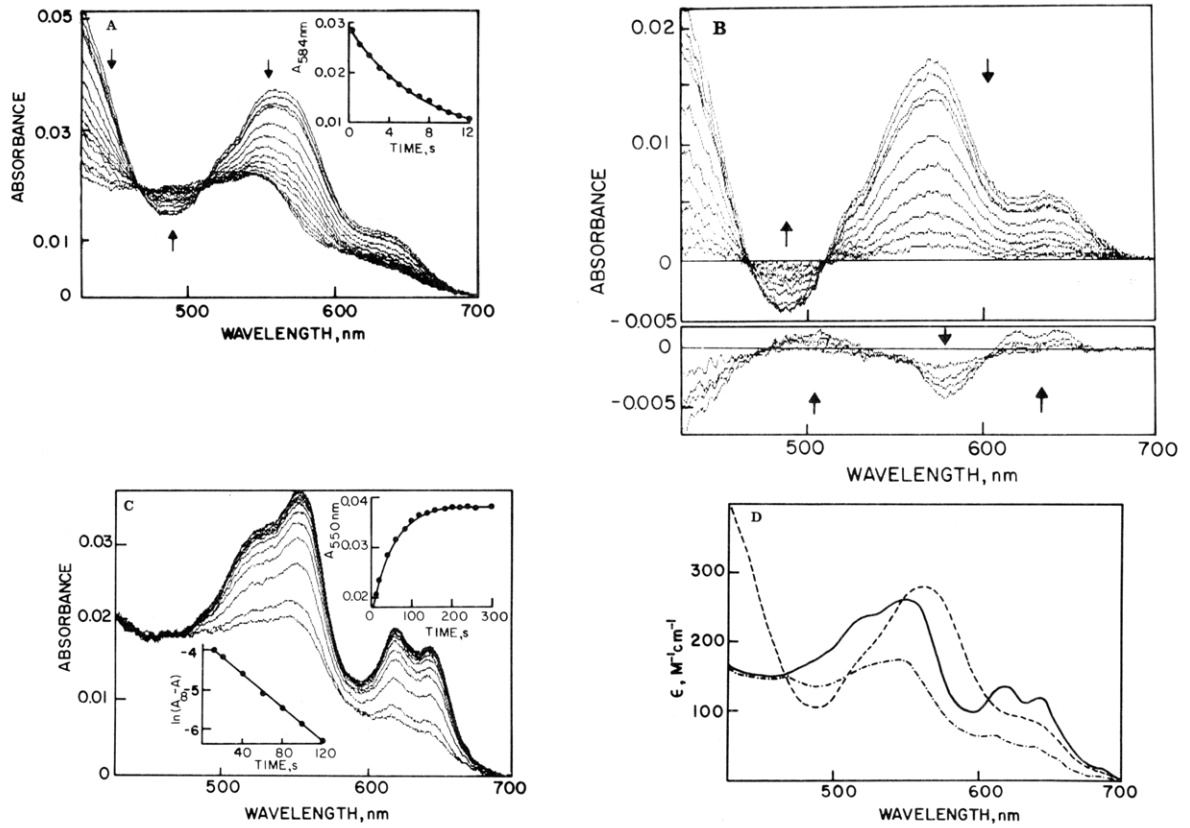


FIGURE 1: Rapid-scanning stopped-flow spectra of cobalt(II) β-lactamase II (75 μM) during catalysis of benzylpenicillin (20 mM) hydrolysis. (A) Spectral change corresponding to the decay of the pre-steady-state intermediate ES[1] between 0.25 and 12 s after mixing (scans were recorded 0.25, 0.5, 0.75, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, and 12 s after mixing). The inset shows the time course of the spectral change at 584 nm. (B) Difference spectra of those shown in (A) using the 8-s scan as reference. (Top) Scans recorded at 0.25, 0.5, 0.75, 1, 2, 3, 4, 5, 6, and 7 s. (Bottom) Scans recorded at 9, 10, 11, and 12 s after mixing. (C) Spectral change showing the recovery of resting enzyme from ES[2] as the substrate was depleted between 12 and 200 s after mixing (scans were recorded 12, 20, 40, 60, 80, 100, 120, 140, 160, 180, 200, 220, 240, 260 and 300 s after mixing). The inset shows the time course of the spectral change at 550 nm. Conditions: 0.2 M NaCl, 0.2 M MES, and 1 mM $CoCl_2$, pH 6 and 3 °C. (D) Comparison of the visible absorption spectra of cobalt(II) β-lactamase II (—), ES[1] (recorded 0.25 s after mixing (– –), and ES[2] (recorded 8 s after mixing) (–·–). The arrows indicate the direction of the absorbance change.

morpholino)ethanesulfonic acid (MES)[1]/sodium salt (pH 6); 0.2 M MOPS/sodium salt (pH 7); 0.1 M boric acid/sodium borate (pH 10). Cobalt(II) chloride was present at a concentration of 1 mM in stopped-flow studies at pH 6 and 7, but no excess cobalt was required for full activity at pH 10. Enzyme spectra recorded at pH 6 and 7 were corrected for the absorption of excess cobalt(II) chloride. Standard precautions were taken to avoid adventitious metal ion contamination (Thiers, 1957).

Details of the stopped-flow rapid-scanning instrument used in this study have been reported (Hanahan & Auld, 1980; Geoghegan et al., 1983a). Each scan took 16.48 ms. Circular dichroic and magnetic circular dichroic spectra were recorded with a Cary 61 circular dichroic spectropolarimeter, equipped with a Varian 4145 superconducting magnet and a Varian 4106 superconducting power supply. A magnetic field of 4 T was used in MCD measurements. The Cary 61 is interfaced to an Apple IIe computer to enable direct data acquisition and storage on floppy disks together with routine spectral manipulation. MCD spectra were corrected for CD absorption by computerized subtraction of the CD from the MCD spectrum. X-band EPR spectra were obtained with a Varian E-109 EPR spectrometer. An Air Products Heli-Tran liquid helium transfer line was employed to obtain sample temperatures of 4 K. The magnetic field was calibrated with a Radiopan NMR magnetometer, and the microwave frequency was measured with an Autonet Model 331 microwave frequency counter.

The cryostabilization of intermediates present during hydrolysis of benzylpenicillin by cobalt(II) β-lactamase II has been described (Bicknell & Waley, 1985b). Thus, to obtain CD and MCD spectra of ES[2], the intermediate was generated by addition of benzylpenicillin (40 mM) to the cobalt(II) enzyme (220 μM) in 60% ethylene glycol and 0.1 M sodium cacodylate at pH* 6 and −16 °C. The spectra were recorded 15 min later, at which point kinetic and spectral data had shown the enzyme to be present to >95% as ES[2]. Similar conditions are used for characterization of ES[1] at −56 °C except that the spectra were recorded 5 min after the addition of substrate, at which point the enzyme was present to >95% as ES[1].

RESULTS

Rapid-Scanning Characterization of Metallointermediates at pH 6 over the Temperature Range 3–20 °C. The spectral changes that occur in cobalt(II)-substituted β-lactamase II during binding and hydrolysis of benzylpenicillin (300-fold above K_m) at +3 °C are shown in Figure 1. The spectra are very similar to those of the intermediates present during binding and hydrolysis of benzylpenicillin in 60% ethylene glycol at subzero temperatures (Bicknell, 1984; Bicknell & Waley, 1985b). Two metallointermediates can be identified by their characteristic absorption spectra. The initial spectrum

[1] Abbreviations: Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; MOPS, 2-(*N*-morpholino)propanesulfonic acid; CD, circular dichroic; MCD, magnetic circular dichroic; EPR, electron paramagnetic resonance.

Scheme I

$$E + S \rightleftharpoons (ES^* \rightleftharpoons ES^1) \xrightarrow{k_2} E + P$$
$$\downarrow k_3$$
$$ES^2 \xrightarrow{k_4} E + P$$

Scheme II

$$E + S \rightleftharpoons (ES^* \rightleftharpoons ES^1) \xrightarrow{k_2} E + P$$
$$\downarrow k_3 \uparrow k_4$$
$$ES^2$$

(Figure 1A) is assigned to the previously detected pre-steady-state intermediate ES^1 (Bicknell & Waley, 1985) characterized by a λ_{max} at 563 nm (ϵ = 282 M^{-1} cm^{-1}) and the distinct cobalt(II)–cysteine charge transfer absorbance at 428 nm. Formation of ES^1 from cobalt(II) β-lactamase II and benzylpenicillin was complete within 250 ms after mixing (see below). The collapse of the ES^1 spectrum to that of the steady-state metallointermediate, ES^2, with λ_{max} 551 nm (ϵ = 176 M^{-1} cm^{-1}), occurs between 250 ms and 8 s after mixing, with a first-order rate constant of 0.183 s^{-1} (Figure 1A).

Shown in Figure 1B are difference spectra corresponding to those in Figure 1A referenced to the scan obtained at 8 s after mixing. The spectra identify two distinct chemical steps. Thus, between 0.25 and 7 s after mixing (Figure 1B, top), the spectra correspond solely to decay of ES^1 to ES^2 as indicated above. The isosbestic points at 465 and 509 nm are sharp. Between 9 and 12 s after mixing, a second small-amplitude process occurs (Figure 1B, bottom) characterized by a reversal of the spectral change (now an increase) between 603 and 665 nm and sharp isosbestic points at 465, 546, and 603 nm. Shown for comparison in Figure 1D are spectra of cobalt(II) β-lactamase II (E), ES^1, and ES^2. An isosbestic point at 465 nm is common to all three while isosbestic points at 546 and 603 nm are common to E and ES^1 and at 509 nm to ES^1 and ES^2. It is clear from the identity of the isosbestic points between E and ES^1 that the spectral change above 603 nm between 9 and 12 s (Figure 1B, bottom) signals decay of ES^1 to E. Thus, the substrate concentration has fallen below the level at which all resting enzyme released after a catalytic cycle is rapidly converted back to ES^1, and accumulation of E is now observed. Between 12 and 300 s after mixing, the major process is (slow) decay of ES^2 to E (Figure 1C). A logarithmic plot of $A_\infty - A$ at 550 nm (lower insert, Figure 1C) shows that this is a first-order process with a rate constant of 0.021 s^{-1} (see below).

The rate of conversion of ES^1 to ES^2 at 3 °C (k_3 = 0.183 s^{-1}) is too slow to be accommodated by a linear kinetic pathway with the known k_{cat} of 2.25 s^{-1} (Bicknell & Waley, 1985b). Thus, a branched kinetic pathway (Schemes I and II), previously established in the earlier cryokinetic study, is confirmed here. The two (kinetically indistinguishable) alternatives (Bicknell & Waley, 1985b) are depicted in Schemes I and II where ES^1 and ES^2 are the pre-steady-state and steady-state intermediates, respectively. The spectral properties of ES^1 and ES^2 are illustrated in Figure 1D and summarized in Table I.

Rapid scanning was repeated at temperatures up to 20 °C. In each case, ES^1 and ES^2 were observed, and k_3 (the branching rate constant) was determined from single-wavelength plots of ln ($A_\infty - A$) against t. Figure 2 compares Arrhenius plots of k_3 and k_{cat}. The Arrhenius parameters of k_3 are similar to those previously found for this step at subzero temperatures in a 60% ethylene glycol solvent (Table II). The low preexponential factor suggests a conformational change. Thus, it is clear that the mechanistic pathway in aqueous

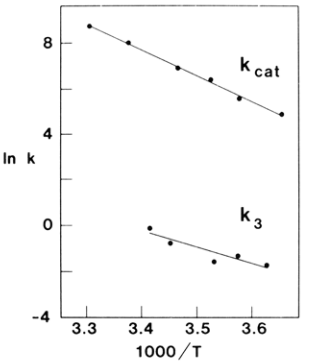


FIGURE 2: Arrhenius plot of k_{cat} and the rate constant for the branching step (k_3). Conditions: k_3 determinations as for Figure 1. The k_{cat} data are taken from Bicknell and Waley (1985b).

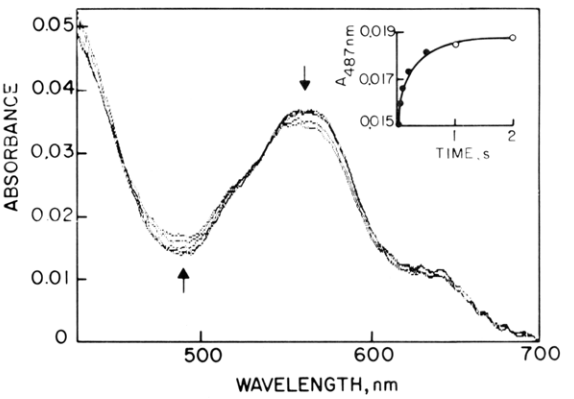


FIGURE 3: (a) Rapid-scanning stopped-flow visible spectra of cobalt(II) β-lactamase II during the first 500 ms of benzylpenicillin hydrolysis at 3 °C. Successive scans were recorded at 20, 40, 60, 180, and 500 ms after mixing. The arrows indicate the direction of the change. The inset shows the time course for the conversion of ES* to ES^1 at 487 nm. The scans at the time points (O) are omitted for clarity; they correspond to the subsequent conversion of ES^1 to ES^2 which occurs with a half-life of 3.8 s.

solution shows all of the essential features previously observed in a mixed aqueous/organic solvent at subzero temperatures (Bicknell & Waley, 1985b).

Detection of a Transient Metallointermediate Preceding ES^1. Extension of the Kinetic Mechanism. Rapid scans of the visible absorption spectrum of cobalt(II) β-lactamase II during the first 2 s after addition of benzylpencillin at 3 °C showed a biphasic spectral change at, e.g., 487 nm, indicating at least two chemical steps. The fast transient is shown in Figure 3. An additional transient intermediate (hereafter labeled ES*) preceding ES^1 is clearly apparent. This result demands inclusion of an additional species, ES*, in the kinetic Schemes I and II.

The visible absorption spectrum of ES* shows very close similarity to ES^1; however, the λ_{max} of the most intense band is 5 nm higher in ES* and the molar absorptivity 15 M^{-1} cm^{-1} greater (Figure 3 and Table I). The close similarity of the ES* and ES^1 spectra leads to only small spectral changes; however, an isosbestic point is apparent at 539 nm. The possibility of the fast transient in the biphasic process corresponding to the formation of ES^1 from E and substrate was ruled out by a comparison of the spectrum of ES^1 with that of E (Figure 1D). The formation of ES^1 from E leads to an increase in absorbance at 570 nm whereas the fast transient is observed as a decrease in absorbance at this wavelength (Figure 3). Above 5 °C, the conversion of ES^1 to ES^2 is the only pre-steady-state exponential process observed, since the lifetime of ES* is now too short to permit detection within the time frame of the rapid-scanning stopped-flow instrument.

Table I: Electronic Absorption, CD, and MCD Spectral Characteristics of Cobalt(II) β-Lactamase II (E) and the Intermediates ES*, ES^1, and ES^2 That Occur during Hydrolysis of Benzylpenicillin at pH 6[a]

(A) Cobalt(II) β-Lactamase II (E) in Aqueous Solution

electronic absorption		CD		MCD	
λ_{max}	ϵ_M	$\lambda_{extrema}$	$\Delta\epsilon_M$	$\lambda_{extrema}$	$\Delta\epsilon_M$
		308	+0.207		
348	864	340	+0.207	350	+0.056
516 (s)	242			520	+0.025
551	274	551	−0.11	554	−0.135
616	152			614	−0.113
641	141	641	+0.151	638	−0.105

(B) Electronic Absorption Spectra of Intermediates in Aqueous Solution

ES*		ES^1		ES^2	
λ_{max}	ϵ_M	λ_{max}	ϵ_M	λ_{max}	ϵ_M
518 (s)	214	521 (s)	212		
568	297	563	282	551	176
521 (s)	115	630 (s)	114	616 (s)	80

(C) CD Spectral Characteristics of Cryostabilized Intermediates ES^1, ES^2, and E under the Same Conditions

E (T = −16 °C)		ES^2		E (T = −56 °C)		ES^1	
$\lambda_{extrema}$	$\Delta\epsilon_M$	$\lambda_{extrema}$	$\Delta\epsilon_M$	$\lambda_{extrema}$	$\Delta\epsilon_M$	$\lambda_{extrema}$	$\Delta\epsilon_M$
308	+0.301	310	+0.855	308	+0.28		
340	+0.869	339	+0.455	343	+0.687	339	+0.103
						392	+0.444
		407	−0.552				
						486	+0.139
553	−0.527			550	−0.542	541	−0.132
636	+0.387	654	+0.31	637	+0.277	622	+0.357

(D) MCD Spectral Characteristics of Cryostabilized Intermediates ES^1, ES^2, and E under the Same Conditions

E (T = −16 °C)		ES^2		E (T = −56 °C)		ES^1	
$\lambda_{extrema}$	$\Delta\epsilon_M$	$\lambda_{extrema}$	$\Delta\epsilon_M$	$\lambda_{extrema}$	$\Delta\epsilon_M$	$\lambda_{extrema}$	$\Delta\epsilon_M$
365	+0.147	365	+0.137	365	+0.242	378	+0.196
		456	−0.104			445	−0.196
517	+0.013			517	+0.028		
551	−0.38	552	−0.276	551	−0.537	560	−0.57
612	−0.164	612	−0.122	612	−0.259		
633	−0.137	633	−0.088	633	−0.215		

[a] Wavelengths are given in nanometers. Units for absorption (ϵ), CD ($\Delta\epsilon_M$), and MCD ($\Delta\epsilon_M$) are M^{-1} cm^{-1}, M^{-1} cm^{-1}, and M^{-1} cm^{-1} T^{-1}, respectively. Experimental conditions are given in the appropriate figure legends.

Table II: Comparison of Arrhenius Parameters for the Branching Step (k_3) in the Hydrolysis of Benzylpenicillin by Cobalt(II) β-Lactamase II in Aqueous and 60% Ethylene Glycol Solution

solvent	temp range (°C)	E_A (kcal/mol)	A (s^{-1})
water, pH 6	+3 to +25	9.7	1.27×10^7
60% ethylene glycol, pH* 6[a]	−55 to −10	8.2	9.5×10^3

[a] From Bicknell and Waley (1985b).

Stopped-Flow Rapid Scanning at pH 7 and 10. Stopped-flow rapid scanning of cobalt(II) β-lactamase II during binding and hydrolysis of benzylpenicillin has also been carried out at pH 7 and 10. The pH optimum for both k_{cat} and k_{cat}/K_m during hydrolysis of benzylpenicillin at 30 °C is 7 (Bicknell et al., 1983). At this pH, the rapid scan data at 3 °C were very similar to those recorded at pH 6, and a branched catalytic pathway operates. At pH 10, with a substrate concentration of 20 mM (200-fold above the K_m at this pH), two intermediates were also detected, and their spectra are compared to that of E in Figure 4. While the visible absorption spectrum of cobalt(II) β-lactamase II is pH insensitive over the range of pH 5–10, the charge transfer absorption has an increased molar absorptivity at high pH (Baldwin et al., 1980b; and cf. Figures 1D and 4). Similarly, at pH 10, the spectrum of ES^2 is somewhat different from that observed at lower pH. Thus, as in ES^1 the charge transfer absorption is enhanced. However, the molar absorptivity of the d–d* transitions is less than those of the resting enzyme, as found for the low-pH form of ES^2. It is possible that the steady state at pH 10 comprises a mixture of ES^1 and ES^2.

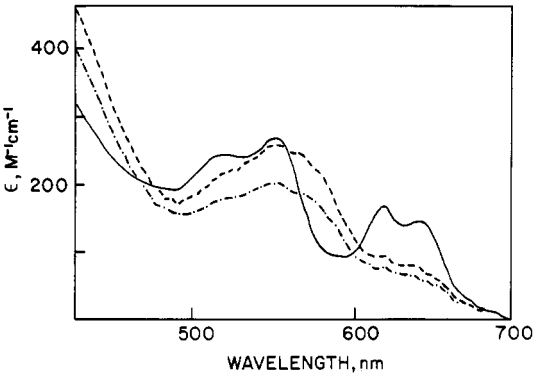


FIGURE 4: Electronic absorption spectra of cobalt(II) β-lactamase II (60 μM) (—) and the intermediates ES^1 (– –) (0.5-s scan) and ES^2 (–·–) (5-s scan) during catalysis of benzylpenicillin (40 mM) hydrolysis at pH 10 in 0.1 M sodium borate and 0.2 M NaCl and at 3 °C.

CD and MCD Spectra of Cobalt(II) β-Lactamase II, ES^1, and ES^2. The earlier cryostudy of β-lactamase II (Bicknell & Waley, 1985b) defined experimental conditions that enable cryostabilization of the intermediates ES^1 and ES^2 for periods of several hours. Shown in Figure 5 are the CD and MCD spectra of ES^1 and ES^2 recorded with cryostabilized samples; the major bands are listed in Table I. The spectra of cobalt(II) β-lactamase II recorded under identical conditions are included for comparison. The CD and MCD spectra of ES^1 and ES^2 are distinct both from each other and from those of the resting enzyme. Incubation of the enzyme permitted the spectral time course of catalysis to be followed by MCD spectroscopy, as was done by rapid-scanning visible spectroscopy at higher

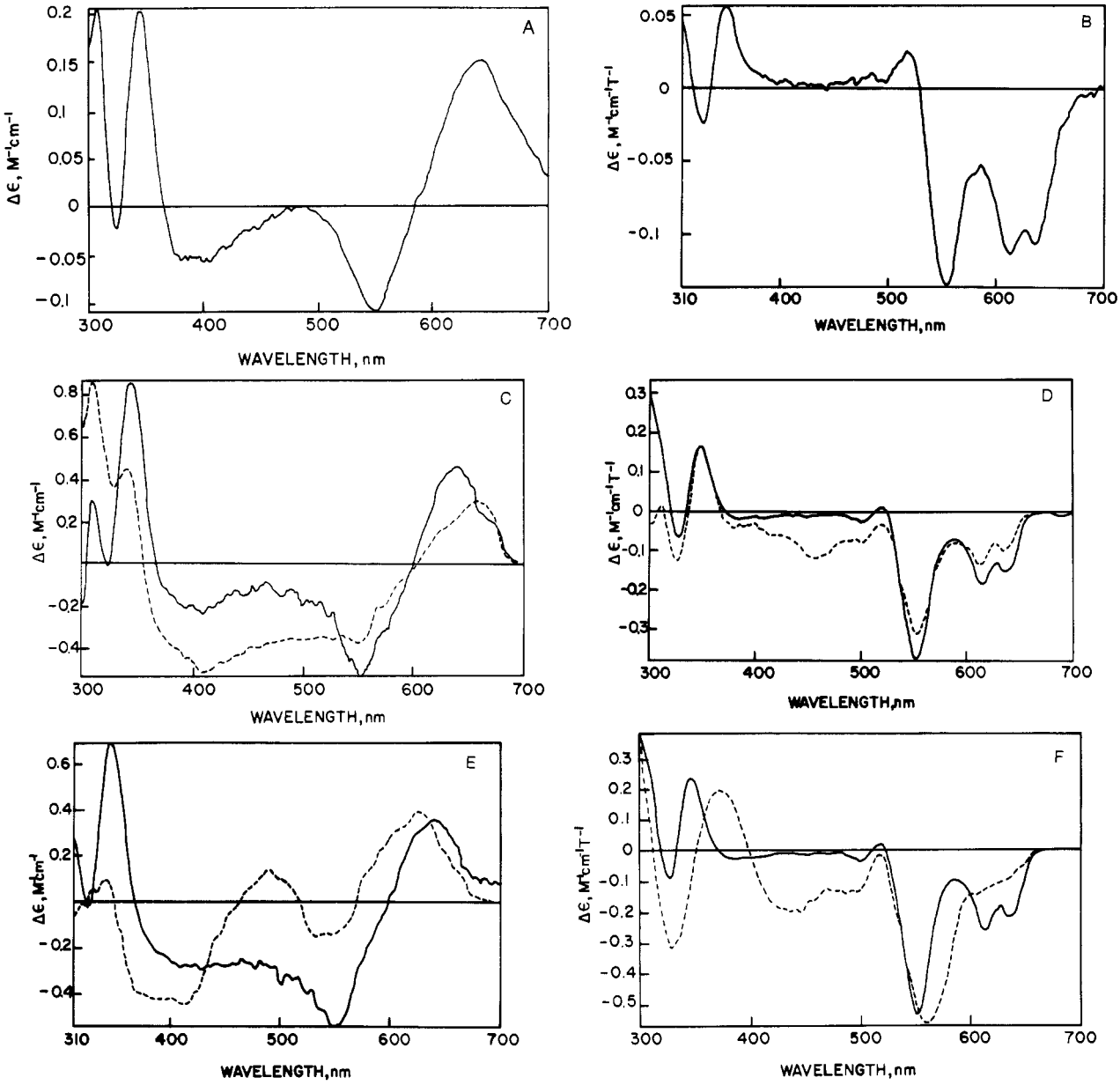


FIGURE 5: Circular dichroic and magnetic circular dichroic spectra of cobalt(II) β-lactamase II and intermediates during catalysis of benzylpenicillin hydrolysis. (A) CD and (B) MCD spectra of cobalt(II) enzyme (527 μM) in 0.05 M MES, pH 6 and 25 °C. (C) CD and (D) MCD spectra of cobalt(II) enzyme (220 μM) (—) and the cryostabilized steady-state intermediate ES^2 (---) recorded in 60% ethylene glycol at −16 °C. (E) CD and (F) MCD spectra of cobalt(II) enzyme (220 μM) (—) and the pre-steady-state intermediate ES^1 (---) recorded in 60% ethylene glycol at −56 °C.

temperatures. The identity between the final and original spectra and the generation of ES^1 upon addition of a second aliquot of substrate confirmed that no irreversible changes had occurred.

EPR Spectrum of Cobalt(II)-Substituted β-Lactamase II. The EPR spectrum of cobalt(II) β-lactamase II was recorded at 4 K and is shown in Figure 6.

Discussion

Cryoenzymology is a well-established technique for the study of enzyme catalysis (Douzou, 1977; Cartwright & Fink, 1981). However, evidence for the normal behavior of enzymes under cryoconditions is often circumstantial. This study shows that the mechanism of β-lactamase II catalysis elucidated in the presence of an organic cosolvent at subzero temperatures is identical with that in aqueous solution at normal temperatures. This is the first instance in which extensive studies of the intimate mechanistic details of metalloenzyme catalysis have been determined under such very different experimental conditions and provides a good case in which results obtained from cryostudies carried out in the presence of an organic cosolvent coincide with those obtained in aqueous solution at ambient temperatures.

The principle findings are (i) that catalysis proceeds by way of a branched kinetic pathway, (ii) that discrete metallo-intermediates are formed during catalysis, and (iii) that changes in the coordination number of the active-site metal occur during catalysis.

Branched Kinetic Pathways and β-Lactamase II. The stopped-flow rapid-scanning study shows that catalysis of benzylpenicillin hydrolysis by cobalt(II) β-lactamase II proceeds by way of the branched kinetic pathway of either Scheme I or Scheme II. Similar results were previously obtained with the cobalt(II) and zinc(II) enzymes under cryoconditions, and with the manganese(II) enzyme at room temperature (Bicknell & Waley, 1985b). At 3 °C, k_2 and $K_{S'}$ ($K_{S'}$ is the equilibrium

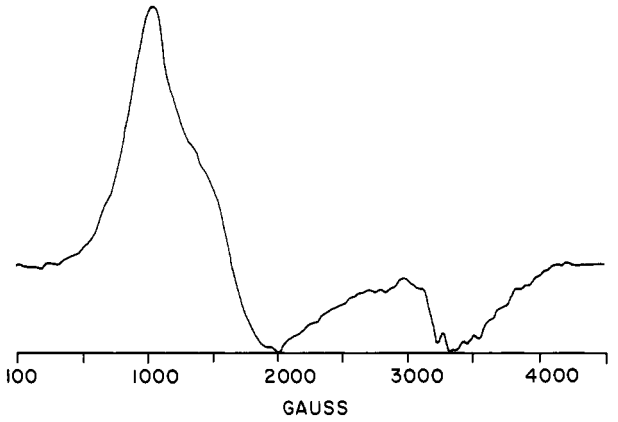


FIGURE 6: EPR spectrum of cobalt(II) β-lactamase II (1 mM). The spectrum is characterized by the following g values: $g_1 = 6.293$, $g_2 = 4.21$, and $g_3 = 1.988$; and a value for A_3 of 0.0079 (±0.0003) cm^{-1}. Conditions: 0.05 M Hepes, pH 7 (25 °C), frozen to 4 K. Spectrometer settings: microwave frequency, 9.3021 GHz, microwave power, 200 mW; modulation amplitude, 10 G; and modulation frequency, 100 kHz. Seventeen scans were averaged to obtain a final spectrum.

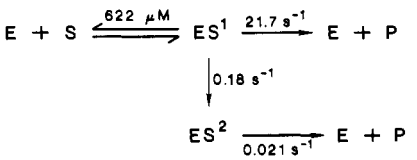
constant between E and ES^1; K_S is that between E and ES*) may be evaluated from the known values of $k_{cat} = 2.25\ s^{-1}$ and $k_{cat}/K_m = 3.52 \times 10^4\ M^{-1}\ s^{-1}$ (Bicknell & Waley, 1985b) and of $k_3(ES^1 \rightarrow ES^2) = 0.18\ s^{-1}$ and $k_4(ES^2 \rightarrow E) = 0.021\ s^{-1}$, together with the transient phase equations relating the kinetic constants to the kinetic parameters. Thus, for Scheme I:

$$k_{cat} = (k_2 + k_3)k_4/(k_3 + k_4)$$

and

$$k_{cat}/K_m = (k_2 + k_3)/K_S$$

(Bicknell & Waley, 1985b), and the complete scheme at pH 6 and 3 °C is



For the alternative mechanism of Scheme II:

$$k_{cat} = k_2k_4/(k_3 + k_4)$$

and $k_{cat}/K_m = k_2/K_S$ from which $k_2 = 21.5\ s^{-1}$ and $K_S = 611\ \mu M$.

The branched pathway is shown to occur with the cobalt(II) enzyme at 3 °C over a pH range of 6–10, and at temperatures between 3 and 30 °C at pH 6. In addition, within these temperature and pH limits, the spectral data show near-stoichiometric accumulation of ES^1 followed by ES^2, which means that the relative magnitudes of the rate constants are $k_2 > k_3 > k_4$, as was found to be the case in the earlier cryostudy and from the above analysis of the 3 °C, pH 6 data.

Steady-state pH studies (Bicknell et al., 1983) have shown that the hydrolysis of benzylpenicillin by cobalt(II) β-lactamase II follows a branched proton pathway. In the steady state, there are two species present that differ in their state of protonation (designated XH and XH_2). Breakdown of XH_2 to product is faster than that of XH. The pK_a value of 8.3 for the equilibrium between XH_2 and XH is not only temperature insensitive but also active-site metal ion insensitive.

The stopped-flow study described here shows that at pH 6, where XH_2 is in large excess over XH, the predominant steady-state intermediate is ES^2. However, there is no simple correspondence between the intermediates ES^1 and ES^2 and the protomers XH and XH_2. This becomes clear when the relative rates of breakdown of each intermediate to product are considered. Thus, in the steady state at pH 6, the enzyme is present as ES^2 and XH_2; ES^1 breaks down faster than ES^2, but XH_2 breaks down faster than XH. In addition, at pH 10 the branched metallointermediate pathway was observed (Figure 4), and yet only XH is present during steady-state turnover. It is concluded that XH and XH_2 are probably different protomers of ES^2 and that the branched proton pathway operates within the branched metallointermediate pathway.

Spectral Characterization of Intermediates. The visible absorption, CD, and MCD spectra of cobalt(II) β-lactamase II indicate that the active-site cobalt(II) in the resting enzyme is entatic; that is, the metal coordination sphere is distorted (Vallee & Williams, 1968). The MCD spectrum (Figure 5B), which signals the overall geometry of the metal ion (Holmquist et al., 1975), indicates pentacoordinate geometry. The spectrum shows close similarity to that of the pentacoordinate model complex $Co(Me_6tren)Br_2$ (Holmquist et al., 1975) and to carbonic anhydrase at high pH. Four enzyme ligands to the cobalt(II) ion in β-lactamase II have been identified, namely, three histidine residues (Baldwin et al., 1978) that have been located in the amino acid sequence (Baldwin et al., 1979; Ambler et al., 1985) and a single cysteine residue (Sabath & Finland, 1968; Baldwin et al., 1980b). The latter gives rise to the prominent cobalt(II)–sulfur charge transfer absorption seen at low wavelength in the absorption (Davies & Abraham, 1974), CD (Figure 4), and MCD (Figure 5) spectra.

The fifth ligand is by analogy to other zinc hydrolases tentatively suggested to be a metal-coordinated water molecule or hydroxy anion (Bicknell & Waley, 1985b). Pentacoordination of the active-site cobalt(II) ion is supported by the EPR spectrum of the enzyme (Figure 7) (Bicknell, 1984). The spectrum shows close similarity to that of the 1:1 adduct between cobalt(II)-substituted carbonic anhydrase B and azide, referred to as type A spectra (Bencini et al., 1981), characterized by a broad low-field signal with a large hyperfine coupling characteristic of pentacoordination to cobalt(II).

Despite the similarity of the visible absorption spectrum of β-lactamase II to that of carbonic anhydrase, unlike the latter it is both pH (R. Bicknell, unpublished experiments) and anion insensitive (Baldwin et al., 1980b). The increase in absorption below 500 nm reported by Baldwin et al. (1980b) on titration of cobalt(II) β-lactamase II with cyanide is due to removal of cobalt(II) from the enzyme and formation of an aquo-cobalt(II) cyanide complex which has a wavelength maximum at 370 nm (R. Bicknell et al., unpublished results). Cyanide has also been shown to remove the cobalt(II) from cobalt(II) carboxypeptidase A at pH 7 (R. Bicknell et al., unpublished results). Anion insensitivity of visible spectra over the pH range 6.5–10 has been found in other cobalt(II)-substituted zinc hydrolases including carboxypeptidase A (Geoghegan et al., 1983), which like β-lactamase II (Little et al., 1986) has an essential active-site glutamate residue.

In carboxypeptidase A, the active-site glutamate is thought to hydrogen bond to the metal-coordinated water molecule to give a stable metal ion–water–carboxylate structure which renders the metal ion spectra insensitive to both pH and anions (Geoghegan et al., 1983b). The metal-bound water may well be present in β-lactamase II as a glutamate-37-stabilized water molecule or hydroxide anion, analogous to the glutamate-270-stabilized water molecule that has been implicated in carboxypeptidase A (Stephens et al., 1974; Geoghegan et al., 1983b; Auld et al., 1986a).

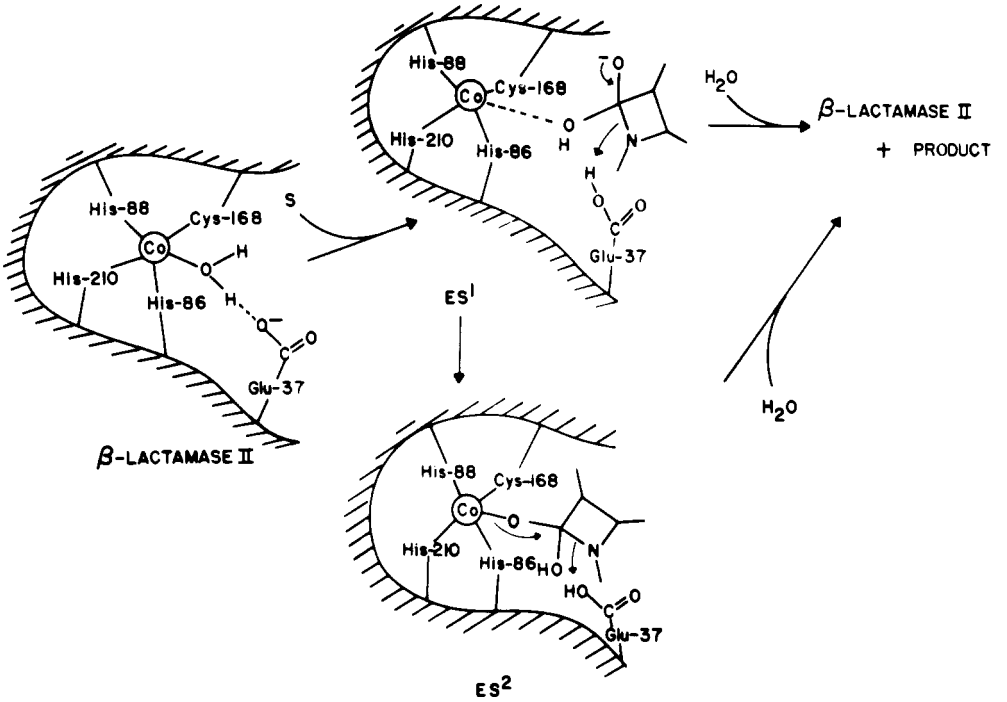


FIGURE 7: Postulated mechanism of β-lactamase II action consistent with current data.

The electronic spectral properties of ES*, ES^1, and ES^2, the intermediates formed during the binding and hydrolysis of benzylpenicillin, are also reported (Table I). The MCD spectrum of ES^2 is very similar to that of the resting enzyme but shows an additional band at 456 nm. The spectrum is consistent with pentacoordinate geometry for the cobalt(II) ion in ES^2. In contrast, that of ES^1 shows close resemblance to MCD spectra of 4-coordinate model complexes (Holmquist et al., 1975). The exceptional similarity between the absorption spectra of ES* and ES^1 suggests that the metal ion coordination number in ES* is also 4-coordinate, indicating loss of a metal ligand as E is converted to ES*.

The lower λ_{max} for the cobalt(II)–sulfur charge transfer absorption in ES^1 (333 nm) relative to that of resting enzyme (348 nm) has been interpreted as a general tightening of the metal coordination sphere on substrate binding (Bicknell & Waley, 1985b). A tightening of the metal coordination sphere is consistent with a change in coordination number from 5 to 4. The presence of a sulfur ligand renders the molar absorptivities of the visible absorption spectra uninformative regarding the coordination number of the metal ion (Banci et al., 1982).

Possible Mechanism. In the absence of any evidence for a covalent intermediate in β-lactamase II catalysis (Bicknell & Waley, 1985b), the simplest mechanism involves nucleophilic attack of water on the β-lactam carbonyl. Low-pH trapping (Bicknell & Waley, 1985b) has clearly shown that the low molecular weight component of ES^1 collapses to substrate. In addition, low-temperature single-turnover assays with zinc β-lactamase II and the chromophoric substrate nitrocefin confirm that the spectral change on hydrolysis, which in this case corresponds to collapse of the tetrahedral intermediate to the ring-opened product, occurs in the k_2 step (Bicknell & Waley, 1985b). It follows that ES^1 is either (i) a noncovalent enzyme–substrate complex or (ii) an enzyme-stabilized tetrahedral intermediate, which irreversibly undergoes conversion to product (k_2). Collapse of ES^1 back to substrate is known to be faster than conversion to products ($k_{-1} >> k_2$) at pH* 6 but under otherwise identical conditions with those used for the trapping experiment. Low-pH "trapping" of a tetrahedral intermediate would give substrate if, at that pH, collapse of ES^1 back to substrate and E were faster than either conversion to product ($k_{-1} >> k_2$) or enzyme denaturation. ES* is the first detected intermediates but may be preceded by, as yet, undetected intermediates such as the Michaelis complex. As ES* precedes ES^1 on the kinetic pathway, it follows that this also must be an enzyme–substrate complex or a tetrahedral intermediate.

The identity of the low molecular weight component of ES^2 has not been identified, however, on the basis of the low value of both the activation energy and preexponential factor for the step converting ES^1 to ES^2; together with lack of evidence for a covalent intermediate from trapping experiments, ES^2 was thought to be a conformer of ES^1 (Bicknell & Waley, 1985b). Similar activation parameters (Table II) and the close similarity of ES^2 visible spectra in both studies suggest that ES^2 is also a conformer of ES^1 in room temperature hydrolysis.

The MCD spectra indicate that the coordination number of the active-site cobalt(II) decreases from 5 to 4 when benzylpenicillin binds to the enzyme but returns to 5 in the steady-state intermediate (ES^2). The decrease in metal coordination number on substrate binding is somewhat surprising and suggests that substrate does not coordinate to the metal in either ES* or ES^1.

A tentative mechanistic possibility encompassing the current data is now briefly outlined (Figure 7). In the proposed scheme, glutamate-37 acts as a general acid/base catalyst, as previously postulated for β-lactamase II (Bicknell & Waley, 1985b; Little et al., 1986) and for carboxypeptidase A (Galdes et al., 1986) by analogy to thermolysin (Mozingo & Matthews, 1984). Concurrent with substrate binding to the enzyme, the metal-coordinated water is delivered as a hydroxide to the β-lactam via general base catalysis by glutamate-37. Thus, the metal may function in catalysis by supplying the water during a critical step early in the pre steady state. ES^1 is a putative tetrahedral intermediate in which the metal has 4-coordinate-like ligand geometry. Distortion from pure tetrahedral geometry could arise from an attenuated interaction with the β-lactam (shown as a dashed line in Figure 7).

The partitioning of ES^1 to ES^2 identifies collapse to a less

reactive intermediate. The accompanying return to 5-coordination increases the likelihood that a substrate group coordinates to the metal in ES^2. It is possible that the newly generated oxyanion is trapped by the metal, accounting for the return to a pentacoordinate metal and weakening of the metal–cysteine-168 bond. In addition, the extra charge stabilization afforded by chelation of the oxyanion to the positively charged metal would account for the lower rate constant for β-lactam ring opening upon decay of ES^2 (k_4) relative to ES^1 (k_2). However, the exact identity of ES^2 and whether or not substrates [and if so which group(s) on the substrate] coordinate the active-site metal ion are not known. NMR studies could provide answers to these questions, should suitably labeled β-lactams become available.

The enzyme–product complex does not accumulate; this is consistent with the weak binding of benzylpenicilloic acid (product) to the enzyme (K_i for zinc β-lactamase II ~50 mM; R. Bicknell, unpublished results). Additional steps, including proton transfers, product dissociation, and uptake of another water molecule, must also take place.

A branched pathway may give some indication of the evolutionary history of the enzyme. Thus, the route via ES^2 is less efficient ($k_4 << k_2$) but also involves less reorganization of the active site (pentacoordination of the metal in both E and ES^2). It is possible that catalysis proceeded exclusively via ES^2 in a less evolved β-lactamase II.

The proposed mechanism of β-lactamase II action (Figure 7) is radically different from the acyl-enzyme mechanism believed to operate with class A and class C β-lactamases [Fisher et al., 1980; Knott-Hunziker et al., 1982; see Bicknell & Waley (1985c) and references cited therein] and illustrates why the many mechanism based serine β-lactamase inhibitors (Cartwright & Waley, 1983) are totally ineffective as inhibitors of the metallo-β-lactamases (Bicknell et al., 1985). At present, the only pathogen known to produce a zinc metallo-β-lactamase is *Pseudomonas maltophilia* (Bicknell et al., 1985); however, it seems likely that more will be discovered. Metallo-β-lactamases will clearly require chemically different inhibitors (none are known at present) should these enzymes cause widespread resistance to β-lactam therapy.

ACKNOWLEDGMENTS

We thank Joan Monks for isolation of β-lactamase II and Drs. Graeme Hanson and Barton Holmquist for helpful comments.

Registry No. Benzylpenicillin, 61-33-6.

REFERENCES

Ambler, R. P., Daniel, M., Fleming, J., Hermoso, J.-M., Pang, C., & Waley, S. G. (1985) *FEBS Lett. 189*, 207–211.

Auld, D. S., Galdes, A., Geoghegan, K. F., Holmquist, B., Martinelli, R. A., & Vallee, B. L. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 5041–5045.

Auld, D. S., Larsen, K. S., & Vallee, B. L. (1986a) in *Zinc Enzymes* (Bertini, I., Luchinat, C., Maret, W., & Zeppezauer, M., Eds.) Birkhauser Verlag, Basel and Boston.

Auld, D. S., Geoghegan, K. F., Galdes, A., & Vallee, B. L. (1986b) *Biochemistry 25*, 5156–5159.

Baldwin, G. S., Galdes, A., Hill, H. A. O., Smith, B. E., Waley, S. G., & Abraham, E. P. (1978) *Biochem. J. 175*, 441–447.

Baldwin, G. S., Waley, S. G., & Abraham, E. P. (1979) *Biochem. J. 179*, 459–463.

Baldwin, G. S., Edwards, G. F. St. L., Keiner, P. A., Tully, M. J., Waley, S. G., & Abraham, E. P. (1980a) *Biochem. J. 191*, 111–116.

Baldwin, G. S., Galdes, A., Hill, H. A. O., Waley, S. G., & Abraham, E. P. (1980b) *J. Inorg. Biochem. 13*, 189–204.

Banci, L., Bencini, A., Benelli, C., Gatteschi, D., & Zanchini, C. (1982) *Struct. Bonding (Berlin) 52*, 37–86.

Bencini, A., Bertini, I., Canti, G., Gatteschi, D., & Luchinat, C. (1981) *J. Inorg. Biochem. 14*, 81–93.

Bicknell, R. (1984) Ph.D. Thesis, University of Oxford, England.

Bicknell, R., & Waley, S. G. (1985a) *Biochem. Soc. Trans. 13*, 766–767.

Bicknell, R., & Waley, S. G. (1985b) *Biochemistry 24*, 6876–6887.

Bicknell, R., & Waley, S. G. (1985c) *Biochem. J. 231*, 83–88.

Bicknell, R., Knott-Hunziker, V., & Waley, S. G. (1983) *Biochem. J. 213*, 61–66.

Bicknell, R., Emanuel, E. L., Gagnon, J., & Waley, S. G. (1985) *Biochem. J. 229*, 791–797.

Cartwright, S. J., & Fink, A. L. (1981) *CRC Crit. Rev. Biochem. 11*, 145–207.

Cartwright, S. J., & Waley, S. G. (1983) *Med. Res. Rev. 3*, 341–382.

Davies, R. B., & Abraham, E. P. (1974) *Biochem. J. 143*, 129–135.

Douzou, P. (1977) *Cryobiochemistry: An Introduction*, Academic Press, London and New York.

Fisher, J., Belasco, J. G., Khosla, S., & Knowles, J. R. (1980) *Biochemistry 19*, 2895–2901.

Galdes, A., Auld, D. S., & Vallee, B. L. (1986) *Biochemistry 25*, 646–651.

Geoghegan, K. F., Galdes, A., Martinelli, R. A., Holmquist, B., Auld, D. S., & Vallee, B. L. (1983a) *Biochemistry 22*, 2255–2262.

Geoghegan, K. F., Holmquist, B., Spilburg, C. A., & Vallee, B. L. (1983b) *Biochemistry 22*, 1847–1852.

Hanahan, D., & Auld, D. S. (1980) *Anal. Biochem. 108*, 86–95.

Holmquist, B., Kaden, T. A., & Vallee, B. L. (1975) *Biochemistry 14*, 1454–1461.

Knott-Hunziker, V., Petursson, S., Waley, S. G., Jaurin, B., & Grundstrom, T. (1982) *Biochem. J. 207*, 315–322.

Little, C., Emanuel, E. L., Gagnon, J., & Waley, S. G. (1986) *Biochem. J. 233*, 465–469.

Mozingo, A. F., & Matthews, B. W. (1984) *Biochemistry 23*, 5724–5729.

Sabath, L. D., & Abraham, E. P. (1966) *Biochem. J. 98*, 11c–13c.

Sabath, L. D., & Finland, M. (1968) *J. Bacteriol. 95*, 273–355.

Stephens, R. S., Jentoft, J. E., & Bryant, R. G. (1974) *J. Am. Chem. Soc. 96*, 8041–8045.

Thiers, R. E. (1957) *Methods Biochem. Anal. 5*, 273–355.

Vallee, B. L., & Holmquist, B. (1980) in *Methods for Determining Metal Ion Environments in Proteins: Structure and Function of Metalloproteins* (Darnell, D. W., & Wilkins, R. G., Eds.) pp 27–74, Elsevier/North-Holland, New York.