Role of Zinc(II) in β-Lactamase II: A Model Study with a Zinc(II)–Macrocyclic Tetraamine (1,4,7,10-Tetraazacyclododecane, Cyclen) Complex

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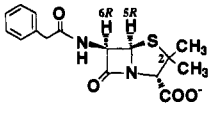
Abstract: Cleavage of the β-lactam ring of benzylpenicillin (**1**) by a zinc(II) complex of the macrocyclic tetraamine 1,4,7,10-tetraazacyclododecane (cyclen) (**8**) has been studied in aqueous solution as a functional model of a zinc-containing hydrolytic enzyme, β-lactamase II. β-Lactam hydrolysis by **8** yielding (5*R*)-benzylpenicilloate (**9**) is a second-order reaction (the second-order rate constant k is $(4.1 \pm 0.1) \times 10^{-2}$ M^{-1} s^{-1} at 25 °C and $I = 0.10$ ($NaNO_3$)), and a plot of the hydrolysis rate vs pH (6.6–9.6) gives a sigmoidal curve with an inflection point at pH 7.9, which is identical to the pK_a value for the Zn^{II}-bound water of Zn^{II}–cyclen–OH_2 (**8a**). Thus, Zn^{II}–cyclen–OH^- (**8b**) must play a crucial role in the hydrolysis of the β-lactam. The activation energy E_a for the hydrolysis by **8b** was determined to be 49 kJ mol^{-1}, lower than the value of 61 kJ mol^{-1} by aqueous OH^- ion. The lower E_a value for **8b** is due to the acidic nature of Zn^{II} that stabilizes the anionic tetrahedral intermediate **15**. The hydrolysis is subject to anion inhibition by deprotonated succinimide$^-$, SCN^-, CH_3COO^-, or Cl^- in the same order as their binding affinity for **8a**. The Zn^{II}–cyclen **8** simultaneously catalyzes the isomerization of (5*R*)-benzylpenicilloate (**9**) to the 5*S*-epimer **10** at pH 6.5–9.5. The pH dependency of the catalytic activity discloses that the reactive species is **8a** and the kinetically obtained pK_a value of 8.0 is almost the same as that obtained thermodynamically. The second-order rate constant k_{ep} with **8a** is 2.5 ± 0.1 M^{-1} s^{-1} at 25 °C and $I = 0.10$ ($NaNO_3$). Presumably the reaction involves C—S bond rupture due to coordination of Zn^{II}–cyclen, followed by recombination to form **10**. While the reaction mechanism of β-lactamase II is still not known in detail, analogies may be drawn with the common role of the Zn^{II}–OH^- species in other zinc enzymes such as carbonic anhydrase.

Introduction

β-Lactamases are clinically important bacterial enzymes, some of which play an important part in the resistance of pathogens to β-lactam antibiotics.[1] β-Lactamase II produced by *Bacillus cereus* is classified as a Zn^{II}-containing enzyme (with 227 amino acid residues) that unselectively hydrolyzes the β-lactam ring of a variety of penicillins (e.g., benzylpenicillin (**1**)) and cephalosporins.[2] At pH 7 and 30 °C the half-life of benzylpenicillin



benzylpenicillin (BP)

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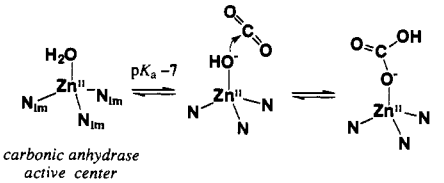
bound to β-lactamase II is ca. 0.5 ms.[3] Recent studies on the Cd^{II}-substituted enzyme disclosed the structure of the enzyme active center, where the metal ion is closely sequestered by three imidazoles (His_{86}, His_{88}, and His_{210}) and S (Cys_{168}).[4] The location of the Zn^{II} site is at the surface of the enzyme in the environment of the hydrophobic residue. This structure around Zn^{II} is similar to that of a typical zinc enzyme, carbonic anhydrase (CA), where

Scheme 1



carbonic anhydrase active center

the Zn^{II} binds to three imidazoles.[5] The role of Zn^{II} in CO_2 hydration with CA (see Scheme 1) is now accounted for on the basis of enzyme[6] and model studies.[7-9] At physiological pH the water at the fourth coordination site deprotonates to yield the strong nucleophile OH^- which attacks the electrophilic center of the substrate CO_2. In the study of β-lactam hydrolysis with Co^{II}-substituted β-lactamase II in aqueous/organic mixed solvents at low temperature, the substrate–enzyme complexes were detected, and from several possible mechanisms, one similar to that of CA was proposed for β-lactam hydrolysis by β-lactamase II (see Scheme 2).[10] However, the role of the Zn^{II} ion in this reaction still remains unclear.

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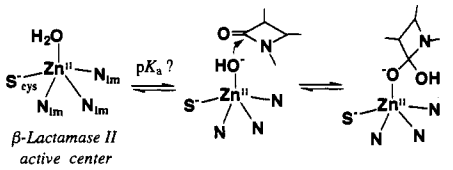
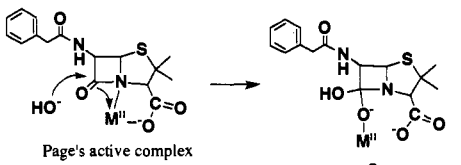
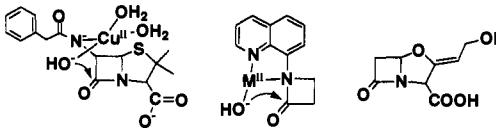
Scheme 2



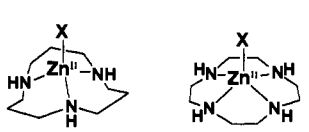
Hydrolysis of β-lactams is also promoted by transition-metal ions, such as Cu^{II}, Zn^{II}, Co^{II}, and Ni^{II}.[3,11-14] The mechanism of metal ion-promoted hydrolysis of benzylpenicillin (**1**) was studied by Page et al.,[3,11] whereby metal ions were found to initially bind to the carboxylate group and the β-lactam nitrogen to form the active species **2**. The metal ions were considered to enhance C—N bond cleavage by stabilizing the tetrahedral intermediate **3** for nucleophilic attack by an *external* OH^- ion. Thus, Cu^{II} and Zn^{II} ions were found to increase the raet of OH^--catalyzed hydrolysis of **1** by as much as 8×10^7- and 4×10^4-fold, respectively.[3] A more detailed kinetic study by Hay et al.[12] led to the suggestion that Cu^{II} binds to the deprotonated amide nitrogen of benzylpenicillin (see **4**), and that subsequent hydrolysis proceeds by intramolecular attack of Cu^{II}–OH^- at the carbonyl. A similar intramolecular OH^- attack by the M^{II}-bound OH^- was concluded by Fife et al. with the β-lactam hydrolysis of *N*-(8-quinolyl)azetidin-2-one using Ni^{II} and Zn^{II} ions (see **5**).[13] Metal ion-promoted β-lactam hydrolysis was also carried out on clavulanic acid (**6**), a potent inhibitor of β-lactamases, where a mechanism similar to **2** → **3** was proposed.[14] In all these studies,





however, clear-cut kinetic results were difficult to obtain, because of the coexistence of various metal species such as free M^{II}_{aq}, M^{II}–β-lactam, M^{II}–OH^-, etc., in aqueous solution. Moreover, it is doubtful whether benzylpenicillin (**1**) actually coordinates to the Zn^{II} of β-lactamase II. Studies with structurally more refined β-lactamase II models would give a better insight into the role of the Zn^{II} in the enzyme.

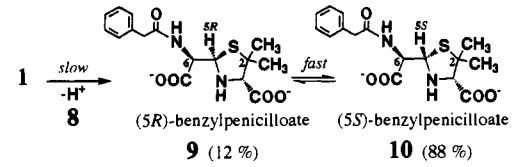
Recently, we have demonstrated that 1,5,9-triazacyclododecane ([12]aneN$_3$)–(**7**) and 1,4,7,10-tetraazacyclododecane (cyclen)–Zn^{II} (**8**) complexes are good models for the active center of CA[8,9] and alkaline phosphatase (AP).[15] The water-bound forms **7a** and **8a** can provide the reactive (nucleophilic) Zn^{II}–OH^- species **7b** and **8b** with pK_a values of 7.3 and 7.9 at 25 °C, respectively.[8]



7a: X = H_2O **8a**: X = H_2O
b: X = HO^- **b**: X = HO^-

These nucleophiles, **7b** and **8b**, in a way similar to that of the active sites of CA and AP, attack carbonyls and phosphates at physiological pH. In view of this, we hoped that these macrocyclic polyamine–Zn^{II} complexes might also become functional models for β-lactamase II. The tetraamine–Zn^{II} complex **8** seemed more appropriate, since it is much more stable (K' = [Zn^{II} complex]/[Zn^{II}][uncomplexed ligand] = $10^{10.8}$ M^{-1} at pH 7 and 25 °C[9a]) with respect to Zn^{II}–benzylpenicillin ($K' = 10^{2.3}$ M^{-1} at pH 7 and 30 °C[3]), while the triamine complex **7** ($K' = 10^{2.4}$ M^{-1} at pH 7 and 25 °C[9a]) is less stable. Furthermore, the five-coordinate complex **8** may be viewed as having a structure closer to that of the β-lactamase II active center. We thus chose the Zn^{II}–cyclen complex **8** as a model for β-lactamase II in a hope that a much more revealing study as to the nature of Zn^{II} in this enzyme could be achieved.

Scheme 3



Results

Reaction of Zn^{II}–Cyclen 8 with Benzylpenicillin (1). The reaction of Zn^{II}–cyclen **8** (1 mM) with twice its amount of benzylpenicillin (BP, **1**) (2 mM as its sodium salt) in D_2O solution at pD 9 (0.1 M borate buffer) and 25 °C was followed by ^1H NMR. As the 2α- and 2β-methyl signals of BP at δ 1.50 and 1.58 decreased, the formation of two new signal pairs (at δ 1.23 and 1.50 and δ 1.04 and 1.57) were observed, and this process was completed within 7 h; i.e., the excess (2 equiv) substrate **1** is completely hydrolyzed with Zn^{II}–cyclen (1 equiv). These new methyl proton peaks and other changeable ones such as HC_6 and $ArCH_2$ could all be assigned to those of (5*R*)-benzylpenicilloate (**9**) and (5*S*)-benzylpenicilloate (**10**), and were consistent with the reported ones[16] (see Scheme 3 and Experimental Section). A first-order dependence of the hydrolysis rate on [benzylpenicillin] in the presence of Zn^{II}–cyclen (until the reaction finished) was disclosed by the log-plot method. The hydrolysis product (5*R*)-benzylpenicilloate (**9**) (as its monosodium salt) was independently prepared as colorless needles from BP by Munro's method.[17] The synthesized 5*R*-epimer **9** was isomerized to the 5*S*-epimer **10** upon treatment with 1 mM Zn^{II}–cyclen **8**, and equilibrium was reached within 1 h under the same conditions as before. Any other possible products such as a benzylpenicilloate–Zn^{II}–cyclen complex or a C_5-deuteride of benzylpenicilloate etc. were undetected. The product ratios of **9** and **10** from BP hydrolysis after 2 and 7 h remained at a constant value of 12:88. In the absence of Zn^{II}–cyclen, BP hydrolysis and the epimerization of **9** to **10** were very slow (half-life time > 100 and > 5 h, respectively). These facts indicate that (i) both hydrolysis of benzylpenicillin (**1**) and epimerization of the product (5*R*)-benzylpenicilloate (**9**) *are catalyzed* by Zn^{II}–cyclen and (ii) no sooner is **1** hydrolyzed than the product **9** epimerizes to form **10**.

Kinetics: Hydrolysis of Benzylpenicillin (BP, 1) by Zn^{II}–Cyclen 8 and OH^- Ion. The hydrolysis of **1** catalyzed by Zn^{II}–cyclen **8** was determined at pH 6.6–9.6 using a pH-stat technique. The

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Table 1. A Comparison of Hydrolysis Rate Constants (M^{-1} s^{-1}) and Activation Energies E_a (kJ M^{-1}) for Zn^{II}–Cyclen-OH^- (**8b**) and Aqueous OH^- Ion

substrate		**8b** k	**8b** E_a	OH^- k_{OH}	OH^- E_a
benzylpenicillin[a] (BP)	15 °C	$(2.2 \pm 0.1) \times 10^{-2}$	49 ± 2	$(6.6 \pm 0.1) \times 10^{-2}$	61 ± 2
	25 °C	$(4.1 \pm 0.1) \times 10^{-2}$		$(1.6 \pm 0.1) \times 10^{-1}$	
	35 °C	$(8.3 \pm 0.2) \times 10^{-2}$		$(3.5 \pm 0.1) \times 10^{-1}$	
4-nitrophenyl acetate[b] (NA)	15 °C	$(5.3 \pm 0.1) \times 10^{-2}$	45 ± 2	4.4 ± 0.1	43 ± 2
	25 °C	$(1.0 \pm 0.1) \times 10^{-1}$		8.1 ± 0.1	
	35 °C	$(1.8 \pm 0.1) \times 10^{-1}$		14.7 ± 0.1	
BNP[c]	35 °C	2.1×10^{-5}		2.4×10^{-5}	

[a] I = 0.10 ($NaNO_3$). [b] [**8b**] = 1, 2, and 3 mM, [OH^-] = 1, 2, and 4 mM, and [4-nitrophenyl acetate] = 0.1 and 0.2 mM at I = 0.10 ($NaNO_3$) in 10% (v/v) CH_3CN. [c] From ref 15 at I = 0.2 ($NaClO_4$).

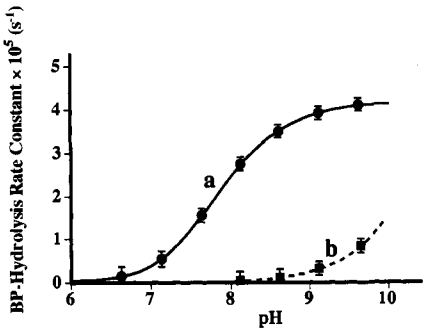

Figure 1. pH–rate profiles for the first-order rate constants at 25 °C and I = 0.10 ($NaNO_3$): (a) Zn^{II}–cyclen-catalyzed BP hydrolysis with 1 mM Zn^{II}–cyclen (k_{ZnL}, ●); (b) background BP hydrolysis (k_b, ■).

BP hydrolysis rate was followed by the evolution of H^+ released from benzylpenicilloic acid **9** (pK_a of 5.20 ± 0.05) at 25 °C and I = 0.10 ($NaNO_3$). Since benzylpenicillin was partially hydrolyzed without Zn^{II}–cyclen at alkaline pH, the observed rate was a composite of v_b and v_{ZnL}, where v_b and v_{ZnL} are the rates of background and Zn^{II}–cyclen-catalyzed hydrolysis, respectively (see eqs 1 and 2). The second-order dependence of the Zn^{II}–cyclen-catalyzed hydrolysis rate on the total concentration (=0.5, 1, and 2 mM) of Zn^{II}–cyclen complexes **8a** and **8b** and [benzylpenicillin] (=4 and 8 mM) is consistent with the kinetic equation 3. The first-order rate constants, k_{ZnL} (with 1 mM Zn^{II}–cyclen) and k_b, are plotted as a function of pH (see Figure 1). The sigmoidal curve for k_{ZnL} (Figure 1a) is characteristic of a kinetic process controlled by an acid–base equilibrium and exhibits an inflection point at pH 7,9, which is identical to the pK_a value for the coordinate water of **8a**. Therefore, the reactive species is Zn^{II}–cyclen–OH^- (**8b**) (or its equivalent species), which should be a good nucleophile to attack at the β-lactam carbonyl group at physiological pH. Thus, the second-order rate constant k of $(4.1 \pm 0.1) \times 10^{-2}$ M^{-1} s^{-1} was determined from the maximum kh_{ZnL} value at 25 °C and I = 0.10 ($NaNO_3$) (see eq 4). The same Zn^{II}–OH^- species (**8b**) worked on hydrolysis of carboxyesters[9a] and phosphates,[15] and **7b** on hydration of CO_2[9b] and acetaldehyde.[9a]

$$v_b = k_b[\text{benzylpenicillin}] \quad (1)$$

$$v_{ZnL} = k_{ZnL}[\text{benzylpenicillin}] \quad (2)$$

$$= k'_{ZnL}([\mathbf{8a}] + [\mathbf{8b}])[\text{benzylpenicillin}] \quad (3)$$

$$= k[\mathbf{8b}][\text{benzylpenicillin}] \quad (4)$$

The temperature dependence of the hydrolysis of benzylpenicillin by Zn^{II}–cyclen–OH^- (**8b**) was determined at I = 0.10 ($NaNO_3$). The obtained second-order rate constants of **8b** are listed in Table 1. The activation energy E_a for the hydrolysis by **8b** was determined to be 49 ± 2 kJ mol^{-1} by an Arrhenius plot.

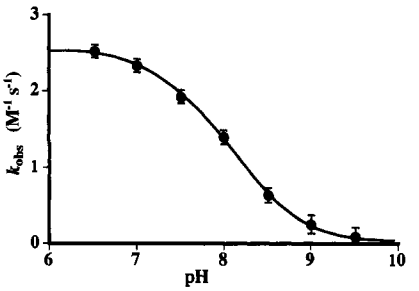

Figure 2. pH–rate profile for the Zn^{II}–cyclen-catalyzed epimerization of (5*R*)-benzylpenicilloate to the 5*S*-epimer at 25 °C and I = 0.10 ($NaNO_3$).

For comparison, aqueous OH^--catalyzed BP hydrolysis was determined under the same conditions by the same pH-stat method at pH 9.5, 10.4, and 10.8. The kinetics followed a second-order dependence on [benzylpenicillin] and [OH^-], eq 5. The rate constants k_{OH} and the activation energy E_a for the hydrolysis by OH^- are listed in Table 1.

$$v = k_{OH}[OH^-][\text{benzylpenicillin}] \quad (5)$$

For comparison, aqueous OH^--catalyzed hydrolysis of 4-nitrophenyl acetate (NA) was also determined at 15, 25, and 35 °C by the same method as described previously.[15] The kinetics followed a second-order dependence on [4-nitrophenyl acetate] and [OH^-]. The rate constants and the activation energy E_a are listed in Table 1.

Epimerization of (5*R*)-Benzylpenicilloate (9) to the 5*S*-Epimer 10 by Zn^{II}–Cyclen 8. Zn^{II}–cyclen **8** simultaneously catalyzes the isomerization of (5*R*)-benzylpenicilloate (**9**) to the 5*S*-epimer **10** at pH 6.5–9.5, 25 °C, and I = 0.10 ($NaNO_3$). (5*R*)-Benzylpenicilloate (**9**) was independently prepared and fully characterized (see Experimental Section). The epimer **10** was identified by ^1H NMR, which is consistent with the literature data.[17,18] The initial pH was attained by a pH-stat method with 20 mM NaOH, but no more NaOH was consumed during the isomerization. This fact indicates that the pK_a value of the product 5*S*-epimer **10** is almost identical to that of **9** (5.20 ± 0.05 at 25 °C and I = 0.10 ($NaNO_3$)). The isomerization rate was measured by following the decrease in the UV absorption at 240 nm ($\Delta\epsilon$ = 65 M^{-1} cm^{-1}). The change in the amount of Zn^{II}–cyclen added (=0.33, 0.67, and 1.0 mM) had no appreciable effect on the epimerization equilibrium. The epimerization process was reversible, and its equilibrium constant K_e (=[**9**]/[**10**]) remained at a constant value of 0.14 with or without Zn^{II}–cyclen. The second-order dependence of the rate constant k_{obs} on the total concentration of Zn^{II}–cyclen complexes **8a** and **8b** and [benzylpenicilloate] (=6 mM) is consistent with the kinetic equation 6. The k_{obs} values are plotted as a function of pH (see Figure 2).

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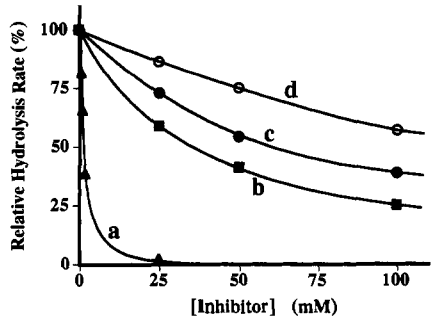


Figure 3. Relative benzylpenicillin hydrolysis rate as a function of the concentration of inhibitor at 25 °C and I = 0.10 ($NaNO_3$): (a) for succinimide; (b) for SCN^-; (c) for CH_3COO^-; (d) for Cl^-.

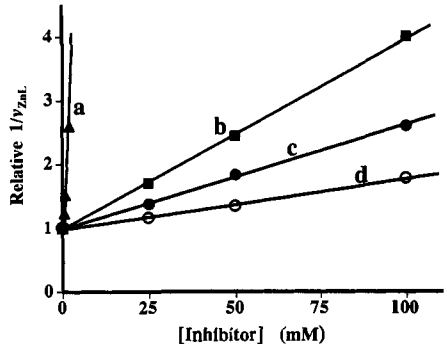


Figure 4. Relative $1/v_{ZnL}$ values as a function of the conentration of inhibitor at 25 °C and I = 0.10 ($NaNO_3$): (a) for succinimide; (b) for SCN^-, (c) for CH_3COO^-; (d) for Cl^-.

In good contrast to the earlier benzylpenicillin hydrolysis, the pH–rate profile shows an antisigmoidal curve with an inflection point at pH 8.0 which is almost the same as the pK_a value of **8a**. Therefore, the reactive species is concluded to be Zn^{II}–cyclen–OH_2 (**8a**) (or its equivalent species). The second-order rate constant k_{ep} with **8a** is 2.5 ± 0.1 M^{-1} s^{-1} (see eq 7). The first-order rate constant for the epimerization in the absence of Zn^{II}–cyclen was determined as a pH-independent value of (3.0 ± 0.5) × 10^{-5} s^{-1} at 25 °C, I = 0.10 ($NaNO_3$), and pH 6.5–9.5.

$$d[(5S)\text{-benzylpenicilloate}]/dt = k_{obs}[(5R)\text{-benzylpenicilloate}]([\mathbf{8a}] + [\mathbf{8b}]) \quad (6)$$

$$= k_{ep}[(5R)\text{-benzylpenicilloate}][\mathbf{8a}] \quad (7)$$

Anion Inhibition of the Benzylpenicillin Hydrolysis by Zn^{II}–Cyclen 8. The hydrolysis of benzylpenicillin is subject to anion inhibition by a number of deprotonated inhibitors (A^-), namely, succinimide$^-$ (pK_a = 9.52 ± 0.02 at 25 °C and I = 0.10 ($NaClO_4$)), SCN^-, CH_3COO^-, or Cl^- at pH 8, 25 °C and I = 0.10 ($NaNO_3$). As the inhibitor concentration increases, the Zn^{II}–cyclen-catalyzed BP hydrolysis rate v_{ZnL} decreases (see Figure 3).

Plots of $1/v_{ZnL}$ against the concentration of inhibitor give straight lines for each inhibitor (see Figure 4). Earlier, we discovered a similar relationship in the inhibition kinetics of **7b**-catalyzed 4-nitrophenyl acetate (NA) hydrolysis in the presence of inhibitors (e.g., sulfonamides and inorganic anions), in which 1:1 anion complexes, Zn^{II}–[12]aneN_3–A^-, are formed to reversibly block the formation of the active Zn^{II}–OH^- species **7b**.[19] From the slopes of the lines, the anion binding constants (K = [Zn^{II}–[12]aneN_3–A^-]/[**7a**][A^-]) were determined. Thus, a similar analysis could be applied to the benzylpenicillin hydrolysis catalyzed by **8b** in the presence of anionic inhibitors. From the slopes of the lines in Figure 4, the anion binding constants (K = [Zn^{II}–cyclen–A^-]/[**8a**][A^-]) were calculated by the same method as previously described.[19] All the log K values are summarized in Table 2. These values are in good agreement with the anion binding affinities determined by potentiometric pH titration at 25 °C and I = 0.10 ($NaClO_4$) (see Table 2).

(19) Koike, T.; Kimura, E.; Nakamura, I.; Hashimoto, Y.; Shiro, M. *J. Am. Chem. Soc.* **1992**, *114*, 7338.

Table 2. A Comparison of Anion Affinity Constants, log K, of Zn^{II}–Cyclen at 25 °C

inhibitor	log K[a] by inhibition kinetics[b]	log K[a] by pH titration[c]
OH^-		6.00 ± 0.02[f]
succinimide$^-$	5.2 ± 0.2[d]	5.60 ± 0.05[g]
SCN^-	1.8 ± 0.2[e]	2.2 ± 0.1[h]
CH_3COO^-	1.6 ± 0.2[e]	1.9 ± 0.1[h]
Cl^-	1.2 ± 0.2[e]	1.5 ± 0.1[h]
captopril^{2-}		7.0 ± 0.1[i]
acetylproline$^-$		2.2 ± 0.1[j]

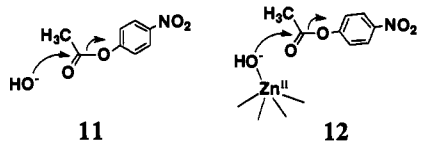
[a] K = [Zn^{II}–cyclen–A^-]/[**8a**][A^-] (M^{-1}). [b] Determined at I = 0.10 ($NaNO_3$). [c] Determined at I = 0.10 ($NaClO_4$). [d] Determined with 0.5, 1.0, and 2.0 mM succinimide and 2.0 mM Zn^{II}–cyclen. [e] Determined with 25, 50, and 100 mM sodium salt and 2.0 mM Zn^{II}–cyclen. [f] Determined with 0.5, 1.0, and 2.0 mM Zn^{II}–cyclen. [g] Determined with 1.0, 2.0, and 5.0 mM succinimide and 1.0 mM Zn^{II}–cyclen. The pK_a value of the imide is 9.52 ± 0.02. [h] Determined with 10, 20, and 50 mM sodium salt and 1.0 mM Zn^{II}–cyclen. [i] Determined with 1.0 mM captopril and 1.0 mM Zn^{II}–cyclen. The pK_a values of the SH and COOH groups of captopfil are 9.97 ± 0.03 and 3.58 ± 0.05, respectively. [j] Determined with 10, 20, and 50 mM acetylproline sodium salt and 1.0 mM Zn^{II}–cyclen. The pK_a value of the carboxyl group is 3.40 ± 0.05.

Discussion

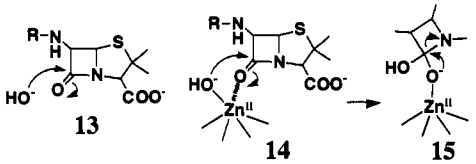
Significance of Zinc(II) in β-Lactam Hydrolysis by Zn^{II}–Cyclen 8. The results show that benzylpenicillin is *catalytically* hydrolyzed by the Zn^{II}–cyclen **8** complex at physiological pH and 15, 25, and 35 °C, and the kinetic study using a pH-stat technique shows an unambiguous rate law of second order: first order each in [benzylpenicillin] and [Zn^{II}–cyclen]. No significant interaction between Zn^{II}–cyclen and the β-lactam N (as reported with aqueous Zn^{II} [13]) or the side chain amide N (as reported with aqueous Cu^{II} [12]) was observed during the hydrolysis reaction. A plot of the BP hydrolysis rate constant k_{ZnL} against pH gave a sigmoidal curve (Figure 1a) with an inflection pH of 7.9, which is identical to the pK_a value of the Zn^{II}-bound water. The reactive species in the benzylpenicillin hydrolysis is thus concluded to be Zn^{II}–cyclen–OH^- (**8b**), and the second-order rate with [benzylpenicillin] and [**8b**] can be established.

Exactly the same rate law was found for the hydrolyses of 4-nitrophenyl acetate and bis(4-nitrophenyl) phosphate (BNP$^-$, a phosphodiester) by **8b**.[15] It is of interest to compare these second-order rate constants with the aqueous OH^--catalyzed rate constants in these hydrolysis reactions (see Table 1). Interestingly, with Zn^{II}–cyclen–OH^- (**8b**) at 25 °C, benzylpenicillin is hydrolyzed only 2.4 times slower than the hydrolysis of an activated ester, 4-nitrophenyl acetate, whereas with aqueous OH^-, benzylpenicillin is hydrolyzed much slower (51 times at 25 °C) than 4-nitrophenyl acetate. This fact suggests that the Zn^{II}–cyclen–OH^- species (**8b**) may act as more than a mere OH^- species (at physiological pH) when it reacts with benzylpenicillin. We thus compared the Arrhenius activation energies (E_a) for benzylpenicillin and 4-nitrophenyl acetate hydrolysis by OH^- and **8b** (see Table 1). Their similar E_a values indicate that both aqueous OH^- and Zn^{II}–cyclen–OH^- (**8b**) react with 4-nitrophenyl acetate via a similar mechanism. Namely, the Zn^{II}-bound OH^- in **8b** is simply OH^- that is avilable at lower pH to react with the carboxyester (see **11** and **12**). On the other hand, **8b** serves to lower the E_a value to 49 kJ M^{-1} from 61 kJ M^{-1} with aqueous OH^- in benzylpenicillin hydrolysis. We thus propose that the transition state, which develops anionic character after the attack

of OH^-, is stabilized by the coordination of the adjacent Zn^{II} uion to O^- (see **14** → **15**). Due to the strain of the β-lactam ring, the following C–N bond cleavage of **15** would occur easily.

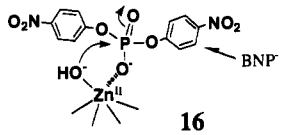


11 **12**



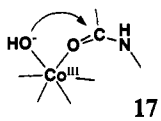
13 **14** **15**

This Zn^{II}–OH^- reaction mechanism is somewhat similar to the earlier proposed one for BNP^- hydrolysis (see **16**),[15] where the Zn^{II} ion in **8b** not only provides OH^- ion (as a nucleophile) at physiological pH but also stabilizes the anionic transition state by electrophilic interaction between the Zn^{II} and the phosphate O^- anion. In this case, the hydrolysis rate constant with **8b** is almost the same as the value with aqueous OH^- at 35 °C (see Table 1).



16

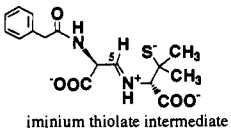
It will be of interest to consider whether the same mechanism holds in the hydrolysis of ordinary amides or peptides. In Co^{III}-promoted hydrolysis, the distinct coordination of both the reactant amide oxygen and OH^- in the *cis* position greatly enhances amide hydrolysis (see **17**).[20] The more acidic and higher valent Co^{III} can accommodate both an OH^- ion and the carbonyl substrate. In light of the less acidic and limited valency of Zn^{II}, **14** may be viewed as an incomplete form of **17**.



17

Epimerization of the β-Lactam Hydrolyzed Product (5*R*)-Benzylpenicilloate (9). Initially, we did not anticipate epimerization of the product (5*R*)-benzylpenicilloate (**9**) to the 5*S*-epimer **10**. This was disclosed by NMR, which revealed that the epimerization was faster than the initial β-lactam hydrolysis. This is the first example of benzylpenicilloate isomerization catalyzed by metal complexes. Earlier, the H^+-promoted (pH < 6) and OH^--catalyzed (pH > 11) epimerizations of **9** to **10** were reported.[21] The epimerization should take place, provided that the C_5—S bond rupture and reformation rapidly occur.

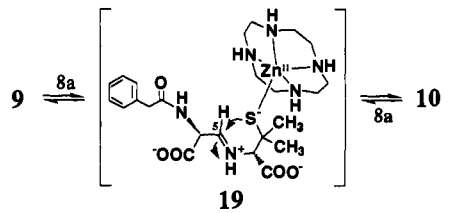
The rates were determined at pH 6.5–9.5 and 25 °C by measuring the change in the UV absorbance of the independently prepared **9** at 240 nm. In the absence of Zn^{II}–cyclen, a much slower UV change occurred. Its first-order rate constant was an extremely small value of $(3.0 \pm 0.5) \times 10^{-5}$ s^{-1} at pH 6.5–9.5 and 25 °C. A similar pH-independent rate constant of 5.8×10^{-5} s^{-1} at pH 6–12 and 30 °C was reported by Page et al.[21] This epimerization was explained by the ring opening of the thiazolidine by C—S bond cleavage to an iminium thiolate intermediate (**18**).



iminium thiolate intermediate

18

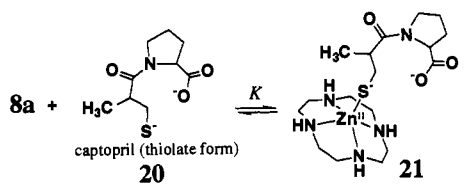
The catalytic rate with Zn^{II}–cyclen was found to increase as the pH was lowered. The second-order rate constants k_{obs} (see eq 6) were plotted against pH (see Figure 2), and the shape was found to be just the opposite of that of the pH–rate plot for benzylpenicillin hydrolysis with Zn^{II}–cyclen (see Figure 1a). Since the inflection point of this sigmoidal curve is pH 8.0, almost the same value as the pK_a for **8a** ⇌ **8b**, we conclude that the reactive catalyst is Zn^{II}–cyclen–OH_2 (**8a**). The acidic Zn^{II} in **8a** attacks the thioether of **9** to assist the C—S bond cleavage and stabilizes Page's thiolate intermediate **18** by Zn^{II}–S^- coordination (see **19**).



19

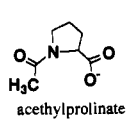
This is followed by intramolecular nucleophilic attack of Zn^{II}–S^- at the iminium cation to reclose the thiazole ring to give either the 5*S*-epimer **10** or the starting 5*R*-epimer **9**. The sterically more crowded and hence less stable 5*R*-form **9** is likely to be isomerized to the more stable 5*S*-epimer **10**. Thus, the ratio of the concentration of **9** to that of **10** was 12:88 in our case (with or without **8** at 25 °C) and 15:85 in Page's case (at 30 °C).[21]

In order to check the strong preference of Zn^{II}–cyclen for a thiolate anion, we determined the affinity of **8a** for captopril (**20**) a drug that inhibits angiotensin converting enzyme II,[22] which also contains Zn^{II} at the active center (see the pH titration curve in the supplementary material). An extraordinary strong affinity (log K = 7.0 for the 1:1 complex **21**) was found at 25 °C and I = 0.10 ($NaClO_4$) (see Table 2). For reference, the affinity of **8a** for acetylprolinate anion (**22**) was much weaker (log K = 2.2). These facts support the feasibility of the thiolate-bound Zn^{II}–cyclen intermediate **19** in benzylpenicilloate epimerization catalyzed by **8a**.



captopril (thiolate form)

20 **21**



acethylprolinate

22

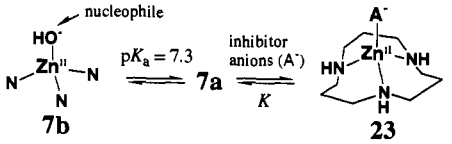
Anion Inhibition of β-Lactam Hydrolysis by Zn^{II}–Cyclen–OH^- (8b). Earlier, we showed that the hydrolysis of 4-nitrophenyl acetate by **7b** was subject to inhibition by anions and (neutral) aromatic sulfonamides.[19] The mechanism involves reversible displacement of the nucleophile OH^- with anions on the Zn^{II}

(20) Takasaki, B. K.; Kim, J. H.; Rubin, E.; Chin, J. *J. Am. Chem. Soc.* **1993**, *115*, 1157.

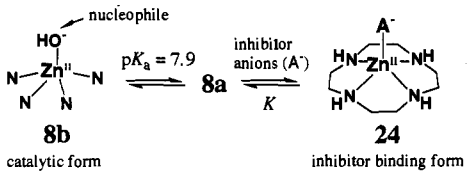
(21) Davis, A. M.; Jones, M.; Page, M. I. *J. Chem. Soc., Parkin Trans. 2*, **1991**, 1219.

(22) Ondetti, M. A.; Cushman, D. W. *Annu. Rev. Biochem.* **1982**, *51*, 283.

coordination site (7 ⇌ **23**), which was the first chemical model of the anion inhibition of carbonic anhydrase.[6]



From the present inhibition kinetics with various anions and succinimide,[23] we have calculated the stability constants K for 1:1 anion–Zn^{II}–cyclen complexation (**24**), and these are in good agreement with the K values obtained by potentiometric pH titration (see Table 2). All these facts lend support to the reactive species being Zn^{II}–OH^- **8b**, as was found in 4-nitrophenyl acetate hydrolysis with **7b**.[19]



The extremely high affinity (log K = 6.0) of Zn^{II}–cyclen for OH^- ion should be emphasized. This consideration will lend support to the proposed β-lactam hydrolysis mechanism involving **14**, rather than an alternative mechanism, where the Zn^{II}-bound OH^- ion first dissociates to give room for the amide O and then the freed exrernal OH^- attacks the Zn^{II}-bound β-lactam.

All of these conclusions from the study of benzylpenicillin hydrolysis with Zn^{II}–cyclen complexes remain as yet to be applied to β-lactamase II itself. Because of the few number of studies, little is known about its reaction mechanism. It is of interest, for instance, to see whether a similar sigmoidal pH–rate profile can be drawn, as found for the catalytic reactions with carbonican-hydrase[6] and the present Zn^{II}–cyclen **8**. If this were the case, then the postulate shown in Scheme 2 could be verified. Also if anion inhibition does indeed occur with β-lactamase II reactions, one could design inhibitory drugs, as has been the case with carbonic anhydrase.[6]

Experimental Section

General Information. All reagents and solvents used were of analytical grade. IR and UV spectra were recorded on a Shimadzu FTIR-4200 and a Hitachi U-3500 spectrophotometer, respectively. Melting points were determined by using a Yanaco micro melting apparatus. Thin-layer chromatography (TLC) was carried out on Merck Art. 5554 (silica gel) TLC plates. ^1H (399.7 MHz) and ^{13}C (100.4 MHz) NMR spectra were determined on a JEOL α-400 spectrometer. 3-(Trimethylsilyl)propionic-*2,2,3,3-d*$_4$ acid sodium salt was used as the internal standard. The elemental analyses (CHN) of the following compounds were found to be within 0.3% of the theoretical values.

The water-bound Zn^{II}–cyclen complex **8a**·$(ClO_4)_2$ was isolated as colorless crystals from an EtOH solution of cyclen and one equivalent amount of $Zn^{II}(ClO_4)_2$·$6H_2O$. ^1H NMR in D_2O: δ 2.75–2.82 (m, 8 H), 2.88–2.95 (m, 8 H). IR (KBr pellet): 3395, 3177, 2955, 2919, 2870, 2840, 1482, 1445, 1375, 1354, 1279, 1242, 1143, 1115, 1090, 1011, 993, 965, 903, 862, 806, 627, 573 cm^{-1}. The deprotonation constant, pK_a of the Zn^{II}-bound H_2O (=–log([**8b**]a_{H^+}/[**8a**])) was 8.06 ± 0.02, 7.86 ± 0.02, and 7.64 ± 0.02 at I = 0.10 ($NaClO_4$) and 15, 25, and 35 °C, respectively.

The benzylpenicillin sodium salt (**1**·Na^+) was purchased from Sigma Chemical Co. and was recrystallized from H_2O/CH_3CN. ^1H NMR (D_2O): δ 1.50 (3 H, s, CH_3), 1.58 (3 H, s, CH_3), 3.67 and 3.73 (2 H, ABq, J = 14.9 Hz, $ArCH_2$), 4.24 (1 H, s, $CHCOO^-$), 5.45 (1 H, d, J = 3.9 Hz, CH), 5.54 (1 H, d, J = 3.9 Hz, CH), 7.34–7.45 (5 H, m, ArH). ^{13}C NMR (D_2O): δ 29.2, 33.4, 44.7, 61.0, 67.1, 69.4, 76.0, 130.3, 131.8, 132.1, 137.4, 177.3, 177.5.

The (5*R*)-benzylpenicilloic acid monosodium salt (**9**·H^+·Na^+) (ammonium form) was prepared as colorless needles by Munro's method.[17] ^1H NMR (D_2O): δ 1.39 (3 H, s, CH_3), 1.57 (3 H, s, CH_3), 3.68 and 3.73 (2 H, ABq, J = 15.1 Hz, $ArCH_2$), 3.92 (1 H, s, $CHCOO^-$), 4.64 (1 H, d, J = 5.2 Hz, CH), 5.36 (1 H, d, J = 5.2 Hz, CH), 7.35–7.45 (5 H, m, ArH). The deprotonation constant, pK_a value of **9**·H^+ (=–log([**9**]a_{H^+}/[**9**·H^+])) was 5.20 ± 0.05 at 25 °C and I = 0.10 ($NaNO_3$).

Potentiometric pH Titrations. The preparation of the test solutions and the calibration method of the electrode system were the same as described earlier.[24] All test solutions were kept under argon (>99.99% purity) atmosphere at 25.0 ± 0.1 °C and I = 0.10 ($NaClO_4$). The calculation methods for the deprotonation constants of Zn^{II}–cyclen–OH_2 (**8a**), (5*R*)-benzylpenicilloic acid monosodium salt (**9**·H^+·Na^+), captopril, and acetylproline and for the anion affinity constants of **8a** were almost the same as described previously for **7a**.[9a,19] For the determination of these constants, at least three independent titrations were always made. The values of K_w' (=[H^+][OH^-]) and f_{H^+} were $10^{-14.15}$ and 0.827 at 15 °C, $10^{-13.79}$ and 0.825 at 25 °C, and $10^{-13.48}$ and 0.823 at 35 °C, respectively.

Benzylpenicillin Hydrolysis Catalyzed by Zn^{II}–Cyclen 8 and Aqueous OH^- Ion. In order to prevent complications caused by buffer catalysis, the hydrolysis reaction of benzylpenicillin (BP) was followed by a pH-stat method at 15.0, 25.0, and 35.0 ± 0.5 °C and I = 0.10 ($NaNO_3$). A solution of 20 mM NaOH was used to keep the pH constant with an automatic titrator (Kyoto Electronics AT-400) in a nitrogen atmosphere. The hydrolysis of benzylpenicillin (4 and 8 mM) catalyzed by Zn^{II}–cyclen ([**8a**] + [**8b**] = 0.5, 1, and 2 mM) or aqueous OH^- ion (1, 2, and 3 mM) was determined by the NaOH consumption rate v. The observed first-order rate constants, k_b and k_{ZnL}, at the given pH (6.6–9.6) were obtained from v/[BP] in the presence and absence of Zn^{II}–cyclen. The second-order rate constant k_{OH} for OH^--catalyzed BP hydrolysis was obtained as k_b/[OH^-]. A typical procedure for the kinetic measurement was as follows: After BP (8 mM) and Zn^{II}–cyclen (2 mM) were mixed in the presence or in the absence of inhibitor (i.e., succinimide, CH_3COO^-, SCN^-, and Cl^-), the solution was adjusted to the desired pH with 10 M NaOH. The pH-stat was then started, and the consumption of OH^- was recorded immediately, which was followed generally until ca. 5% hydrolysis of BP. The reaction products were identified as (5*R*)-benzylpenicilloate (**9**) and its epimerized product (5*S*)-benzylpenicilloate (**10**) by ^1H NMR analysis. Characteristic peaks of the 5*R*-epimer **9** and 5*S*-epimer **10** were assigned at pD 9 as follows, respectively: δ 1.23 and 1.04 (CH_3), 1.50 and 1.57 (CH_3), 3.71 and 3.80 ($ArCH_2$), 4.25 and 4.78 (CH). All kinetic experiments including those described in the following paragraph were run in triplicate, and the obtained rate constants were reproducible to ±5%.

Epimerization of (5*R*)-Benzylpenicilloate (9) to (5*S*)-Benzylpenicilloate (10) Catalyzed by Zn^{II}–Cyclen. The epimerization rate of (5*R*)-benzylpenicilloate (**9**) to (5*S*)-benzylpenicilloate (**10**) catalyzed by Zn^{II}–cyclen ([**8a**] + [**8b**] = 0.33, 0.67, and 1.0 mM) in aqueous solution at 26.0 ± 0.5 °C and I = 0.10 ($NaNO_3$) was measured by following the decrease of the UV absorption at 240 nm ($\Delta\epsilon$ = 65 M^{-1} cm^{-1} in both D_2O and H_2O). The desired pH was maintained by a pH-stat method with 20 mM NaOH solution. The reaction followed excellent pseudo-first-order kinetics. The pseudo-first-order rate constants k_p (s^{-1}) at various pH values (6.5–9.5) were obtained by a log plot method. The epimerization process was reversible, and its equilibrium constant K_e (=[**9**]/[**10**]) remained a constant value of 0.14 with or without Zn^{II}–cyclen. The equation k_p/[Zn^{II}–cyclen](1 + K_e) gave the observed second-order rate constant k_{obs} (M^{-1} s^{-1}) for the catalytic reaction of **9** → **10** with Zn^{II}–cyclen. A typical procedure for the kinetic measurement was as follows: After (5*R*)-benzylpenicilloate (6 mM) and Zn^{II}–cyclen (1 mM) were mixed at the desired pH adjusted with 10 M NaOH, the UV absorption decay was recorded immediately and followed for more than five half-lives. The reaction product was identified as (5*S*)-benzylpenuicilloate (**10**), and the epimer ratio was the same as that determined in the preceding paragraph.

(23) Recently, the highly selective recognition of imide derivatives (e.g., thymidine, riboflavin, 3′-azido-3′-deoxythymidine, etc.) by **8a** was discovered. In this case, the novel complementary associations of a Zn^{II}—N^- coordination bond and the NH···O (carbonyl group) hydrogen bonds were found in these 1:1 imide–bound Zn^{II}–cyclen complexes. See: Shionoya, M.; Kimura, E.; Shiro, M. *J. Am. Chem. Soc.* **1993**, *115*, 6730.

(24) (a) Kimura, E.; Koike, T.; Uenishi, K.; Hediger, M.; Kuramoto, M.; Joko, S.; Arai, Y.; Kodama, M.; Iitaka, Y. *Inorg. Chem.* **1987**, *26*, 2975. (b) Kimura, E.; Nakamura, I.; Koike, T.; Shionoya, M.; Kodama, Y.; Ikeda, T.; Shiro, M. *J. Am. Chem. Soc.* **1994**, *116*, 4764.

Kinetics. 4-Nitrophenyl Acetate Hydrolysis Catalyzed by Zn^{II}–Cyclen–OH^- (8b) and Aqueous OH^- Ion. The hydrolysis rate of 4-nitrophenyl acetate (NA) was measured by an initial slope method (following the increase in 400-nm absorption of released 4-nitrophenolate) in 10% (v/v) CH_3CN aqueous solution at 15.0, 25.0, and 35.0 ± 0.5 °C, as previously described for **7b**-catalyzed NA hydrolysis.[19] Buffered solutions containing 20 mM CHES buffer (pH 9.5, 9.3, and 9.1, respectively) were used for **8b**-catalyzed NA hydrolysis ([**8b**] = 0.5, 1.0, and 2.0 mM), and 1, 2, and 3 mM NaOH for OH^--catalyzed NA hydrolysis. The ionic strength was adjusted to 0.10 with $NaNO_3$. For the initial rate determination, the following procedure was employed: NA (final concentration of 0.5, 1.0, and 2.0 mM) was mixed with the test solution and the UV absorption increase recorded immediately and then followed generally until ca. 2% formation of 4-nitrophenolate, where log ε of 4-nitrophenolate was 4.24 at 400 nm. The observed rate constant k_{obs} (s^{-1}) was calculated from the decay slope. The value of $k_{obs}/[OH^-$ species] gave the second-order rate constants k and k_{OH} (M^{-1} s^{-1}) for NA hydrolysis.

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Supplementary Material Available: Figure of the potentiometric pH titration curve for 1 mM captopryl in the presence of 1 mM Zn^{II}–cyclen (1 page). This material is contained in many libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.